G-protein from *Medicago sativa*: functional association to photoreceptors

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G-protein subunits were characterized from *Medicago sativa* (alfalfa) seedlings. Crude membranes and GTP-Sepharose-purified fractions were electrophoresed on SDS/polyacrylamide gels and analysed by Western blotting with 9193 (anti- α_{common}) and AS/7 (anti- α_i , anti- α_{11} and anti- α_{12}) polyclonal antibodies. These procedures led to the identification of a specific polypeptide band of about 43 kDa. Another polypeptide reacting with the SW/1 (anti- β) antibody, of about 37 kDa, was also detected. The 43 kDa polypeptide bound specifically [α - ^{32}P]GTP by a photoaffinity reaction and was ADP-ribosylated by activated

cholera toxin, but not by pertussis toxin. Irradiation of etiolated *Medicago sativa* protoplast preparations at 660 nm for 1 min produced a maximal increase in the guanosine 5'-[γ -thio]triphosphate (GTP[^{35}S]) binding rate. After this period of irradiation, the binding rate tended to decrease. The effect of a red-light (660 nm) pulse on the binding rate was reversed when it was immediately followed by a period of far-red (> 730 nm) illumination. These results may suggest that activation of GTP[S]-binding rate was a consequence of conversion of phytochrome P_r into the P_{fr} form.

INTRODUCTION

Light, as a primary stimulus in plants for germination, leaf growth, plastid differentiation, pigment biosynthesis, vascular differentiation and flowering, interacts with several wavelength-specific receptors. Phytochrome, the most characterized photoreceptor, exists in two interconvertible forms, P_r and P_{fr} , with maximal absorption at 660 nm (red) and 730 nm (far red) respectively (for review, see Quail, 1991). Some of the light effects mediated by the phytochrome P_{fr} form are exerted at the level of gene expression (Kühlemeier et al., 1987; Couzzo et al., 1988).

A variety of second messengers and effector entities involved in intracellular signalling events have been postulated as putative transducers for both physical and chemical stimuli in plants (Trewavas and Gilroy, 1991; Einspahr and Thompson, 1990). As occurs with all other eukaryotic organisms, Ca^{2+} (Owen, 1988; Marmé, 1989; Schroeder and Hagiwara, 1989), phosphoinositide metabolites (Morse et al., 1987; Ettlinger and Lehle, 1988; Boss, 1989) and, to a lesser extent, cyclic AMP (Kurosaki et al., 1987; Brown and Newton, 1981) and pH (Felle, 1988), may be considered the most important second messengers in plants.

Some effectors and transducer proteins involved in the control of intracellular levels of these second messengers, or their effects, have been described in plant tissues. Ca^{2+} -dependent protein kinase activities have been found in soluble or membrane fractions of wheat embryo and pea, respectively (Polya and Davies, 1982; Hetherington and Trewavas, 1982). A Ca^{2+} - and calmodulin-dependent protein kinase activity was described in membrane fractions from zucchini hypocotyls (Salimath and Marmé, 1983); Ca^{2+} - and phospholipid-dependent type C protein kinase activities were found in zucchini and in spinach chloroplast fractions (Schäfer et al., 1985; Zherelova, 1989); Ca^{2+} -activated phospholipase-C and phosphatidylinositol kinase were found in membrane fractions from wheat seedlings (Sandelius and Sommarin, 1986; Melin et al., 1987; Pfaffmann et al., 1987; Tate et al., 1989; Einspahr et al., 1989); and a Ca^{2+} - and calmodulin-dependent adenylate cyclase activity associated with soluble fractions of alfalfa roots was found in our laboratory (Carricarte

et al., 1988). Moreover a sedimentable GTP-stimulated adenylate cyclase has been discovered in the roots of *Ricinus communis* (Luisini et al., 1991). All these results indicate that most of the regulatory mechanisms in animal tissues, involving intracellular signalling by second messengers, are highly conserved in plants.

On the other hand, specific guanine-nucleotide-binding activities were described in higher plants (Hasunuma et al., 1987; Jacobs et al., 1988; Blum et al., 1988). These binding activities might be associated with the blue-light phototransduction system (Warpeha et al., 1991), or with the phytochrome-mediated transduction mechanism (Romero et al., 1991), or with an inward K^+ channel in guard cells (Fairley-Grenot and Assmann, 1991). In addition, the molecular cloning of *ras*-like and G-protein α genes (Matsui et al., 1989; Ma et al., 1990; Anuntalabhochai et al., 1991; Palme et al., 1992) has also been reported in higher plants.

This paper describes the characterization of G-protein subunits from *Medicago sativa* (alfalfa) seedlings and their relationship to phytochrome in the transduction of light signals.

MATERIALS AND METHODS

Materials

The sources of *Medicago sativa* L. adult plants and other materials have been indicated in previous work (Carricarte et al., 1988). Bovine rod-outer-segment membranes and transducin were prepared as described by Muschietti et al. (1989).

Epicotyls of 8-day-old alfalfa seedlings were grown at 28 °C in darkness, or for periods under white fluorescent light for 8 h followed by 16 h of darkness, on 0.9% (w/v) agar with 1 mM CaSO_4 . Harvesting and further manipulations of seedlings were performed under a dim-green safety-light.

Antisera raised against synthetic peptides that correspond to defined regions of G-protein subunits (Mumby et al., 1986; Goldsmith et al., 1988; Simonds et al., 1989) were kindly provided by Dr. Allen M. Spiegel (National Institutes of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD, U.S.A.), Dr. Daniel Altschuler (Burroughs Wellcome, Research Triangle

Park, NC, U.S.A.) and Dr. Lutz Birnbaumer (Baylor College of Medicine, Houston, TX, U.S.A.). These antisera correspond to the following antigens: AS/7 (C-terminus of α_i , α_{11} and α_{12}), SW/1 (C-terminus of β -subunit) and 9193 (GTP-binding site; α_{common}). Synthetic peptides with amino acid sequences corresponding to α subunits were provided by Dr. Daniel Altschuler; these are: AS/7 peptide (KENLKDCGLF, C-terminus of α_i , α_{11} and α_{12}), used to raise AS/7 antiserum), α_8 -peptide (RMHLRQYELL, C-terminus of rat α_8) and α_{common} -peptide (CGAGESGKSTIVKQMK).

Guanosine 5'-[γ - ^{35}S]thiotriphosphate (GTP[^{35}S]), [adenylate- ^{32}P]NAD $^{+}$ and [α - ^{32}P]GTP were purchased from New England Nuclear (Boston, MA, U.S.A.). GTP-Sepharose, phenylmethanesulphonyl fluoride, leupeptin, aprotinin, pectolyase, nystatin, cholera toxin and pertussis toxin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and cellulase and Macerozyme were from Yakult Honsha Co. (Tokyo, Japan). Light filters from Schott and Gen (Mainz, Germany) were kindly provided by Dr. Silvia Braslavsky (Max Planck Institute, Muhlheim-at-Ruhr, Germany).

Protoplast preparation

All operations were done under dim-green safety-light. Epicotyls of 7-day-old alfalfa seedlings grown under sterile darkness conditions at 28 °C were dissected, cut into 1 mm cross-sections, and incubated in CPW-13M medium (Power and Chapman, 1985; 10 ml/g of tissue) for 60 min at 22 °C and then for 18 h at 22 °C with gentle shaking in the same medium but containing 1.0% cellulase, 0.5% Macerozyme and 0.1% pectolyase (all w/v). The mixture was then filtered through a nylon cloth (45 μm mesh) and centrifuged for 10 min at 100 g . The supernatant was then layered on to 21% (w/v) sucrose in CPW-13M medium and centrifuged at 120 g for 10 min. Protoplasts were collected from the surface and immediately used for the irradiation experiments.

Protoplasts were the only observable structures under a light microscope. Protoplast counting and viability (over 90%) were determined as indicated by Power and Chapman (1985).

Light treatment

Portions of the protoplast suspension in CPW-13M medium (0.03 ml; 5 mg of protein/ml) in 12 mm $\times$ 75 mm borosilicate glass test tubes were irradiated (or not) for the indicated times at 30 °C with a Kodak Ektagraphic 300 W slide projector (model B-2AR) supplemented with a 660 nm interference filter or a 730 nm cut filter in the slide slot. Flux rate was about 7 W/m ^{2} . After irradiation, the suspension was assayed for GTP[^{35}S] binding as indicated below.

Membrane preparation

Plasma membranes were prepared by the procedure of Hodges and Mills (1986) with some modifications. *M. sativa* leaves from 1–2-year-old plants or 8-day-old seedlings were excised and stored at –70 °C. The plant material was ground in a frozen mortar under liquid N $_2$ and further homogenized with 4 ml (per g of tissue) of 25 mM Tris/HCl buffer, pH 7.4, containing 0.25 M sucrose, 3 mM EDTA, 2.5 mM dithiothreitol, 0.5 mM phenylmethanesulphonyl fluoride, 50 μM leupeptin and 25 units/ml aprotinin. The homogenate was squeezed through several pieces of gauze and centrifuged at 12000 g for 10 min.

The supernatant was then centrifuged at 105000 g for 60 min, and the pellet was resuspended in the same buffer solution (one-tenth of the initial volume) but containing 20% (w/w) sucrose. The suspension was layered on to a discontinuous gradient continuing 30, 35 and 45% sucrose. The gradient was centrifuged at 95000 g for 60 min in a Beckman SW-28 rotor. Membranes were removed by aspiration from the 35–45%-sucrose interface and stored at –70 °C. The cytosolic fraction was obtained by further centrifugation of the 105000 g supernatant at 150000 g for 90 min.

DEAE-cellulose chromatography of G-protein

Partial purification of G-protein from *M. sativa* membranes was carried out by procedures developed for purification of mammalian G-proteins as described by Sternweis et al. (1981) and Codina et al. (1984). The thawed membrane preparation was diluted in 10 vol. of 50 mM Tris/HCl buffer, pH 7.4, containing 2 mM β -mercaptoethanol, 5% (v/v) glycerol, and 1% (w/v) sodium cholate, left for 90 min at 4 °C, and centrifuged at 105000 g for 60 min. The supernatant, designated 'cholate extract' (20 ml; 5.5 mg/ml protein; specific activity 10 pmol of GTP[S] bound/mg of protein) was loaded on a DEAE-cellulose column (Whatman DE-52; 25 cm $\times$ 0.6 cm) equilibrated with 20 mM Tris/HCl buffer, pH 8.0, containing 1% (v/v) Lubrol PX, 1 mM EDTA and 1 mM β -mercaptoethanol (Buffer A). After washing with 200 ml of this buffer solution, the column was eluted with 350 ml of a linear 0–200 mM NaCl gradient in Buffer A. Fractions (5 ml) were collected at 0.5 ml/min.

GTP-Sepharose affinity chromatography

A GTP-Sepharose column (1 cm $\times$ 11.5 cm) was equilibrated with 50 ml of Buffer B, containing 20 mM Tris/HCl buffer, pH 7.4, 1 mM EDTA, 2 mM MgCl $_2$, 100 mM NaCl and 2% glycerol, at 4 °C. The column was loaded with 50 ml of a 'cholate extract' adjusted to 0.4 mg of protein/ml with Buffer B, at 0.1 ml/min, and washed with Buffer B until the A_{280} of the effluent was below 0.02. Elution was performed with 30 ml of the same buffer solution containing 0.1 mM GTP. The eluate was dialysed overnight against 10 mM Tris/HCl buffer, pH 7.4, containing 10 mg of activated charcoal/ml, and freeze-dried.

GTP-binding assays

The concentration of GTP[^{35}S]-binding sites in purified fractions was determined by the procedure of Northup et al. (1982) as modified by Waldo et al. (1987). Under the assay conditions GTP[S] binding was maximal after a 30 min incubation and proportional to the protein concentration. Scatchard analysis of GTP[^{35}S] binding to the seedling-membrane fraction was performed by varying the concentration of non-radioactive GTP[S] from 1 to 1000 nM in the presence of a constant amount of GTP[^{35}S]. Binding was not affected by the presence of 1 μM ATP.

In protoplast lysates, GTP[^{35}S] binding rate was measured in 0.03 ml samples, after dilution with 0.17 ml of 20 mM Tris/HCl buffer, pH 8.0, containing 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 0.1% Lubrol PX, 10 mM MgCl $_2$ and 10 nM GTP[^{35}S] (sp. radioactivity 150000 c.p.m./pmol), for 2 min at 30 °C. Reactions were stopped and processed as indicated by Waldo et al. (1987). Microscopic examination showed that protoplasts were instantaneously lysed under the assay conditions. All the assays were performed in triplicate. Non-specific binding was measured in the presence of 100 μM GTP[S],

and was about 3 % of the radioactivity measured in the absence of unlabelled nucleotide.

GTP photoaffinity labelling

Direct photoaffinity labelling of membranes with [α - ^{32} P]GTP was performed as described by Linse and Mandelkow (1988). The gel was blotted on to a nitrocellulose membrane and exposed to a radioautographic film.

NAD⁺ and cholera- or pertussis-toxin treatment of membrane proteins

Plant membranes or bovine rod-outer-segment control membranes (0.1 mg of protein/assay) were incubated for 45 min at 30 °C in 50 mM Tris/HCl buffer, pH 7.4, containing 2 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.1 % Lubrol PX, 1 mM ATP, 0.2 mM GTP, 1 μ M [adenylate- ^{32} P]NAD⁺ (sp. radioactivity 40000 c.p.m./pmol) and 12 μ g of activated cholera or pertussis toxin (0.4 mg/ml, preincubated in the presence of 20 mM dithiothreitol). Total volume was 0.06 ml. After incubation, a 0.025 ml portion was diluted in 0.5 ml of 3 % (w/v) SDS and precipitated by addition of 0.5 ml of 30 % (w/v) trichloroacetic acid. The precipitate was collected on a nitrocellulose filter, washed with 5 % trichloroacetic acid and counted for radioactivity. The rest of the incubation mixture was adjusted to final concentrations of ATP and NAD⁺ of 10 and 5 mM respectively, and subjected to SDS/PAGE. The resolved polypeptides were blotted on to a nitrocellulose membrane to be further exposed to a radioautographic film.

Analytical methods

Protein was determined by the method of Lowry et al. (1951). Procedures for SDS/PAGE of protein samples were described elsewhere (Kornblihtt et al., 1981). Polypeptide transfer from polyacrylamide gels to nitrocellulose membranes was carried out by diffusion and solvent flow (Schaltman and Pongs, 1980; Bowen et al., 1980) or by electrotransference (Khyse-Andersen, 1984). Before reaction with antibodies, the membranes were blocked with a suspension of non-fat milk (Johnson et al., 1984). After the transfer, the individual lanes were incubated with 1:1000 dilutions of different antisera. Then the immune complexes were detected with the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA, U.S.A.) by following the manufacturer's instructions. Pre-immune rabbit sera and blocking peptides were used as controls in the immunological assays. In the competition assays, immunoreactions were performed in the presence of 100 μ M of the corresponding peptide for 2 h at room temperature and then overnight at 4 °C.

RESULTS

Preliminary characterization

Proteins from plasma membrane of *Medicago sativa* leaf cells were solubilized with 1 % sodium cholate and chromatographed on DEAE-cellulose. A GTP[^{35} S]-binding activity was eluted as a single peak at about 70 mM NaCl. This chromatographic behaviour was similar to that of mammalian G-proteins (Sternweis et al., 1981; Codina et al., 1984). Scatchard analysis of the GTP-binding activity showed the existence of only one binding component with a K_d of 171 nM (results not shown), which is

roughly similar to other reported values for plant tissues (Hasunuma et al., 1987; Zbell et al., 1989).

In order to establish a correlation between studies on *M. sativa* GTP[^{35} S]-binding activity and immunochemical characterization of transducing proteins (see below), 'cholate extracts' were also subjected to affinity chromatography on GTP-Sepharose.

On the other hand, photoaffinity labelling of *M. sativa* leaf membranes with [α - ^{32} P]GTP and further resolution of the photoproducts by SDS/PAGE showed two labelled bands of 41–45 and 27–30 kDa (Figure 1, lane 2). These labelled bands were not observed when reactions were performed in the presence of 100 μ M unlabelled GTP (Figure 1, lane 3), but only the 27–30 kDa-labelled band disappeared when 100 μ M ATP was added (Figure 1, lane 1).

Subunit Identification

Polypeptides from seedling membranes and GTP-Sepharose-affinity-chromatography-eluted fractions were resolved by SDS/PAGE and analysed after Western blotting by reaction with the AS/7 (anti- α , anti- α_{11} and anti- α_{12}) polyclonal antibody. These procedures led to the identification of a specific polypeptide band of about 43 kDa in membrane and GTP-Sepharose-eluate fractions (Figure 2a, lanes 1 and 2 respectively). The specificity of the reaction between the 43 kDa band and the AS/7 antibody was supported by the following evidence. (1) The band was not detected by preimmune rabbit serum (Figure 2a, lane 5); (2) it was not observed after preincubation of the AS/7 antiserum with 100 μ M of AS/7-peptide (Figure 2a, lane 4); and (3) preincubation of the AS/7 antibody with 100 μ M of α_s -peptide did not block the immunoreaction with the 43 kDa band in the Western blot (Figure 2a, lane 3). The same result was obtained by using 9193 anti- α_{common} antibody (Figure 2b). The 43 kDa AS/7- and 9193-immunoreactive polypeptide was also detected in membrane preparations from etiolated seedlings or adult plant leaves, but not in leaf cytosolic extracts (results not shown). The positive band in the GTP-Sepharose eluate strongly indicated the presence of a G-protein in *Medicago sativa* microsomal fractions.

In addition, the reaction of the 'cholate extract' from membrane seedlings with the SW/1 anti- β -subunit antibody revealed another specific polypeptide band of about 37 kDa (Figure 2c, lane 1), not detected by pre-immune serum (Figure 2c, lane 2). This polypeptide also appeared in homogenates from etiolated and white-light-grown seedlings (results not shown).

Figure 1 Photoaffinity binding of [α - ^{32} P]GTP by a leaf membrane fraction

Incubation mixtures containing 1 mM MgCl₂ were supplemented with 100 μ M GTP (lane 3) or 100 μ M ATP (lane 1) (lane 2, control). Other conditions are described in the Materials and methods section. DF, dye front. The arrow indicates the position of the 41–45 kDa band.

Figure 2 Reaction of leaf fractions with anti-G α and anti-G β polyclonal antibodies

(a) reaction of the AS/7 antibody (anti- α_1 , anti- α_{11} and anti- α_{12}) to membrane (50 μ g of protein; lanes 1, 3 and 4) and GTP-Sepharose-eluate (1 μ g of protein; lane 2) polypeptides. In lanes 3 and 4, reactions were performed with membrane polypeptides (50 μ g of protein), and with the AS/7 antibody preincubated with 100 μ M of α_s and AS/7 peptide, respectively. In lane 5, reaction was performed with membrane polypeptides and normal rabbit serum. (b) Reaction of the 9193 antibody (anti- α_{common}) with membrane polypeptides (50 μ g of protein). (c) Reaction of seedling 'cholate fraction' polypeptides (25 μ g of protein) with normal rabbit serum (lane 2) and the SW/1 anti- β antibody (lane 1). Details are given in the Materials and methods section and in the text. Arrows indicate the positions of 43 kDa (a and b) and 37 kDa (c) bands respectively.

Table 1 Requirements for ADP-ribosylation of *Medicago sativa* leaf membrane fraction by cholera toxin

Details are given in the Materials and methods section.

Reaction mixture	Trichloroacetic acid-insoluble fraction (c.p.m.)
Complete	4500
Minus cholera toxin	130
Minus membrane protein	100
Complete, plus unlabelled 1 mM NAD ⁺	500
Minus cholera toxin, plus pertussis toxin	120

ADP-ribosylation with cholera toxin

Incorporation of radioactivity into a trichloroacetic acid-insoluble product was detected after incubation of *M. sativa* membranes with activated cholera toxin and [adenylate-³²P]NAD⁺. As shown in Table 1, this ADP-ribosylating activity was dependent on the presence of cholera toxin and could not be replaced by pertussis toxin. The labelled product showed an electrophoretic mobility on SDS/PAGE (Figure 3, lane 2) slightly

Figure 3 ADP-ribosylation by cholera toxin of leaf membrane polypeptides

Lane 1, leaf membranes plus 100 μ M NAD⁺; lane 2, leaf membranes; lane 3, bovine transducin.

slower than that of ADP-ribosylated transducin (Figure 3, lane 3). The apparent molecular mass of this product was approx. 43 kDa.

GTP[S] binding in irradiated protoplast preparations

A putative role for G-proteins in leaves could be to mediate phytochrome-dependent responses to red light. In theory, the irradiation should be immediately followed by variations in the rate of guanine nucleotide exchange at the level of these G-proteins. A similar approach was used by Warpeha et al. (1991) to study the effect of blue-light irradiation on GTPase activity associated with pea apical buds.

In order to test this hypothesis, two relevant facts must be considered. (1) The phytochrome of dark-grown plants (P_r form) is localized in the cytoplasmic fraction (Quail, 1991); therefore, it is imperative to work with the whole plant cell, and not with membrane fractions, to find any possible G-protein activation due to the P_r form (Warpeha et al., 1991). (2) As phytochrome activation occurs in a few seconds, it is impossible to work with whole seedlings because, after irradiation, processes occurring during the period required for preparation of cell-free extracts or membranes would mask any phytochrome response (Romero et al., 1991). Therefore protoplasts from etiolated seedlings were used because they allowed us to measure GTP[S]-binding variations immediately after red-light irradiation.

The effect of protoplast irradiation with red (660 nm) or far-red (> 730 nm) light on the GTP[³⁵S]-binding reaction rate was studied. Figure 4(a) shows that irradiation at 660 nm for 1 min produced a maximal increase in the binding rate. After this period of irradiation, the binding rate tended to decrease. These results suggest that: (1) activation of GTP[³⁵S]-binding rate was the consequence of conversion of phytochrome P_r into the P_{fr} form; (2) the P_{fr} form reaches a maximum during the first 1 min of illumination; (3) this phytochrome form was responsible for the binding-rate activation; under these conditions, the binding was proportional to the assay time up to 5 min of incubation (Figure 4b); and (4) the activation effect was transient and disappeared when the transduction event was completed (later than 5 min). This behaviour is coincident with that described for

Figure 4 Effect of red light (660 nm) illumination on GTP[³⁵S]-binding rate by a protoplast preparation

(a) Binding rate as a function of illumination time. Triplicate samples of protoplast samples were subjected to the indicated periods of illumination. The assay time was 5 min. (b) Binding as a function of assay time: protoplasts were irradiated (●) or not (○) for 1 min at 660 nm and assayed for binding in triplicate samples. Other conditions are described in the Materials and methods section.

Figure 5 Reversion of the red-light effect on GTP[³⁵S]-binding rate in protoplasts by far-red light (> 730 nm)

The sequences of light and dark periods are indicated. The horizontal broken line indicates the background binding rate determined in the dark.

the light effects on Ca²⁺ uptake by maize protoplasts (Das and Sopory, 1985).

If phytochrome P_{fr} form is involved in the activation of GTP[S]-binding rate, reversion to the P_r form should decrease this rate. To explore this possibility, two facts were taken in consideration: one is that the effect of a red-light (660 nm) pulse on a photoresponse is reversed when it is immediately followed by a far-red (> 730 nm) pulse; the other fact is that reversion does not occur if a brief dark period is intercalated between these two pulses (Warner et al., 1981; Oelze-Karow and Mohr, 1982; Das and Sopory, 1985; for reviews see Sharma, 1984; Schäfer and Briggs, 1986). As shown in Figure 5, a partial reversion

occurs only if the red-light pulse is immediately followed by a far-red-light period, and intercalation of a dark period between red and far-red irradiation blocked this reversion. In this experiment, the binding rate measured in the dark, before the red-light pulse, was considered as a non-specific background, not involved in the phytochrome-mediated transduction event.

DISCUSSION

Results reported in this paper indicate the presence in *Medicago sativa* seedlings of a G α - α_{11-2} protein which can be ADP-ribosylated in the presence of NAD⁺ and preactivated cholera toxin. This polypeptide was detected in membrane fractions, but not in cytosolic fractions.

In addition, this α - α_{11-2} -like polypeptide may be part of a heteromultimeric protein of the G-family, since the presence of a polypeptide of about 37 kDa, bearing the epitope characteristic of G-protein β -subunit, was also detected in membrane detergent extracts.

The finding of an α - α_{11-2} -like polypeptide in *Medicago sativa* leaves is strikingly coincident with the finding by Ma et al. (1990) of cloned-genomic and cDNA sequences from *Arabidopsis thaliana*, coding for a 44583 Da polypeptide designated *GPa1*, whose mRNA is most abundant in leaves and roots. The *Arabidopsis* polypeptide amino acid sequence is similar to those of α subunits of bovine transducin and G₁₁₋₂ proteins. In addition it has potential sites for N-myristoylation and ADP-ribosylation by cholera toxin, but lacks the site for ADP-ribosylation by pertussis toxin.

It is worth mentioning the finding of polypeptides of 33 and 50 kDa in *Cucurbita pepo* (Jacobs et al., 1988), and of 31–38 kDa in *Vicia faba*, *Commelina communis* and *Arabidopsis thaliana*. These polypeptides are immunoreactive with a variety of antisera specific for α -subunits (Blum et al., 1988).

The hypothesis that a G-protein could mediate P_{fr} responses was advanced by Couzzo et al. (1988). This hypothesis is supported by the following evidence. (1) GTP and non-hydrolysable GTP analogues stimulated the formation of inositol phosphates by *Acer pseudoplatanus* membranes (Dillenschneider et al., 1986). (2) A Ca²⁺-stimulated phosphoinositide phospholipase C was identified in wheat seedlings (Melin et al., 1987). (3) Red-light treatment of maize leaf protoplasts for 1–2 min increased the rate of Ca²⁺ uptake. (4) This effect was reversed by far red light (Das and Sopory, 1985). (5) Red light induced swelling of mesophyll protoplasts from dark-grown wheat leaves. (6) This process was inhibited by guanosine 5'-[β -thio]diphosphate. (7) GTP[S] induced swelling to the same extent as after red-light treatment (Bossen et al., 1990).

Some of these P_{fr}-associated responses appear to involve calmodulin, since the phytochrome-dependent expression of chlorophyll *a*- and *b*-binding proteins was blocked by phenothiazine derivatives (Lam et al., 1989). Additional support for the hypothesis that P_{fr} responses are mediated by a G-protein is provided by the evidence reported here. Illumination of alfalfa protoplasts during a short period with red light increased the GTP[³⁵S]-binding reaction rate; this effect was reversed by far-red illumination. Similarly to other phytochrome-mediated responses, the introduction of a dark period between red and far-red illuminations blocked the reversion process. Under this condition, as was postulated by Sharma (1984) and Schäfer and Briggs (1986), the signal event becomes independent of the phytochrome condition, since it is transferred to other components of the transduction pathway ('escape of the signal').

In addition, it is relevant to point out that protoplasts are the most suitable material in which to analyse biochemical changes

of the plant transduction pathway occurring in short periods. Other possibilities, such as membrane preparations or whole etiolated-seedling extracts, were not satisfactory for this purpose. From the results of Warpeha et al. (1991) it is very clear that procedures for plasma-membrane preparation eliminated red-light effects. The obvious interpretation is that these membranes have no associated phytochrome protein. On the other hand, only small GTP[³⁵S]-binding changes could be measured, after red-light irradiation of whole etiolated oat seedlings, in assays performed on total homogenates (Romero et al., 1991).

Further studies on the interaction of photoreceptors and transducing proteins with plant membranes will improve understanding of the participation of plant G-proteins in the light-transduction pathway. With animal tissues, cell-free reconstituted systems containing receptors, transducing proteins and effector entities (Flawiá et al., 1983; Eisenschlos et al., 1986; Muschietti et al., 1989), provided a better knowledge of the participation of G-proteins in light- or hormone-dependent transduction mechanisms.

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