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Plant Science 166 (2004) 163–168
PLANT SCIENCE
www.elsevier.com/locate/plantsci

Involvement of G-proteins, calmodulin and tagetitoxin-sensitive RNA polymerase in light-regulated expression of plastid genes (*psb*A, *psa*A and *rbc*L) in rice (*Oryza sativa* L.)

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Received 19 June 2003; received in revised form 30 July 2003; accepted 2 September 2003

Abstract

The regulation of chloroplast gene expression by light involves multiple signaling components. In an earlier study, we demonstrated the role of calcium and phosphorylation in regulating the expression of photosynthesis-related plastid genes, *psb*A, *psa*A and *rbc*L, using rice as a model monocot system. This work has been extended further to examine the possible involvement of heterotrimeric GTP-binding proteins and calmodulin. Vacuum infiltration of 5-day-old etiolated rice seedlings with G-protein agonists, cholera toxin and GTPγS, increased the steady-state transcript levels of the plastid genes. The antagonists/inhibitors of calmodulin action, trifluoperazine and W7, inhibited the light-induced increase in steady-state transcript levels of these genes. The light-regulated expression of photosynthetic genes was also adversely affected by tagetitoxin, a specific inhibitor of plastid-encoded RNA polymerase. These results indicate the involvement of various signaling components in transduction of light signal that probably also recruits PEP to regulate plastid gene expression.

Keywords: Calmodulin; Gene expression; G-proteins; Light; *Oryza sativa* L.; Plastid-encoded RNA polymerase (PEP)

1. Introduction

Light is unarguably the most important environmental signal that not only acts as a source of energy for photosynthesis but also triggers photomorphogenesis in plants [1,2]. The photosynthetic machinery is constituted by proteins encoded both by the nuclear and chloroplast genes, whose expression is known to be regulated by light. Light signal is perceived by a rather large number of sensory photoreceptors in higher plants. These include five phytochromes, two cryptochromes and two phototropins [2–4]. Both, phytochromes and cryptochromes have been shown to influence the expression of chloroplast- and nuclear-encoded photosynthesis-related genes in several plant species [5]. How the light signal perceived is relayed to the downstream signaling components has been addressed by employing both molecular genetic and pharmacological approaches. Several photomorphogenic loci [6] and signal cascade intermediates [7] that influence and participate in the expression of photosynthesis-related nuclear genes have been identified. But, the signaling pathways involved in relaying the light signal to the chloroplast gene expression machinery are not understood completely.

Genetic approaches have resulted in the identification of several light signal transduction mutants. These mutants have an altered response to light with apparent effects on the photosynthetic apparatus [1,6]. However, the proteins encoded by these photomorphogenic loci are localized in the nucleus or the cytosol and their molecular analysis has not yet revealed any direct interaction with the plastid genetic machinery. It is not clear if protein–protein or protein–DNA interactions similar to those seen in case of nuclear gene expression play a specific role in regulating chloroplast gene expression. There is evidence that light quality directly

Abbreviations: cab, gene for chlorophyll a/b binding protein; CTX, cholera toxin; D_1, 32 kDa polypeptide of photosystem II; GTPγS, guanosine 5′-(γ-thio) triphosphate; NEP, nuclear-encoded RNA polymerase; P_{700}, reaction center of photosystem I; PEP, plastid-encoded RNA polymerase; *psa*A, gene for P_{700} chlorophyll a apoprotein A; *psb*A, gene for 32 kDa D_1 polypeptide; *rbc*L, gene for large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; *rbc*S, gene for small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; TFP, trifluoperazine

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doi:10.1016/j.plantsci.2003.09.003

influences transcriptional activity of photosystem I and II genes by affecting the redox state of plastoquinone that is a component of the photosynthetic electron transport [8]. Phosphorylation status of the *trans*-acting factors affects plastid gene transcription [9] and role of kinases and phosphatases has also been implicated in linking signal perception to gene expression in barley [10] and rice [11,12].

Simultaneously, pharmacological approaches have provided evidence for the involvement of heterotrimeric GTP-binding proteins, cGMP, calcium and calmodulin in light signal transduction pathway that regulates the expression of photosynthesis-related nuclear genes [1,6,7,13]. Utilizing similar approach, we provided evidence for the involvement of calcium and phosphorylation in the regulation of steady-state transcript levels of photosynthesis-related plastid genes representing photosystem I (*psa*A), photosystem II (*psb*A) and a stromal protein (*rbc*L) in rice [11,12]. In the present investigation, certain agonists/antagonists of G-protein and calmodulin action were employed to ascertain if these signaling components are also involved in the regulation of chloroplast gene expression.

Chloroplast gene expression is complex owing to the presence of at least two different types of RNA polymerases, plastid-encoded RNA polymerase (PEP) and nuclear-encoded RNA polymerase (NEP) [14]; there is evidence for one more form of nuclear-encoded RNA polymerase from spinach [15]. NEP represents a phage-type RNA polymerase [16], which preferentially drives the expression of housekeeping plastid genes including genes that encode for plastid-encoded RNA polymerase. On the other hand, PEP is known to drive the expression of photosynthesis-related plastid genes and its own expression is regulated by nuclear-encoded sigma factors that are under the influence of light [17]. Recent evidence has revealed complex nature of functional integration of different RNA polymerases in plastids [18]. Earlier, plastid genes in rice were shown to be light-regulated [19]. We have made an attempt to investigate if there is any cross-talk between the light signal and the PEP in rice. The effect of PEP-specific inhibitor, tagetitoxin, was thus examined on the expression of photosynthesis-related plastid genes, namely *psb*A and *rbc*L, and a housekeeping gene, 16S rDNA.

2. Materials and methods

The growth conditions for *Oryza sativa* L. ssp. *indica* cv. Pusa 169 and chemical treatments were essentially the same as described earlier [12]. Five-day-old dark-grown seedlings, submerged under MilliQ water (Millipore), were excised just below the junction of the coleoptile and the mesocotyl in complete darkness. A maximum of 25 seedlings were transferred to each vial containing 5 ml of 5 mM potassium phosphate buffer (pH 6.0) along with the desired agonists and antagonists/inhibitors of the signaling intermediate. To ensure proper uptake of the chemicals, the vials containing the seedlings were subjected to vacuum infiltration for 60 min. Following vacuum infiltration in the dark, samples containing the agonists were maintained in the dark for 12 h while the samples containing the antagonists were placed under light for the same duration before being harvested and frozen for subsequent northern analysis. For treatment with the PEP-specific inhibitor, tagetitoxin, after vacuum infiltration for 60 min, the seedlings were incubated in dark, with or without tagetitoxin, for 8 h and then maintained in dark or transferred to light for desired duration before freezing them for RNA isolation. As for the agonists of heterotrimeric G-proteins, cholera toxin (CTX) was used at a final concentration of 0.5, 1 and 2 μg/ml, from a stock of 1 mg/ml, and GTPγS, at a final concentration of 50, 100 and 200 μM, from a 100 mM stock. The antagonists/inhibitors for calmodulin action, trifluoperazine (TFP), was used at a concentration of 5, 25 and 50 μM from a stock solution of 1 mM, and W7 was used at a final concentration of 0.1, 0.2 and 0.4 mM from a stock of 20 mM. Tagetitoxin was procured from Epicenter Technologies, Madison, USA, as a stock solution of 20 U/μl and used at a final concentration of 800 U/ml. All the stock solutions were prepared in MilliQ water. The agonists and antagonists used in this study were procured from Sigma–Aldrich, USA.

Northern analysis on frozen plant samples was performed by using *psb*A, *psa*A and *rbc*L gene-specific probes as reported earlier [12]. For 16S rDNA, *Bam*HI fragment of the plasmid pRP7 [20] representing the requisite gene was used. Each experiment was repeated at least three times with essentially similar results. Observed variation in basal transcript levels for individual genes under dark or light among different figures is due to varied exposure of the autoradiograms and does not indicate the in vivo difference in relative transcript levels of the genes studied.

3. Results and discussion

Earlier studies from our laboratory have demonstrated that light, perceived by phytochrome, and developmental cues interact to establish the steady-state transcript levels of photosynthesis-related plastid genes in rice; calcium and the phosphorylation status of the regulatory intermediates also contribute in this process [12,19]. As an extension of this work, experiments were performed to find out the probable involvement of other signal transduction components and the nature of the RNA polymerase involved in the regulation of plastid gene expression by light.

3.1. Involvement of G-proteins

Heterotrimeric G-proteins are composed of three subunits: α, β and γ. The inactive state is represented by the GDP bound form wherein the three subunits are held together. Activation is brought about by the exchange of GDP with GTP and the dissociation of α subunit from the βγ dimer

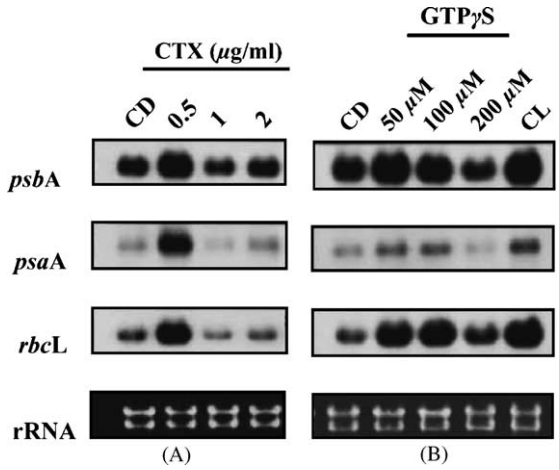

Fig. 1. Effect of G-protein agonists on light-dependent accumulation of plastid gene transcripts. (A) Effect of cholera toxin (CTX). Autoradiograms show the steady-state transcript levels of plastid genes, *psb*A, *psa*A and *rbc*L, in rice seedlings treated with 0.5, 1 and 2 μg/ml of CTX. (B) Effect of GTPγS, a non-hydrolysable analogue of GTP. Autoradiograms show the steady-state transcript levels of plastid genes, *psb*A, *psa*A and *rbc*L, in rice seedlings treated with 50, 100 and 200 μM GTPγS. Lower panels show ethidium bromide-stained bands of rRNA. CD and CL represent the untreated control samples, kept in dark throughout and exposed to light for 12 h, respectively.

[21]. The involvement of heterotrimeric G-proteins has been demonstrated earlier in phytochrome-regulated expression of photosynthesis-related nuclear genes [13]. In the present investigation, the involvement of heterotrimeric G-proteins in regulation of plastid gene expression in rice was examined by using the diagnostic agonists (CTX and GTPγS) of heterotrimeric G-protein action. Cholera toxin irreversibly activates the G-protein by catalyzing the ADP-ribosylation of α subunit [22]. Five-day-old etiolated seedlings of rice were treated with 0.5, 1 and 2 μg/ml of CTX. Northern analysis of the samples revealed that the transcript levels of the plastid genes, *psb*A, *psa*A and *rbc*L, are significantly enhanced, over dark control, at the concentration of 0.5 μg/ml CTX (Fig. 1). Increase in the steady-state transcript levels of plastid genes in the dark-grown samples treated with CTX was essentially similar to the dark-grown samples transferred to light for the same duration (12 h).

The second agonist, GTPγS (a non-hydrolysable analogue of GTP), binds to the α-subunit of G-proteins blocking its GTPase activity, thereby maintaining it in an active state [21]. GTPγS was used at concentrations of 50, 100 and 200 μM. For *psb*A, the enhancement in the transcript levels was more with 50 μM GTPγS, whereas, in case of *rbc*L and *psa*A, both 50 and 100 μM levels resulted in essentially the same degree of enhancement (Fig. 1B). Higher concentration of the agonists (CTX, GTPγS) showed no effect and the transcript levels of all three genes were somewhat comparable with the untreated dark controls. This could be due to the desensitization phenomenon, a general feature of sensory systems [23].

The involvement of GTP-binding proteins in various signaling pathways in plants is well documented [21]. Our results are reminiscent of the observed enhancement in the transcript levels of the photosynthetic nuclear gene, *cab*, in etiolated *Avena* seedlings, *aurea* mutant of tomato and soybean suspension cells treated with CTX or GTPγS [1,13]. In *Avena* seedlings, maximal enhancement was obtained at a much higher level of 30 μg/ml, as opposed to only 0.5 μg/ml CTX required for the plastid genes (present study); this may probably be due to more efficient vacuum-assisted uptake of the agonist carried out in the present study. Recent evidence for the occurrence and regulation of heterotrimeric GTP-binding proteins in spinach chloroplast envelope [24] has raised the possibility that these proteins may be involved in communication with signaling components to bring about chloroplast biogenesis and development. Our observations on rice, where the agonists of G-protein action were able to enhance the steady-state transcript levels of the plastid genes in the dark, simulating the effect of light, corroborate these recent findings.

3.2. Involvement of calmodulin

We had demonstrated earlier that the light-mediated increase in the steady-state transcript levels of plastid genes, *psb*A, *psa*A and *rbc*L, in dark, could be simulated by vacuum infiltrating the etiolated rice seedlings with external calcium and calcium ionophore, A23187 [12]. Calcium ion fluxes have also been observed in the chloroplast stroma and cytosol of transgenic tobacco harboring plastid-targeted aequorin, indicating towards the modulation of calcium levels by dark/light transition [25]. It is generally accepted that extracellular signals communicate with the target proteins by bringing about changes in the cellular calcium and, downstream sensors perceive and interpret these changes. There are three types of proteins that respond to calcium ion fluxes, namely, calmodulin (CaM), calcineurin B-like proteins (CBL) and calcium-dependent protein kinases (CDPKs). CaM is a highly conserved eukaryotic protein and is a ubiquitous calcium sensor [26] that brings about transcriptional regulation either via direct interaction with transcription factors or indirectly by affecting the protein kinases and phosphatases that regulate transcription factor activity.

The calcium-mediated effect observed earlier in our system [12] could have possibly involved calmodulin since in plants there is evidence for the participation of calmodulin in phytochrome signal transduction pathway [27]. The antagonists of calmodulin action, namely, trifluoperazine and W7, which also inhibit calcium/calmodulin-dependent phosphodiesterase [28], were thus employed to investigate the involvement of calmodulin in light-mediated enhancement of plastid gene expression. The seedlings were vacuum-infiltrated with 5, 25 and 50 μM TFP, or 0.1, 0.2 and 0.4 mM W7, for 1 h. Significant suppression in the accumulation of mRNA was observed for *psb*A, *psa*A and

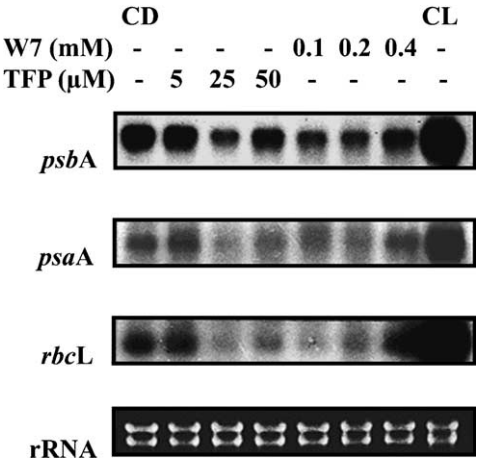

Fig. 2. Effect of calmodulin antagonists, trifluoperazine (TFP) and W7, on light-dependent accumulation of plastid gene transcripts. Autoradiograms show the steady-state transcript levels of plastid genes, *psb*A, *psa*A and *rbc*L in rice seedlings treated with 5, 25 and 50 μM TFP and 0.1, 0.2 and 0.4 mM W7. Lower panel shows ethidium bromide-stained bands of rRNA. CD and CL represent the untreated control samples as in Fig. 1.

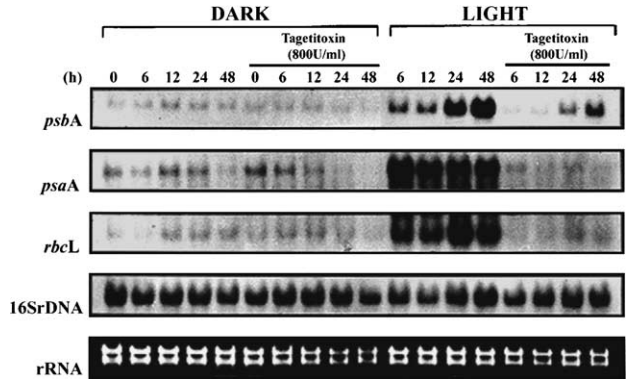

Fig. 3. Effect of PEP specific inhibitor, tagetitoxin, on plastid gene expression in rice. Autoradiograms show steady-state transcript levels of *psb*A, *psa*A, *rbc*L and 16S rRNA. Lower panel shows ethidium bromide-stained bands of rRNA in total RNA. Samples were treated with 800 U of tagetitoxin for 8 h in dark and transferred to light or dark for 0, 6, 12, 24 or 48 h before freezing for northern analysis.

*rbc*L in the samples treated with calmodulin inhibitors and incubated under light, as compared to the respective controls (Fig. 2). The treatment with TFP, 25 μM, and W7, at concentrations of 0.1 and 0.2 mM, decreased the mRNA accumulation corresponding to *psb*A, *psa*A and *rbc*L genes. The increase in transcript abundance at a higher concentration of 0.4 mM W7 could be due to desensitization phenomenon [23]. These antagonists of calmodulin action have been employed earlier at similar concentrations to provide evidence for the possible involvement of calmodulin in photosynthesis-related nuclear gene (*cab*) expression in photoautotrophic cell suspensions of soybean [13]. The role of calcium-activated calmodulin in enhancing the expression of photosynthesis-related nuclear genes in *aurea* hypocotyls has also been demonstrated [13,21]. The observed suppression of mRNA accumulation for plastid genes in the presence of TFP and W7 thus substantiates the earlier observations, although on nuclear genes, for the possible involvement of calmodulin in light signal transduction pathway operative within the plastids.

3.3. Role of PEP

To investigate the light-induced increase in plastid gene expression and PEP activity in rice, tagetitoxin, a specific inhibitor of PEP [29–31] was used. Apparently, tagetitoxin does not influence accumulation of transcripts from promoters known to be used by NEP [32]. In dark, tagetitoxin (800 U/ml) had no discernible affect on the steady state transcript levels of the photosynthesis-related genes indicating that PEP activity is minimal or undetectable at this developmental stage. On the other hand, light-dependent transcript accumulation for the photosynthetic genes was markedly arrested by tagetitoxin (Fig. 3). The effect was more prominent in case of *psa*A and *rbc*L as compared to *psb*A. No significant effect of tagetitoxin was observed on the expression of 16S rRNA in dark or light. The 16S promoter is known to possess both NEP and PEP recognition sites but from this observation it is apparent that the contribution of PEP derived transcription is negligible in the early developmental stages of etiolated rice seedlings. Similarly, based on observations from Δ*rpo*B transplastomic tobacco plants, it was proposed that NEP transcribes the housekeeping genes during early developmental stages [33]. It is, therefore, evident that, in rice, PEP activity is mainly responsible for the observed light-dependent expression of photosynthesis-related plastid genes. The presence of low levels of transcripts for such genes even in dark and after tagetitoxin treatment requires further investigation particularly in view of recent work revealing the complexity of functional integration of the activities of various plastid RNA polymerases as well as post-transcriptional processing in plastid gene expression [18].

It has been demonstrated that tagetitoxin treatment resulted in selective depletion of plastid ribosomes in wheat leaves while at the same time no deleterious effect was observed on the cytosolic counterparts. Consequently, accumulation of *rbc*L and *psb*A transcripts was arrested. The only nuclear gene transcript that was adversely affected by the toxin was that of *rbc*S [34] and that could be attributed to the failure of *rbc*L transcript accumulation [35]. The PEP-derived chloroplast transcriptional activity was also strongly inhibited by tagetitoxin during recovery of light-deprived barley leaves [36]. In both cases, no other adverse affect of tagetitoxin was observed in plants even though the toxin also inhibits eukaryotic RNA polymerase III [37]. Inhibition of the light-mediated transcript enhancement in the presence of tagetitoxin observed in the present study indicates towards a probable cross-talk between PEP and the light signal.

Our results have given a glimpse of the possible circuitry that connects light signal perception and plastid gene expression. The light signal perceived by phytochorme is transduced downstream via the involvement of G-proteins, calcium, calmodulin and phosphatases/kinases that in turn regulate the effector proteins to bring about the observed changes in the expression of photosynthesis-related plastid genes (present findings and [12]). Further, from the available genetic and biochemical data, it appears that a repressor/derepressor complex exists in the nucleus, which controls the expression of photosynthesis-related nuclear genes [38]. It would be interesting to investigate the possible existence of a similar regulatory complex that influences plastid gene expression and/or identify the components that establish a communication network between the plastid and extraplastidic compartments, which could also involve regulation via signal transduction controlling nuclear gene expression.

Acknowledgements

The Department of Science and Technology, Government of India, and the Rockefeller Foundation, USA, financially supported research in our lab. UGC fellowship to AD is gratefully acknowledged. We also acknowledge the generous help of Dr. S. Raghuvanshi, IIRGS, UDSC, New Delhi, India.

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