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INHIBITION OF CHLOROPLAST PROTEIN PHOSPHORYLATION BY cAMP IN *LEMNA PAUCICOSTATA* 6746

LAXMAN GANGWANI, JITENDRA P. KHURANA and SATISH C. MAHESHWARI*†

Department of Plant Molecular Biology, University of Delhi South Campus, New Delhi-110021, India; *Department of Botany, University of Delhi, Delhi-110007, India

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Key Word Index—*Lemna paucicostata*; Lemnaceae; duckweed; protein phosphorylation; chloroplast; light harvesting chlorophyll a/b-binding proteins; 3′,5′-cyclic AMP; sodium fluoride; staurosporine.

Abstract—Phosphorylation was carried out in chloroplasts, purified on sucrose density gradient, using [γ-^{32}P]ATP. Among several phosphopolypeptides detected, polypeptides in the M_r range of 24–22 k, which are known to constitute the light-harvesting chlorophyll a/b-binding protein complex (LHCP), and 16–18 k were highly phosphorylated. Cyclic AMP (100 μM) decreased the overall phosphorylation of chloroplast polypeptides and its effect was more striking on LHCP. Other nucleotides such as cGMP, 2′,3′-cAMP, 2′,3′-cGMP and 5′-AMP, at equimolar level, did not show any significant effect on phosphorylation of chloroplast polypeptides. The effect of cAMP was also studied on light-dependent *in vitro* phosphorylation of chloroplast polypeptides by incubating a crude preparation of intact chloroplasts with [^{32}Pi]. In dark control, two polypeptides of M_r 66 and 64 k were distinctly phosphorylated. On illumination, several other polypeptides in the range of 97–12 k were also phosphorylated, with the 26–24 k LHCP fraction being the major phosphopolypeptides. Cyclic AMP (at 100 μM and 1 mM) specifically caused a decrease in phosphorylation of the 26–24, 21–20 and 12 k polypeptides. The phosphorylation status of the 66–64 k doublet, as attained in dark, remained unaffected in the presence of light and/or cAMP. Sodium fluoride, a protein phosphatase inhibitor, enhanced the phosphorylation of 26–24, 21–20 and 12 k polypeptides but did not affect the inhibitory action of cAMP. It is thus likely that cAMP down-regulates protein kinase(s) responsible for the phosphorylation of these polypeptides. The present study also provides evidence that this protein kinase is sensitive to staurosporine, a protein kinase inhibitor.

INTRODUCTION

The cyclic AMP-adenylate cyclase system is ubiquitously present in prokaryotes and eukaryotes and is known to regulate various diverse functions in these organisms [1]. The presence of various elements of cAMP regulatory system has also been demonstrated in higher plants, including the presence of cAMP, enzymes of its metabolism, adenylate cyclase and phosphodiesterase [2], high affinity cAMP-binding proteins [3], phosphatases with high affinity for cGMP and cAMP [4], and 5′-nucleotidases that are competitively inhibited by cAMP and cGMP [5]. In addition, cAMP has been shown to affect a spectrum of biochemical and physiological processes in higher plants. However, despite these advances, it has not been possible to assign a general regulatory role to cAMP in higher plants.

Cyclic AMP is known to bring about most of its effects through protein phosphorylation by activating cAMP-dependent protein kinase (PKA) in animals and lower eukaryotes such as *Saccharomyces cerevisiae* and *Dictyostelium discoideum* [6, 7]. In higher plants too, there are a few reports indicative of cyclic nucleotide-dependent protein kinases. Two soluble protein kinase fractions from maize were found to be inhibited by 10–100 μM cAMP, using casein as substrate [8]. In addition, the presence of a protein kinase activated by 6 μM cAMP has also been reported in maize [9]. Likewise, *Lemna* has been found to contain soluble protein kinases, PI and PII, which are inhibited and activated, respectively, by 10 μM cAMP, using histone as a substrate [10]; in this study, cGMP and cIMP were as effective as cAMP. However, these protein kinases resolved from higher plants do not exhibit substrate specificity and subunit composition similar to PKA from animals. Only recently, a protein kinase similar to the catalytic subunit of PKA has been purified from *Petunia* petals [11]. In an earlier study too, although the enzyme was not characterized biochemically, the sequences of cDNA clones from *Phaseolus* and rice were shown to have homology with the catalytic subunit of mammalian PKA [12].

In order to understand the mode of action of cAMP in higher plants, attempts have also been made to study the

†Author to whom correspondence should be addressed.

effect of cAMP on phosphorylation of endogenous proteins. Most of the earlier studies somehow failed to support a role of cAMP in protein phosphorylation [13–17]. Lately, however, there is a report claiming that the addition of cAMP (10 μM) to dialysed coconut milk results in stimulation of phosphorylation of its constituent polypeptides in the M_r range of 60–70 k, and also decreases the phosphorylation of polypeptides in the range of 27–30 k [18]. Besides this, plants have also been shown to contain substrate proteins for PKA [19].

As a part of a larger effort to investigate a possible role of cAMP in higher plants, we have earlier demonstrated the presence of cAMP [20] and cyclic nucleotide phosphodiesterase [21] in the duckweed, *Lemna paucicostata* 6746, which is an aquatic plant and can be clonally propagated as well as maintained under aseptic conditions. In this strain, flowering is induced by exogenously supplied cAMP, under non-inductive photoperiods [22]. In the present investigation, we demonstrate the direct effect of cAMP on *in vitro* phosphorylation of chloroplast polypeptides of *L. paucicostata* 6746. The effect of cAMP was also studied on light-dependent *in vitro* phosphorylation of chloroplast polypeptides. Chloroplasts have been chosen because they have been shown to contain cAMP as well as enzymes adenylate cyclase and cyclic nucleotide phosphodiesterase [2, 23, 24]. In addition, chloroplasts have also been shown to contain the highest concentration of plant phosphoproteins [25].

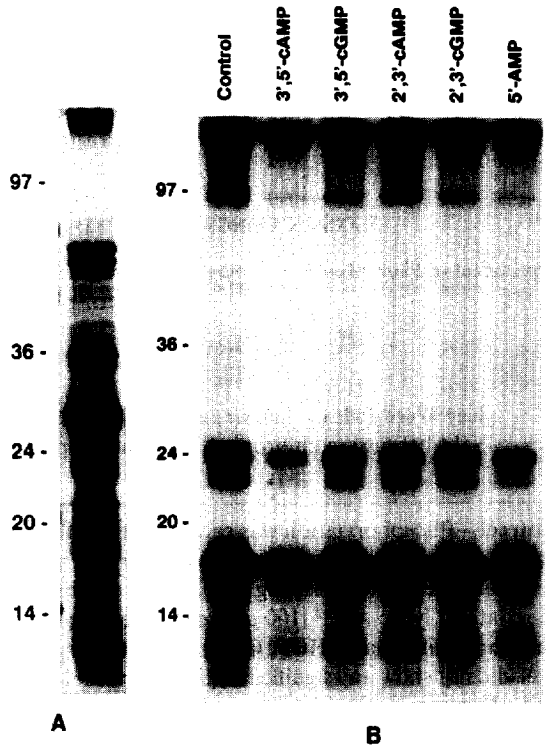

Fig. 1. (A) Photograph of gel stained with Coomassie Brilliant Blue R showing SDS-PAGE profile of chloroplasts purified by sucrose density gradient. (B) Autoradiogram showing *in vitro* phosphorylation of *Lemna* chloroplast proteins using [γ-^{32}P]ATP. The first lane (control) shows chloroplast phosphoproteins whereas other lanes show the effect of different nucleotides (100 μM) on phosphorylation of chloroplast polypeptides. The position and relative M_rs in k of standard proteins is indicated on the left.

RESULTS

Effect of cAMP

The intact chloroplasts, purified by sucrose density gradient, were lysed and subjected to phosphorylation reaction (as described in Experimental). The proteins were then resolved by SDS-PAGE and the phosphorylated polypeptides detected by autoradiography. The stained gel in Fig. 1A represents a profile of purified chloroplast polypeptides. The autoradiogram in Fig. 1B (lane 1) shows *in vitro* phosphorylation of chloroplast polypeptides of *Lemna* using [γ-^{32}P]ATP as a substrate. Under these experimental conditions, several (> 15) polypeptides in the M_r range of 97–8 k were found to be phosphorylated. Polypeptides in the M_r range of 24–22 k, which are known to be constituents of light-harvesting chlorophyll-protein complex (LHCP), and polypeptides in the range of 18–16 k were highly phosphorylated. Cyclic AMP (100 μM) decreased (by 50–60%) the overall incorporation of the label into chloroplast phosphopolypeptides and its inhibitory effect was more striking on phosphorylation of LHCP (Fig. 1B, lane 2). Cyclic AMP was also tried at 10 μM level and it decreased phosphorylation by 20–30% (data not presented).

Effect of other nucleotides

Fig. 1B (lanes 2–6) shows the effect of 2',3'- and 3',5'-cyclic nucleotides of both adenine and guanine and also 5'-AMP (all tested at 100 μM) on *in vitro* phosphorylation of chloroplast proteins. In comparison with 3',5'-cyclic AMP, 3',5'-cyclic GMP, 2',3'-cyclic AMP and 5'-AMP showed a marginal effect, whereas 2',3'-cyclic GMP was ineffective. These results suggest that cAMP specifically affects the phosphorylation of chloroplast proteins either by inhibiting a protein kinase or by activating a phosphatase.

Effect of cAMP on light-dependent in vitro *phosphorylation*

Light plays a pivotal role in photosynthesis by regulating the phosphorylation of chloroplast proteins and is known to induce reversible phosphorylation of chloroplast proteins by activating light-dependent protein kinase(s) located in thylakoid membranes [25]. To examine the effect of cAMP on light-dependent *in vitro* phosphorylation of chloroplast proteins, a crude preparation of intact chloroplasts, isolated by method B (see Experimental), was used. A crude preparation was preferred to prevent any modifications that may occur in membrane structure during purification, which in turn may affect

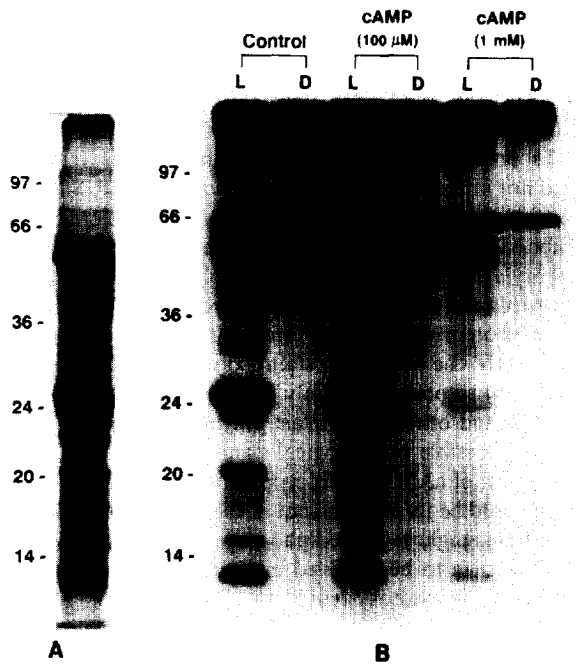

Fig. 2. (A) Photograph of stained gel showing protein profile of crude chloroplast preparation. (B) Autoradiogram showing *in vitro* incorporation of orthophosphate [^{32}Pi] into chloroplast phosphoproteins under the conditions of dark (D) and light (L) and effect of cAMP on phosphorylation.

the photosynthetic ability of chloroplasts. Intact chloroplasts have the unique ability to synthesize [γ-^{32}P]ATP from inorganic phosphate [^{32}Pi] by photophosphorylation. Therefore, in order to selectively phosphorylate chloroplast proteins in a crude preparation, ^{32}Pi-orthophosphate was supplied in the incubation reaction instead of [γ-^{32}P]ATP, which is generally employed as a substrate for protein kinases in *in vitro* assays.

The photograph of a stained gel in Fig. 2A shows the polypeptide profile of the crude chloroplast preparation. The autoradiogram in Fig. 2B shows the *in vitro* incorporation of [^{32}Pi] into chloroplast phosphopolypeptides under light and dark conditions. In dark control, two polypeptides of 66 and 64 k were distinctly phosphorylated. On high intensity illumination, several other polypeptides were also phosphorylated in the M_r range of 97 to 12 k. The major phosphorylated polypeptides were in the range of 26–24 k which are known to be components of LHCP. Addition of cAMP (100 μM and 1 mM) to the incubation mixture specifically caused a decrease in the light-stimulated phosphorylation of 26–24, 21–20 and 12 k polypeptides, but it did not affect phosphorylation in darkness. The minimum concentration of cAMP required to produce detectable change in phosphorylation status of polypeptides of intact chloroplasts (crude preparation) was 100 μM, which is 10-fold higher than the amount required in case of *in vitro* phosphorylation of polypeptides in the lysed extract of purified chloroplasts. This may be due to limited transport of cAMP across the chloroplast double envelope membrane because of its polar nature and/or the probable presence of cyclic nucleotide phosphodiesterase in intact chloroplast [2], which may simultaneously hydrolyse it during the course of reaction.

Strikingly, the phosphorylation status of 66–64 k doublet, as attained in dark, remained unaffected in the presence of light and/or cAMP (Fig. 2B). These results suggest that cAMP specifically inhibits protein kinase(s) or activates protein phosphatase(s) responsible for regulating the phosphorylation status of chloroplast polypeptides including LHCP.

Effect of sodium fluoride and staurosporine on light-dependent in vitro *phosphorylation*

The effect of sodium fluoride, a protein phosphatase inhibitor, was studied together with cAMP on light-stimulated phosphorylation. The autoradiogram in Fig. 3 shows the effect of increasing concentration of cAMP in the absence and presence of 10 mM NaF. Sodium fluoride enhanced the phosphorylation of 26–24, 21–20 and 12 k polypeptides. However, the presence of NaF did not affect the inhibitory action of cAMP. Staurosporine, a protein kinase inhibitor, was tried at 10 μM and it specifically decreased the phosphorylation of 26–24, 21–20 and 12 k polypeptides (Fig. 4). These results suggest that cAMP is either directly or indirectly inhibiting protein kinase(s) responsible for the phosphorylation of chloroplast polypeptides including LHCP.

Endogenous level of cAMP in chloroplasts

The level of cAMP was estimated in chloroplasts, purified on sucrose density gradient (Method A), by

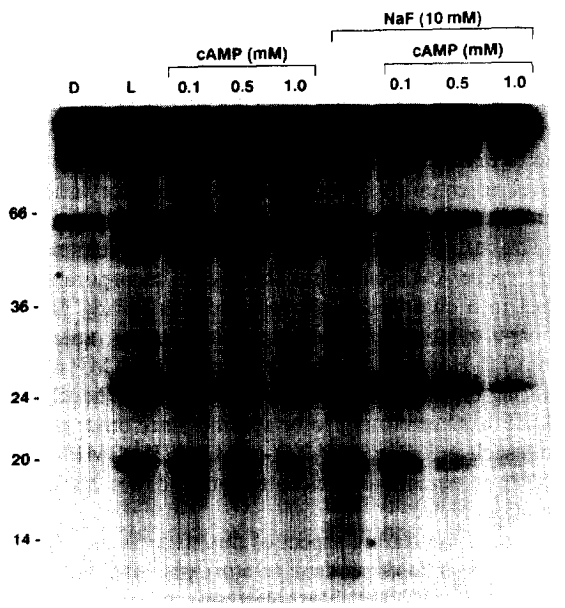

Fig. 3. Autoradiogram showing effect of increasing concentration of cAMP alone and in the presence of NaF (10 mM) on *in vitro* incorporation of [^{32}Pi] in chloroplast phosphoproteins.

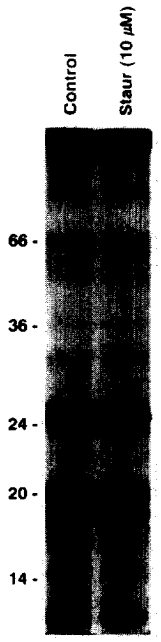

Fig. 4. Autoradiogram showing effect of staurosporine (10 μM) on phosphorylation of chloroplast proteins using [^{32}Pi], under conditions of light.

radioimmunoassay. The amount of cAMP was found to be 8–12 pmol (mg protein)$^{-1}$.

DISCUSSION

Cyclic AMP brings about most of its effects through protein phosphorylation which is now recognized as a universal regulatory mechanism and exists in almost every form of life [26]. In general, protein phosphorylation is also involved in regulation of several metabolic processes in higher plants [27, 28] but the role of cAMP in this regulatory mechanism is not clearly established. In order to determine whether cAMP plays a role analogous to that in animals or is operative through some other novel mechanism(s), its effect on phosphorylation of chloroplast proteins of *Lemna* was studied.

The results of the present investigation demonstrate that cyclic AMP decreases the overall incorporation of label into chloroplast phosphoproteins during *in vitro* phosphorylation of lysed chloroplasts using radiolabelled ATP. However, the inhibitory effect of cAMP is most striking on the phosphorylation of LHCP. The effect of cAMP has been found to be specific, since other nucleotides such as 3′,5′-cGMP-2′,3′-cAMP, 2′,3′-cGMP and 5′-AMP, at equimolar concentration, did not show any significant effect.

A more interesting aspect of this study is the effect of cAMP on light-dependent *in vitro* phosphorylation of proteins in the intact chloroplast. Among several chloroplast polypeptides which were phosphorylated on illumination, cAMP specifically decreased the phosphorylation of LHCP (26–24 k) and polypeptides in the range of 21–20 k and a 12 k polypeptide. Sodium fluoride (NaF), which has been shown to inhibit a thylakoid-bound phosphoprotein phosphatase responsible for dephosphorylation of LHCP in pea [29], enhanced the light-stimulated phosphorylation of 26–24 (LHCP), 21–20 and 12 k polypeptides of *Lemna* chloroplast. However, NaF did not influence the inhibitory effect of cAMP. Staurosporine, a potent protein kinase inhibitor, selectively inhibited the protein kinase(s) responsible for the phosphorylation of these chloroplast polypeptides. These results suggest that the cAMP is not regulating the activity of a protein phosphatase but is inhibiting, directly or indirectly, the activity of staurosporine-sensitive protein kinase(s) responsible mainly for the phosphorylation of LHCP and other chloroplast polypeptides.

The possible physiological significance of the inhibitory effect of cAMP on chloroplast protein phosphorylation could be its involvement in the regulation of photosynthesis. Light regulated phosphorylation of LHCP has been shown to be involved in the transfer of excitation energy from photosystem II to photosystem I [30, 31]. However, there are several questions that remain to be answered about the role of protein phosphorylation in the regulation of photosynthesis [32, 33]. For example, how is phosphorylation of LHCP proteins regulated under continuous light, since a few minutes exposure to light is sufficient enough to bring about phosphorylation of the whole population of LHCP. How a balance is maintained between phosphorylated and non-phosphorylated forms of proteins under continuous light, is another question that remains to be answered. There is a possibility that light regulates the activity of both a phosphatase and a protein kinase in order to maintain an equilibrium between the phosphorylated and non-phosphorylated forms of LHCP. There is, however, no evidence as yet available for the presence of a light-activated protein phosphatase. The rate of dephosphorylation of LHCP by a NaF-sensitive, thylakoid-bound protein phosphatase has been found to be same in light and dark conditions [29]. Therefore, control of the activity of protein kinase in the presence of continuous light appears to be more plausible.

Under conditions favourable to LHCP phosphorylation, ATP causes a decrease in chlorophyll fluoresence and under dephosphorylation conditions fluorescence decline is reversed and this reversal can be blocked by NaF [30]. Cyclic AMP has earlier been shown to inhibit the ATP induced decrease in chlorophyll fluorescense and also to decrease the incorporation of label into LHCP in pea [34]. Light has also been shown to increase the levels of cAMP by 3–4 folds in *Avena* etioplasts [35] and *Phaseolus* chloroplasts [36]. Keeping in view earlier findings and results of the present investigation, we suggest that the cAMP may be involved in modulating the phosphorylation status of LHCP and other chloroplast polypeptides (function yet to be ascertained) by repressing the activity of protein kinase(s).

The phosphorylation of the 66–64 k doublet in dark, which is not affected by NaF, deserves special consideration. The kinase responsible for this phosphorylation

appears to be light-independent and insensitive to cAMP and staurosporine, or else this may be a case of autophosphorylation. The radiolabelled ATP utilized under these situations may be synthesized in the dark from [^{32}Pi] by phosphate exchange and/or due to residual activity of ATP synthase in dark. The point to be noted is that the phosphorylation of these polypeptides was not obtained in lysed chloroplasts using [γ-^{32}P]ATP (Fig. 1B) or [^{32}Pi] (data not presented). Therefore, it is suggested that the phosphorylation of this doublet may represent the presence of photosynthetically active intact chloroplasts. Another noteworthy point is the difference in phosphorylation pattern of LHCP in lysed and intact chloroplasts using [γ-^{32}P]ATP and [^{32}Pi], respectively. This result indicates the presence of more than one protein kinase, or more likely, different isoforms of protein kinase responsible for the phosphorylation of LHCP. However, more work is needed to conclusively establish the presence and characteristics of these protein kinases.

EXPERIMENTAL

Plant material and growth conditions. Aseptic cultures of *Lemna paucicostata* 6746 were grown in Bonner–Devirian medium [37], pH 5.5, under a photoperiodic regime of 16 hr light and 8 hr darkness, as described before [22]. The experimental cultures were initiated with the transfer of two 3-frond colonies per 250 ml conical flask, containing 100 ml nutrient medium, from a 12–14 days old stock culture. Temp. was maintained at 25 ± 2° and the light energy provided by Cool Daylight fluorescent tubes (Philips, 40 W) varied from 5.0–5.2 W m^{-2}.

Isolation and purification of chloroplasts (Method A). Chloroplasts were isolated and purified following the procedures developed earlier by others [38, 39], with some modifications. Aseptically grown 14–15 days old *Lemna* plants (40 g fr. wt) frozen in liquid N_2 were homogenized to a fine powder using a mortar and a pestle. After the liquid N_2 had evaporated, the powdered leaf material was suspended in 5 vol. of grinding buffer [40 mM Tris-HCl at pH 8], 0.4 M sucrose, 40 mM EDTA, 0.1% 2-mercaptoethanol (v/v), 0.1% BSA (w/v)]. All subsequent steps were carried out on ice or at 4° in a cold room. The suspension was filtered through 8 layers of muslin cloth and then centrifuged at 2000 g for 10 min. The pellet was resuspended in 8 ml of resuspension buffer (40 mM Tris-HCl at pH 8, 25 mM EDTA, 0.35 M sorbitol) and loaded on top of preformed 25–45–60% sucrose gradients prepared in the resuspension buffer in such a way that every tube contained material corresponding to 20 g fr. wt of the tissue. The samples were centrifuged at 90 000 *g* for 1 hr. The chloroplast band at the interface of 25% and 45% sucrose was collected and diluted 10-fold with the resuspension buffer and centrifuged at 2000 *g* for 10 min at 4°. The pellet of chloroplasts from 40 g leaf material was resuspended in 3 ml (*ca.* 10 mg protein ml^{-1}) of reaction buffer containing 0.01% Triton X-100 and used for phosphorylation of chloroplast proteins employing [γ-^{32}]ATP.

Method B. Chloroplasts were isolated as described in ref. [40] with some modifications. Plants (40 g fr. wt) were homogenized with 6 vol. of chilled isolation medium [40 mM Tris-HCl at pH 8, 0.4 M sucrose, 40 mM EDTA, 0.1% 2-mercaptoethanol (v/v), 0.1% BSA (w/v)] in an electric mixer for 15–20 sec with 5 sec pulses at the lowest speed setting, in the cold room at 4°. The homogenate was squeezed through 8 layers of muslin cloth and centrifuged at 2000 *g* for 10 min at 4°. The pellet was resuspended in 40 ml sorbitol resuspension medium (40 mM Tris-HCl at pH 8, 25 mM EDTA, 0.35 M sorbitol) and used for phosphorylation of chloroplast proteins by inorganic phosphate [^{32}Pi].

In vitro *phosphorylation of chloroplast proteins using* [γ-^{32}*P*]*ATP.* Chloroplasts purified by method A were resuspended in reaction buffer [50 mM Tris-HCl (pH 7.5), 9 mM $MgCl_2$, 4 mM $NaHCO_3$, 5 mM 2-mercaptoethanol, 1 mM PMSF, 50 μM DTT, 20 μM EDTA, 0.01% Triton X-100] and incubated on ice for 15 min. Aliquots of equal vol. were transferred to reaction tubes. The reaction was started by adding 10 μCi of [γ-^{32}P]ATP (sp. act. 3000 Ci mmol^{-1}) and 0.5 nmol of ATP. Final reaction vol. was 100 μl. The reaction was stopped by precipitating proteins with 8 vol. of Me_2CO and tubes were kept at − 20° for 2 hr. Samples were centrifuged to 15 000 *g* for 10 min at 4°. The protein pellet was washed twice with 1 ml of 80% Me_2CO and air dried. Pellet was resuspended in sample electrophoresis buffer containing 62.5 mM Tris-HCl buffer (pH 6.8), 5% 2-mercaptoethanol, 10% glycerol and 2% SDS and heated to 100° for 3 min. Samples were allowed to cool and centrifuged to 15 000 *g* for 15 min at 25° and used for gel electrophoresis.

In vitro *phosphorylation of chloroplast proteins using inorganic phosphate* [^{32}*Pi*]. Chloroplasts isolated by method B were resuspended in sorbitol buffer. Aliquots of equal vol. (75 μl) were transferred to reaction tubes. The reaction was started by adding 100 μCi carrier-free ^{32}P-orthophosphate [^{32}Pi]. The final reaction vol. was 100 μl. The reaction mixture was incubated at 27° for 10 min illuminated from bottom with Single Coil 150 W (Philips, Comptalux) lamp at a distance of 10 cm. The reaction was stopped by adding 8 vol. of Me_2CO and samples were processed for gel electrophoresis in a similar way as described above. Phosphorylation reaction was also carried out in dark. The reaction was initiated after dark adaptation of chloroplasts for 5 min and terminated in dark.

Estimation of cAMP in chloroplasts. Amount of cAMP was estimated in the chloroplasts purified by sucrose density gradient method (method A). Chloroplasts were resuspended in 50 mM Tris-HCl (pH 7.5) and vortexed for disruption. Suspension was heated at 100° for 3 min and then centrifuged at 15 000 *g* for 15 min at 4°. An aliquot of 100 μl was used for the determination of cAMP level by radioimmunoassay, using cyclic AMP assay kit with [^{125}I]cAMP (Yamasa Shoyu Co. Ltd., Japan). The succinylation procedure was used and sepn of bound and free cAMP was carried out using dextran-coated charcoal. Cyclic AMP levels were

estimated using a standard curve in the range of 0.625–80 pmol ml^{-1}. Each sample was analysed in duplicate.

Gel electrophoresis and autoradiography. Labelled polypeptides were resolved by SDS-PAGE following the method described in ref. [41]. The separation gel contained 12.5% acrylamide (w/v) while the stacking gel contained 5% acrylamide (w/v). Aliquots of 50 μl containing equal amount of the dissolved proteins (200–300 μg) were loaded in each well on 1.5 mm thick slab gel and electrophoresed at 2 mA per lane until the sample reached the separation gel boundary and then at 3 mA per lane through separation gel. The gels were stained for 1–2 hr in a soln containing 0.3% Coomassie Brilliant Blue R-250 (w/v) dissolved in 40% MeOH (v/v), 10% HOAc (v/v) and 50% H_2O. Destaining was carried out in a soln of 40% MeOH (v/v), 10% HOAc (v/v) and 50% H_2O. The gels were mounted on Whatman filter paper (3 MM) and dried using a vacuum slab-gel dryer. Dried gels were laid on X-ray films and exposed in a cassette with intensifying screen at -20° and developed after 10–12 days.

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