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Protein kinase activity in *Cucumis sativus* cotyledons: Effect of calcium and light

Dolores Vidal, M. Teresa Gil, Fagua Alvarez-Flórez, Luisa Moysset, Esther Simón *

Departamento de Biologia Vegetal, Facultad de Biologia, Universidad de Barcelona, 08028 Barcelona, Spain

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Abstract

Light signals received by phytochromes in plants may be transduced through protein phosphorylation. Ca^{2+} as second messenger was involved in phytochrome-mediated cellular events. Our experiments with *Cucumis sativus* cotyledons, treated with red (R) and far-red (FR) light, showed a stimulatory effect on *in vitro* protein phosphorylation of histone, added as exogenous substrate to the cotyledon extracts, and also modified the phosphorylation of endogenous polypeptides. The effect of light treatments was mimicked by the addition of Ca^{2+} to the phosphorylation buffer, indicating phytochrome- and Ca^{2+}-dependence on activity of some protein kinases (PKs). In-gel kinase assays were performed to characterize the PKs involved at the cotyledon stage of cucumber plants. Three proteins of about 75, 57 and 47 kDa with PK activity were detected between M_r markers of 94 and 45 kDa. All three were able to phosphorylate histone and undergo autophosphorylation. However, only the 75 and 57 kDa proteins autophosphorylated and phosphorylated the substrate in a Ca^{2+}-dependent manner, and were inhibited when calmodulin (CaM) antagonists were added to the incubation buffer. Western-blot analysis with polyclonal antibodies directed against calcium-dependent protein kinase of rice (OsCDPK11) or *Arabidopsis* (AtCPK2) recognised 57 and 75 kDa polypeptides, respectively. These results indicate the presence in cucumber cotyledons of at least two proteins (ca. 75 and 57 kDa) with activity of PKs that could be calcium-dependent protein kinases (CDPKs). Both CDPKs could be modulated by phytochromes throughout FR-HIR and VLFR responses.

Keywords: *Cucumis sativus* L.; Cucurbitaceae; Cucumber; Protein phosphorylation; Ca^{2+}-dependent protein kinase; Phytochromes; Signal transduction

1. Introduction

Reversible protein phosphorylation has long been known to play a key role in eukaryotic cell signalling and to be involved in the regulation of many fundamental cellular events (Hardie, 1999; Harmon et al., 2000). Signal transduction in plant systems modulates phosphorylation of target proteins by protein kinases (PKs) in response to various internal and external stimuli, including developmental processes, light, phytohormones, environmental stress, fungal elicitors and cell damage (Anil and Rao, 2001; Cheng et al., 2002).

Most environmental stimuli elicit an increase in cytosolic Ca^{2+} and it has also been suggested that Ca^{2+} may be an important node at which cross-talk between pathways signalling can occur (Sanders et al., 2002; Ludwig et al., 2004). In spite of the growing body of evidence that Ca^{2+} can function in many plant systems as a second messenger, the mechanism by which Ca^{2+} controls processes is only beginning to be understood. PKs are one of its main targets, which by reversible phosphorylation/dephosphorylation activates other constituents of signal transduction pathways (Cheng et al., 2002; Harper et al., 2004). In plants, a wide variety of calcium-regulated PKs are involved in Ca^{2+} signalling, which differ in whether they are regulated by binding Ca^{2+}, Ca^{2+}/calmodulin (CaM), a combination of both, or neither (Harmon et al., 2000). Calcium-dependent PKs (CDPKs), which belong to the

* Corresponding author. Tel.: +34 93 402 14 67; fax: +34 93 411 28 42.
E-mail address: esimon@ub.edu (E. Simón).

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CDPK/SnRK superfamily of PKs, are a class of serine/threonine PKs regulated by Ca^{2+} but not by CaM. They have acquired CaM independence due to binding of Ca^{2+} to its intrinsic CaM-like domain (Hrabak, 2000). They are ubiquitous in plants and exist as multiple isoforms. The members of the CDPK family differ in their biochemical properties and can potentially be activated by different levels of Ca^{2+}, which supports the hypothesis that each isoform has a distinct role in calcium signal transduction (Harmon et al., 2000). CDPKs may function as sensors of fluctuations in cytosolic Ca^{2+} and initiate downstream signalling events.

Many light-dependent processes are under the control of the phytochrome photoreceptor system, which mainly senses red (R)/far-red (FR) light. Phytochromes are R/FR photoreversible chromoproteins which form dimmers with a M_r of ca. 120 kDa per monomer and which have a covalently linked open-chain tetrapyrrole as chromophore. Red light-induced formation of the active form of phytochrome (Pfr) initiates a signalling cascade which controls plant photomorphogenesis. In *Arabidopsis* five members of a small gene family (*PHYA–PHYE*) encode the photoreceptor phytochromes, with phy A and phy B as the main ones (Clack et al., 1994). Phy A has a very specific mode of action in controlling very low fluence response (VLFR) and FR high irradiance response (HIR) (Furuya and Schäfer, 1996). VLFR is initiated by even a few seconds of starlight and is saturated at about 1 $\mu mol\ m^{-2}$, whereas HIR requires prolonged irradiation with FR light. Phy B–E mediates responses to continuous R light and shows the R/FR reversible induction responses (Nagy and Schäfer, 2002). Increasing evidence has established the involvement of Ca^{2+}-signaling during phytochrome action (Roux, 1994). Thus, Bowler et al. (1994) and Neuhaus et al. (1997) demonstrated the involvement of Ca^{2+} in phytochrome-mediated responses during the development of tomato chloroplasts.

Light signals perceived by phytochromes may be transduced through protein phosphorylation. Thus, Datta et al. (1985) demonstrated that protein phosphorylation is regulated by phytochrome and calcium. It was also shown that R and FR light controls the phosphorylation level of some proteins both in the cytoplasm (Park and Chae, 1989; Singh and Song, 1990) and in the nucleus (Romero et al., 1991). In addition, Chandok and Sopory (1996), Tong et al. (1996) and Malec et al. (2002) described protein phosphorylation/dephosphorylation mediated by phytochromes. Moreover, phytochromes are phosphoproteins and light-regulated PKs that catalyze the phosphorylation of their own molecule and possibly other proteins (Kim et al., 2005).

Phytochromes control the nitrate reductase (NR) activity of *Cucumis sativus* cotyledons only in the presence of external calcium; and calcium ionophore mimics the effect of R light (Bergareche et al., 1994). In addition, phosphoinositides and protein phosphorylation are involved in the phototransduction pathway of NR response in cucumber cotyledons (Vidal et al., 2001). Thus, 15 min R light irradiation of etiolated seedlings of cucumber increases the amount of ^{32}P incorporated in the proteins of the cotyledon extracts, as measured with a liquid scintillation counter. Furthermore, the addition of Ca^{2+} to the phosphorylation buffer mimics the effect of R light (Vidal et al., 2001).

In the study reported here, we tried to elucidate the effect of calcium and light on protein phosphorylation in etiolated or light-treated *C. sativus* cotyledons in order to detect changes in protein phosphorylation dependent on phytochromes. Attempts were also made to identify PKs from cucumber cotyledon extracts that responded to changes in Ca^{2+} concentrations and phytochrome photoconversion, in order to understand phytochrome signal transduction better.

2. Results and discussion

2.1. Effect of light on in vitro protein phosphorylation

To determine the polypeptides phosphorylated by the PKs present in cucumber cotyledon extracts, activated in response to light, assays of *in vitro* protein phosphorylation, added to the extracts of histone III-S as exogenous substrate, were carried out.

Fig. 1a shows several *in vitro* phosphorylated proteins in the extracts of etiolated (E) cucumber cotyledons. The irradiation of seedlings with 15 min of R light enhanced (32% ± 5) phosphorylation of histone added to the extract (Fig. 1a, lane R). This effect was reverted by 5 min of FR light treatment (Fig. 1a, lane R-FR). The irradiation of seedlings with 3 min of R light pulses applied hourly also had a stimulatory effect on cotyledon protein phosphoryla-

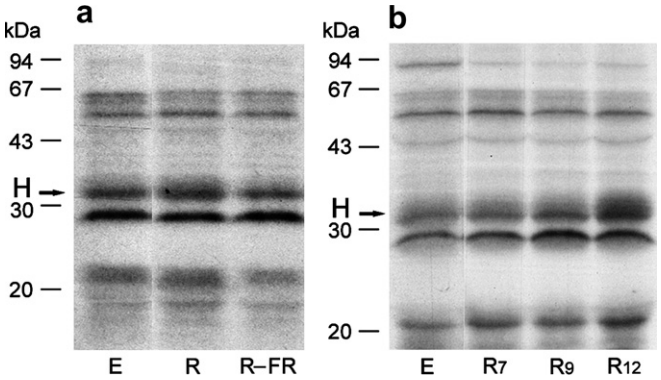

Fig. 1. *In vitro* protein phosphorylation of *Cucumis sativus* cotyledon extracts obtained from etiolated cotyledons (lane E) and cotyledons subjected to different light treatments before cutting. (a) Proteins from cotyledons treated with 15 min of red (lane R) or 15 min R followed by 5 min of far-red light (lane R-FR). (b) Proteins from cotyledons irradiated with seven (lane R_7), nine (lane R_9) or 12 (lane R_{12}) pulses of 3 min R light applied hourly. Extracts containing 1 mg ml^{-1} of exogenous histone were incubated with [γ^{32}P]ATP and electrophoresed in 13.5% (w/v) SDS–polyacrylamide gels. Radiolabelled proteins were detected by autoradiography of the dried gels. M_r markers are indicated on the left of the figure.

tion, which was mainly seen when the number of R light pulses rose to 12 (Fig. 1b). This stimulatory effect clearly affected the exogenous histone added to the extracts, whose phosphorylation rate increased 13% ± 3.1 after seven pulses (lane R_7), 64% ± 5.5 after nine pulses (lane R_9) and 146% ± 14.8 after 12 pulses (lane R_{12}) over control values (E). In addition, R light pulses decreased the phosphorylation of ca. 90 kDa bands and increased ca. 22 kDa bands (Fig. 1b).

The reversion of the R light effect by subsequent FR light treatment suggests the regulation by LFR of phytochrome, presumably sensed by phy B, on the activity of PK(s) involved in histone phosphorylation. Datta et al. (1985) presented the first report of the modulation of protein phosphorylation in pea nuclei by R light and indicated that this effect could be reversed by FR light treatment. Doshi and Sopory (1992) also reported this effect on etiolated *Sorghum* coleoptiles, indicating that protein phosphorylation was affected by phytochromes and suggesting that Pfr regulates the phosphorylation of the proteins by affecting the activity of PK(s).

In addition, a single treatment of 5 min FR light (Fig. 2a, lane FR_5) applied to the etiolated seedlings, also enhanced the phosphorylation of exogenous histone (81.4% ± 7.1) and modified the profile of *in vitro* protein phosphorylation of cucumber cotyledon extracts. Thus, bands of ca. 90 and 45 kDa were enhanced and bands of ca. 55, 28 and 22 kDa were inhibited by FR light treatment. The stimulatory FR light effect was also observed when FR light treatment was maintained for 120 min (Fig. 2a, lane FR_{120}). The effect on protein phosphorylation of a single 5 min FR light irradiation, suggests a VLFR of phytochrome, as was also described by Romero et al. (1991) for nuclear protein phosphorylation from etiolated *Avena* seedlings. Besides, the effects of 120 min of FR light indicate that a FR-HIR response of phytochrome was also involved. FR light effects throughout VLFR and HIR were probably sensed by phy A.

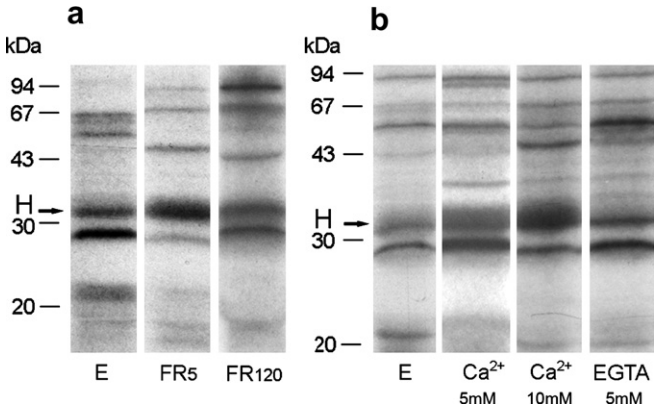

Fig. 2. Effect of far-red light (FR) and Ca^{2+} on protein phosphorylation of cucumber cotyledon extracts. The extracts, with exogenous histone, were incubated and electrophoresed as indicated in Fig. 1. (a) Phosphorylation was performed in the extracts from etiolated cotyledons (lane E) and from cotyledons previously treated with 5 min of FR light (lane FR_5) or with 120 min of continuous FR light (lane FR_{120}). (b) The effect of calcium was analysed by adding 5 mM, 10 mM $CaCl_2$ or 5 mM EGTA to the phosphorylation buffer. Phosphorylation of etiolated cotyledon extracts used as control, lane E. M_r markers are indicated on the left of the figure.

2.2. Effect of calcium on in vitro protein phosphorylation

To assess changes in protein phosphorylation depending on Ca^{2+}, exogenous histone was added to the etiolated cotyledon extracts before phosphorylation in the presence of Ca^{2+} or EGTA. The addition of 5 mM or 10 mM $CaCl_2$ to phosphorylation buffer increased the phosphorylation of the histone (74% ± 6 and 112% ± 18, respectively, respect to the phosphorylation in the presence of EGTA) and of some other polypeptides (ca. 90 and 45 kDa) (Fig. 2b) as was induced by 5 min of FR light treatment (compare Fig. 2b with Fig. 2a). In the presence of 1 mM $CaCl_2$ histone phosphorylation was also strongly enhanced (data not shown). The addition of 5 mM EGTA did not significantly alter the exogenous histone phosphorylation respect to the control (E), but activated the phosphorylation of other proteins, such as 55 kDa and 28 kDa (Fig. 2b). This unusual stimulatory effect of EGTA on 55 and 28 kDa protein phosphorylation could be explained by the fact that casein kinase activity inhibited by Ca^{2+} was detected in cucumber cotyledon extracts (data not shown), as also described by Vetter et al. (2003).

The stimulatory effect of Ca^{2+} on *in vitro* protein phosphorylation (Fig. 2b) indicated Ca^{2+}-dependent PK activity in the extracts. The Ca^{2+}-enhanced effect on histone phosphorylation coincides with the same stimulatory effect observed with R and FR light treatments (Figs. 1 and 2a) suggesting that Ca^{2+} could be involved in this phytochrome response, as was suggested by Bergareche et al. (1994) in their study of phytochrome control of NR activity in cucumber cotyledons.

Fallon et al. (1993) also provided evidence for the involvement of Ca^{2+}-sensitive protein phosphorylation in the transduction sequence of R light signal in etiolated wheat leaf protoplasts. Bowler et al. (1994) supported the hypothesis that phy A signalling involved a rapid increase in cellular Ca^{2+} levels in tomato chloroplasts. According to Malec et al. (2002), the phytochrome-mediated increase in Ca^{2+} allows rapid activation of CDPKs in *Arabidopsis* seedlings.

2.3. Detection of in-gel kinase activity and CaM antagonist effects

The etiolated cucumber cotyledon extracts were subjected to in-gel kinase assays in gels containing histone (Fig. 3a) or without histone (Fig. 3b), and PK activity between M_r of 97 and 45 kDa was analysed. Three polypeptides of ca. 75, 57 and 47 kDa with PK activity were detected (Fig. 3a, lane 1). These three proteins also showed

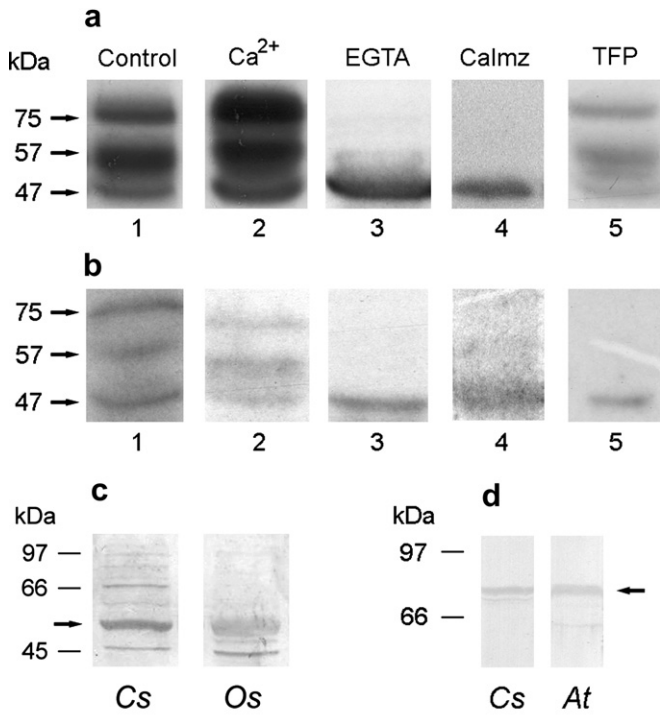

Fig. 3. Detection of kinase activity by in-gel kinase assays and characterization of CDPKs by immunoblot. Extracts from etiolated cucumber cotyledons were electrophoresed in 10% (w/v) SDS–polyacrylamide gels, polymerized with 0.5 mg ml^{-1} of histone as exogenous substrate (a), or with no substrate for autophosphorylation (b) and incubated with [γ^{32}P]ATP (lane 1 as control). The effect of calcium on PK activity was assayed by adding to the incubation buffer 1 mM $CaCl_2$ (lane 2) or 5 mM EGTA (lane 3). The effect of CaM antagonists was assayed by adding 50 μM calmidazolium (lane 4) or 350 μM TFP (lane 5) to the buffer containing 1 mM $CaCl_2$. For immunoblotting, the extracts of etiolated cucumber cotyledons (Cs) and rice (Os) were probed with a polyclonal antibody against a rice CDPK (OsCDPK11) (c); the extracts of etiolated cucumber cotyledons (Cs) and *Arabidopsis thaliana* (At) were also probed with polyclonal antibody against an *Arabidopsis* CDPK (AtCPK2) (d), as described in Section 3. M_r markers are indicated on the left of the figure.

autophosphorylating activity (Fig. 3b, lane 1), but lower than the phosphorylating activity of histone.

The assays, conducted in-gel containing histone and with 1 mM $CaCl_2$ in the incubation buffer, showed an increase in PK activity of 75 kDa and 57 kDa proteins (Fig. 3a, lane 2) respect to the control (Fig. 3a, lane 1). In the presence of 5 mM EGTA, the PK activity of both proteins was inhibited almost completely (Fig. 3a, lane 3), indicating strong Ca^{2+}-dependency.

The Ca^{2+}-dependency of PK activity of 75 and 57 kDa proteins was also observed on autophosphorylation (Fig. 3b, lanes 2 and 3), but the stimulatory effect of 1 mM $CaCl_2$ was less evident on autophosphorylation than on histone phosphorylation (Fig. 3, compare a and b). This may be explained by the quite small Ca^{2+} content (ca. 5 μM), present in the incubation buffers as contaminant, increasing the low autophosphorylating activity, so masking the stimulatory effect of Ca^{2+} addition.

We next examined the effect of CaM antagonists on PK activity in the presence of 1 mM $CaCl_2$. Histone phosphorylation by the proteins of 75 and 57 kDa was inhibited by the addition to the incubation buffer, as well as by 50 μM calmidazolium (Fig. 3a, lane 4) and 350 μM TFP (Fig. 3a, lane 5). The addition of 250 μM W_7 also caused an inhibitory effect on PK activity of 75 and 57 kDa proteins (data not shown). The inhibitory effect of calmidazolium and TFP was also observed in the autophosphorylation assays of the same extracts (Fig. 3b, lanes 4 and 5).

Nevertheless, neither PK activity in histone gels nor autophosphorylation of 47 kDa protein were inhibited by Ca^{2+} or CaM antagonists (Fig. 3a and b), indicating that they are not Ca^{2+}-dependent. Preliminary data indicated that this 47 kDa protein showed characteristics of a mitogen-activated protein kinase (MAPK) (data not shown). At present, studies to characterize this PK are under way.

Our data, obtained from in-gel assays, indicated that proteins of ca. 75 and 57 kDa, whose PK activity was considerably enhanced by the addition of calcium, are Ca^{2+}-dependent protein kinases. The inhibitory effect of calmidazolium, TFP and W_7 on the PK activity of these proteins, both in histone gels and in autophosphorylation assays, indicated CaM involvement and may suggest their correspondence with Ca^{2+}-dependent PK with a CaM-like domain (CDPKs). Ganguly and Singh (1998) described a soluble CDPK of about 70 kDa in a winged bean shoot with capacity of autophosphorylation. Calcium-dependent PKs with M_r ca. 75 kDa have been described in isolated nuclei of etiolated pea (Datta et al., 1985) and oats (Romero et al., 1991). A predicted structure of 57 kDa soybean PK established the prototype for the CDPK family (Harper et al., 1991) and several reports described similar CDPKs in plant systems. Thus, Abo-El-Saad and Wu (1995) described rice CDPK of 58 kDa, whose activity was strongly inhibited by TFP and W_7. Li et al. (1998) found a CDPK of 57 kDa in guard cell protoplasts of *Vicia faba*, close to that found in our *C. sativus* cotyledons and whose autophosphorylation was Ca^{2+}-dependent. Chico et al. (2002) also found a CDPK of about 58 kDa in tomato leaves, probably related to the plant's defence mechanisms against biotic or abiotic attacks. CDPKs of M_r close to 57 kDa PK found in cucumber cotyledons were also described in oat roots (Schaller et al., 1992), zucchini seeds (Verhey et al., 1993), silver beet leaves (Klucis and Polya, 1988), groundnut seeds (DasGupta, 1994) and sandalwood (Anil et al., 2000). In addition, other close CDPKs were described in rice plants inhibited by light (Frattini et al., 1999) or induced by low temperature (Martin and Busconi, 2001). More recently, Ullanat and Jayabaskaran (2002) cloned four CDPKs from cucumber in relation to light and phytohormones, and their results point towards the possibility that individual CDPK isoforms may play distinct roles in different tissues, in response to the same stimulus.

2.4. Immunoblot of soluble CDPKs

Immunoblot analysis using anti-OsCDPK11, polyclonal antibody directed against OsCDPK11, detected a major band, among several other minor bands, in both cucumber- and rice-etiolated extracts (positive control), with similar electrophoretic mobility (Fig. 3c). No cross-reactivity was observed in controls in which anti-OsCDPK11 was omitted. Anti-OsCDPK11 was obtained against the amino acids residues 23–183 (55 from variable domain and 105 from kinase domain) of OsCDPK11 (Frattini et al., 1999). Thus, our results would indicate that the protein of ca. 57 kDa with Ca^{2+}-dependent PK activity of cucumber cotyledon extracts would correspond to a CDPK immunologically related to the OsCDPK11 described in rice (Frattini et al., 1999; Breviario et al., 1995). These authors reported two CDPK isoforms (OsCDPK11 and OsCDPK2) with predicted M_r of 61 and 59 kDa, respectively, and different responses to light. In our experimental conditions, anti-OsCDPK11 detected the PK band at a M_r slightly lower that the predicted one (61 kDa). However, PK bands in both cucumber and rice extracts showed exactly the same electrophoretic mobility. The minor immunolabelling polypeptides probably resulted from the extracts proteolytic activity as reported by Frattini et al. (1999).

Immunoblot analysis using anti-AtCPK2, polyclonal antibody directed against the first 90 amino acids (variable domain) of the N-terminal tail of *Arabidopsis* CDPK, detected a single band ca. 75 kDa in cucumber cotyledon extracts as well as in the positive control (Fig. 3d). No cross-reactivity was observed in controls in which anti-AtCPK2 was omitted. This indicates that the protein of ca. 75 kDa with Ca^{2+}-dependent PK activity of cucumber cotyledon extracts is a CDPK immunologically related to the AtCPK2, described in *Arabidopsis* (Hrabak et al., 1996).

2.5. In-gel kinase activity affected by light

To determine the kinetics involved in PK activation after light treatments of cucumber cotyledons, different irradiation times with continuous FR light were assayed. As can be seen in Fig. 4, the in-gel kinase activity of 75 and 57 kDa proteins slightly increased after 30 min of FR (30% and 28%, respectively); this increase in activity was maintained after 60 or 120 min of FR irradiation. This finding implicates a FR-HIR of phytochrome in PK activity of the 75 and 57 kDa proteins. To test the possible involvement of a VLFR, shorter irradiation treatments were also assayed since VLFR of phytochrome can be induced by irradiation times of only a few seconds (Schäfer and Nagy, 2006). Thus, 5 and 15 min of FR light increased PK activity of the 75 kDa protein (36% and 48%, respectively) and that of the 57 kDa protein (53% and 59%, respectively).

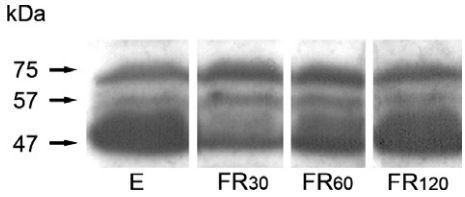

Fig. 4. Effect of FR light on kinase activity. Extracts from etiolated cucumber cotyledons (lane E), treated with 30 min (lane FR_{30}), 60 min (lane FR_{60}) or 120 min (lane FR_{120}) of FR light, were electrophoresed in 10% SDS–polyacrylamide gels polymerized with 0.5 mg ml^{-1} histone and incubated with [γ^{32}P]ATP. M_r markers are indicated on the left of the figure.

Datta et al. (1985) and Tong et al. (1996) also detected two light-dependent phosphorylated proteins of 77–64 kDa and 72–62 kDa, respectively. Romero et al. (1991) found, in etiolated *Avena* seedlings, two nuclear proteins of ca. 75 and 60 kDa whose phosphorylation was enhanced by R or FR light probably through a VLFR of phytochrome. These proteins could correspond to our PKs of 75 and 57 kDa detected in in-gel phosphorylation, which also autophosphorylated (Fig. 3). However, we detected no phytochrome effect on autophosphorylation (data not shown), probably because the level of autophosphorylation was very low even in the presence of Ca^{2+}.

The 47 kDa PK activity in the dark (E) decreased after 30 min of FR (about 70%), but fully recovered when FR light irradiation rose to 60 min. In cucumber cotyledons treated with 120 min of FR light, the 47 kDa protein showed almost the same PK activity as in etiolated cotyledons (Fig. 4). Thus, FR light treatment caused a transient inhibitory effect on 47 kDa PK activity, which disappeared when FR irradiation was prolonged. This FR light effect is almost opposite to that described by Malec et al. (2002). These authors found in *Arabidopsis thaliana* a PK activity with an apparent M_r of 50 kDa, whose activity increased transiently after irradiation of dark-grown seedlings with continuous FR light. As FR is classically involved in the HIR, they referred to this kinase as HIR kinase (HIRK) and proposed a model whereby HIRK could be a CDPK because it was activated by increased levels of free Ca^{2+}mediated by phytochrome. However, as was indicated above, the PK activity of the 47 kDa protein detected in our cucumber cotyledon extracts was scarcely affected by Ca^{2+} and CaM antagonists.

In summary, as the proteins of 75 and 57 kDa phosphorylate with in-gel histone substrate and autophosphorylate in a Ca^{2+}-dependent manner, are sensitive to CaM-antagonists, and cross-reacted with antibodies anti-CDPKs, we suggest that they are CDPKs. Our results also indicate that these putative CDPKs could be, at least in part, responsible for the phosphorylation of 90 and 45 kDa proteins in cucumber cotyledon extracts, and also for phosphorylation of the histone added to the extracts. FR light treatments on *in vitro* protein phosphorylation and in-gel kinase assays also indicated that both CDPKs (ca. 75 and 57 kDa) were modulated by phytochrome

through a FR-HIR response and probably also a VLFR, since 5 min FR light irradiation affected *in vitro* protein phosphorylation. Thus, our data suggest that both CDPKs' activity could be involved in phytochrome signal transduction responses during the de-etiolation of cucumber cotyledons.

3. Experimental

3.1. Plant material and light treatments

Cucumis sativus cv. Bellpuig seeds (Semillas Fitó, Barcelona, Spain) were germinated on moist vermiculite at 25 °C in complete darkness. After 6 days in these conditions, one set of etiolated seedlings was kept as dark control (E), while the other sets were irradiated with red (R), far-red (FR) or R followed by FR light. Light treatments consisted of R 15 min, FR 5 min or R light pulses of 3 min applied hourly for 12 h. To test the effect of longer FR light irradiations, treatments of 30, 60 and 120 min of FR light were also applied.

R light source was a bank of eight 15 W Gro-lux fluorescent lamps (F15/T8/N, Sylvania), each wrapped in three layers of medium-red filter (No. 27 Rosco Supergel, Sydenham, London, UK). This assembly allowed the transmission of a broad-band from 590 to 700 nm (λ_{max} from 670 nm). The FR light source was a bank of five 150 W incandescent lamps (Sylvania BRS), with the light filtered through 10 cm of water, a layer of a blue filter (No. 20 Cinemoid, Rank Strands Electrics Ltd., London, UK) and a deep-orange filter (No. 5A Cinemoid). This assembly allowed the transmission from 728 nm. The fluence rates were 17.4 and 26 $\mu E\, m^{-2}\, s^{-1}$for R and FR, respectively. A dim-green safe light was used in the dark (15 W daylight fluorescent lamp, Sylvania, covered with three layers of No. 39 green filter, Cinemoid). The fluence rate was measured at the level of plants using a quantum radiometer photometer Li-Cor 188 B (Lincoln, NE, USA), fitted with a quantum sensor Li-190 SB for R and a near-infrared sensor Li-220 SB for FR.

3.2. Protein extraction

The cotyledons of the etiolated and light-treated seedlings were excised under dim green safe light, frozen on liquid nitrogen and homogenized in extraction medium at 4 °C, using a Polytron (Kinematica GmbH Kriens, Switzerland) homogenizer. The medium, modified from Ganguly and Singh (1998), contained 20 mM Tris–HCl pH 7.5, 2 mM EDTA, 5 mM EGTA, 20% glycerol, 40 mM β-mercaptoethanol (β-ME), 2 mM PMSF and 2.5 $\mu g\ ml^{-1}$ of leupeptin, aprotinin and pepstatine. The homogenate was centrifuged at 17,000*g* at 4 °C for 30 min in a Beckman J-21-C. Then the pellet was discarded, and the supernatant was centrifuged again at 100,000*g* at 4 °C for 1 h in an ultracentrifuge (Sorvall Pro 80) with a fixed angle rotor. The supernatant was filtered in YM10 Microcon centrifugal filters (Millipore), stored at −20 °C and then used for protein quantification and kinase activity assays. Proteins were quantified according to the dye-binding method of Bradford (1976), using bovine serum albumin (BSA) as standard.

3.3. SDS polyacrylamide gel electrophoresis

Proteins from extracts were subjected to SDS–PAGE in 13.5% or 10% gels, according to the method of Laemmli (1970), using the Mini-Protean II system (Bio-Rad). Electrophoresis was done for 60 min by 30 mA per gel. The gels were stained with Coomassie Brilliant Blue R250 (Sigma), de-stained and then air-dried between two sheets of cellophane. The relative M_r of polypeptides separated by SDS was estimated from the electrophoretic mobility of the following markers: phosphorylase b (97.7 kDa), albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and α-lactalbumin (14.4 kDa).

3.4. In vitro protein phosphorylation

Protein phosphorylation was assayed according to Chico et al. (2002) in a reaction mixture of 100 μl (total volume) containing 20 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, with or without 1 $mg\ ml^{-1}$ histone III-S (as exogenous substrate), 50 μl of sample extract and 80 μM [γ-^{32}P]ATP (3000 $Ci\ mmol^{-1}$). Non-specific binding was determined in the absence of added substrates. Calcium dependence was tested with different concentrations of $CaCl_2$ (1 mM, 5 mM or 10 mM) or in the presence of 5 mM EGTA. The presence of 1 mM, 5 mM or 10 mM $CaCl_2$ in the incubation system is equivalent approximately to 0.075 μM, 1.5 μM and 6.5 mM of free Ca^{2+}, respectively, calculated according to the Max-Chelator program Winmaxc v. 2.05 (Bers et al., 1994). After incubation at 37 °C for 10 min, the enzyme reaction was stopped by addition of one volume of SDS-sample buffer. Protein samples were finally boiled for 5 min and subjected to SDS–PAGE. Gels were dried and radiolabelled polypeptides were viewed by autoradiography after several days of exposure to Kodak X-OMAT-AR films at −80 °C and analysed by quantitative densitometry of the autoradiograms using the Quantity One 1-D program (Bio-Rad). The experiments were repeated at least four times with similar results.

3.5. In-gel assay for protein kinase activity

The enzymatic activity assay was a slight modification of the procedure previously described by Nakamura et al. (1995). Extracts containing 100–150 μg of protein were electrophoresed on 10% SDS–polyacrylamide gels, embedded or not with 0.5 $mg\ ml^{-1}$ of type-III-S histone H1 (Sigma) in separating gel prior to polymerization. After electrophoresis, SDS was removed by washing the gel

2×3 min in 25 mM Tris–HCl pH 7.5 and 20% *iso*-PrOH; then, it was washed once for 60 min with 25 mM Tris–HCl pH 7.5 and 1 mM DTT. The proteins were denaturised in 6 M guanidine-HCl, 25 mM Tris pH 7.5, 1 mM DTT and 2 mM EDTA. To renature the proteins, the gel was soaked (2×20 min) in 25 mM Tris–HCl pH 7.5, 1 mM DTT, 2 mM EDTA and 0.04% Tween 20 and then left for 16 h at 4 °C without shaking. After this, the gel was pre-incubated at 22 °C for 1 h in 25 mM Tris–HCl pH 7.5, 1 mM DTT, 1 mM EDTA and 20 mM $MgCl_2$. Phosphorylation was carried out by incubation of the gel at room temp. for 1 h in the same buffer, with 100 μM ATP containing 5 μCi ml^{-1} [γ-^{32}P]ATP (3000 Ci $mmol^{-1}$). Calcium dependency was assayed by adding 1 mM, 5 mM, 10 mM $CaCl_2$ or 5 mM EGTA to the incubation buffer. The reaction was stopped and the excess of [γ-^{32}P]ATP removed by extensive washes (4×10 min, 1×16 h) with a solution containing 5% TCA and 1% Na-pyrophosphate (until radioactivity in the used wash solution was barely detectable). The gels were then stained with Coomassie, de-stained, air-dried and exposed to Kodak films for 10 days at −80 °C. Bands with PK activity were viewed by autoradiography and quantified as indicated before. The experiments were repeated at least four times with similar results.

To determine the inhibitory effect of CaM antagonists on PK activity, phosphorylation assays were also performed in the presence of 50 μM calmidazolium or 350 μM TFP added to the incubation buffer.

[γ-^{32}P]ATP (3000 Ci $mmol^{-1}$) was purchased from Amersham and protein markers were from Bio-Rad. Type-III-S histone H1, protein-kinase inhibitors and the analytical-grade reagents were obtained from Sigma and Bio-Rad.

3.6. Immunoblotting analysis

Protein cotyledon *Cucumis* extracts were run on 10% SDS–polyacrylamide gels and transferred (in 25 mM Tris, 129 mM glycine and 20% MeOH, pH 8.4) to nitrocellulose membranes (pore size 0.45 μm) at 20 mA, for 2 h at 4 °C. The membranes were stained briefly with 0.05% Ponceau S to monitor M_r markers and then were blocked for 2 h at room temperature in PBS-T (140 mM NaCl, 7 mM disodium hydrogen phosphate, 3 mM sodium dihydrogen phosphate, and 0.05% Tween 20, pH 7.4) containing 5% non-fat dry milk blocking solution. After washing, the membranes were incubated in blocking solution containing the primary polyclonal anti-CDPK antibody directed against rice CDPK (OsCDPK 11) (Frattini et al., 1999) or directed against *Arabidopsis* CDPK (AtCPK2) (Lu and Hrabak, 2002) at dilutions of 1:250 for 1 h at room temp. and 1:1000 overnight at 4 °C, respectively. Membranes were then washed in PBS-T three times for 30 min, followed by incubation with the secondary antibody anti-rabbit IgG (H+L) alkaline phosphatase conjugate diluted 1:2000 in PBS-T plus 5% non-fat dry milk for 1 h at room temp. Immunoreactive protein bands were detected by staining alkaline phosphatase with BCIP/NTB for 5–10 min. Reaction was stopped with water and membranes were air-dried. Experiments were repeated at least four times.

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