Results

Protein Purification

Role of calcium as one of the most important second messengers in signalling pathways has been very well established. One of the ways calcium mediates these diverse signalling processes is via a phosphorylation/dephosphorylation cascade involving various kinases and phosphatases. Present study has been undertaken to study the role of calcium in plant signalling via phosphorylation reactions. Towards this goal a novel protein kinase has been purified and subsequently characterised from etiolated maize seedlings.

Purification of Ca^{2+}/CaM-stimulated protein kinase

A Ca^{2+}-dependent and CaM-stimulated protein kinase (ZmCCaMK) has been purified to homogeneity from 8 days old etiolated maize coleoptiles as described in the flow chart (Fig 1). The total soluble protein extract (~ 400 mg of total protein) was fractionated with 0-40%, 40-50% and 50-80% ammonium sulphate precipitation and dialysed extensively. All the precipitated protein fractions were analysed separately for Ca^{2+}-dependent kinase activity in presence and absence of calcium. Proteins precipitated by 40-50% ammonium sulphate showed a major calcium-dependent kinase activity compared to the proteins precipitated by 0-40% or 50-80% ammonium sulfate. This fraction containing ~135 mg protein was further analysed by separating different proteins based on their elution profile from the DEAE Sephacel column. Proteins bound to the column were eluted using a salt gradient of 0-0.4 M KCl. Every alternate fraction was assayed for Ca^{2+}-dependent protein kinase activity using histone IIIS as an exogenous substrate. Protein elution profile from the DEAE Sephacel column, Ca^{2+}-dependent kinase activity profile and SDS-PAGE electrophoretic profile of active fraction is shown in Fig 2. Approximately 20 fold purification of protein kinase activity was achieved at this step compared to the crude protein extract. Active fractions eluted from the DEAE
Sephacel column (~ 40 mg protein) were dialysed extensively in an EGTA free buffer and loaded on a CaM Sepharose affinity column in the presence of CaCl₂. The column was washed with washing buffer containing 1M NaCl, to remove all the proteins which bind non-specifically. Specifically bound proteins were step eluted with different concentrations of EGTA (0-4 mM). Each fraction was evaluated for a Ca²⁺-dependent as well as CaM-stimulated activity using bovine CaM (in nM concentration range) and histone IIIS as substrate. Proteins eluted with 2 mM EGTA showed a Ca²⁺-dependent/CaM-stimulated activity. Protein elution profile from CaM Sepharose column, Ca²⁺-dependent/CaM-stimulated activity profile and protein electrophoretic profile of active fraction on a 10% SDS-PAGE is shown in Fig 3. Approximately 115 fold purification was achieved at this stage.

Four major polypeptides eluted from the CaM Sepharose column with 2 mM EGTA, were eluted individually from a 10% SDS polyacrylamide gel. Their ability to phosphorylate histone IIIS as a substrate under in vitro assay conditions and autophosphorylation was checked separately. The 72 kDa polypeptide, which was the major protein eluted from the CaM Sepharose column showed autophosphorylation as well as histone phosphorylation activity under in vitro conditions in a Ca²⁺-dependent/CaM-stimulated manner. The yield and fold purification achieved is given in Table 1. A gel showing protein profile at different stages of purification is shown in Fig 4.

Purity and homogeneity of the protein

The purity of the protein was checked by running different amount of the eluted protein on a 12.5 % SDS-PAGE. 1-5 µg of gel eluted protein was separated on SDS-PAGE and silver stained. The eluted protein showed a single band of 72 kDa at all the concentrations checked (Fig 5) confirming that it is a single, homogenous polypeptide.
TABLE I

Purification protocol for ZmCCaMK from Zea mays

Protein extract at different steps of purification were used for protein quantification. Kinase assay was performed in presence of Ca\textsuperscript{2+} and Ca\textsuperscript{2+}/CaM and specific activity was calculated as described in "Experimental Procedures". Yield (%) and fold purification were calculated based on these values. Yield was calculated from the ammonium sulfate precipitated protein fraction onwards as the total activity of the crude protein extract and total soluble protein fractions was very less possibly due to presence of various phosphatases or kinase inhibitors in these fractions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Protein (mg)</th>
<th>Total activity (pmols/min)</th>
<th>Specific Activity (pmols/min/mg)</th>
<th>Yield (%)</th>
<th>Fold Purification</th>
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<tr>
<td>Crude Extract</td>
<td>412.086</td>
<td>24.642</td>
<td>0.0598</td>
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<td>1</td>
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<tr>
<td>Soluble Fraction</td>
<td>403.761</td>
<td>41.466</td>
<td>0.1027</td>
<td>-</td>
<td>1.75</td>
</tr>
<tr>
<td>Ammonium Sulfate</td>
<td>134.328</td>
<td>69.514</td>
<td>0.5175</td>
<td>100</td>
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<td>DEAE Fraction</td>
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<td>48.082</td>
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<td>CaM Sepharose</td>
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<td>6.8900</td>
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<td>114.80</td>
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<tr>
<td>Gel purified protein</td>
<td>0.075</td>
<td>1.165</td>
<td>21.534</td>
<td>1.6</td>
<td>358.83</td>
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</table>
Biochemical Characterization of ZmCCaMK

Substrate phosphorylation studies

Purified protein was checked for its activity to phosphorylate various substrates under *in vitro* assay conditions. Most of the studies were performed with histone IIIS and syntide-2 as substrates except in few experiments where dephosphorylated casein was also used.

Effect of different physiological factors: Effect of various physiological factors was checked on the activity of purified protein by its ability to phosphorylate histone IIIS as a substrate. *In vitro* assays were performed in presence of different physiological factors. As is clear by the autoradiogram (Fig 6A) and specific activity data (Fig 6B), in presence of 2 mM EGTA, the kinase activity decreased down to the basal levels, showing its complete calcium-dependency. Calcium at 1 μM concentration stimulated the kinase activity by ~6.5 folds. Calmodulin (at 100 nM concentration) in presence of 1 μM calcium, stimulated the kinase activity over and above calcium stimulated levels, bringing about 12 folds stimulation in comparison to without calcium control. In absence of calcium, calmodulin had no effect on kinase activity showing that the kinase is a calcium-dependent, CaM-stimulated kinase.

To check lipid-dependence of kinase, *in vitro* assays were also performed in presence of PS (1-25 μM) and PMA (10-100 nM). The autoradiogram (Fig 6A) and specific activity data (Fig 6B) show that both PS and PMA either alone or in combination, failed to stimulate kinase activity above the basal level. In assays performed with PS and PMA along with Ca²⁺, stimulation equivalent to the only Ca²⁺-dependent level was obtained.

A similar trend was obtained using syntide-2 as exogenous substrate but it was a more favoured substrate than histone IIIS. As is evident from the autoradiogram (Fig 7A)
Results

and specific activity data (Fig 7B), Ca\(^{2+}\) alone caused about 30 fold stimulation of the activity compared to basal level which is ~5 times higher than in case of histone IIIa. Addition of calmodulin stimulated the kinase activity ~62 folds above the basal level which is about 2.0 folds of Ca\(^{2+}\)-dependent stimulation. Besides it is very high (more than 5 fold) compared to the histone IIIa phosphorylation under similar conditions. PS and PMA had no effect on stimulation of enzyme activity either alone or in combination.

**Effect of pH:** Kinase activity was assayed in buffers of different pH values to determine the optimum pH value for the enzyme. Assay was performed with histone IIIa as substrate using citrate buffer for pH range 3-6 and Tris buffer for pH range 7-10. As shown in autoradiogram (Fig 8A) and specific activity data (Fig 8B), the kinase was active in pH range 6-8, and showed an optimum pH value of 7.5.

**Effect of magnesium:** As most of the phosphorylation reactions require presence of Mg\(^{2+}\) in the assay mixture, a range of Mg\(^{2+}\) concentration was used to determine the optimum value for Mg\(^{2+}\) in presence of Ca\(^{2+}\) and CaM. In assays performed with histone IIIa as substrate, the enzyme showed a complete dependence on Mg\(^{2+}\) showing optimum activity at 5 mM (Fig 9A). No activity could be detected in the absence of Mg\(^{2+}\).

**Effect of calcium:** Since the kinase has been purified on the basis of a Ca\(^{2+}\)-dependent activity, effect of varying concentrations of free Ca\(^{2+}\) was checked to determine the optimum value. Using the Ca\(^{2+}\)/EGTA buffer system a linear free calcium concentration ranging from 10 nM to 100 µM was used in the assay mixture using histone IIIa as substrate. Calcium was found to be essential for the activity of kinase and only basal activity could be obtained in absence of calcium. 1 µM free calcium was obtained as optimum value for kinase activity (Fig 9B), stimulating the activity by ~6.5 folds above basal level.
Results

**Effect of CaM:** Addition of exogenous CaM (in nM concentration range) stimulated the kinase activity over and above the Ca$^{2+}$ stimulated level with histone IIIS as substrate. As shown in Fig 9C, in presence of 1 μM free calcium, 100 nM CaM concentration was found to be optimum for maximum stimulation of kinase activity stimulating it ~ 12 folds above the basal level.

**Effect of CaBP:** As the kinase showed stimulation with CaM, another calcium-binding protein, *E. histolytica* CaBP which has very similar properties as CaM, was also checked for its effect on ZmCCaMK activity. CaBP concentration ranging from 0-250 nM did not stimulate the kinase activity over and above the Ca$^{2+}$ alone stimulated levels (Fig 9D), showing that the kinase is specifically stimulated by CaM and not by any other calcium-binding protein in general.

**CaM specific stimulation of kinase activity:** To further confirm that the stimulation of kinase activity by Ca$^{2+}$/CaM was fully Ca$^{2+}$ dependent, and specific to calmodulin, kinase assay was performed in presence of 2 mM EGTA (as control), 1 μM Ca$^{2+}$, 1 μM Ca$^{2+}$ and 100 nM CaM, 1 μM Ca$^{2+}$ and a 100 nM CaBP (Ca$^{2+}$ binding protein from *E. histolytica*) and Ca$^{2+}$/CaM with saturating concentration of moss CaM antibodies using histone IIIS, casein and syntide-2 as substrates. As is evident from the autoradiogram and the specific activity data (Figs. 10-12), for all the three substrates, the kinase activity was Ca$^{2+}$-dependent as EGTA decreased the activity, although the extent of stimulation was different in all the cases. The autoradiogram (Fig 10A) and specific activity data (Fig 10B) show that free calcium at 1 μM concentration stimulated the kinase activity to ~6.5 folds with histone as substrate, 2 folds with casein as substrate (Fig 11A and B) and ~30 folds with syntide-2 as substrate (Fig 12A and B). The stimulation by CaM was specific as CaBP, a protein similar to CaM from *E. histolytica*, did not stimulate the enzyme activity over and above the Ca$^{2+}$-stimulated level in any case. Addition of CaBP in presence of
calcium stimulated the kinase activity to calcium stimulated levels only whereas, addition of CaM stimulated the kinase activity over and above the Ca$^{2+}$-stimulated levels by 2-2.5 folds depending on the substrate used. Anti CaM antibodies raised against moss calmodulin also inhibited the kinase activity decreasing it down to the only Ca$^{2+}$-stimulated level with all the substrates. This data gave direct evidence that the maize kinase has a Ca$^{2+}$/CaM-stimulated kinase activity.

$^{45}$Ca$^{2+}$ binding and Ca$^{2+}$ mobility gel shift assay: As the kinase showed a Ca$^{2+}$-dependent activity, to show the direct binding of kinase with calcium; $^{45}$Ca$^{2+}$ binding and gel mobility shift on SDS-PAGE was done in presence of CaCl$_2$.

For binding of $^{45}$Ca$^{2+}$ to the enzyme, purified protein was run on a 10 % SDS-PAGE, transferred to nitro-cellulose membrane and incubated with $^{45}$Ca$^{2+}$ at a final concentration 1 mCi/litre. The membrane on autoradiography showed a clear signal at 72 kDa showing direct Ca$^{2+}$ binding to the protein (Fig 13) thus confirming that the kinase itself is a calcium binding protein.

Calcium-binding to proteins is known to cause a shift in mobility of protein on SDS-PAGE. As shown in Fig 14, addition of calcium caused a shift in mobility which was reversed by addition of EGTA, confirming that the shift is actually caused by interaction of protein with Ca$^{2+}$.

Kinetic studies using different substrates: Preliminary studies showed that ZmCCaMK phosphorylated different substrates with varying efficiency. For comparison, the Ca$^{2+}$-dependent and CaM-stimulated kinase activity was determined with various substrates i.e. with syntide-2, a specific CaM kinase substrate shown in animal system, histone IIIS and dephosphorylated casein. For kinetic studies, assays were performed with different amounts of all the three substrates and their Km and Vmax values were determined using the Michaelis-Menton equation and Lineweaver-Burk plot. The kinase phosphorylated syntide-2 with maximum efficiency showing a Km value of 3.3 μg and Vmax of 250.
pmols/minutes/mg in presence of Ca\(^{2+}\) and CaM (Fig 15) which was ~10 folds higher than that of histone. With histone as a substrate, kinase showed Km value 40 µg and Vmax value 25 pmols/minutes/mg, many folds less than syntide-2 (Fig 16). The kinase phosphorylated casein, but the efficiency was low as is clear by the comparison of Km and Vmax values (Fig 17). Although the Km value was a little lower compared to histone IIIS, the Vmax was also very low (8 pmols/minutes/mg protein). Km and Vmax values were also determined for γ\(^{-32}\)PATP (Fig 18). A comparison of Km and Vmax values for all the three substrates is given in Table 2.

**Effect of inhibitors:** Effect of different Ca\(^{2+}\)/CaM antagonists and kinase inhibitors was checked on ZmCCaMK activity using both histone IIIS and syntide-2 as substrates. As clear by autoradiogram (Fig 19A) and specific activity data (Fig 19B) EGTA decreased the kinase activity to the basal level in both the cases, showing complete calcium-dependence of kinase. Various CaM inhibitors as CPZ and TFP inhibited the CaM stimulated activity specifically, bringing it down to the Ca\(^{2+}\) alone stimulated levels. Histone phosphorylation decreased down from ~12 folds to ~5 fold with CPZ and 4 folds with TFP which was comparable to Ca\(^{2+}\) alone stimulated level i.e. ~6.5 folds. Similarly, syntide-2 phosphorylation decreased down from ~62 folds to ~26 folds with both CPZ and TFP which was comparable to the calcium stimulated levels. Other CaM kinase/CDPK specific inhibitors such as compound 48/80, staurosporine, W7 and compound KN-62 also inhibited the kinase activity bringing it down further, which was very less compared to the calcium alone stimulated levels itself, whereas H7 which is a specific inhibitor of calcium/phospholipid-dependent kinases had no effect on kinase activity as is evident from the autoradiogram (Fig 20A) and specific activity data (Fig 20B).

**Concentration kinetics with different inhibitors:** All the inhibitors were checked for their ability to inhibit the kinase activity in a concentration dependent manner and their IC
Kinase assay was performed with different substrates as described in "Materials and Methods" and Km and Vmax values were calculated on basis of Lineweaver-Burk plot.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km (µg)</th>
<th>Vmax (pmols/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syntide-2</td>
<td>3.3</td>
<td>250</td>
</tr>
<tr>
<td>Histone IIIS</td>
<td>40</td>
<td>29</td>
</tr>
<tr>
<td>Casein</td>
<td>18.2</td>
<td>8</td>
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</table>
50 values were determined using histone IIIS as substrate. EGTA inhibited the kinase activity in a concentration dependent manner with an IC 50 value of 1 mM (Fig 21A). CaM antagonists such as CPZ and TFP inhibited the kinase activity with IC 50 values of 100 µM and 75 µM respectively (Fig 21B, C). More specific inhibitors as KN-62, which is a very specific inhibitor of Ca²⁺/CaM-dependent kinases in animal systems, compound 48/80, W7 and staurosporine also inhibited kinase activity, in a concentration dependent manner with IC 50 values of 5 µg for compound 48/80 (Fig 21D), 2 nM for staurosporine (Fig 22A), 20 µM for KN-62 (Fig 22B) and 50 µM for W7 (Fig 22C). Compound H7 which generally inhibits PKC activity did not inhibit the ZmCCaMK activity (Fig 22D) at all the concentrations checked. Thus the inhibitor data provided more evidence of ZmCCaMK being a Ca²⁺/CaM-dependent kinase.

**Autophosphorylation studies**

**Autophosphorylation of purified kinase:** As autophosphorylation is an inherent property of the protein kinases so purified ZmCCaMK autophosphorylated in presence of Ca²⁺ as described in materials and methods. As shown in Fig 23A, the purified protein showed a single band on silver staining. Similar protein, on autophosphorylation showed presence of a 72 kDa phosphorylated band on autoradiogram (Fig 23B).

**Time kinetics of autophosphorylation and substrate phosphorylation activities:** Time kinetic studies of both substrate phosphorylation and autophosphorylation activities were performed with ZmCCaMK. Fig 24 shows that the autophosphorylation activity of the kinase was faster, attaining the saturation level at approximately 1 minute and then going down slightly over a period of 30 minutes. The substrate phosphorylation activity however, was comparatively slower, attaining the saturation level at 4 minutes and remained constant over a period of 30 minutes. This gives an indication that the
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autophosphorylation activity of the kinase is probably preceded by the substrate phosphorylation activity.

**Effect of different physiological factors:** Autophosphorylation of purified kinase was checked in presence of various exogenous physiological factors. As is clear from the autoradiogram (Fig 25A) and specific activity data (Fig 25B), the autophosphorylation activity was completely calcium-dependent and no activity could be detected in presence of EGTA. Addition of 10 μM calcium stimulated the autophosphorylation activity to the optimum level. But in contrast to substrate phosphorylation activity which was calcium-dependent and CaM stimulated, autophosphorylation activity was unaffected by addition of exogenous CaM at all the concentrations tested. CaM alone also had no effect on autophosphorylation activity. PS and PMA either alone or together failed to show any effect on autophosphorylation activity of ZmCCaMK, but in combination with Ca²⁺, a stimulation level equivalent to calcium alone stimulated levels could be achieved.

**Effect of pH:** Effect of pH on autophosphorylation activity of ZmCCaMK was determined using different pH buffers for autophosphorylation. As clear by autoradiogram (Fig 26A) and specific activity data (Fig 26B), the autophosphorylation was highly pH-dependent, and no activity could be detected below pH 5 and above pH 8. In contrast to substrate phosphorylation activity which shows a pH optimum of 7.5, autophosphorylation was more active in the acidic pH range showing an optimum value of pH 6 (Fig 26).

**Effect of inhibitors:** Effect of various Ca²⁺, CaM antagonists and kinase inhibitors was checked on autophosphorylation activity of ZmCCaMK. As is evident from autoradiogram (Fig 27A) and specific activity data (Fig 27B), EGTA inhibited the kinase activity almost completely. But, in sharp contrast to the substrate phosphorylation activity, which was inhibited to different extents with different CaM antagonists and kinase inhibitors, CaM
antagonists as CPZ and TFP, W7 as well as various kinase inhibitors as 48/80, H7, KN-62 and staurosporine had no effect on autophosphorylation activity of the kinase.

**Dual regulation of ZmCCaMK activity by Ca$^{2+}$ and CaM**

As the data showed differential regulation of autophosphorylation and substrate phosphorylation activities by calcium and CaM, both the autophosphorylation (Fig 28A) and substrate phosphorylation activities (Fig 28B) of ZmCCaMK were checked in presence of equimolar quantities of EGTA, Ca$^{2+}$ and CaM. The autoradiogram clearly showed a complete calcium-dependence of the kinase as both the activities were negligible in presence of EGTA. But, CaM affected the two activities differently, stimulating the substrate phosphorylation activity of the kinase over and above the calcium alone stimulated levels and showing no affect on autophosphorylation activity. This further confirms the dual regulation of kinase activity by Ca$^{2+}$ and CaM.

**Effect of autophosphorylation on substrate phosphorylation activity of ZmCCaMK**

It is known in animal systems, that autophosphorylation modulates the activity of CaM kinases and make them calcium-independent. This possibility was checked with ZmCCaMK, by phosphorylating histone IIIS using autophosphorylated and non-autophosphorylated purified protein in presence of EGTA. Using non-autophosphorylated kinase for assay, no substrate phosphorylation could be achieved in presence of 2 mM EGTA (Fig 29A), whereas if autophosphorylated kinase was used for assay, even in presence of 2 mM EGTA, histone IIIS phosphorylation could be achieved (Fig 29B). This thus showed that autophosphorylation induced calcium-independence in ZmCCaMK activity.
Stability of ZmCCaMK

Purified ZmCCaMK is not a very stable enzyme as on storage at -20°C the protein showed rapid autolysis. After storage at -20°C for more than two days, the polypeptide degraded first into two and then four polypeptides. On autophosphorylation, all these polypeptides gave signal on autoradiogram (Fig 30) showing either all of them have the autophosphorylation activity or some of the degradation products are being used as substrate for others.

Phospho amino acid analysis

The phosphoaminoacid analysis was done with autophosphorylated kinase as well as phosphorylated histone IIIS and syntide-2. The autoradiogram (Fig 31), showed that serine was the phosphorylated residue(s) in case of histone (1) and syntide-2 (2) however, the enzyme was autophosphorylated at threonine residue (3). This showed that the kinase belong to the serine/threonine family of protein kinases.

Immunological Characterization of ZmCCaMK

Raising antibodies and titration

Polyclonal antibodies were raised in 6 months old rabbits over a period of three months as given in Fig 32. Pre-immune serum was collected from the rabbits after keeping them for 15 days in animal house. Rabbits were injected first with 250 μg of protein and the boosters were given thrice at 15 days intervals with 100 μg of antigen (purified ZmCCaMK). After final bleeding of the rabbit, titre of antibodies was checked by ELISA. Formation of a coloured complex was detected up to 1: 100000 dilution on ELISA showing a very good titre of antibodies.
Results

Titre and specificity of antibodies was also checked on western blots using 10, 25, 50 and 100 µg of total maize protein extract and 1:5000, 1:10000 and 1:20000 dilution of antibodies. The antibodies cross-reacted with total maize protein extract to give a single band at 72 kDa at all the concentrations of protein checked and all the dilution of antibodies used whereas no signal could be obtained with pre-immune serum (Fig 33), proving that the antibodies were monospecific.

Cross-reactivity of ZmCCaMK antibodies with purified kinase

As the antibodies cross-reacted with total protein to give a single band, there cross-reactivity was also checked with purified ZmCCaMK. Western blotting with 1 µg of purified kinase using 1:20000 dilution of antibodies showed presence of a single band of 72 kDa (Fig 34).

Immuno-precipitation of kinase activity by antibodies

ZmCCaMK antibodies were checked for their activity to precipitate down ZmCCaMK activity at both substrate phosphorylation and autophosphorylation level. As seen by the autoradiogram and specific activity data, antibodies precipitated both the activities. Precipitation of substrate phosphorylation was checked using histone IIIS as substrate. As is evident from the autoradiogram (Fig 35A) and specific activity data (Fig 35B), the antibodies precipitated this activity very effectively as 1 µl of antibodies could decrease the activity by ~80%. Addition of more antibodies decreased the kinase activity to the basal levels. The antibodies precipitated this activity specifically as no effect could be seen using either pre-immune serum or a non relevant antibody, Brassica glyoxalase I (Fig 36).

Autophosphorylation activity of the kinase was also inhibited by ZmCCaMK antibodies in a similar manner. Addition of 1 µl of antibodies could decrease the autophosphorylation activity by 20 % and 80 % inhibition of the activity could be achieved with 10 µl of antibodies. Addition of more antibodies completely inhibited the
autophosphorylation activity of the kinase as seen by the autoradiogram (Fig 37A) and specific activity data (Fig 37B). Higher amount of antibodies required to precipitate down autophosphorylation activity of ZmCCaMK is in accordance with the amount of protein used for autophosphorylation (1 μg) compared to substrate phosphorylation (100 ng).

**Precipitation of rat brain CaM kinase activity by ZmCCaMK antibodies**

Rat brain is a very rich source of CaM kinase so an attempt was made to precipitate a CaMK activity in brain extract using ZmCCaMK antibodies. *In vitro* phosphorylation assays performed with total protein extract from rat brain in presence of EGTA and Ca$^{2+}$, showed presence of some specific Ca$^{2+}$-dependent phosphorylations on SDS-PAGE. Addition of moss CaM antibodies to assay mixture, inhibited activity of a 54 kDa polypeptide specifically. Addition of exogenous CaM to the same assay mixture restored the phosphorylation of that polypeptide confirming that it was a CaM specific kinase phosphorylated polypeptide (Fig 38). Addition of ZmCCaMK antibodies in assay mixture inhibited the phosphorylation of this 54 kDa polypeptide, showing that ZmCCaMK antibodies could block the activity of rat brain CaM kinase (Fig 38).

**Detection of immuno-homologue of ZmCCaMK in other plants**

Using ZmCCaMK antibodies, presence of immuno-homologue of ZmCCaMK was checked in other plant species as well. Total protein extracts of various monocot as well as dicot plants as *Triticum aestivum, Oriza sativa, Sorghum bicolor, Brassica juncea and Arabidopsis thaliana* cross reacted with ZmCCaMK antibodies to give a single band at 72 kDa position (Fig 39) on the western blot. This showed that the kinase possibly had a ubiquitous presence.
**Results**

*Distribution pattern of ZmCCaMK in different plant parts*

As ZmCCaMK was purified from coleoptiles of maize plants, its presence was checked in other plant parts as well. Western blot of protein extract from leaf, hypocotyl, stem and roots of maize showed that the kinase has differential expression in different plant parts tested (Fig 40). Leaves showed maximum expression of kinase while the level was minimum in roots. The stem portion and hypocotyl also expressed lesser amount of protein compared to leaf. This study also showed that although the level of expression of this kinase was different in different plant parts, it is not localized specifically to a particular tissue or organ.

*Developmental expression of ZmCCaMK*

Expression of ZmCCaMK was also studied with different developmental stages of maize seedlings. Probing total protein extract of maize seeds (taken as 0 days), 3 days, 5 days, 8 days and 10 days old maize coleoptiles with ZmCCaMK antibodies showed an age dependent expression of ZmCCaMK. Seeds had very low level of protein, followed by 3 days old coleoptiles. By 5 days the level of protein was high which remained constant up to 10 days (Fig 41).

*Effect of stress on ZmCCaMK*

As expression level and activity of a number of kinases is effected by different stress treatments, effect of different stresses was checked on ZmCCaMK expression levels. Etiolated maize plants were given different stress treatments in dark itself. Temperature stress was given by placing the 7 days old plants at 42° C for 24 hours for heat shock and at 4° C for 24 hours for cold shock. 8 days old plants grown at 25° C served as control. Osmotic stress was given by transferring 5 days old etiolated plants in 0.3 M mannitol solution for 3 days while 0.3 M NaCl solution was given to 5 days old plants for 3 days to give salt stress. 8 days old water grown plants served as controls. Equal amount
of proteins separated on SDS-PAGE were probed with ZmCCaMK antibodies. The blot showed no difference in the level of ZmCCaMK, compared to the control (Fig 42), depicting no effect of stress on ZmCCaMK expression.

**Effect of light on ZmCCaMK**

Light is one of the main environmental signals which effect the growth and development of plants at every stage. Light regulates various processes via changing the phosphorylation pattern of some proteins. Besides, ZmCCaMK was purified from etiolated maize coleoptiles, it was all the more important to see the effect of light on expression level of this kinase.

8 days old etiolated cut coleoptiles were given different light treatments (as described in materials and methods) and non-treated etiolated, cut coleoptiles served as control. Equal quantity of total protein extract of all the treatments was separated on SDS-PAGE, blotted and probed with ZmCCaMK antibodies. The blot showed a clear difference in expression level of the kinase with different light treatments. Red light treatment for 5 minutes 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. This lack of red/far-red reversibility showed that this red light regulated response was not mediated by phytochrome. Far red light alone had no effect on level of kinase. Incubating 5 minutes red light treated coleoptiles in dark for 2 hours decreased the kinase level further down. White light treatment for 5 minutes also decreased the ZmCCaMK level (Fig 43).

As red light was decreasing the ZmCCaMK level a time kinetics of red light treatment was performed. Etiolated maize coleoptiles were treated with red light for 5 minutes, 15 minutes, 30 minutes and 2 hours continuously and dark grown, non-treated plants served as control. Blot containing equal amount of total protein probed with ZmCCaMK antibodies showed that red light lowered the level of kinase with time period
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(Fig 44) and very little amount of ZmCCaMK could be detected after 2 hours of red light treatment.

As red light lowered the level of ZmCCaMK effect of blue light was also tested on the expression level of this kinase. A blue light kinetics was performed starting from 5 minutes to 15 minutes, 30 minutes and 2 hours similar to red light kinetics. Equal quantity of protein separated on SDS-PAGE, blotted and probed with ZmCCaMK antibodies showed no effect of blue light treatment (Fig 45) and the level remained same in dark and blue light treated plants.

Effect of hormones on ZmCCaMK

As ZmCCaMK was down regulated by red light and light and hormone signals might transduce via some common messengers, an attempt was made to study the role of different hormones on ZmCCaMK level. 6 days old, dark grown plants were treated with IAA, BAP, GA and ABA. Water grown plants served as control. Probing equal amount of total soluble protein extract separated on SDS-PAGE and blotted on nitro-cellulose did not show any major change in level of ZmCCaMK in comparison to control plants (Fig 46) in all the cases except ABA. Like red light, ABA also decreased the level of ZmCCaMK compared to the control plants.

Molecular Characterization of ZmCCaMK

As ZmCCaMK turned out to be a novel protein kinase having properties similar to both plant CDPKs and animal system CaM kinases an attempt was made to fish out the gene for it and characterise it further. For this three different cDNA libraries were screened using the ZmCCaMK antibodies. To eliminate the possibility of cross reactivity
of ZmCCaMK antibodies with bacterial host cells, the antibodies were pre-treated with host cell lysate. As shown in Fig 47, both XL-1 Blue and Y-1090 cross reacted with antibodies to give several bands. Using lysate treated antibodies, no signal could be detected on the blot with host cell total protein whereas maize total protein extract gave a single band.

**Screening of Sorghum cDNA library**

Five days old, light grown *Sorghum bicolor* leaves cDNA expression library, constructed in λ ZapII vector, showing a titre of $10^8$ pfu/ml was screened with ZmCCaMK antibodies. A total 3.8x10^5 plaques were screened for the primary screening using conditions standardised for western blotting. Out of these only one plaque showed cross-reactivity with the ZmCCaMK antibodies. This plaque was picked up, amplified and proceeded further up to fourth round of screening till a homogenous populations of the plaques was obtained. Homogeneity was confirmed where all the descendent plaques gave positive signal in the final round of screening (Fig 48). pBluescript containing the insert was excised *in vivo* from this phage stock by infecting with helper phage and recombinant colonies were selected on ampicillin containing growth media.

The insert was excised by digesting the plasmid with EcoR1 and Xho1, and separated on 0.8% agarose gel. On excision a 0.8 kb insert was obtained (Fig 49). This clone was named as *pSbSp1*. The plasmid was digested with several restriction enzymes to map the sites on the insert but no site for commonly used restriction enzymes could be detected.

**Sequencing of the *pSbSp1* clone and homology search**

The 0.8 kb insert was sequenced using T7 and T3 primers from both the ends using the Sanger’s dideoxy method for sequencing. The sequence was further confirmed by automated sequencing. The complete nucleotide sequence (812 bp) of the *pSbSp1* is
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given in Fig 50. Analysis of the sequence was done by various available softwares. At the DNA level the clone shows some homology with few calcium-binding proteins. The translated putative peptide sequence consists of 270 amino acids and shows some homology with dual specificity serine/threonine kinases from yeast and mammalian system at the amino acid level. Comparison of nucleic acid and amino acid sequence with the available data bases is given in Fig 51.

Screening of maize cDNA library

A maize cDNA library constructed in λ Zap II vector using mRNA isolated from 8 days old etiolated maize coleoptiles (10^9 pfu/ml) was screened using the ZmCCaMK antibodies. A total 4x10^5 plaques were screened for the primary screening using conditions standardised for western blotting. Out of these, 9 plaques cross-reacting with the antibodies were picked up, amplified and proceeded for further screening. All these plaques were followed separately up to third and fourth round of screening till a homogenous population of the plaques was obtained. Homogeneity was confirmed where all the descendent plaques gave positive signal (Fig 52).

pBluescript containing the insert was excised in vivo from all the nine phage stocks by infecting with helper phage and recombinant colonies were selected on ampicillin containing growth media.

The insert was excised from all the 9 clones and separated on 0.8 % agarose gel. In all the 9 cases, a 2.5 kb insert was obtained. Out of all these clones, clone #9, named as pZmSp9 was taken for further studies.

Restriction mapping of pZmSp9

Plasmid DNA was extracted out from pZmSp9 and 5 μg of plasmid DNA was digested with different restriction enzymes and separated on 1.2% agarose gel. A
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restriction map was made based on the sites of different enzyme present on the cloned fragment (Fig 53).

Sequencing of the ZmSp9 and homology search

Using the Sanger's dideoxy method for sequencing, 1.3 kb of ZmSp9 was sequenced from both the ends with T7 and T3 primers and confirmed by automated sequencing. Fig 54, shows an representative autoradiogram for the sequencing reaction. Partial nucleotide sequence of the clone is given in figure (Fig 55). Analysis of sequence was done by various available softwares. Partial sequence of pZmSp9 shows homology various reported HSP70s from plant systems. Comparison of nucleic acid and amino acid sequence with the available data bases is given in Fig 56.

Screening of Brassica library

*Brassica juncea* cDNA library constructed from 7 days old, 0.3 M mannitol treated plants was screened for presence of ZmCCaMK using the antibodies. 7.2x10^5 plaques were screened for the primary screening but no positive signal could be obtained.

Amino Acid Sequencing of Purified ZmCCaMK

Purified ZmCCaMK (5 μg) was electroblotted on PVDF membrane and used for sequencing. The protein after digestion with trypsin showed 17 fragments on HPLC (Fig 57). Out of this fragment # 7 of molecular weight 14.746 kDa was sequenced (Fig 58). The sequence analysis showed it consists of 13 amino acid residues viz. Thr-Thr-Pro-Ser-Tyr-Val-Ala-Phe-Thr-Asp-Ser-Glu-Arg. Database analysis shows it matches 100% with a tryptic peptide derived from heat shock protein 70 (HSP70). Results of blast analysis is shown in Fig 59.