Plant cells receive various signals via a change in concentration and activity of a large number of second messengers as IP$_3$, cGMP, cADPR, cAMP, phospholipids and calcium, all of which participate in the transduction of a variety of signals. Calcium ions are one of the most widely accepted second messengers which couple a large number of diverse stimuli to their characteristic responses in a very specific and precise manner. This specificity is probably provided by the whole network of cellular calcium signalling, at every level and by the interaction of all the factors involved in the signalling cascade. These include the receptors, the spatial and temporal pattern of calcium distribution, phosphorylation status and activity of various protein kinases and phosphatases with which calcium interacts, along with the developmental stage of the plant and the environment it is growing in.

The information encoded in the calcium signals can be de-coded downstream 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 pathway, working either independently or jointly. Thus all these components i.e. CaM, CaM-binding proteins and calcium-dependent kinases, interact with each other at different levels and form a very complex network, which may be required for the multidimensional role played by calcium in plant signalling.

Calcium is known to regulate three different families of protein kinases i.e. calcium-dependent kinases (CDPKs), the Ca$^{2+}$/phospholipid-dependent protein kinases and Ca$^{2+}$/calmodulin-dependent protein kinases. CDPKs are a family of plant specific protein kinases which require only calcium for their activation. Ca$^{2+}$/CaM-dependent kinases and Ca$^{2+}$/phospholipid-dependent kinases are very well characterized in animal systems, but in plants, existence of these two families of kinases is shown by presence of a limited number of cDNA homologues only and they are not very well characterized at the biochemical level (Stone and Walker, 1995).

Ca$^{2+}$/CaM-dependent kinases are the main transducers of various calcium signals in animal systems and a number of recent reviews have appeared emphasising the important
role played by these kinases (Hanson and Schulman, 1992; Braun and Schulman, 1995; Hidaka and Yokokura, 1996; Picciotto et al., 1996; Soderling, 1996). CaM regulates different families of kinases in animal systems, some of them are multifunctional as CaMK II, CaMK IV and CaMK Ia/Ib and they phosphorylate a variety of substrates in vivo. Others are more dedicated and phosphorylate specific substrates as myosin light chain kinase (MLCK) whose primary task is to phosphorylate the 20 kDa light chain of myosin or CaMK III, which regulates protein synthesis via phosphorylation of elongation factor-2 (eEF-2). In plant systems, main transducers of calcium signals known till date are kinases from CDPK family, but presence of calmodulin in plants, its induction in response to various stimuli (Jena et al., 1989; Braam et al., 1992), and evidence for presence of calmodulin-dependent kinases (Watillon et al., 1992; 1993; 1995; Lu et al., 1996; Takezawa et al., 1996) suggests the possibility of involvement of these CaM kinases in a novel signalling pathways parallel to CDPK mediated signalling.

The present study has been undertaken to inquest the Ca\(^{2+}\)/CaM-dependent signalling pathways in plants. Towards this goal, as an initial effort, a novel Ca\(^{2+}\)-dependent, CaM-stimulated kinase has been purified and characterized from maize. Besides, antibodies raised against the purified kinase have been used to study the distribution, localization and expression pattern of this kinase. Finally, an attempt has been made to clone the gene for it and characterize it further.

**Purification of ZmCCaMK**

A large number of CaM kinases have been purified from the animal systems; but when we started this work there was no report for the purification and biochemical characterization of a CaM kinase from plant systems, except a few studies where existence of CaM kinases has been shown at the cDNA level (Watillon et al., 1992; 1993; 1995). Till now only one report is there for the biochemical characterization of a CaM kinase from plants. A cDNA isolated from lily anthers showing homology to existing CaM
Discussion

kinases from animal systems has been over expressed in *E. Coli.* and biochemically characterized (Patil et al., 1995; Takezawa et al., 1996).

In animal systems, CaM kinases have been purified from various tissues such as brain, which is the richest source of CaM kinases (Goldenring et al., 1983; Ohmstede et al., 1989; Mochizuki et al., 1993; Okuno et al., 1994), spleen (Sato et al., 1990), smooth muscles (Ikebe et al., 1990), skeletal muscles (Woodgett et al., 1983), liver (Payne et al., 1983; Schworer et al., 1983; Sorge et al., 1985), lung (Schulman et al., 1985), heart (Iwasa et al., 1986) and retina (Cohen et al., 1987). These kinases have been purified from both soluble as well as membrane fractions (see Schulman, 1988). Homogenous preparation of CaM kinases have been obtained in different cases using a combination of ammonium sulfate precipitation and various chromatographic steps depending on the system used. But in most of the cases, the primary strategy has been to first concentrate the kinase activity using fractionation by ammonium sulfate, gel filtration, ion exchange (DEAE Sepharose or DE-52) and phosphocellulose columns. The fractionated proteins which show kinase activity have been invariably passed through a CaM Sepharose affinity column. The proteins bound to the column in a calcium-dependent manner were eluted from the column with EGTA, ranging from 1-2 mM in different preparations. The proteins eluted from the CaM Sepharose column have been purified to homogeneity using a number of matrices as hydroxyapatite, blue sepharose or Sephacryl S300. A similar protocol has been followed for purification of a CaM kinase from *Dictyostelium* where a combination of Q Sepharose and CaM Sepharose columns have been used (Dunbar and Wheldrake, 1994). For the purification of lily anther CaM kinase (CCA MK), the proteins over expressed in *E. Coli* were fractionated by 50% ammonium sulfate and then purified using CaM Sepharose affinity column (Patil et al., 1995; Takezawa et al., 1996).

Taking clues from the purification of CaM kinases from animal systems, we standardised a protocol for the purification of CaM kinase (ZmCCaMK) from maize. In the crude extract and the soluble fractions of total proteins, a very low total activity was detected. This could possibly be due to the presence of some phosphatases, kinase inhibitors or specific proteases. The total soluble protein extract was fractionated using
Discussion

ammonium sulfate precipitation and all the fractions were assayed. The protein fractions showing a major calcium-dependent kinase activity were further fractionated on DEAE Sephalcel column to enrich the kinase activity in particular fractions, before loading it on an affinity column. At this stage approximately 20 fold purification of kinase activity could be achieved compared to the crude protein extract. Till this step effect of CaM could not be taken into account as a large number of proteins were present in all the fractions and effect of addition of exogenous CaM could not be interpreted clearly.

To isolate a CaM-dependent kinase from this pool of Ca$^{2+}$-dependent kinases, the proteins were loaded on a CaM Sepharose affinity column in a calcium-dependent manner. Specific binding of proteins with column was ensured by washing the column with buffer containing 1 M NaCl. This washing step would elute all the non-specific and weakly bound proteins from the column. Proteins specifically bound to the CaM Sepharose column were eluted with EGTA and all the fractions were analysed for CaM kinase activity. Proteins eluted with 2 mM EGTA could phosphorylate histone IIIS in a calcium-dependent/CaM-stimulated manner under in vitro assay conditions showing the possibility of presence of a CaM-binding protein with calcium-dependent kinase activity. At this stage ~115 fold purification of kinase activity was obtained compared to the crude protein extract.

Though this pool of proteins eluted from the column had a calcium/CaM-dependent activity, SDS-PAGE profile of this fraction showed presence of 4 polypeptides and we did not know which amongst the four proteins eluted out was the kinase. To determine this, all the polypeptides were eluted individually from the gel according to the method followed by Harmon et al. (1987), and autophosphorylated in presence of Ca$^{2+}$/CaM. The 72 kDa polypeptide, which was the major polypeptide eluted from the CaM Sepharose column showed autophosphorylation giving evidence about it could be the active protein kinase in the eluted fraction. Other proteins with molecular weights 78 kDa, 82 kDa and 54 kDa, which were eluted from the column could be some other CaM-binding proteins however, their nature and properties have not been evaluated in the present study and we concentrated only on the 72 kDa polypeptide having
autophosphorylation activity. Since this polypeptide was eluted from SDS-PAGE, we checked if it retained its activity. *In vitro* assays performed with Histone IIIS as substrate showed the kinase gets renatured during the process of elution and retained its activity. Thus we could get a homogenous and active kinase preparation after elution of protein from SDS-PAGE. Besides, the gel elution of kinase at the final step of purification assured that the eluted protein preparation did not have contaminating CaM present with it. Purity and integrity of this eluted protein was checked on re-running the proteins on SDS-PAGE and silver staining the gel. At all the concentrations, the protein showed presence of a single band confirming the purity and homogeneity of the kinase. The only drawback of this method was that the recovery of the proteins after gel elution was only 30-40%. This thus led to a low yield of the purified kinase at the final stage of purification. We could obtain 1.6% yield compared to the most of the protocols followed in animal systems where 0.4-20% yield has been reported. The fold purification achieved at the final stage was ~ 360 folds compared to animal system preparations where 400-20,000 fold purification of the kinase has been reported from various preparations. The low value fold purification and yield is due to the fact that the activity data obtained at the crude extract and total soluble protein extract step were very less.

**Biochemical Characterization of ZmCCaMK**

Gel purified ZmCCaMK was used for phosphorylating various substrates under *in vitro* assay conditions, to biochemically characterize it.

**Effect of various physiological factors on ZmCCaMK activation**

Kinases are affected by a large number of physiological parameters and are classified on the basis of factors required for stimulation of their activity. Calcium is one of the most important factors affecting the kinase activity, either alone or in combination with CaM/CaBPs or lipids. In animal systems, Ca$^{2+}$ along with CaM regulates many kinases,
which are the main transducers of signalling processes. These kinases form a very well characterized family of CaM kinases. In plant systems Ca\(^{2+}\) alone regulates a large group of kinases belonging to the CDPK family. CDPKs are similar to CaM kinases in some of their properties like absolute calcium-dependence and inhibition of activity by CaM inhibitors. CDPKs require \(\mu\)M range of calcium for their activation (Harmon et al., 1987; Li et al., 1991; Das-Gupta et al., 1994; Nakamura et al., 1995; Saha and Singh, 1995; Stone and Walker, 1995). In some cases, however, millimolar concentration range is also reported (Polya et al., 1987; Michiello et al., 1989). Addition of very high concentration of CaM also could not stimulate the CDPK activity above the Ca\(^{2+}\) stimulated level. In contrast, CaM kinases do not respond to Ca\(^{2+}\) alone and CaM in nM range concentration is always required for their activity. Various studies have shown that calcium concentration varying from 0.5-1 \(\mu\)M, which is low compared to CDPKs, and CaM concentration ranging from 25-100 nM is the optimum for activation of these kinases (Sato et al., 1990; Mochizuchi et al., 1992; Rostas and Dunkley, 1992; see Harmon and Schulman, 1992 and references therein).

As ZmCCaMK has been purified on the basis of a calcium-dependent kinase activity and a specific binding to the CaM Sepharose affinity column we expected it to be a Ca\(^{2+}\)/CaM-dependent kinase. This fact was confirmed by performing assays in presence of EGTA, Ca\(^{2+}\) and CaM either alone or in combination. Preliminary studies showed that the kinase activity is fully calcium-dependent, and only basal level of activity could be achieved in presence of EGTA.

Comparison of properties of ZmCCaMK with CDPKs and CaM kinases show that it gets activated by Ca\(^{2+}\) alone like CDPKs and the calcium requirement is 1 \(\mu\)M which is comparable to the CDPKs and CaM kinases. ZmCCaMK, besides showing total calcium dependence for its activity, is a calcium-binding protein as was shown by direct \(^{45}\)Ca\(^{2+}\)-binding studies (see Fig. 13) and calcium induced mobility shift on the gel, which could be reversed by addition of EGTA (see Fig 14). This property of ZmCCaMK is similar to CDPKs which also show direct calcium binding whereas it is not the case with CaM kinases. But in contrast to CDPKs, 100 nM CaM (a concentration range falling well
within the animal systems CaM kinases) affected the ZmCCaMK activity. CaM alone had no effect on ZmCCaMK activation, but along with Ca\(^{2+}\) it increased the activity more than 2-2.5 folds in comparison to calcium, depending on the substrate used. Addition of PS and PMA, the stimulators of Ca\(^{2+}\)/lipid-dependent kinases had no effect on ZmCCaMK activity either alone or in presence of calcium. Although we have not checked ZmCCaMK for direct CaM-binding, it is one of the proteins very tightly bound to the CaM Sepharose column, in a calcium-dependent manner. As the protein remained bound to the column even after washing with 1 M NaCl containing buffer it suggests that the binding is not due to ionic or any other weak interactions but possibly because of presence of a CaM-binding domain in the kinase. Thus ZmCCaMK seems to have both Ca\(^{2+}\)-binding and CaM-binding domains.

Mg\(^{2+}\) is another divalent cation, besides Ca\(^{2+}\), which was required for the activity of ZmCCaMK. Though absolutely essential, it could not stimulate the kinase activity above the basal level in absence of Ca\(^{2+}\) and CaM. In absence of Mg\(^{2+}\) however, no stimulation of kinase activity could be obtained even at the optimum concentration of Ca\(^{2+}\) and CaM. The necessity of Mg\(^{2+}\), even though it is not stimulating the kinase activity shows that it might be involved in the phosphorylation reactions itself, as it is the case with most of the ~P transfer reactions. All these properties of ZmCCaMK thus show that it might be a dual specificity kinase, showing similarity to both CDPKs as well as CaM kinases.

**CaM specific stimulation of kinase activity**

In animal systems, the CaM kinases do not show any stimulation with Ca\(^{2+}\) alone, and CaM is absolutely essential for the activity. In plant systems, some earlier reports related to stimulation of kinase activity by micromolar concentration of CaM (Blowers et al., 1985) turned out to be non-specific as this effect could be achieved with other proteins like BSA (Harmon et al., 1987). Besides, CDPKs have a CaM like domain present which also make the interpretation of results difficult. To ensure that the stimulation of
ZmCCaMK activity by CaM is specific, two strategies were followed. In vitro assays performed in presence of *E. histolytica* CaBP (EhCaBP), a protein very similar to CaM (66% homologous to *E. histolytica* CaM, and *Petunia hybrida* CaM gene, and 72% with *Solanum tuberosum* gene), yet showing different biochemical properties. When added in place of CaM, the EhCaBP did not show any stimulation over and above that was obtained with Ca$^{2+}$ alone. This confirmed that the stimulation of ZmCCaMK by CaM is specific and other CaM like proteins or calcium-binding proteins could not replace the CaM requirement. To further confirm this we used CaM antibodies to block this CaM specific stimulation. As shown in the result (see Fig 10, 11 and 12), CaM antibodies did block the CaM mediated stimulation to the level equal to that achieved with Ca$^{2+}$ alone. Thus it is clear that the activation of ZmCCaMK activity is a two step process. At the first step, it gets activated/stimulated with Ca$^{2+}$ and then CaM further stimulates this Ca$^{2+}$ dependent activity. This trend was obtained with all the three substrates tested in this study viz. syntide-2, histone IIIS and casein. With all the substrates used, calcium stimulated the kinase activity differentially and CaM enhanced this Ca$^{2+}$-dependent phosphorylation to 2-2.5 folds. Based on this two step activation with calcium and CaM, ZmCCaMK could be categorized as a novel plant kinase showing dual specificity. The CCaMK from lily anthers, also showed dual regulation by Ca$^{2+}$/CaM however, this was shown in context of autophosphorylation and substrate phosphorylation (Takezawa et al., 1996).

**Kinetic studies with ZmCCaMK**

In animal systems the CaM kinases are classified on the basis of their substrate specificity and except the MLCK and CaMK III, all the other classes of CaM kinases are multifunctional and phosphorylate a number of substrates with varying efficiency (see Braun and Schulman, 1994). Depending on the substrate used, different mechanisms have been proposed for the kinetic properties of CaM kinases e.g. using casein or MAP-2 (microtubule associated protein-2) as substrates, a rapid equilibrium random bi bi mechanism has been identified with rat brain kinase (Kuret and Schulman, 1984; Katoh
Discussion

and Fujisawa, 1991) whereas with syntide-2 as substrate, a rapid equilibrium ordered bi bi mechanism has been identified (Kwiatkowski et al., 1990).

Preliminary studies using different substrates showed that the ZmCCaMK does not phosphorylate any particular substrate but different substrates were phosphorylated with varying efficiency in a Ca\(^{2+}\)-dependent and CaM Specific manner. Syntide-2, which is a specific peptide substrate used routinely for animal Ca\(^{2+}\)/CaM kinase assays, was phosphorylated with maximum efficiency compared to histone IIIS and casein. The fact that ZmCCaMK phosphorylates syntide-2 most efficiently gives further evidence for it being similar to animal system CaM-kinase homologues.

In the present study we found that all the three substrates are phosphorylated by ZmCCaMK in a Ca\(^{2+}\)/CaM-dependent manner and the Km values for all the substrates including ATP are comparable with animal systems. With CaM kinase II as well as with CaMK IV from different sources, syntide-2 was phosphorylated with a Km value ranging from 4-20 $\mu$M (Mukherji and Soderling, 1994 and references therein). The Km value for syntide-2 with ZmCCaMK was 3.3 $\mu$g corresponding to 20 $\mu$M is comparable to animal systems kinases. This was also the case with histone IIIS and casein. For ATP also, a Km value 75 $\mu$M was obtained which is comparable to animal system CaM kinases where it ranges from 50-150 $\mu$M (Sato et al., 1990; Mukherji et al., 1994; Okuno et al., 1994).

While Km values are comparable, Vmax value for ZmCCaMK are many folds lower than animal CaM kinases varying from 200-250 pmols/min/mg with all the substrates tested. With animal system CaMK II, Vmax value is generally reported to be between 4-20 $\mu$M/min/mg protein (Mukherji and Soderling, 1994 and references therein). With CaM kinase IV also, though the Vmax value is generally low, ranging between 150-450 nM/min/mg protein (Okuno et al., 1994; Tokumitsu et al., 1994) it is still higher than ZmCCaMK. This fact shows that though the rate of reaction is slow with ZmCCaMK, compared to animal systems, the affinity of ZmCCaMK for substrates is comparable.
Effect of inhibitors on ZmCCaMK activity

A number of kinase inhibitors are known which either generally or specifically inhibit the activity of various kinases. Ca²⁺/CaM-dependent kinases are inhibited by different families of inhibitors/antagonists (see Hanson and Schulman, 1992). Calcium chelators such as EGTA invariably inhibit the activity of all the calcium-dependent kinases both from animal systems and plant systems and in most of the cases only basal level of activity could be obtained in the presence of EGTA. Besides, a variety of CaM antagonists and CaM-binding peptides also inhibit the CaM kinase activation. These include compounds like chlorpromazine (CPZ), neuroleptics as trifluoperazine, miconazoles as calmidazolium and napthalenesulphonamides as W7. Inhibitors designed to interact with ATP-binding site, as staurosporine are also effective but not as selective for CaM kinases. Isoquinolinesulfonamides such as H7 also inhibit the activity of CaM kinases but these have higher affinity for PKC than for CaM kinases. Other compounds which bind directly to the kinase also act as inhibitors as KN-62 which is a very specific inhibitor of CaM kinases. This compound binds to some unidentified site on CaM kinase and interferes with CaM binding (Tokumitsu et al., 1992), though it does not inhibit the autophosphorylation activity of the kinases. Besides these, synthetic peptides derived from the autoinhibitory sequence of the CaM kinases also act as potent and selective inhibitors of CaM kinases in animal systems (see Braun and Schulman, 1994). In plant systems, although CDPKs show no stimulation with addition of exogenous CaM, these also show inhibition with CaM antagonists as these inhibitors might interact with the CaMLD (CaM like domain) present in these kinases.

ZmCCaMK activity was also checked in the presence of different Ca²⁺/CaM antagonists and kinase inhibitors. As discussed earlier, 2 mM EGTA completely inhibited the activity showing a complete calcium-dependence of the kinase. CaM inhibitors such as 48/80, CPZ, TFP and W7 also inhibited the ZmCCaMK activity with IC₅₀ values of 5 μg, 100 μM, 75 μM and 50 μM respectively which is comparable to the IC₅₀ values obtained in case of various animal and plant kinases. All these compounds decreased ZmCaMMK activity to the Ca²⁺ alone stimulated level. This fact again showed that the kinase activity
though CaM-stimulated was mainly regulated by Ca$^{2+}$, and CaM is not essential for its activity. Kinase inhibitors such as staurosporine and KN-62, a very specific inhibitor of animal system CaM kinases also inhibited the ZmCCaMK activity up to different extents whereas H7 which is a more specific inhibitor of Ca$^{2+}$/phospholipid-dependent kinases had no effect on ZmCCaMK activity, giving additional proof for it being a Ca$^{2+}$/CaM-dependent kinase.

**Autophosphorylation of ZmCCaMK and its possible regulation**

Autophosphorylation is an important property of kinases reported both from the plants as well as animal systems. Ca$^{2+}$ and CaM affect the autophosphorylation of kinases in three different ways. In animal system CaM kinases, both Ca$^{2+}$ and CaM are required for autophosphorylation and this activity is inhibited by EGTA as well as by various CaM inhibitors.

In plant systems, the calcium dependent protein kinases (CDPKs) undergo the autophosphorylation in a fully calcium-dependent manner and though addition of exogenous CaM has no effect on autophosphorylation activity, it could be inhibited by EGTA as well as various CaM inhibitors probably due to presence of CaMLD in these kinases (Harmon et al., 1987; Putnam-Evans et al., 1990; Das-Gupta, 1994). The known CaM kinase from plants have some unique characteristics. In CCaMK, the only other biochemically characterised kinase from plant, though autophosphorylation is Ca$^{2+}$ dependent/stimulated but interestingly CaM inhibits this activity in a concentration-dependent manner.

Ca$^{2+}$/CaM stimulated autophosphorylation is a prominent characteristic of multifunctional CaM kinases in animal systems and its role in regulation of kinase activity has been extensively studied. The regulatory domain of CaM kinase II comprised of two overlapping motifs, a CaM binding domain (residues 296-309) and an autoinhibitory domain (residues 281-302) which occupies the catalytic domain and inactivates the kinase (Brickey et al., 1994). Binding of Ca$^{2+}$/CaM to residues 296-309 neutralizes the inhibitory
potency of autoinhibitory domain presumably by a structure/conformational change promoting its dissociation from the catalytic domain (Colbran et al., 1988). The activated kinase binds its first substrate Mg\(^{2+}\)-ATP resulting in extremely rapid autophosphorylation of Thr\(^{286}\), which precedes the phosphorylation of exogenous proteins (Kwiatkowski et al., 1988; Kalon and Fujisawa, 1991). Autophosphorylation occurs at multiple sites as an intramolecular reaction within the holoenzyme and thus the reaction rates are not dependent on enzyme concentration. One of the most important characteristics of autophosphorylation is that it converts the CaM kinase to a Ca\(^{2+}\) independent form (Lai et al., 1986; Lou et al., 1986; Miller and Kennedy, 1986; Schworer et al., 1986; Hanson et al, 1989). The Ca\(^{2+}\)/CaM independent activity is generally 20 to 80\% of the activity in presence of Ca\(^{2+}\)/CaM depending on the substrate used. This Ca\(^{2+}\)-independent autophosphorylated kinase could be fully restored to Ca\(^{2+}\)- dependent form by treatment with protein phosphatases 1 or 2A (Miller and Kennedy, 1986; Schworer et al., 1986). Besides, it has been shown that autophosphorylation traps the CaM bound to CaM kinase by markedly reducing its dissociation rate and both autophosphorylation and trapping are co-operatively stimulated by CaM.

During purification of ZmCCaMK, autophosphorylation property was used to identify this kinase from the protein fractions eluted from CaM Sepharose column. ZmCCaMK, also showed autophosphorylation in a Ca\(^{2+}\) dependent manner (optimum concentration 10 \(\mu\)M) like CDPKs. But in contrast to CDPKs and substrate phosphorylation activity of ZmCCaMK itself, CaM had no effect on autophosphorylation. Various CaM and kinase inhibitors tested on ZmCCaMK autophosphorylation activity also showed no affect (see Fig. 25 and 27). These data show that the autophosphorylation and substrate phosphorylation activities of ZmCCaMK are differentially regulated.

Besides showing a dual regulation by Ca\(^{2+}\) and CaM, the autophosphorylation and substrate phosphorylation activities of ZmCCaMK showed some other unique features. Even though both the activities are pH dependent, the pH optimum is different for both. The autophosphorylation was obtained at pH 6, whereas pH optimum for substrate phosphorylation reaction was 7.5. A comparison of time kinetics of both the activities
Discussion

showed that the autophosphorylation activity is very fast and gets saturated within 1 minute after the start of the reaction whereas substrate phosphorylation is comparatively slower and saturation was obtained 5 minutes after the start of reaction. Thus it seems that autophosphorylation of ZmCCaMK precedes the substrate phosphorylation reaction. Autophosphorylation of ZmCCaMK was at threonine residue(s), which is the case with animal CaM kinases as well as CCaMK. In contrast, in CDPKs, autophosphorylation has been reported to occur at both serine and threonine residues (Putnam-Even et al., 1990; Saha and Singh, 1995; Yuasa et al., 1995).

All these biochemical properties of ZmCCaMK enable us to ask some important questions about the activity and possible regulation of this kinase. One important point is dual regulation of kinase by Ca\textsuperscript{2+} and CaM. The biochemical data show that calcium alone is sufficient for autophosphorylation as well as substrate phosphorylation activity of the kinase. Besides, CaM could also affect the kinase activity though, only at the substrate phosphorylation level. Effect of CaM could be explained at the two levels. One possible explanation is that CaM after binding with ZmCCaMK confers some change in the conformation of the ZmCCaMK in a way that it makes more substrates available for the kinase to act on i.e. CaM binding could be effecting the substrate binding site of the kinase, and thus after initial calcium-dependent substrate phosphorylation, CaM is merely enhancing the activity. Another explanation could be related with the stability of ZmCCaMK. As we know, most of the kinases are not very stable after phosphorylation and get degraded very fast. As the purified ZmCCaMK itself is not a very stable protein and shows autodegradation, CaM might be having a role to play in its stability. We have found that purified ZmCCaMK on storage at -20\textdegree C autodegrades first in to 2 polypeptides and then into 4 polypeptides, and all of these showed autophosphorylation activity. Further studies are required in this direction to study if CaM could stabilize the kinase under in vitro conditions.

Another important point is regulation of substrate phosphorylation activity of ZmCCaMK by its autophosphorylation which is the case with most of the animal system CaM kinases. From the time kinetics of both the reactions, it is clear in the present study
that the autophosphorylation is preceded by substrate phosphorylation activity. In animal systems, autophosphorylation has been shown to cause calcium independence in the kinase at the substrate phosphorylation level. This possibility was checked with ZmCCaMK also, using the pre-autophosphorylated enzyme for substrate phosphorylation. Autophosphorylated enzyme could phosphorylate substrate in presence of EGTA, showing no calcium requirement (see Fig 29). This fact points towards the possible mechanism of regulation of ZmCCaMK activity. It looks like that 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 requirement of calcium. CaM probably could bind to the autophosphorylated form of ZmCCaMK and either stabilize it or somehow enhances the substrate availability. Further studies are required in this direction to study how calcium and CaM are regulating the autophosphorylation and the 2-step substrate phosphorylation.

**Immunological Characterization of ZmCCaMK**

As ZmCCaMK has turned out to be a novel kinase showing dual regulation by Ca\(^{2+}\) and CaM, antibodies were raised against purified protein to characterize it further.

*Immuno-precipitation of kinase activity using ZmCCaMK antibodies*

Polyclonal but monospecific antibodies raised against ZmCCaMK could precipitate both the substrate phosphorylation and autophosphorylation activity of ZmCCaMK in a concentration dependent manner. To further check if ZmCCaMK is in fact a CaM-stimulated kinase and not a CDPK, we tried checking if ZmCCaMK antibodies could block a similar kinase activity in some animal system. For this we used rat brain extract which is a very rich source of Ca\(^{2+}\)/CaM dependent kinases. The brain extract, on *in vitro* phosphorylation showed presence of a polypeptide which is phosphorylated in a Ca\(^{2+}\)/CaM dependent manner; as both EGTA and CaM antibodies could block the phosphorylation of this polypeptide. ZmCCaMK antibodies could also block the phosphorylation of this
polypeptide giving further proof for it being both biochemically and immunologically similar to animal system Ca\textsuperscript{2+}/CaM dependent kinases.

**Localization and developmental regulation of ZmCCaMK**

In animal systems, the CaM kinases are ubiquitous in nature and have been found present in all the cells thus far examined. But the brain and neuronal tissues are richest sources of CaM kinases where a 20-50 times higher concentration could be detected (Fukunaga et al., 1988; Ouimet et al., 1991). Studies of expression levels as quantified by radioimmunoassay showed that CaM kinases could constitute 1-2% of total brain proteins. Immunodetection assays with ZmCCaMK antibodies also showed its ubiquitous presence in all the plant tested (both dicots and monocots) showing that it has some very basic role to play. Besides, the kinase is not present in some specific plant part as it is the case with CaM kinase from lily where it specifically localized in anthers (Takezawa et al., 1996) or the CaM kinase from maize where it has been shown to be present in roots and involved in gravitropic responses (Lu et al., 1996). Though ZmCCaMK is present in all the plant parts tested, its expression level was not same in different plant parts. It showed a very low level of expression in roots compared to leaves.

The level of ZmCCaMK was found to be developmentally regulated too. Seeds had very low level of expression of this kinase which increased up to five days and then remained constant up to ten days of growth. As the plants were grown on moist germination paper, normal growth could not be seen after ten days and thus the expression could not be studied. This expression of ZmCCaMK could be compared with developmentally regulated expression of CaM kinases from animal systems where changes have been noted in total CaM kinase activity, subunit ratio and subcellular distribution during development (Kelly and Vernon, 1985; Sahyoun et al., 1985; Kelly et al., 1987).
Effect of exogenous factors on ZmCCaMK

In plants, though phosphorylation/dephosphorylation have been shown to be altered in response to a variety of exogenous factors, only a few cases are known where activity/expression of respective kinases is directly affected. In wheat embryo a strong upregulation of PKABA1 transcript is obtained by ABA and dehydration whereas another kinase WPK4 is regulated by cytokinins. Similarly, expression of Vigna radiata kinase (VrCDPK1) is upregulated by auxins and a rice membrane protein kinase is regulated by GA (Abo-El-Saad and Wu, 1995). Stress also has been shown to modulate the expression of various protein kinases (Sheen, 1996). We checked the effect of different exogenous factors as stress, hormones and light on ZmCCaMK level. All the different kinds of stress treatments including heat shock, cold shock and osmotic stress did not effect the level of ZmCCaMK. Similarly, treatment with different plant hormones also did not show any change in ZmCCaMK level except in the case of ABA where a slight decrease was observed.

The only exogenous factor which significantly affected the level of ZmCCaMK was light. Light is one of the most important environmental cues which regulates a number of processes in plants. In many cases, light has been shown to change the pattern of phosphorylation/dephosphorylation (Romero et al., 1991; Fallon et al., 1993; Short and Briggs, 1994; Sharma et al., 1997), but no study to show a direct change in level of kinase/phosphatase, in response to light has yet been reported. ZmCCaMK thus turned out to be a specific example of a protein kinase showing down regulation by red light. Red light treatment for five minutes decreased the kinase level 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 Phy B mediated phenomenon. Red light treatment for different time periods decreased the level of the kinase in a time dependent manner. Possibility of deleterious effect of long duration of red light treatment is ruled out as a five minutes of red light immediately followed by 2 hours dark incubation showed same level of ZmCCaMK as 30 minutes continuous red light treatment.
In *in vivo* systems, in response to any signal such as light the kinases and phosphatases play in concert to each other and same response could be brought about by either affecting kinase or phosphatase. The phytochrome, (Pr form) after absorbing the red light gets converted to Pfr form and the phytochrome itself has recently been shown to be present in a phosphorylated form (see 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 increase in activity/level of kinase or due to decrease in activity/level of phosphatase and vice-versa. Many pathways which show an increase in phosphorylation are reported but existence of a kinase which is down regulated by red light shows the possibility of existence of other complex regulatory pathways as well.

Though level of ZmCCaMK is down regulated with red light, in white light grown plants decrease in the level of ZmCCaMK was less. In fact, blue light had no affect on ZmCCaMK level. The low level of ZmCCaMK in response to red light treatment could possibly be explained by three different ways. Light has been shown to affect expression of a number of genes in both positive as well as negative manner (Neuhaus et al., 1997). Such responses are generally very fast and a change in transcript level could be detected within 1 to 5 minutes. So ZmCCaMK could be a kinase showing down regulation by light and thus might be involved in the light signalling pathway. Other possibilities could be that red light could have an affect on the post translational level. It could either lead to the degradation of the protein or kinase could be relocalized in response to red light. At present we do not have proof in support of either of the possibilities but the very fast nature of the response seems to be due to the relocalization rather than such a fast turnover of protein. In animal systems, interestingly there are some reports related to the relocalization of CaM kinases. In *Drosophilla*, the brain CaM kinase gets more and more membrane bound in parallel with the blue light adaptation (Willmund et al., 1986). Similarly, in rats the retinal CaM kinase becomes membrane bound after dark incubation (Bronstein et al., 1989). Whether such is the case with decrease in the level of ZmCCaMK on red light irradiation is not known and more experiments are required in this direction.
Why ZmCCaMK is a novel kinase?

A comparison of the biochemical properties of ZmCCaMK with other known Ca\(^{2+}\)/CaM-dependent kinases both from plant system as well as animal systems and also with plant CDPKs, suggests that ZmCCaMK may be a novel kinase. It showed a dual regulation by Ca\(^{2+}\)/CaM, has ubiquitous presence, developmentally regulated and is down regulated by red light.

The dual regulation of phosphorylation activity is one of the most important properties of ZmCCaMK. Calcium seems to be essential and sufficient for the activity of the kinase and regulates both autophosphorylation as well as substrate phosphorylation, like the CDPKs from plant systems, but unlike CaM kinases from animal systems. However, in contrast to the CDPKs the ZmCCaMK activity is stimulated by CaM, making it more similar to the CaM kinases. This means that at the substrate phosphorylation level the kinase shows a two step regulation. At the first step Ca\(^{2+}\) stimulates the kinase activity and then at the second step addition of CaM stimulates this activity further. Interestingly autophosphorylation of the kinase is calcium dependent but CaM independent. This two step stimulation of kinase activity makes it different from both the CDPKs as well as CaM kinases. The CDPKs have no affect of CaM on their substrate phosphorylation as well as autophosphorylation activity and animal CaM kinases are distinct as in these kinases though Ca\(^{2+}\)/CaM stimulate the kinase activity at both substrate phosphorylation level as well as autophosphorylation level, the kinase binds with calcium activated calmodulin and thus no stimulation could be seen with calcium alone.

The only Ca\(^{2+}\)/CaM-dependent plant kinase that has been characterized biochemically is an over expressed protein in E. coli. from cDNA from lily anthers (Takezawa et al., 1996). This kinase also shows dual regulation by Ca\(^{2+}\)/CaM where substrate phosphorylation is regulated by both Ca\(^{2+}\) and CaM but autophosphorylation is only Ca\(^{2+}\)-dependent. This dual regulation of CCaMK activity is distinct from ZmCCaMK dual regulation, as Ca\(^{2+}\) alone has no affect on substrate phosphorylation activity of CCaMK, which is the not the case with ZmCCaMK. CaM is essential for substrate
phosphorylation activity of CCaMK whereas in ZmCCaMK it only stimulates the activity over and above the Ca\(^{2+}\) alone stimulated levels. Autophosphorylation activity of both these kinases is calcium-dependent, and show involvement of threonine residue(s), like animal CaM kinases, but CaM affects both the kinases differentially. Ca\(^{2+}\) is sufficient for autophosphorylation of ZmCCaMK and addition of CaM has no effect whereas, autophosphorylation of CCaMK decreases with addition of exogenous CaM. Based on these properties of ZmCCaMK it looks like that immediately after direct binding of calcium to the kinase it autophosphorylates. This autophosphorylated form of the kinase phosphorylates various other substrates with varying efficiency. The autophosphorylation of the kinase makes it calcium independent. Calmodulin, on the other hand is needed to further stimulate the calcium dependent activity either by changing the conformation which lowers its \(K_m\) for the substrates, or it some how stabilizes the kinase.

All these properties mentioned above indicate that ZmCCaMK, like CCaMK belongs to a family of kinases which share properties of both animal system CaM kinases and plant CDPKs and opens the possibility of existence of conventional as well as non-conventional kinases in plants.

Molecular Characterization of ZmCCaMK

As ZmCCaMK turned out to be a novel CaM kinase we tried to clone the cDNA encoding for it from *Sorghum* and maize. Antibodies raised against ZmCCaMK were used to screen *Sorghum* and maize cDNA libraries. The *Sorghum* clone pSbSp1 (0.8 kb) was fully sequenced. The sequence analysis and comparison with available databases showed that it is a new gene having some homology with a human K\(^+\) channel gene hSKCa3 (Chandy et al., 1998) and with an aspartate aminotransferase gene from *Medicago sativa* (Gregerson et al., 1994). At the protein level the clone showed some matching with a calcium-binding protein from *Dictyostelium discoideum* (Wenningtun et al., 1993), with serine/threonine specific protein kinase mini brain homologue (Guimera et al., 1996) and dual specificity Dyrk type kinase from rat (Kentrup et al., 1996). Antibodies raised against
ZmCCaMK picked up this unique gene possibly due to presence of these calcium-binding or kinase specific domain.

The maize cDNA clone (2.5 kb insert) partially sequenced (1.3 kb). The sequence homology search of this clone with available databases showed a high degree of homology with HSP70s from various plant sources. Automated sequencing of a tryptic peptide fragment also showed that it was 100% matching with HSP70s at the amino acid levels.

At present one could argue that the protein we have purified could have an HSP70 contamination especially due to the correspondence in the molecular mass of the proteins. However, we rule out this as the protein after gel elution when re-run on gel showed no other contaminating polypeptide. Further, the peptide used for sequencing after trypsin digestion when checked on HPLC did not show any contamination. Antibodies raised against ZmCCaMK showed presence of a single band at all the concentration of proteins and all the dilutions of antibodies checked. This shows that the antibodies are monospecific. These antibodies could also precipitate down both the substrate phosphorylation as well as autophosphorylation activity of ZmCCaMK. Besides, these antibodies could precipitate down a similar CaM kinase activity in rat brain. These antibodies also cross react with a moss Ca\(^{2+}\)/CaM-dependent kinase (Personal communication with M. M. Johri, T.I.F.R., Bombay). Besides, as both the peptide sequence as well as DNA sequence show homology with HSP70, it means that the cDNA clone picked up does code for the protein used for raising antibodies. These observations could be explained in two possible ways i.e. either ZmCCaMK is an isoform of HSP70s or it is a chimeric kinase having HSP70 domain evolved for some specialized role.

**Is ZmCCaMK an HSP70?**

HSP70s are a family of proteins which mostly act as chaperonins and are not very well characterized at the functional level. Most of the plant HSPs are thus named only on their sequence homology with the animal system HSPs. Even though some of the HSPs get induced during the heat shock treatments to the plants, there are others which belong to the cognate family (HSCs), do not show any such induction. The constitutive
expression of these proteins might have some other roles to play related to the basic functioning of the plants, which is still unexplored.

As is clear from the experiments done in this work, the ZmCCaMK expression level, it is not heat inducible and therefore it possibly belongs to the cognate HSP family of proteins (HSCs). cDNA sequence homology search also shows that ZmCCaMK matches more with the cognate heat shock proteins e.g. 97% homology with *Oryza sativa* cognate HSP70 gene (Qun et al., 1996), 87% with *Zea mays* HSP70 gene (Bates et al., 1994), 83% with *Lycopersicon esculentum* (Lin et al., 1991; Sun et al., 1996) 84% with *Spinacia oleracea* HSC70 gene (Li et al., 1994), 83% with *Arabidopsis thaliana* HSC70 gene (Wu et al., 1994). Similarly, at the protein level also it shows a high degree of homology with different known HSP70s.

In plants systems various HSP homologues have been reported to be differentially expressed in different organs (DeRocher and Vierling, 1995) and subjected to developmental regulation (Dhankher et al., 1997; Dudley et al., 1997) along with their role in stress response and as chaperonins. However, no HSP70 from plants has been fully characterized at the biochemical level.

Search of available literature on animal systems HSP70s show some very important properties which could be correlated with the properties of ZmCCaMK. ZmCCaMK shows direct $\text{Ca}^{2+}$-binding, and a $\text{Ca}^{2+}$/CaM-dependent phosphorylation of various substrates. Some proteins of HSP70 family also have a calcium-binding domain present. Crystal structure of human HSP70 shows that it has two calcium-binding sites. One of the sites appears to be important for ATP hydrolysis and might be involved in *in vitro* phosphorylation. It has been shown that a small but significant movement of metal ions and side chains could position the catalytically important threonine residue for phosphorylation. This *in vitro* phosphorylation may be facilitated by local changes in protein structure due to calcium-binding. The second calcium-binding site is proposed to play a role in stabilization of this protein (Sriram et al., 1997). Besides, many members of HSP70 family have a conserved CaM-binding domain and a 21 amino acid sequence forms a potential amphipathic alpha helix which binds calmodulin with high affinity (Stevenson
Autophosphorylation is another very important property generally associated with kinases. ZmCCaMK like animal CaM kinases shows autophosphorylation at threonine residue(s). Many proteins belonging to HSP70 family also show autophosphorylation solely at the threonine residues(s) (Leustek et al., 1992; Csermely et al., 1995). Besides, presence of an ATPase domain has been shown in case of all the HSP70s (Bork et al., 1992; Chamberlain and Burgoyne, 1997). Apart from these properties associated with HSP70 at the protein level, the activity and expression of hsp70s at the gene level has also been shown to be affected by calcium, various calcium antagonists and protein kinase inhibitors (Erdos and Lee, 1994; Lee et al., 1994; Jacquier-sarlin et al., 1995; Elia et al., 1996; Morris et al., 1996; Holmberg et al., 1997; Usui et al., 1997). Expression of hsp70 gene has been shown to be developmentally regulated in some cases (Olson et al., 1994). Although all these facts indicate towards the possibility of HSP70s associated with kinase activity, no reports are available till date in this regard in plant systems. In animal system however, a Ca$^{2+}$/CaM-dependent kinase III which specifically phosphorylates elongation factor II (eEF-2) has been shown to be correlated with HSPs. Nygard et al. (1991) have purified an eEF-2 kinase from rat liver (MW 90 kDa) to homogeneity and show that this purified preparation could specifically phosphorylate EF-2 under in vitro assay conditions. Amino acid analysis of this kinase showed that it is homologous to HSP90. Purified HSP90 as such showed no kinase activity but on treatment with alkaline phosphatase, it showed a kinase activity and phosphorylated eEF-2. This thus show that HSP90 is an inactive homologue of CaMK III, and the activity is regulated by phosphorylation.

In plants also, it has been shown that the clatherin coated vesicles from the Zucchini hypocotyl have a number of phosphorylatable polypeptides. The fraction having kinase activity after the gel filtration step showed phosphorylation of two major polypeptides; a 45 kDa heat stable and calcium-binding clatherin light chain and a 70 kDa polypeptide which was inhibited by staurosporine. The same polypeptide also cross reacted with HSP70 antibodies giving evidence for it being an immunohomologue of HSP70 with kinase activity (Drucker et al., 1996).
In view of foregoing discussion, we would like to explain the prospect of ZmCCaMK being a HSP70 homologue in two possible ways. One probability is that ZmCCaMK is a functional homologue of a cognate HSP70s with Ca\(^{2+}\)-binding and CaM-binding domains. Comparison of autophosphorylation activity shows that HSP70 has a fully calcium-dependent autophosphorylation at threonine residue as it is the case with ZmCCaMK also. A possible mechanism could be that in this particular case due to some modification, the ATPase domain of HSP70 is able to transfer the \(\sim P\) moiety to some other proteins using them as substrates and this then could not be the case with other HSP70s.

Another possibility is that ZmCCaMK is a chimeric protein like CCaMK of lily anthers and has a HSP70 domain fused with it along with kinase domains. The peptide used for sequencing could be from this part itself. This may be so since the partial sequence of the clone does not match fully with HSP70 sequence. In animal systems a novel ER localized calcium-binding protein CBP140 which has a carboxy terminal NDEL sequence showing partial homology with HSP70 (Naved et al., 1995) has been reported, though it is not induced by heat shock. It is proposed to be a novel gene, coding for a chimeric gene containing HSP70 domain.

Both the possibilities mentioned above could be checked by isolating a full length clone for this kinase, sequencing it and further over expressing it in bacterial system. The over expressed protein could then be used for confirming the possibility of ZmCCaMK being a HSP70 homologue and characterizing it further.