Zeae mays CCMK: autonom phosphorylation-dependent substrate phosphorylation and down-regulation by red light

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Abstract
The role of protein kinases has been extensively studied in various signal transduction pathways and they are one of the most important components that link the signal perception to the final response. However, not many studies have been reported, especially from the plant systems, that show the regulation pattern of the kinase itself under different conditions. A calcium calmodulin-dependent protein kinase has already been purified and characterized from etiolated maize coleoptiles. In this paper a detailed study of how the kinase itself is regulated at the autophosphorylation level is provided. Evidence is also given that the autophosphorylation of kinase affects its activity towards substrate phosphorylation. It is further shown that the kinase is an important component of the light signalling pathway as the level of the kinase itself decreases by red light irradiation.

Key words: Autophosphorylation, Ca2+ /CaM-dependent protein kinase, red light, Zeae mays.

Introduction
Plant cells can respond to various signals in a precise and specific manner due to the presence of a complex but efficient perception and signal transduction apparatus. Following perception, a signal is decoded via a change in concentration of a plethora of second messengers such as inositol phosphates, cADPR, diacyl glycerol, and calcium. Calcium ions are the ubiquitous signal molecules and their role is central to the theme of signalling in both plants and animal systems. They are the most widely accepted second messengers, which couple a large number of diverse stimuli to their characteristic responses (Trewavas and Malho, 1997; Wu et al., 1996, 1997; Pandey et al., 2000).

The information encoded in the calcium signals can be de-coded downstream generally by two distinct signal transduction pathways, i.e. one involving calmodulin, which is an universal calcium receptor and other involving calcium-dependent protein kinases. Both these pathways are inter-linked and they also interact with a large number of other factors down the signal transduction cascade, working either independently or jointly. Thus all these components, i.e. CaM, CaM-binding proteins and calcium-dependent protein kinases interact with each other at different levels and form a very complex network (Poovaiah and Reddy, 1993; Bush, 1995). Calcium-dependent protein kinases are one of the most important components of this signalling network as they transduce different signals mediated by calcium in a specific and precise manner (Stone and Walker, 1995).

Calcium-dependent protein kinases can be divided into two major categories based on the components they require for their activation, i.e. Ca2+/phospholipid-dependent protein kinases and Ca2+/calmodulin-dependent protein kinases. Ca2+/CaM-dependent kinases...
are the main transducers of various calcium signals in animal systems. Some of them are multifunctional, for example, CaMK II, CaMK IV and CaM Ia/Ib as they phosphorylate a variety of substrates in vivo whereas others are more dedicated (Hanson and Schulman, 1992). In plants, the main transducers of calcium signals known to date are kinases from the CDPK family, but recent evidence for the presence of calmodulin-dependent kinases (Watillon et al., 1995; Lu et al., 1996; Takezawa et al., 1996), suggest the possibility of involvement of these CaM kinases in a novel signalling pathway parallel to the CDPK-mediated signalling.

It is very well known that most of the Ca\(^{2+}\) signal transduction processes are mediated by a change in the phosphorylation/dephosphorylation pattern of related proteins in plants (Huber et al., 1994; Monroy et al., 1997), but only in a few cases have the Ca\(^{2+}\)-dependent kinases been identified and characterized. Most of the studies are based on the observations that transcript levels of the protein kinase homologues change in response to a particular stimulus. (Anderberg and Walker-Simmons, 1992; Sano and Youssefien, 1994; Botella et al., 1996). Various stress signals which act through the modulation of [Ca\(^{2+}\)]\(_{cyt}\) level, have also been shown to act by modulating CDPKs (Sheen, 1996). Light-mediated responses have been shown to be accompanied by changes in cytoplasmic calcium concentration (Tretyn et al., 1991; Chory, 1994, 1997). Red light-mediated responses have been studied in maize coleoptiles and mesophyll protoplasts of wheat and a fast transient increase in calcium levels have been observed (Shacklock et al., 1992). In Chlamydomonas both calcium channel blockers and calmodulin antagonists affect the induction of the gsa gene suggesting that calcium and calmodulin are involved in a signal transduction pathway linking the blue light perception and induction of the gene (Im et al., 1996). Ca\(^{2+}\) and CaM have been shown to be directly involved in the regulation of gene expression in phytochrome-mediated responses (Neuhau et al., 1993; Boller et al., 1994; Wu et al., 1996; Mustilli and Boller, 1997; Neuhau et al., 1997), but so far no report for direct modulation of a kinase level in response to light has been observed.

Earlier, the biochemical characterization of a Ca\(^{2+}\)/CaM-dependent protein kinase from etiolated maize coleoptiles was reported (Pandey and Sopory, 1998). This kinase (ZmCCaMK) has turned out to be novel, showing similarity to both animal system CaM kinases and plant CDPKs. The autophosphorylation of ZmCCaMK was studied in detail, and compared with the autophosphorylation activity of other known kinases as well as with its own substrate phosphorylation activity. Ca\(^{2+}\)/CaM-stimulated autophosphorylation is a prominent characteristic of multifunctional CaM kinases in animal systems and it has been shown conclusively to play a role in the regulation of kinase activity (Kwiatkowski et al., 1988). In plants, there are few studies related to the regulation of a kinase activity by its autophosphorylation. In this study, it is shown that autophosphorylation of ZmCCaMK regulates the kinase activity towards its substrate phosphorylation. In order to elucidate the possible functional role(s) played by this kinase in signalling, its expression level was studied under different developmental conditions. It is shown for the first time that the ZmCCaMK level itself changes in response to red light, indicating that this kinase may be involved in the light-dependent signal transduction pathway.

**Materials and methods**

**Materials**

\(\gamma\)-\(^{32}\)P ATP (specific activity 3000 Ci mM\(^{-1}\)) and \(^{45}\)Ca\(^{2+}\) (specific activity 29.08 mCi mg\(^{-1}\)) was obtained from Bhabha Atomic Research Centre, India. DEAE Sephacel and MW markers were obtained from Pharmacia. Histone IIIS, syntide-2, BSA, chlorpromazine (CPZ), trifluoromorazine (TFP), compound 48/80, W7, H7, staurosporine, KN-62, PMSF (phenyl methyl sulphonyl fluoride), aprotinin, dithiothreitol (DTT), HEPES, EGTA, EDTA, CaM, CaM Sepharose, BCP/NBT (5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium), and alkaline phosphatase linked anti-rabbit IgG were from Sigma chemical Co. All the other chemicals were of AR grade.

**Plant material and light treatments**

Maize seeds (Zea mays var. Ganga 5) were grown at 25 ± 2 °C for 8 d in dark on moist germination paper. Etiolated coleoptiles were harvested in dark under green safe light and frozen immediately in liquid N\(_2\).

Different light treatments were given to 8-d-old plants, grown in continuous darkness. Red (\(\lambda_{max}\) 600 nm), far red (\(\lambda_{max}\) 730 nm) and blue light (\(\lambda_{max}\) 427 nm) of desired intensities were obtained from respective light emitting diodes (Quantron devices, Wisconsin USA). White light was obtained from fluorescent tubes (intensity 1200 µW cm\(^{-2}\)). All the treatments were given under green safe light obtained from a white fluorescent tube covered with several layers of green cellophane paper (\(\lambda_{max}\) 500 nm).

**Protein purification**

The kinase was purified from 8-d-old, etiolated, maize coleoptiles essentially as reported earlier (Pandey and Sopory, 1998). Briefly, the tissue was ground in the presence of liquid N\(_2\) to a fine powder and mixed with 3 vols of extraction buffer (20 mM HEPES, pH 7.5, 5 mM EGTA, 2 mM EDTA, 2 mM PMSF, 5 mM DTT, and 10% glycerol v/v). The slurry was centrifuged at 15000 g, 4 °C for 45 min and the pellet of cell debris was discarded. The supernatant was ultracentrifuged at 100000 g, 4 °C for 1 h to remove the membranes and undissolved material. Crude protein extract thus obtained was further fractionated by 40–50% ammonium sulphate precipitation. Precipitated proteins were proceeded for ion exchange chromatography.

A DEAE Sephacel column was pre-equilibrated with equilibration buffer (10 mM HEPES, pH 7.5, 5 mM EGTA, 2 mM EDTA, and 10% glycerol v/v). The proteins after binding were washed extensively with washing buffer (equilibration buffer with 10 mM\_mercaptoethanol) and eluted with a linear
gradient of 0–0.4 M KCl. Alternate fractions were assayed for Ca\(^{2+}\)-dependent protein kinase activity. The active fractions were pooled and proceeded for affinity chromatography using CaM Sepharose.

Pooled proteins were dialysed extensively against 40 vols of dialysis buffer (10 mM HEPES, pH 7.5, 200 mM CaCl\(_2\), 100 mM NaCl, 1 mM DTT, 0.2 mM PMSF, 10 mg ml\(^{-1}\) aprotinin, and 10% glycerol (v/v)) and bound on a CaM Sepharose column pre-equilibrated with the same dialysis buffer. Non-specifically bound proteins were removed by washing the column with 1 M NaCl. Specifically bound proteins were step eluted with one column volume of elution buffer containing 1–4 mM EGTA and assayed for Ca\(^{2+}\)/CaM-dependent kinase activity.

Active fractions were run on 10% SDS-PAGE and all the four polypeptides were excised separately. Gel containing the 72 kDa polypeptide was cut into small pieces and transferred to elution buffer (20 mM HEPES, pH 7.5, 5 mM DTT, 2 mM PMSF, 10 mg ml\(^{-1}\) aprotinin, and 20% v/v glycerol). After overnight incubation at 4 °C, the slurry was centrifuged and supernatant containing eluted protein was run on 12% SDS-PAGE to check the purity and used for further characterization and for raising antibodies as described earlier (Pandey and Sopory, 1998).

**Protein kinase assay**

Protein kinase assay was performed as reported earlier (Pandey and Sopory, 1998). Purified enzyme (0.2 mg µl\(^{-1}\)) was incubated in 30 mM HEPES (pH 7.5) buffer containing 5 mM MgCl\(_2\), 0.5 mM DTT and 100 µM of γ\(_{32}\)P-ATP with syntide-2 (20 µM) in a total reaction volume of 100 µl. Assay was performed at 30 °C for 5 min. The reaction was stopped either by spotting 10 µl of reaction mixture on P81 phospho-cellulose paper (Whatman) or by adding equal volume of SDS sample buffer to it. P81 paper was washed thoroughly with 75 mM phosphoric acid for 45 min with frequent changes. The paper was dried under a lamp and placed in scintillation vial containing cocktail-O and counts were recorded in a liquid scintillation counter (Beckman). When the reaction was stopped by the addition of SDS sample buffer, the mixture was boiled at 100 °C for 5 min and run on a 10% SDS-PAGE. The gel was dried and exposed for autoradiography. For inhibitor studies various Ca\(^{2+}\)/CaM inhibitors were incubated with the reaction mixture with proper controls. To check the effect of different pH buffers, the reaction was performed in presence of 0.25 M citrate buffer (for pH 3–6) and 0.25 M TRIS buffer (for pH 7–10).

**Autophosphorylation**

Purified protein (5 µg) was incubated in autophosphorylation buffer (20 mM HEPES, pH 7.5, 3 mM MnCl\(_2\), 10 µM ZnCl\(_2\), 50 µM DTT, 0.2% NP-40, 10 mg ml\(^{-1}\) aprotinin). To start the reaction, 10 µM of γ\(_{32}\)P-ATP was added and the reaction mixture was incubated at 30 °C for 5 min. The reaction was stopped by adding equal volume of SDS sample buffer; boiled for 5 min, and run on 10% SDS-PAGE. The gel was dried and exposed for autoradiography (Dasgupta, 1994). For inhibitor studies, the inhibitors were included in the reaction mixture and to check the effect of pH the reaction was performed in the presence of buffers of different pH values as in the case of substrate phosphorylation.

To check the effect of autophosphorylation on substrate phosphorylation activity of ZmCCaMK, purified kinase was autophosphorylated, and then acetone precipitated. The protein was air-dried and then used for substrate phosphorylation studies in the presence or absence of 2 mM EGTA.

For kinetic studies, tubes containing both autophosphorylation and substrate phosphorylation reaction mixes were simultaneously incubated at 30 °C and small aliquots were taken out on P81 paper, at different time intervals, starting from 1 min up to 30 min. Counts incorporated were determined using scintillation counter.

**Phospho amino acid analysis**

Phospho amino acid analysis of phosphorylated proteins was performed as described previously (Cooper et al., 1983) to

![Fig. 1. (A) Autophosphorylation of purified ZmCCaMK: purified ZmCCaMK (5 µg) was autophosphorylated in the presence of 10 µM Ca\(^{2+}\), run on a 10% SDS-PAGE, dried and exposed for autoradiography. The figure shows silver-stained purified protein and the autoradiogram showing autophosphorylation of the purified kinase. (B) Phospho amino acid analysis of phosphorylated proteins: phosphorylated histone (1), syntide-2 (2) and ZmCCaMK (3), were run on a paper chromatogram after digestion with 5.7 N HCl along with phospho amino acid standards; phosphoserine (S), phosphothreonine (Y) and phosphothreonine (T). The chromatogram containing phosphorylated samples was exposed for autoradiography. The figure shows a chromatogram showing ninhydrin-stained phospho amino acid standards and an autoradiogram showing phospho amino acids of phosphorylated proteins.](image-url)
determine which amino acid residue was getting phosphorylated. Briefly the phosphorylated band was cut from the gel and digested with 5.7 N HCl at 110 °C for 2 h. Digested protein was dried under vacuum and run on an ascending paper chromatogram using propionic acid, 1 M NH₄OH, isopropanol (45:17.5:17.5, by vol.) as solvent, along with phosphoserine, phosphothreonine and phosphotyrosine as standards. Standards were visualized by ninhydrin reagent and the chromatogram containing phosphorylated proteins was autoradiographed.

Preparation of total protein extract and Western blotting

For preparation of total protein extract, the tissue was ground in the presence of liquid N₂ to a fine powder and mixed with 3 vols of extraction buffer (20 mM HEPES, pH 7.5, 5 mM EGTA, 2 mM EDTA, 2 mM PMSF, 5 mM DTT, and 10% glycerol v/v). The slurry was centrifuged at 15000 g, 4 °C for 45 min and the pellet of cell debris was discarded. The clear supernatant obtained was used for Western blotting. For Western blotting 20 μg of total protein was separated on 10% SDS-PAGE and electro-blotted on nitro-cellulose membrane using Semi-dry transfer apparatus for 1.5 h. Membrane was blocked with 3% BSA in TBS (150 mM NaCl and 10 mM TRIS buffer, pH 7.4) for 1 h at 37 °C with shaking and probed with anti-kinase antibodies at a dilution of 1:20000 in TBS containing 1% BSA and 0.05% Tween-20 for 1 h at 37 °C. Alkaline phosphatase linked anti-rabbit IgG (Sigma Chemical Co.) was used as secondary antibody at a dilution of 1:30000 in TBS containing 1% BSA and incubated for 1 h at 37 °C. Blot was developed with BCIP/NBT solution till a purple-blue colour appeared.

Other methods

Protein estimation was done by using BSA as a standard (Bradford, 1976). SDS-PAGE was done according to Laemmli (Laemmli, 1970) and silver staining of proteins was done according to Blum et al. (Blum et al., 1986).

Results

Autophosphorylation of Zea mays CCaMK is at threonine residue(s) whereas substrate phosphorylation is at serine residue(s)

Purified ZmCCaMK was autophosphorylated by incubating it in the presence of 10 μmol of free calcium as described in the Materials and methods. As shown in Fig. 1, the purified kinase autophosphorylated, resulting in the presence of a 72 kDa band on the autoradiogram. To determine which amino acid was phosphorylated, phospho amino acid analysis was done with purified ZmCCaMK, as well as with substrates such as histone IIIS and syntide-2. As seen in Fig. 1, both the substrates were phosphorylated at serine residue(s); ZmCCaMK autophosphorylated at threonine residue(s) showing this kinase belongs to the serine/threonine family of protein kinases.

Autophosphorylation and substrate phosphorylation activities of Zea mays CCaMK are differentially regulated in response to various physiological factors

It has been shown earlier that purified ZmCCaMK undergoes a calcium-dependent but CaM-independent autophosphorylation. In this study it is further shown that calcium is the only physiological factor that affects the autophosphorylation activity of ZmCCaMK. The

Physiological Factors

![Fig. 2](image1)

(A) Effect of different physiological factors: purified ZmCCaMK (5 μg) was autophosphorylated in the presence of various additives (2 mM EGTA, 10 μM calcium, 100 nM CaM, 5 μM PS, 100 nM PMA), run on 10% SDS-PAGE and exposed for autoradiography. Purified ZmCCaMK was also used to phosphorylate syntide-2 under similar conditions. The autoradiogram shows syntide-2 and autophosphorylated ZmCCaMK under different conditions. (B) Effect of different inhibitors: purified ZmCCaMK (5 μg) was autophosphorylated in the presence of different calcium and CaM antagonists and kinase inhibitors (2 mM EGTA, 0.1 μM each of CPZ, TFP, KN-62, and W7, 10 μg 48.80, 5 mM staurosporine, 100 μM H7); syntide-2 was phosphorylated under similar conditions using purified ZmCCaMK. The autoradiogram shows syntide-2 phosphorylation and autophosphorylation. (C) Effect of pH: purified ZmCCaMK (5 μg) was autophosphorylated and syntide-2 was phosphorylated with purified kinase at different pH buffers (0.25 M citrate buffer for pH 3–6 and 0.25 M TRIS buffer for pH 7–10). The autoradiogram shows syntide-2 phosphorylation and autophosphorylation at different pH values. Different pH optimum values were obtained with both the proteins.
purified ZmCCaMK was autophosphorylated in the presence of various exogenous physiological factors. The purified protein kinase was also used to phosphorylate syntide-2 under similar conditions. As evident from the autoradiogram (Fig. 2A), the autophosphorylation activity of the ZmCCaMK was fully calcium-dependent and no activity could be detected in the presence of EGTA (200 μmol). The addition of 10 μmol calcium optimally stimulated the kinase activity. Autophosphorylation was unaffected by the addition of calmodulin at all the concentrations tested. But the syntide-2 phosphorylation activity, which is also fully calcium-dependent, showed a further 2.5-fold stimulation with calmodulin. CaM alone had no effect on autophosphorylation, similar to the substrate phosphorylation activity. Other factors tested such as PS and PMA also failed to affect both the autophosphorylation as well as the substrate phosphorylation activity of the ZmCCaMK. The data show that calcium is the only exogenous factor that affects autophosphorylation of ZmCCaMK. Furthermore, CaM which affects the substrate phosphorylation activity of the same kinase has no effect on autophosphorylation. The activity of this kinase thus shows a distinct dual regulation by calcium and CaM. This is in contrast to other known plant CDPKs where the activity is only calcium dependent, and CaM has no effect. The ZmCCaMK is also distinct from the lily anther kinase, which showed an inhibition of autophosphorylation activity in the presence of CaM (Takezawa et al., 1996).

As autophosphorylation showed absolute calcium dependency, the effect of various calcium and kinase inhibitors on the activity of the kinase was determined. To confirm further if CaM has any role during autophosphorylation, the effect of various CaM antagonists on autophosphorylation activity of ZmCCaMK was also analysed. Syntide-2 phosphorylation using purified kinase was also performed under similar conditions. As evident from autoradiogram (Fig. 2B), EGTA inhibited both the autophosphorylation and syntide-2 phosphorylation activities almost completely. Substrate phosphorylation activity was inhibited to different extents with different CaM antagonists and kinase inhibitors. But in sharp contrast, CaM antagonists as CPZ, TFP and W7, as well as various kinase inhibitors such as 48/80, H7, KN-62, and staurosporine had no effect on autophosphorylation activity of the kinase. These data give further evidence that the autophosphorylation and substrate phosphorylation activities of the kinase are differentially regulated.

The effect of pH on autophosphorylation and substrate phosphorylation activity of ZmCCaMK was determined by performing the reaction under different pH conditions. As is clear from Fig. 2C, both the

Fig. 3. (A) Time kinetics of autophosphorylation and substrate phosphorylation reaction: the autophosphorylation and substrate phosphorylation reactions were started separately by addition of γ-32P ATP to the reaction mix at 30 °C and stopped at different time points by applying small aliquots on PS1 paper. The kinetics shows that autophosphorylation precedes the substrate phosphorylation reaction. (B) Effect of autophosphorylation on substrate phosphorylation activity of ZmCCaMK: in vitro kinase assay was performed with histone III S as substrate in the presence of EGTA (2 mM), using non-autophosphorylated (1) and autophosphorylated (2) ZmCCaMK (1 μg). The autoradiogram shows calcium-independent histone phosphorylation by pre-autophosphorylated ZmCCaMK (2).
autophosphorylation and substrate phosphorylation activities were pH-dependent, but each showed a different optimum pH value. The autophosphorylation activity could not be detected below pH 5 and above pH 8, with an optimum activity at pH 6, whereas syntide-2 was phosphorylated with a pH optimum of 7.5, showing that the two activities are differentially regulated by pH.

**Autophosphorylation precedes the substrate phosphorylation and makes the Zea mays CCAmK $Ca^{2+}$-independent towards substrate phosphorylation**

Time kinetic studies of both substrate phosphorylation and autophosphorylation activities were performed with ZmCCaMK. Figure 3A shows that the autophosphorylation activity of the kinase was faster, attaining the saturation level at approximately 1 min and then going down slightly over a period of 30 min. The substrate phosphorylation activity, however, was comparatively slower, attaining the saturation level at 4 min and remained constant over a period of 30 min. This indicated that the autophosphorylation activity of the kinase is preceded by the substrate phosphorylation activity. It is known from animal systems, that autophosphorylation modulates the activity of CaM kinases and makes them calcium-independent (Lai et al., 1986; Miller and Kennedy, 1986; Hanson et al., 1994). Since ZmCCaMK showed many properties similar to the animal system CaM kinases, it was determined whether autophosphorylation could induce calcium independence towards substrate phosphorylation activity. Using non-autophosphorylated kinase for the assay, no substrate phosphorylation could be achieved in the presence of 2 mM EGTA. If autophosphorylated kinase was used for the assay, even in the presence of 2 mM EGTA, histone IIIS phosphorylation could be achieved (Fig. 3B). This shows that autophosphorylation induced calcium-independence in ZmCCaMK activity.

**Expression of Zea mays CCAmK is tissue specific and development-dependent**

As ZmCCaMK was purified from etiolated coleoptiles of maize plants, its presence was determined in other plant parts as well. Western blot analysis of protein extracts from leaf, hypocotyl, stem and roots of maize showed that the kinase has differential expression (Fig. 4A). Leaves showed the maximum expression of kinase while the level was minimum in roots. The stem portion and hypocotyl also expressed a lesser amount of protein compared to leaf. Although the level of expression of this kinase was different in various plant parts, it was not localized specifically to a particular tissue or organ. Expression of ZmCCaMK was also studied with different ages of maize seedlings. Probing total protein extracts of maize seeds (taken as 0 d), 3 d, 5 d, 8 d, and 10-d-old maize coleoptiles with the antibodies showed an age-dependent expression of ZmCCaMK. Seeds had very low level of protein, followed by 3-d-old coleoptiles. By 5 d the level of protein was high and it remained constant for up to 10 d (Fig. 4B).

**Zea mays CCaMK expression level is down-regulated by red light**

Light is one of the main environmental signals, which affect the growth and development of plants at every stage, mostly via phosphorylation/dephosphorylation. Since ZmCCaMK was initially purified from etiolated tissues, the effect of different light radiation on expression levels of the kinase was sought. Eight-day-old etiolated cut coleoptiles were given different light treatments (as described in the Materials and methods) and non-treated

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**Fig. 4.** Immuno-detection studies (A) in different plant parts and (B) during different ages of development with ZmCCaMK antibodies: Total protein (25 µg) of different plant parts and different day old coleoptiles was separated on 10% SDS-PAGE and Western blotting of similar protein samples was done with 1:20000 dilution of ZmCCaMK antibodies. ZmCCaMK is not specific to any particular plant part, but roots have very low expression compared to leaves. Seeds also have very low level of expression that starts increasing from day 3 and saturates at day 8.
etiolated, cut coleoptiles served as the control. Equal quantities of total protein extract of all the treatments were separated on SDS-PAGE, blotted and probed with the ZmCCaMK antibodies. The blot showed a clear difference in expression level of the kinase with different light treatments (Fig. 5). Red light treatment for 5 min decreased the kinase level in comparison to dark-grown (control) coleoptiles. Red light treatment followed by far-red light treatment did not restore the level of kinase. Far red light alone had no effect on the level of the kinase. Incubating coleoptiles treated with 5 min red light in the dark for 2 h decreased the kinase level even further. White light treatment for 5 min also down-regulated the ZmCCaMK level slightly.

As red light decreased the ZmCCaMK level, a concentration and time kinetics of red light treatment was performed. Etiolated maize coleoptiles were treated with red light for 5 min, 15 min, 30 min, and 2 h continuously; dark-grown, non-treated plants served as the control. Blots containing equal amounts of total protein probed with the ZmCCaMK antibodies showed that red light lowered the level of kinase with time (Fig. 6A) and very little amount of the ZmCCaMK could be detected after 2 h of red light treatment.

To check the intensity of red light required for this effect, the etiolated coleoptiles were treated with 25, 50, 75, and 100 μmol min⁻¹ of red light. As seen in Fig. 6B, red light at 75 and 100 μmol min⁻¹ intensity decreased the level of kinase in a concentration-dependent manner. Because red light lowered the level of ZmCCaMK, but no far-red reversibility was seen, the role of blue light was tested on the expression level of this kinase. A blue light kinetics was performed starting from 5 min to 15 min, 30 min and 2 h similar to red light kinetics. Equal quantities of protein separated on SDS-PAGE, blotted and probed with the ZmCCaMK antibodies showed no effect of blue light treatment (Fig. 7A) and the level remained same in dark and blue light-treated plants. No difference in the level of kinase could be seen at any of the intensities of blue light (Fig. 7B) confirming that the response is specifically mediated at a particular intensity of red light.

**Discussion**

Autophosphorylation is an important property of kinases reported both from the plants as well as animal systems. Ca²⁺ and CaM affect the autophosphorylation of kinases in different ways. In animal systems CaM kinases, both Ca²⁺ and CaM are required for autophosphorylation and this activity is inhibited by EGTA as well as by various CaM inhibitors. In plants, the calcium-dependent protein kinases (CDPKs) undergo autophosphorylation in a fully calcium-dependent manner. Although CDPK autophosphorylation activity is not affected by the addition of exogenous CaM, it can be inhibited by EGTA as well as various CaM inhibitors possibly due to the presence of CaMLD in these kinases (Harmon et al., 1987; Putnam-Evans et al., 1990; Dasgupta, 1994). The known CaM kinases from plants have some unique characteristics. In CCaMK from lily anthers, autophosphorylation
is Ca$^{2+}$-dependent/stimulated but, interestingly, CaM inhibits this activity in a concentration-dependent manner (Takezawa et al., 1996).

During purification of ZmCCaMK, the property of autophosphorylation was used to identify this kinase from the protein fractions eluted from a CaM Sepharose column (Pandey and Sopory, 1998). The kinase also showed autophosphorylation in a Ca$^{2+}$-dependent manner (optimum concentration 10 μM free calcium) like CDPKs. But in contrast to CDPKs and substrate phosphorylation activity of this CCaMK itself, CaM had no effect on autophosphorylation. Various CaM and kinase inhibitors tested on ZmCCaMK autophosphorylation activity also showed no effect. These data show that the autophosphorylation and substrate phosphorylation activities of ZmCCaMK are differentially regulated. Autophosphorylation of the kinase was at threonine residue(s), similar to animal CaM kinases and lily anther CCaMK whereas in CDPKs, autophosphorylation has been reported to occur at both serine and threonine residues (Putnam-Evans et al., 1990; Saha and Singh, 1995).

Like the animal system CaM kinases, ZmCCaMK substrate phosphorylation activity is regulated by its own phosphorylation. As clear by the time kinetics of both the reactions, the autophosphorylation is preceded by substrate phosphorylation activity. Autophosphorylated ZmCCaMK could also phosphorylate substrate in the presence of EGTA, showing no calcium requirement and pointing towards the possible mechanism of regulation of the kinase activity. ZmCCaMK is active in its autophosphorylated form for which calcium is the only requirement. After its activation with calcium, it can phosphorylate different substrates without the requirement of calcium. CaM probably binds to the autophosphorylated form of ZmCCaMK and either stabilize
it or somehow enhance the substrate availability. Further studies are required in this direction to discover how calcium and CaM are regulating the autophosphorylation and the 2-step substrate phosphorylation.

Apart from showing an interesting dual regulation of the activity by calcium and CaM, ZmCCaMK also showed a very specific regulation by light. Red light treatment for 5 min decreased the level of kinase protein, which could not be reversed by far-red light treatment. This loss of far-red light reversibility could either be due to the very fast nature of the response which is somehow escaping the reversal by far red light or it may be a PhB-mediated phenomenon. Red light treatment for different time periods decreased the level of the kinase in a time-dependent manner. The possibility that the long duration of red light treatment had a deleterious effect was ruled out; 5 min of red light immediately followed by 2 h dark incubation showed same level of the ZmCCaMK as 30 min continuous red light treatment.

In in vivo systems, in response to any signal such as light, the kinases and phosphatases play in concert with each other; affecting either of them could bring about the same response. The phytochrome (Pr form) after absorbing the red light gets converted to the Prf form and the phytochrome itself has recently been shown to be present in a phosphorylated form (Elich and Chory, 1997, and references therein). The phytochrome on absorbing light could either affect kinases or phosphatases to bring the same response, i.e. up-regulation of phosphorylation could either be due to an increase in activity/level of kinases or due to a decrease in activity/level of phosphatases and vice versa (Sopory and Munshi, 1998). Many pathways, which show an increase in phosphorylation, are reported, but the existence of a kinase that is down-regulated by red light shows the possibility of existence of other complex regulatory pathways as well.

Though the level of the ZmCCaMK decreases with red light, blue light had no effect on its level at all the time points and intensities studied. Low levels of ZmCCaMK in response to red light treatment could possibly be explained in the following ways. Light has been shown to affect the expression of a number of genes in both positive as well as negative ways (Neuhau et al., 1997). Such responses are generally very fast and a change in transcript level could be detected within 1–5 min. So this kinase could be one such example showing down-regulation by light and thus might be involved in the light-signalling pathway. Other possibilities could be a very fast turnover of the protein or by some kind of relicatalization in response to red light. Recently, a phospho-protein regulatory factor was shown to migrate to the nucleus following light irradiation (Wellmer et al., 1999). At present there is no proof in support of either of the possibilities and further studies are required to confirm anything conclusively.

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References


