A DNA-binding activity for the promoter of the gene encoding C₄ phosphoenolpyruvate carboxylase is modulated by phosphorylation during greening of the *Sorghum* leaf

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Abstract

Electrophoresis mobility shift assay (EMSA) identified nuclear proteins with binding activity to a 430 bp promoter fragment of the *Sorghum* C₄ phosphoenolpyruvate carboxylase gene (*S₆C₄*). The DNA binding activities (two main retarded bands; PC1 and PC2) were high in nuclear extracts from etiolated leaves, decreased during greening and became very low or null in nuclear extracts from green leaves. This process was found to be mediated by phytochrome and was apparently irreversible since the DNA-binding activities were not restored in green plants kept in continuous darkness. The AT-rich region of the promoter fragment was identified to be the interaction domain of PC2. The detection of PC2 with EMSA was markedly reduced by preincubation of nuclear protein extracts with Mg-ATP or Mg-GTP and restored in the presence of a general protein serine/threonine-kinase inhibitor, K252a. The results suggested that the PC2 binding activity was modulated by phosphorylation during the greening process of the *Sorghum* leaf. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is an essential enzyme of photosynthetic CO₂ fixation in C₄ plants [1,2]. In *Sorghum*, PEPC is nuclear encoded by a small multigenic family [3]. We have previously reported the cloning and sequencing of three PEPC genes, *S₆C₃*, *S₆C₃RI* and *S₆C₄* from a *Sorghum* genomic library, including their 5’ flanking sequences and 3’ untranslated regions. *S₆C₄* is highly and specifically expressed in a phytochrome-mediated light dependent and tissue specific (mesophyll cells) manner [4–6] during leaf greening. This was correlated with the accumulation of specific mRNA and C₄ PEPC in the mesophyll-cell cytoplasm [7]. Moreover, it has been shown that the light induction of C₄ PEPC gene expression during the greening process of etiolated maize leaves relies on light-dependent developmental changes [8].

Various *cis*-elements and the corresponding *trans*-acting factors of light regulated photosynthetic gene promoters have been identified [9]. Leaf-specific protein factors, MNF1, MNF2a, MNF2b and PEP1 have been shown to interact with the maize C₄ PEPC gene promoter, among which MNF1 and PEP1 were presumed to act as positive transcriptional effectors [10,11] and MNF2a as a negative transcriptional effector [12,13]. Subsequently, two proteins, MNB1a and MNB1b, which bind the MNF1 box in the maize C₄ PEPC gene promoter have been cloned and sequenced [12–15]. MNB1a markedly enhances this promoter activity in vivo [9].

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From the data summarized above, it is evident that no clear picture has emerged till now concerning the regulatory mechanisms that control transcription of the C₄ PEPC genes. However, there is growing evidence that light-dependent genes are regulated via transduction cascades and protein phosphorylation events [16–20]. In this report, we describe the identification in EMSA and in vitro phosphorylation in nuclear extracts of a protein factor, PC2, which binds to an AT-rich domain of SvC4 promoter.

2. Materials and methods

2.1. Plant material

*Sorghum* plants (*S. vulgare* Pers. cv. Tamaran) were grown in vermiculite watered with Hoagland’s nutrient solution under 16 h of white light (700 μE m⁻² s⁻¹) and an 8 h dark cycle for 11 days, or maintained for the same period in complete darkness. For red/far-red light experiments, etiolated 8-day-old plants were irradiated by red or far-red light pulses using white lamps (Philips TL15) wrapped in a red rhodoid filter (λ_max, 660 nm) or a Kodak filter (Wratten 88A; λ_max, 730 nm), respectively. The light intensity was 2 μE m⁻² s⁻¹ at the leaf level. The plants were treated with red and far-red light in 3 modes; 10 min of red light every 3 h for 3 days; 20 min of far-red light every 3 h for 3 days; 10 min of red light followed by 20 min of far-red light every 3 h for 3 days.

2.2. Preparation of nuclei

This was performed according to the procedure described in [21]. Experiments were carried out at 4°C. *Sorghum* leaves (100 g) were homogenized at maximal speed of a Polytron homogenizer for 4 × 20 s in 100 ml of extraction buffer (15 mM HEPES–NaOH pH 8.0, 110 mM KCl, 5 mM MgCl₂, 1 mM ascorbic acid, 10 mM DTT, 1 mM PMSF, 1 mM benzamidine, and 1 mM leupeptin). The homogenate was filtered through one layer of Miracloth and then 0.1 vol of 4 M (NH₄)₂SO₄ was added to the filtrate. After incubation for 30 min at 4°C, the chromatin fraction was pelleted by ultracentrifugation at 170,000 × g, 15 min. Proteins were salted out of the supernatant by the addition of 0.3 g ml⁻¹ (NH₄)₂SO₄ and incubated for 30 min at 4°C. Following centrifugation at 10,000 × g for 15 min, the pellet was resuspended in 1 ml of dialysis buffer (25 mM HEPES–NaOH pH 8.0, 40 mM KCl, 0.1 mM EDTA, 10% (v/v) glycerol, 10 mM DTT, 1 mM PMSF, 1 mM benzamidine, and 1 mM leupeptin), left for 10 min on ice, and dialyzed twice for 2 h against 350 ml of the same buffer (omitting the protease inhibitors) with 5 mM 2-mercaptoethanol instead of DTT. The preparation was clarified by centrifugation at 12,000 × g for 10 min. Samples were aliquoted, frozen in liquid nitrogen and stored at −80°C.

2.3. Preparation of nuclear protein extracts

Extraction of nuclear proteins was performed by a modification of the method described in [16]. All the steps were carried out at 4°C. The nuclear pellet (10% of nuclei) was resuspended in 1 ml of lysis buffer containing 50 mM HEPES–NaOH pH 8.0, 1 mM EDTA, 0.3% (v/v) Triton X-100, 1 mM ascorbic acid, 10 mM DTT, 1 mM PMSF, 1 mM benzamidine and 1 mM leupeptin. Incubation was carried out on ice for 20 min, and then the lysate was ultracentrifuged at 170,000 × g for 15 min. The chromatin-containing pellet was resuspended in 0.25 ml of high salt buffer containing 50 mM HEPES–NaOH pH 8.0, 400 mM NaCl, 5 mM MgCl₂, 1 mM ascorbic acid, 1 mM PMSF, 1 mM benzamidine, 1 mM leupeptin and 10 mM DTT. The extraction of nuclear proteins was performed on ice for 1 h, and then the insoluble material was eliminated by ultracentrifugation (170,000 × g, 15 min). The nuclear protein extracts were aliquoted, frozen in liquid nitrogen, and stored at −80°C. Protein concentration was determined according to [22].
2.4. Determination of $C_4$ PEPC activity in protein extracts from Sorghum leaves

Leaf tissue (200 mg) was harvested and immediately extracted in 500 µl of extraction medium (100 mM Tris–HCl pH 8, 10 mM MgCl$_2$, 1 mM EDTA, 10% (v/v) glycerol, 10 mM DTT, 2% (w/v) insoluble PVP and some washed sand) in a precooled mortar (0°C). The homogenate was centrifuged for 5 min at maximal speed in an Eppendorf centrifuge. $C_4$ PEPC activity was determined spectrophotometrically from an aliquot of the supernatant in a 1 ml assay mixture containing 100 mM HEPES–KOH pH 8, 5 mM phosphoenolpyruvate, 5 mM MgCl$_2$, 5 mM NaHCO$_3$, 0.2 mM NADH and 5 U of commercial NAD–MDH. The decrease in absorbance was recorded at 340 nm and 30°C. One $C_4$ PEPC unit was defined as that amount of enzyme that catalyzed the transformation of 1 µmol of substrate per min under the described experimental conditions.

2.5. DNA probes

A 430-bp fragment of the SrC4 promoter (−625 to −195 bp from the translation start site) was cut out from the 0.6-GUS-pSK$^+$ plasmid by HindIII/SphI digestion. Probes shorter than 430 bp were prepared by PCR with the 0.6-GUS-pSK$^+$ plasmid as template. Four DNA fragments were synthesized: R36 (−392 to −285 bp), R29R30 (−625 to −520 bp), ATC (−532 to −406 bp) and ATL (−573 to −406 bp) (Fig. 1). The probes were 3'-end labeled with a DIG Gel Shift Kit (Boehringer Mannheim) according to the manufacturer’s instructions and used in EMSA (see below).

2.6. EMSA

A typical binding reaction (20 µl) contained 2 µg of nuclear proteins or 20 µg of leaf proteins, 15 fmol of DNA probe, 0.25 M NaCl, 10 µg double-stranded poly (dI-dC) (Pharmacia), 30 mM HEPES–NaOH pH 8.0, 3 mM MgCl$_2$, 6 mM DTT, 0.6 mM ascorbic acid, 0.6 mM PMSF, 0.6 mM benzamide and 0.6 mM leupeptin. After incubation for 15 min at 25°C, 5 µl of loading buffer (60% 0.25 TBE, 40% (v/v) glycerol) was added and the samples were loaded onto a 1.8% agarose gel in 0.25 TBE [24]. Electrophoresis was carried out for 90 min at 8 V cm$^{-1}$. DNA was blotted onto a positively charged nylon membrane (Amersham) by overnight capillary transfer using 0.25 × TBE buffer. The membrane was soaked in 10 × SSC (1.5 M NaCl, 0.15 M sodium citrate pH 7.0), and the DNA was fixed to the nylon membrane by UV crosslinking at 120 mJ for 40 s. The chemiluminescent detection of probe signals (DIG Gel Shift Kit) was performed according to the manufacturer’s instructions.

2.7. Phosphorylation assays

Phosphorylation of nuclear protein extracts was performed in vitro as described in [16] with the following modifications. Protein extracts (2 µg) were preincubated with 15 mM NaF for 15 min on ice. ATP or GTP (2 mM) was added to the assay (total volume, 12.5 µl) containing the preincubated nuclear protein extract, 15 mM NaF, 5 mM MgCl$_2$, 400 mM NaCl, 10 mM DTT, 1 mM ascorbic acid, 1 mM PMSF, 1 mM benzamide, 1

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Fig. 1. Nucleotide sequence of the promoter fragment and the probes used in EMSA. Putative light-responsive domains (R36, R29, R30, R36B, AT), AT-rich domain, C box (putative CAAT box), T box (putative TATA box) and tsp (transcription start site) are underlined. The following fragments have been used in EMSA: 430-bp (−625 to −195 bp counting from translation start site), R36 (−392 to −285 bp), R29R30 (−625 to −520 bp), ATC (−532 to −406 bp), ATL (−573 to −406 bp).
Fig. 2. EMSA with the S6C4 promoter fragment (430-bp) and nuclear protein extracts from etiolated Sorghum leaves. Nuclear protein (2 µg) and 15 fmol of the DNA probe were used in each assay. Lane 1, free probe; lane 2, probe with nuclear protein extract from etiolated leaves; lane 3, nuclear protein extract from etiolated leaves were preincubated at 50°C in the presence of 10 µg of proteinase K for 20 min, prior to EMSA; lane 4, nuclear protein extract from etiolated leaves were boiled for 3 min prior to EMSA. The free probe and the DNA-protein complexes are indicated as FP, PC1 and PC2, respectively.

There was no detectable signal in EMSA with nuclear protein extracts from light-adapted leaves for four days under photoperiodic conditions (Fig. 3 lane 2). After the return of the greening plants to darkness for 4 additional days, during which the

3. Results

3.1. DNA-binding activities for the S6C4 (430 bp) promoter fragment in nuclear protein extracts from etiolated Sorghum leaves

EMSA has been devised to detect the interaction of nuclear proteins with the S6C4 promoter [25,26]. A S6C4 promoter fragment (430-bp, −625 to −195 bp from the translation start site) was used in these experiments because (a) it contains several consensus domains homologous to light dependent boxes found in other photoregulated plant gene promoters ([3] and Fig. 1) and (b) it was previously shown to direct the mesophyll-specific expression of the marker gene uidA in transient expression experiments with Sorghum leaves (unpublished). The 430-bp probe was labeled with digoxigenin-11-ddUTP and incubated with nuclear protein extracts from etiolated Sorghum leaves. Two retarded bands, PC1 and PC2, the major one (PC2) moving faster, were observed in EMSA of the protein samples from etiolated leaves (Fig. 2 lane 2). Pretreatment of the protein extracts with proteinase K, or boiling, prior to electrophoresis caused the disappearance of the retardation bands thus establishing that the shift resulted from protein-DNA interactions (Fig. 2 lanes 3 and 4).

Other assays were supplemented with 500 U of commercial, rat liver casein kinase II (CKII) or with 100 µM K252a (general protein kinase inhibitor). After incubation for 20 min, 25°C, the reactions were blocked by 1 µl EDTA (final concentration, 20 mM). The other components of the shift assay (poly (dI-dC) and the labeled probe in TE buffer) were added and the mix (total volume, 20 µl) subjected to electrophoresis in agarose gel as described above.
3.2. Binding activities depend on the light quality

Etiolated plants (8-day-old) were irradiated by pulses of red or far-red light for 3 consecutive days. Control plants were either maintained in continuous darkness (etiolated plants), or illuminated with white light (normal photoperiodic condition; green plants). C₄ PEPC activity was measured in protein extracts from leaves of treated and control plants. Compared to etiolated plants, white light- and red-treated plants showed a 7.2 and 5.8-fold enrichment in leaf C₄ PEPC activity, respectively (Fig. 4 lanes 1–3). In far-red treated plants, this C₄ PEPC activity increased to a lower extent (3.2-fold), and far-red given after red blocked the red light effect; the red/far-red photoreversibility of this response was in good agreement with data of previous reports [6,7]. With respect to EMSA, the retarded complexes (PC1 and 2) were observed with protein extracts from etiolated leaves, but not from green leaves (Fig. 4 lanes 1 and 2), as expected. Red and far-red treated plants behaved essentially as the green and etiolated ones, respectively (Fig. 4 lanes 3 and 4). In contrast, far-red given after red pulses, partially but clearly restored the retardation signals (Fig. 4 lane 5). Overall, the results support the view that both the gain in C₄ PEPC activity and the loss in DNA-binding activities to the SrC₄ promoter are mediated by phytochrome during greening of the Sorghum leaf.

3.3. The major nuclear binding activity interacts with an AT-rich domain within the 430-bp fragment of the SrC₄ promoter

To further identify the protein binding sequence(s) on the promoter DNA, four subfragments covering most of the 430-bp probe of the SrC₄ promoter were prepared (Fig. 1) and used in EMSA. Retarded bands with nuclear protein extracts from etiolated leaves were seen with the 430-bp probe (Fig. 5 lane 2; positive control) and the ATL, ATC and R29/R30 subfragments (Fig. 5 lanes 4 and 6). Furthermore, ATC or ATL showed only one retarded complex which, based on signal intensity, should correspond to the faster migrating band in the control (PC2). The very faint retarded R29/R30 band could be due to PC2, or due to a very weak binding of PC1 to this probe. Therefore, one protein-binding domain was en-
tirely located in the shorter ATC element of the \textit{S\textsubscript{6}C\textsubscript{4}} promoter. In a competition experiment, a 25-fold molar excess of the unlabeled ATC probe caused the retarded band to decrease severely (Fig. 6 lane 3) and become almost non-detectable when higher concentrations of the competitor were used (Fig. 6 lane 4–6). In contrast, poly (dI-dC) had no effect on the intensity of the signal (data not shown). Thus, this DNA-protein complex, as detected in the EMSA, resulted from a specific interaction.

So far, reversible phosphorylation is the best documented posttranslational protein modification shown to modulate the binding activity of trans-acting factors and transcriptional activity of photoregulated genes [16,20,27–32]. To test this hypothesis, nuclear protein extracts from etiolated leaves were preincubated in the presence of ATP and \textit{Mg\textsuperscript{2+}}, to stimulate the activity of putative, endogenous protein kinases, and NaF, to simultaneously reduce the activity of protein-phosphatases, before EMSA was performed. Under these experimental conditions, the DNA binding activity (PC2) to the ATL probe was considerably reduced (Fig. 7 lanes 2 and 3). It showed a further decrease if the pretreatment was performed in the presence of commercial, rat liver casein kinase II (Fig. 7 lane 5); however, it was essentially maintained when the preincubation medium contained the general protein serine/threonine kinase inhibitor K252a (Fig. 7 lane 4). Similar results were observed when Mg-GTP was used during preincubation of the nuclear protein extracts (not shown). These findings demonstrated that a protein kinase activity, possibly a CKII-type casein kinase [33], phosphorylating the protein factor is present in nuclear protein extracts from etiolated \textit{Sorghum} leaves.

3.4. The major nuclear binding activity (PC2) is modulated by phosphorylation

In this report, we provide evidence that nuclear protein extracts from etiolated \textit{Sorghum} leaves contain two DNA binding activities (PC1 and PC2) for a promoter fragment of the C\textsubscript{4} PEPC gene (\textit{S\textsubscript{6}C\textsubscript{4}}). The major one (PC2) interacts with an AT-rich region (−473 to −447 bp) within the promoter fragment. AT-rich DNA sequences and corresponding trans-acting protein factors have been reported to regulate gene expression in plants [34]. For example, this \textit{cis}-element plays a role in the transcriptional activation of the pea small subunit of ribulose bisphosphate carboxylase \textit{(rbcS-3A)} gene promoter [35]; in contrast, it acts as a negative regulatory element in the \textit{Nicotiana plumbaginifolia} chlorophyll a/b binding protein \textit{(Cab-E)} gene promoter [36]. Two AT-rich domains are also present in the promoter of the salt
stress-inducible PEPC gene from the CAM plant *Mesembryanthemum crystallinum* [37]. Among the three protein factors, PCAT 1-3, with binding activity in these DNA domains, only PCAT1 is a putative salt stress-dependent regulatory activator.

Both PC1 and PC2 almost completely vanished during the greening process of the etiolated *Sorghum* leaf and cannot be restored in dark-adapted green leaves. Phytochrome and is well correlated with a phytochrome-dependent increase in transcriptional capacity of *Sc*4 [6,7]. In vitro assays have suggested that the DNA binding activity of PC2 to the AT-rich sub-fragment of the *Sc*4 promoter was abolished by a phosphorylation process. Along the same lines, it has been reported that inhibition of DNA binding by phosphorylation is a common mode of regulation of gene expression [20]. This is the case of the nuclear proteins AT-1 (binding an AT-rich cis-element of the Cab gene promoter from pea) and ATBP-1 (binding an AT-rich cis-element from the glutamine synthetase gene (GS2) promoter from tobacco) which show a significant loss of binding activity in EMSA after preincubation with a protein kinase activator, like Mg-GTP [16,34].

The loss of PC2 binding activity to the ATC fragment from *Sc*4 promoter was observed after pretreatment of the nuclear protein extracts from etiolated leaves with protein kinase activators, Mg-ATP and Mg-GTP. Furthermore, this effect was strongly enhanced when CKII was supplemented in the incubation mixture and markedly inhibited in the presence of the general protein kinase threonine kinase inhibitor, K252a. Collectively, the data pointed to a CKII-type casein kinase of nuclear protein extracts from etiolated *Sorghum* leaves. This multifunctional, nuclear protein kinase is involved in central cellular functions including the regulation of gene expression. It has been formerly characterized in nuclear protein extracts from tobacco [38], pea [33,39], *Arabidopsis thaliana* [40] and broccoli [32]. Broccoli CKII was found to co-purify with phosphorylated HMG proteins in in vitro assays [41]. Some subgroups of these chromatin binding proteins interact with AT-rich regions of the promoters [34,42].

Altogether, these data support the view that the trans-acting factor PC2 is present in non-phosphorylated form in etiolated *Sorghum* leaves and binds the ATC domain of the *Sc*4 promoter. Based on these data, PC2 might well be a homolog of AT-1 already shown in pea to bind *rbcs* promoters, also presumably *Cab* promoters, and suggested to be required for the regulation of gene expression [16]. It would come as no surprise if the machinery underlying light-regulated gene expression have some regulatory devices in common. During the greening process of etiolated leaves, PC2 would undergo a phosphorylation-dependent unbinding from its cognate DNA domain. Whether and how the activity of the putative converter enzymes (protein kinase/protein phosphatase) is regulated by light (via phytochrome) in mesophyll cell nuclei to cause the corresponding target protein factor to become phosphorylated remains to be investigated. Since (1) phytochrome apparently mediates both the loss in binding activity of PC2 (and also of PC1) and an increase in the transcriptional efficiency of *Sc*4 [6,7], and (2) PC2 is not restored in dark-adapted green leaves, one hypothesis is that this protein factor at least contributes to the negative control of *Sc*4 as far as the skotomorphogenetic program of the etiolated C4 leaf is pursued.

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