Volume 261, number 2, 445–448 FEBS 08191 February 1990

Calmodulin antagonists inhibit the phytochrome-induced appearance of two nuclear encoded transcripts in radish cotyledons

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Received 28 December 1989

The effects of two calmodulin antagonists on the phytochrome-mediated appearance of two nuclear encoded transcripts in radish cotyledons have been investigated. The extent of inhibition of transcript accumulation was dependent of the time ellapsed between the administration of trifluoperazine and the light stimulus. When 1 mM trifluoperazine was administered to the seedlings 8 h before red light irradiation, the inhibition of transcript accumulation was up to 62% for the chlorophyll *a/b* binding protein mRNA and 56% for the ribulose 1,5-bisphosphate carboxylase small subunit mRNA. Similar results were obtained with W-7 (0.1 mM).

Calmodulin antagonist; Light induction; Phytochrome; Signal transduction; (Radish seedlings)

1. INTRODUCTION

The mechanism of signal transduction in plants is currently a subject of great interest. Light-mediated developmental responses of plants provide an interesting system for studying the transduction of environmental signals. In higher plants many light-dependent processes are under the control of phytochrome [1]. At the molecular level the best studied phytochrome-mediated responses are the control of the expression of two nuclear genes coding for the small subunit of the ribulose 1,5-bisphosphate carboxylase (EC 4.1.1.39; rbcS) and the chlorophyll *a/b* binding protein (cab) [2–5]. However, although many workers have tried to gain information about the light (phytochrome-dependent) signal, little is known about this mechanism [5]; up to now, the light transduction chain remains a 'black box'. Because the transcription stimulation of several genes is obtained at low or very low fluences [6], it is very likely that the initial signal must be amplified in some way [7]. Calcium is thought to play a central role in the transduction of external stimuli [8,9], especially with respect to phytochrome-mediated processes [10–13]. Calcium-binding proteins have been identified in plants and the best characterized is calmodulin [14]. In *Raphanus sativus* the presence of both calmodulin and a calmodulin inhibitor have been reported recently [15]. In this plant the rbcS and cab genes exhibit a positive phytochrome control [16] as in many other plants [3,5]. In the present work we have investigated the effect of two calmodulin antagonists (TFP and W-7) on the phytochrome-induced accumulation of rbcS and cab transcripts in developing radish cotyledons.

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Abbreviations: TFP, trifluoperazine; W-7, (*N*-(6-aminohexyl)-5-chloronaphthalene-1-sulfonamide)

2. MATERIALS AND METHODS

2.1. *Plant growth conditions*

Radish seeds (*Raphanus sativus* L., cv. National) were surface-sterilized with 1% sodium hypochlorite. Seedlings were grown in darkness on moistened filter paper (ashless no.40, Whatman, Maidstone, England) at 25°C. When appropriate, seedlings were transferred under a green safe light to filter paper imbibed with either TFP (1 mM) or W-7 (0.1 mM) in water. Red light (2 $W \cdot m^{-2}$) was given 12 h prior to the harvest of cotyledons. All solutions were prepared with milli-Q water (Millipore Corp., Bedford, MA, USA). TFP and W-7 were from Sigma (St. Louis, MS, USA).

2.2. *RNA extraction and analysis*

Total RNA was extracted from 100 cotyledon pairs as previously described [16]. Equal amounts (2.5 μg) of RNA were denatured in Tris-acetate buffer (20 mM, pH 7.0) containing 50% dimethyl sulfoxide and 1 M glyoxal for 10 min at 50°C. The RNA samples were then fractionated by electrophoresis onto 1.5% agarose gels for 4 h at 4°C. RNA was transferred to a nylon membrane (Genescreen Plus; NEN, Boston, MA, USA). Prehybridization was carried out according to Siegel and Bresnick [18]. Probes were labelled according to the random primed method [19] with [^{32}P]dATP (3000 Ci/mmol, Amersham). The following probes cloned from radish RNA have been used: pFG 139 which encodes the rbcS [20], pFG 124 containing part of the cab sequence and pFR 12 containing a 5.8 S rRNA se-

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00145793/90/$3.50

quence (unpublished data). Hybridization and washing conditions were as previously described [16]. Autoradiograms of the Northern blots were scanned with a Shimadzu CS-930 analyser.

3. RESULTS AND DISCUSSION

We have studied the effects of calmodulin antagonists on the light-induced accumulation of two abundant transcripts in radish seedlings: the rbcS and cab mRNAs. In a first set of experiments, seedlings were grown for 47 h in darkness and transferred onto filter paper imbibed with 1 mM TFP. One hour later seedlings were irradiated for 5 min with the red light. At this developmental stage, radish seedlings exhibited the most pronounced phytochrome-mediated increase of cab and rbcS gene expression [16]. Northern blots of RNA extracted from control or TFP-treated seedlings were probed with radish cab or rbcS sequences. Fig.1 shows that under these conditions, the transcript accumulation, promoted by red light, was inhibited by 45% in the case of cab and 27% for the rbcS. When the drug was given to the whole seedlings 8 h before the red light stimulus, instead of 1 h, the inhibition of transcript accumulation was of 62% for cab and 46% for rbcS (fig.2). This suggests that it takes several hours for TFP to reach cotyledon cells. RNA samples were also probed with a sequence corresponding to the radish 5.8 S rRNA. As depicted on figs 1 and 2 the TFP treatment has no apparent impact on the level of this RNA. Although mRNA and rRNA are synthesized by different systems this result indicates, at least, that the possible non-specific detergent action of TFP does not impair the accumulation of this rRNA species. Additionally, 1 mM TFP neither affects germination nor modifies the pattern of total proteins (not shown). The relatively high TFP concentration was necessary because whole seedlings were used and very likely only a small percentage of the drug can enter the cotyledon cells. Previous studies with calmodulin antagonists in plants were carried out with cell cultures or isolated organs [11,21] but none of these experimental systems is suitable for studying the regulation of the rbcS gene expression [22].

In addition to TFP we used another calmodulin antagonist (W-7) that possesses a high affinity for this protein [23]. When W-7 (0.1 mM) was given to the seedlings 8 h before the red light stimulus, the amount of cab mRNA was reduced by 60% and that of rbcS by 45% (fig.2). Taken together our results suggest that calmodulin is involved in the control of the expression of cab and rbcS genes in radish.

In plants, the role of Ca^{2+} and calmodulin in several physiological or developmental processes has been demonstrated [8]. For instance the response of roots to auxin and gravity involves calmodulin [24–26]. Protein phosphorylation in oats can be inhibited by calmodulin antagonists such as TFP [21] and the phosphorylation of phosphoenolpyruvate is calmodulin-dependent in the leaves of *Sorghum* [27]. It has also been demonstrated that TFP was able to inhibit the phytochrome-mediated germination of *Onoclea sensibilis* [28] and the rotation of chloroplast in *Mougeotia* [29]. In carrot protoplasts TFP prevents the

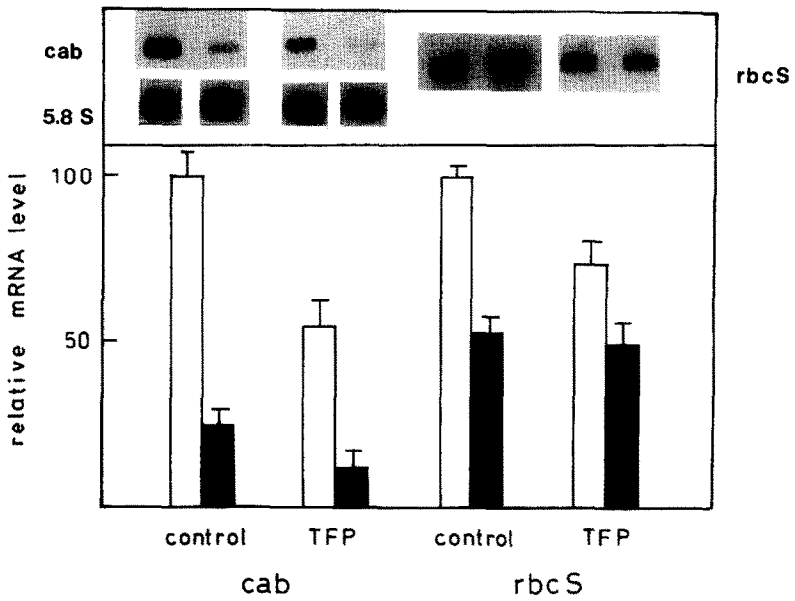

Fig.1. Effect of TFP on the accumulation of cab and rbcS transcripts in radish cotyledons. Seedlings were grown on water in the dark for 47 h and placed onto the TFP solution (1 mM) for 1 h. Seedlings were then either given 5 min red light and returned to darkness for 12 h (open bars) or left in the dark for 12 h (solid bars). RNA was extracted from cotyledons and subjected to Northern analysis. Autoradiograms (top panel) were scanned and the corresponding values are presented as histograms. Values are means of at least 4 independent experiments. In control experiments TFP was replaced by water.

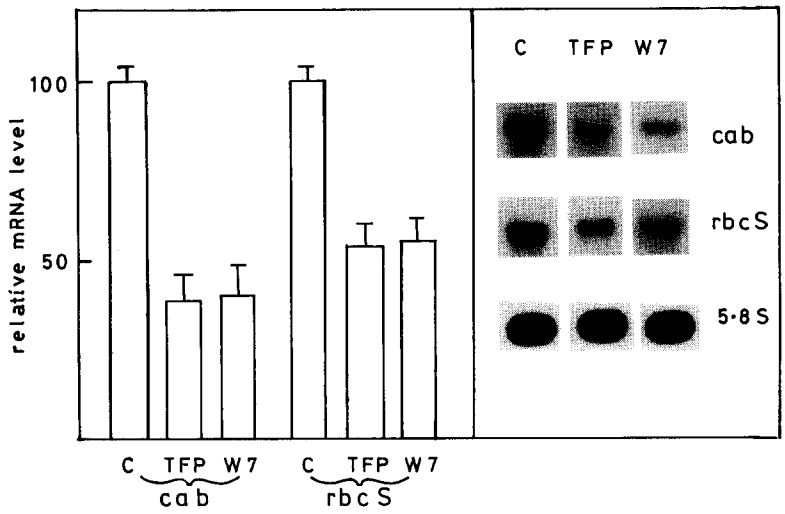

Fig.2. Inhibition of cab and rbcS transcript accumulation in TFP- or W-7-treated radish seedlings. Radish seedlings were grown on water in darkness for 40 h and placed onto the TFP (1 mM) or W-7 (0.1 mM) solution. 8 h later they were given 5 min red light and returned to the dark for 12 h. RNA was extracted from cotyledons and subjected to Northern analysis. Autoradiograms (right panel) were scanned and the corresponding values are presented. C, water control.

myoinositol triphosphate-induced calcium efflux [30]. It has been reported that calmodulin antagonists were able to inhibit the red light-promoted enhancement of protein phosphorylation in isolated nuclei [11]. Besides, the presence of calmodulin [31,32] and phytochrome [33] in plant cell nuclei have been reported. However, the intracellular distribution of phytochrome is still a matter of debate [34]. Our data indicate that calmodulin is a potential candidate for the phytochrome signal transduction chain. It remains to be examined whether calmodulin antagonists increase the transcription rate or decrease the rate of degradation of cab and rbcS mRNAs or both. Run-on transcription experiments with isolated nuclei are often used to measure the transcription rate of a given gene. However, it has been reported that the preparation of nuclei from plant cell led to alterations of their transcriptional activity [35].

To date, to the best of our knowledge, there are no other data available on the effect of calmodulin antagonists on photoregulated genes in plants. Calmodulin could be responsible for the activation of protein kinases that could in turn phosphorylate specific transcription factors. Recently, a nuclear protein factor (GT-1) that specifically recognizes light-responsive elements has been identified in the upstream region of pea rbcS gene [36,37]. It is tempting to think that light, via the photoreceptor phytochrome, could trigger the binding of such factors to DNA control regions by means of activation of these factors through the Ca^{2+}/calmodulin system.

Acknowledgements: We wish to thank Professor A. Boudet and Dr B. Thoiron for stimulating discussions and Dr R. Ranjeva for critical reading of the manuscript.

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