Chapter II

Oscillation of phosphorylation of an endogeneous calcium dependent protein kinase from groundnut (Arachis hypogea)
Evidences for its role in Osmosensory signaling
Fig 2.1 Effect of 0.4M sucrose on *Arachis hypogea* cells

*Arachis* cells grown in aux" cyt" and aux" cyt+ media were treated with 0.4M sucrose for 4 days and their growth monitored in respective solid media as described in Methods.
In the preceding chapter the domain analysis of a CDPK from *Arachis hypogea* (AhCPK2) has been reported. The cDNA of AhCPK2 was developed from *Arachis* cells that were subjected to osmotic stress. It was found that AhCPK2 belongs to a sparsely membered group of CDPKs, all of which contain NLS in their junction domain and this property is obligatorily coupled with the presence of nonconsensus calcium binding EF hand loops in their respective CaMLDs. Among these closely related CDPKs, AtCPK10 from Arabidopsis is 88% homologous to AhCPK2 and its expression is known to be induced in presence of stress (Urao et al., 1994) which is in consistence with the fact that AhCPK2 was expressed in stressed *Arachis* cells. In the present chapter experiments that were done to understand the involvement of AhCPK2 in stress response is described.

The investigation was taken up in *Arachis* cells that are maintained in our laboratory and are reported to have a characteristic stress physiology. The culture was initiated in our lab from cotyledonary explants in the year 1997, and is maintained since then. In an attempt to cryopreserve, they were subjected to prior desiccation under hyperosmotic condition with 0.4M sucrose for 4 days, to counteract the dehydrating conditions that accompany cryoconditions. It was noted that the auxin deprived cells failed to survive such treatments but the auxin supplemented cells were not affected at all (Fig 2.1). Subsequent investigations revealed that unlike the auxin-supplemented counterpart, the auxin-deprived cells were sensitive to all sorts of treatment that led to water loss. Interestingly these cells were sensitive to exogenous treatments to ABA too, which could also be reversed in presence of exogenous auxin. This characteristic physiology of the cultured *Arachis* cells provided us with an *in vitro* system where investigations could be undertaken for understanding the abiotic stress signaling pathways of a plant cell. These cells are subcultured at an interval of 7 days, and even after almost 400 passages performed to date the auxin autotrophy and nature of sensitivity to water loss and ABA treatment remain unchanged for these cells.

Detail investigation revealed that permanent presence of auxin in the growth medium was not a requisite for stress recovery of the *Arachis* cells. Rather transient exposure to auxin for as low as one hour was necessary and sufficient to revive osmotically stressed *Arachis* cells. Qualitative proteome analysis revealed that ubiquitin mediated protein turnover is a necessary downstream event in exogenous auxin mediated stress tolerance in *Arachis* cells (Nag et al 2006). A transcription factor of ABI3 (ABA insensitive 3) family is differentially expressed in presence of auxin in the *Arachis* cells. This factor has dual DNA binding property targeted to both Auxin and ABA responsive elements suggesting it to have a potential role in conferring crosstalk between...
Fig 2.2: Subcellular localization of the endogenous form of AhCPK2. The endogenous form of AhCPK2 was detected in *Arachis* cell extracts using anti-AhCPK2. A. Western blot analysis of total protein preparation treated with either calcium or EGTA. Crossreactive bands are indicated. B. Western blot analysis of the microsomal and soluble protein preparation from normal *Arachis* cells using anti-AhCPK2.
these two phytohormones (Nag et al. 2005). Attempts to look for the role of calcium signaling in the stress response behavior of *Arachis* cells led to the identification of a putative stress responsive calcium dependent protein kinase from osmotically stressed *Arachis* cells. The pertinent questions that naturally arose is whether it is involved in stress signaling at all and if yes whether it is involved directly in (i) stress response (ii) ABA response (iii) stress as well as ABA response (iv) ABA auxin crosstalking. A systematic investigation was undertaken to probe into these possibilities.

**The endogenous AhCPK2**

The JD-CaMLD region of the *Arachis* CDPK contained unique features that distinguished it from canonical CDPKs (Fig. 1.4). Unlike canonical CDPKs it had nonconsensus calcium binding EF hands in its CaMLD and a nuclear localization sequence in its JD. Therefore, to detect the endogenous polypeptide representing the untagged or native *Arachis* CDPK (AhCPK2) in the cultured *Arachis* cells, polyclonal antibodies raised against the JD-CaMLD polypeptide of the kinase (described in section I) was used. This antibody crossreacted with a single 58 kD band in the total protein preparation of normal *Arachis* cells that showed a characteristic calcium dependent shift in mobility (Fig 2.2A). It failed to cross react with the 53 kD CDPK in dry seed extracts of *Arachis hypogea* that was previously characterized in our laboratory (DasGupta, 1994; Chaudhuri et al., 1999) indicating the specificity of its interaction. In view of the fact that most CDPKs are associated with membrane fractions AhCPK2 was also investigated for its subcellular localization in the *Arachis* cells. To probe into the matter *Arachis* cells were resolved into soluble and microsomal fractions and the isolated proteins from these fractions were subjected to western blot analysis using the raised antibody. The crossreactive 58 kD polypeptide was localized in the microsomal fractions as well as the soluble fractions (Fig. 2.2 B) in the *Arachis* cells indicating the kinase to be distributed in both membrane and soluble fractions. In the normal *Arachis* cells therefore AhCPK2 has both membrane and cytoplasmic components.
Fig. 2.3: AhCPK2 expression in *Arachis* cells is unaffected by stress or ABA or Auxin treatment. *Arachis* cells were subjected to osmotic stress (0.4 M sucrose 4 days) and ABA (5mg/L 16 hrs) treatment. A) Growth potential of the indicated cells is shown in (i) auxin autotrophic condition (ii) auxin supplemented (2.5 mg/L) condition (iii) auxin autotrophic condition but after transient exposure to auxin (2.5 mg/L) for 1 hour B) & C) Northern blot and Western blot analysis of indicated cells using AhCPK2 probe and anti-AhCPK2 respectively.
Expression of Arachis CDPK (gi: 67479988) in Arachis cells subjected to osmotic stress and ABA treatments:

To understand the involvement of AhCPK2 in stress signaling the level of expression of this kinase in response to stress, ABA or auxin treatments in the Arachis cells was first investigated. As shown in Fig. 2.3 A Arachis cells subjected to osmotic stress (0.4M sucrose 4 days), or ABA treatments (5 mg.L⁻¹ 1 day) fail to grow under auxin autotrophic conditions. The same cells show normal growth in auxin supplemented condition or under auxin autotrophic conditions if exposed to exogenous auxin transiently for an hour. These observations indicate that the Arachis cells were driven to quiescence when subjected to above treatments in absence of auxin. Northern blot revealed that AhCPK2 is constitutively expressed in the Arachis cell culture and the expression level remains unchanged in the transcriptional level following stress ABA, and/or auxin treatments (Fig. 2.3 B). To check if the expression level of AhCPK2 was affected in the post transcriptional level by these treatments western blot analysis was done using antibodies specific for AhCPK2 with total protein preparation isolated from normal, osmotically stressed and ABA treated cells and their auxin treated counterparts. As indicated in (Fig. 2.3 C) the expression of the kinase remain unchanged following all such treatments indicating that AhCPK2 expression was not affected by stress treatments as well as ABA and Auxin treatments in the Arachis cells. As indicated earlier, AtCPK10 from Arabidopsis is 88% homologous to AhCPK2 and its expression is known to be induced in presence of stress (Urao et al., 1994) But unlike AtCPK10, AhCPK2 fails to respond to stress treatments by changing its level of expression.

Difference in state of phosphorylation of endogenous AhCPK2 in normal and stressed cells.

As indicated in Chapter I the expressed AhCPK2 could not phosphorylate any known exogenous substrate. Therefore purification of the kinase or investigation towards monitoring its differential activity under stressed conditions could not be undertaken. At this juncture it was noticed that the 58 kd band which we believed was putatively representing the AhCPK2 kinase because of its crossreaction with anti-AhCPK2, also crossreacted with antibodies against pThr but not pSer or pTyr both in the soluble protein preparation as well as the microsomal protein preparation (Fig. 2.4A). Crossreactivity of the 58 kD band with anti pThr suggested that the endogenous 58kD kinase was phosphorylated in threonine residues in vivo.
Fig. 2.4: State of phosphorylation of endogenous AhCPK2. A) Western blot of soluble and microsomal proteins (50 μg each) from normal Arachis cells using anti-AhCPK2, anti-pSer, anti-pThr and anti-pTyr. Arachis cells were subjected to osmotic stress (0.4 M sucrose 4 days) and ABA (5mg/L 1 day) treatment with or without subsequent treatment with auxin for an hour. These cells were resolved into soluble and microsomal fractions. B) and C) Western blot of the indicated fractions using anti-pThr and anti-AhCPK2 respectively.

Fig 2.5: Analysing the effect of osmotic stress, ABA treatment and subsequent auxin treatments on the growth potential of the Arachis cells and the associated state of phosphorylation of AhCPK2. Effect of ABA and stress are similar (blue encircled) as far as their effect on the growth potential of Arachis cells are concerned, but they are dissimilar (red encircled) with respect to their effect on the state of phosphorylation of AhCPK2.
An attempt was therefore made to look for the state of phosphorylation of the 58 kD AhCPK2 in normal, stressed and ABA treated cells as well as their auxin treated counterparts, both in their soluble and microsomal fractions by its crossreactivity with anti pThr. In the cells that were subjected to osmotic stress irrespective of whether it was subsequently treated with auxin or not, AhCPK2 was unphosphorylated \textit{in vivo} in the soluble state (Fig. 2.4 B). In the normal as well as the ABA treated cells and their auxin treated counterparts AhCPK2 was present in phosphorylated form. In microsome associated form, AhCPK2 was phosphorylated in normal, stressed and ABA treated cells both in their auxin treated and untreated form. Absence of phosphorylation in the AhCPK2 in its soluble form in stressed cells was not due to absence of the kinase per se as western blot analysis using AhCPK2 antibody clearly showed that the level of the kinase was unaffected by stress, ABA and/or auxin treatments (Fig. 2.4 C).

These results describing the state of phosphorylation of AhCPK2 can now be analysed in the light of the stress physiology of the \textit{Arachis} cells. As indicated in Fig 2.5 the \textit{Arachis} cells are driven to quiescence when subjected to osmotic stress as well as ABA treatments. Therefore signals originating from stress or ABA treatment either in conjunction or independently lead to growth arrest. The growth potential is revived in presence of auxin. Thus ABA and auxin have completely different effect on these cells with respect to growth potential. With respect to influencing the state of phosphorylation of AhCPK2, ABA and auxin appear to be similar as none of them changes the state of phosphorylation of the kinase from its normal state. Interestingly the state of phosphorylation of AhCPK2 dissected the effects of osmotic stress and ABA treatment on the \textit{Arachis} cells. It stays in a phosphorylated form in the ABA treated cells just like it stays in normal and the auxin treated cells but is completely dephosphorylated in osmotically stressed cells. Thus dephosphorylation of AhCPK2 appear to be a direct consequence of perception of stress in the upstream signaling pathways. The downstream signaling originating from ABA is not involved in causing dephosphorylation of AhCPK2 and possibly do not mediate its effects through this kinase.

\begin{itemize}
\item \textbf{In vitro phosphorylation of endogenous AhCPK2: Phosphoproteome analysis of normal and stressed \textit{Arachis} cells}
\end{itemize}

Investigations were then undertaken to check whether AhCPK2 could be phosphorylated \textit{in vitro}. To follow \textit{in vitro} phosphorylation the total protein preparation from normal \textit{Arachis} cells were subjected to phosphorylation reactions and the reaction products were subjected to two dimensional electrophoresis. The separated proteins were then subjected to western blot analysis to identify AhCPK2 using anti-AhCPK2. The blot was then subjected to autoradiography to
Fig 2.6. Phosphoproteome profiles of normal and stressed Arachis cells. A and C are silver stained electrophoregrams of total soluble proteins isolated from normal and stressed Arachis cells. Equal amount of total protein was loaded for isoelectric focusing in pH 3-10, NL strips from Pharmacia. The separated proteins were subjected to 10%SDS-PAGE for second dimensional analysis. B and D are corresponding phosphoproteomes of normal and stressed Arachis cells. Details of phosphoproteome development is described in methods. The position of AhCPK2 is ascertained by western blot analysis with anti-AhCPK2 and indicated by arrows in all panels.

Fig.2.7: Calcium dependent phosphorylation of endogenous AhCPK2 in normal, stressed and ABA treated cells. Arachis cells were subjected to osmotic stress (0.4 M sucrose 4 days) and ABA (5mg/L 1 day) treatment and resolved into soluble and microsomal fractions. Soluble and microsomal proteins from normal, stressed and ABA treated cells were subjected to D) in vitro phosphorylation E) immunoprecipitation followed by phosphorylation, in presence and in absence of calcium as described in methods.
understand the associated phosphorylation with the AhCPK2 spot. Fig 2.6 A shows the silver stained proteome of a normal *Arachis* cell. The arrow indicates the spot that was highlighted in western blot analysis. Fig 2.6 B shows the autoradiogram and represents those proteins that were phosphorylated in the *in vitro* reactions. The AhCPK2 spot is again indicated by an arrow. This analysis clearly showed that in the normal cell extracts AhCPK2 polypeptide is potentially phosphorylated in *in vitro* reactions. The corresponding analysis undertaken with osmotically stressed cells showed that AhCPK2 failed to be phosphorylated in *in vitro* phosphorylation reactions (Fig 2.6 C and D). These results are similar to the observations made on the *in vivo* state of phosphorylation of AhCPK2 in its soluble form (Fig 2.4B) where also the kinase was detected in phosphorylated state in normal cells but in a dephosphorylated state in osmotically stressed cells.

- **Calcium dependence of *in vitro* phosphorylation of endogenous AhCPK2 in normal stressed and ABA treated cells.**

*In vitro* phosphorylation with soluble and the microsomal protein preparations from normal, stressed and ABA treated cells was carried out in the same way as described in Fig 2.6 except that here the reaction mixture was analysed in single dimension. In consistence with *in vivo* results (Fig. 2.4 B), *in vitro* phosphorylation reactions also indicated that AhCPK2 failed to be phosphorylated in soluble protein preparations from cells that were subjected to osmotic stress (Fig. 2.7 A). In similar preparations from the normal and the ABA treated cells AhCPK2 could be phosphorylated *in vitro*. Also similar to what we observed *in vivo*, AhCPK2 got phosphorylated *in vitro* in the microsomal protein preparation from normal stressed, as well as the ABA treated cells. AhCPK2 phosphorylation in all the above cases was found to be sensitive to EGTA indicating the reactions to be calcium dependent. In contrast to the above observations when we immunoprecipitated AhCPK2 and then undertook the *in vitro* phosphorylation reactions, soluble AhCPK2 was phosphorylated even in the osmotically stressed cells (Fig. 2.7 B). Thus irrespective of whether AhCPK2 was collected from normal stressed or ABA treated cells immunoprecipitated AhCPK2 was phosphorylated. Phosphorylation of immunoprecipitated AhCPK2 could be due to its autophosphorylation as the reactions appear to be calcium sensitive but it can very well be due to an upstream calcium dependent protein kinase that have coprecipitated with AhCPK2. What ever be the case in isolation from the milieu of the extract AhCPK2 can successfully be phosphorylated even in the extracts prepared from osmotically stressed cells. The fact that AhCPK2 was found in dephosphorylated form (Fig. 2.4 B, 2.6 and
A Osmotic stress
Days 0 1 2 3 4 5 6 7 8
58 Kd ▶

B Days 6 6' 7 7' 8 8'
58 Kd ▶
Stress Stress Stress Stress Stress Stress
Free Free Free Free Free Free
γ32p incorporation

C ABA
Time 0 min 5 min 10 min 1h 24h 48h
58 Kd ▶
γ32p incorporation

Fig. 2.8: State of phosphorylation of endogenous soluble AhCPK2 oscillates with change of water potential. Soluble proteins from Arachis cells A) that were osmotically stressed for the indicated periods B) that were osmotically stressed for the indicated periods and then moved over to stress free medium for 16 hours C) that were treated with ABA (5mg/L) for the indicated periods were subjected in vitro phosphorylation reaction as described in materials and methods.
2.7 A) in spite of having its potential to be phosphorylated unaffected (Fig. 2.7 B) indicates that the stressed cell has an induced phosphatase activity that leads to dephosphorylation of the *Arachis* kinase. That AhCPK2 is indeed involved in stress response was first evidenced from this set of data.

Similar line of investigation with immunoprecipitated AhCPK2, both in its phosphorylated or dephosphorylated form, indicated that this kinase failed to phosphorylate any common exogenous substrate. This prevents us from making any further comments on the state of activation of the kinase. It should be noted that the expressed AhCPK2 fails to autophosphorylate as well as phosphorylate any exogenous substrate (Raichaudhuri et al., 2006). Interestingly the expressed form of AtCPK10, the closely related homolog of AhCPK2 from *Arabidopsis*, also failed to show any activity (Urao et al., 1994).

- **The state of phosphorylation of AhCPK2 oscillates with change in osmotic potential.**

A systematic investigation was undertaken to monitor the appearance of the dephosphorylated state of the 58 kD AhCPK2 with the progress of osmotic stress. *In vitro* phosphorylation reactions as described in the previous experiments were done with soluble protein preparations from osmotically stressed cells. AhCPK2 was gradually detected in dephosphorylated form with complete dephosphorylation being detected after four days of treatment with 0.4 M sucrose. With further progress of stress AhCPK2 regained its phosphorylated state with maximum phosphorylation attained in six to seven days (Fig. 2.8 A). Observing such oscillation of phosphorylated state of the kinase with increased exposure to stress conditions leading to loss of water it occurred to us that the dephosphorylation of AhCPK2 must be a response to sensing a change in water potential around the cell and rephosphorylation a response to sensing a balance in water potential between inside and outside of the cell. In an attempt to check this proposition we intended to check how the phosphorylated state of AhCPK2 get affected if *Arachis* cells subjected to prolonged stress is moved to normal conditions. As indicated in Fig. 2.8 B, when cells subjected to prolonged osmotic stress was moved over to stress free medium for 16 hours, AhCPK2 was detected in a dephosphorylated state. The corresponding western blots showed that AhCPK2 expression was not affected in response to above treatments. These observations clearly showed that dephosphorylation of AhCPK2 is not a response to water loss per se but a change of water potential around the cell which could either be hyperosmotic or hypoosmotic.

Noting this oscillation of phosphorylation, the unchanged state of phosphorylation of AhCPK2 in presence of ABA became a cause of apprehension as it could be representing a rephosphorylation event. Therefore similar investigations were undertaken with ABA.
Fig 2.9: Subcellular localization and detection of the associated phosphorylation state of the endogenous form of AhCPK2. Western blot analysis of the microsomal, soluble and the nuclear protein preparation from normal, stressed and ABA treated Arachis cell extracts with A) anti-AhCPK2 B) anti-tubulin C) anti-ABI3 and D) anti-pThr
treatment where phosphorylation of AhCPK2 in *Arachis* cells was followed after various periods of the hormone treatment. As shown in Fig. 2.8 C there was no change in the level of AhCPK2 phosphorylation in the *Arachis* cells following ABA treatment indicating the oscillation of phosphorylation of AhCPK2 to be an osmosensory response.

• The dephosphorylated form of AhCPK2 is localized in the nucleus

AhCPK2 contains a functional bipartite nuclear localization sequence (NLS) in its junction domain. In the preceding chapter it has been demonstrated that this kinase can interact with nuclear import factors of the importin family with this NLS. Subcellular fractionation followed by western blot analysis revealed that AhCPK2 was not detectable in the nuclear fraction of the normal cells (Fig. 2.9A) but was clearly detectable in the nuclear fraction of the stressed cells that were subjected to 3.4 M sucrose for 4 days. But similar to normal cells, it was not detected in the nuclear fraction following ABA treatment. These observations suggest that AhCPK2 responds to stress-originated signals by being localized in the nucleus. Similar dynamicity in subcellular localization in response to stress has been noted earlier with McCPK1, a CDPK from *Mesembryanthemum crystallinum* (Chehab et al., 2004). Anti-tubulin antibody cross reacted with soluble and the microsomal protein preparations (Drykova et. al., 2003) but showed no crossreaction with the nuclear protein indicating our fractionation procedures to be effective (Fig. 2.9 B). Antibodies raised against a transcription factor ABI3 (ABA insensitive 3) that is induced in presence of auxin in these cells (Nag et al., 2005) are however strictly localized in the nuclear fractions of the *Arachis* cells (Fig. 2.9 C) indicating the fractionation procedures to be effective.

It has already been shown that AhCPK2 is dephosphorylated in its soluble form in osmotically stressed cells (Figs. 2.4, 2.6 and 2.7). It was therefore pertinent to ask whether nuclear localization of this kinase had any relation with its dephosphorylated state. Investigations revealed that the nuclear localized AhCPK2 in osmotically stressed cells failed to crossreact with anti phosphothreonine indicating it to be in dephosphorylated state (Fig. 2.9 D). This is consistent with the results where AhCPK2 was detected unphosphorylated in the cytoplasmic fraction under osmotic stress treatments (Figs. 2.4, 2.6 and 2.7). In normal cells or in ABA treated cells AhCPK2 do not dephosphorylate in its soluble or cytoplasmic form and was not detected in the nucleus. Thus an interesting correlation between the state of phosphorylation of AhCPK2 with its nuclear localization was noted. It is possible that dephosphorylation is a prerequisite for the kinase to migrate in to the nucleus.
Presently, functional annotation available for our target set of CDPKs having NLS sequence in JD is low and nuclear localization has not been reported for any of them. In fact AtCPK7 and AtCPK8, members of our target group, have been localized in membranes, not in the nucleus (Hrabak et al., 2003). In the next chapter we have demonstrated that specific signatures of stress associated calcium signaling have a role in localizing AhCPK2 in the nucleus. Similar to what has been observed with the Arachis CDPK it is possible that localization of AtCPK7 and 8 are also responsive to specific signature signals. Alternatively, as suggested by the authors, it is also possible that addition of a C-terminal GFP-tag have interfered with proper targeting of these kinases. Another Arabidopsis CDPK, AtCPK13 was previously shown to contain an NLS (PlantsP no. 84110) in its JD, but we were intrigued to find that in its recent curated form (PlantsP no. 137883) the NLS has been removed from its annotation.

**Discussion**

In this chapter the stress responsiveness of the endogenous form of AhCPK2 has been investigated, and biochemical evidences in favour of its participation in osmosensory stress signaling has been put forward. AhCFK2 is 88% homologous to AtCPK10 in Arabidopsis that provided the earliest lines of evidence to link a CDPK to an abiotic stress response pathway. Expression of AtCPK10 was induced in presence of stress or ABA treatment of Arabidopsis plant (Sheen, 1996) and in transient-expression experiments dominant-active AtCPK10 activated a stress regulated promoter that responds to cold, drought, and salt stress (Sheen, 1996). AhCPK2 expression on the other hand appeared to be constitutively expressed and remained unaffected by ABA treatment or osmotic stress (Fig. 2.3). It is however found to be differentially phosphorylated in response to change in water potential in the environment. On the basis of these observations the results are discussed in the light of (i) the present layout of abiotic stress signaling and the unique stress physiology of the Arachis cells. (ii) other CDPKs that are reported to be involved in stress response.

The initial perception of water deficit stress is connected to upstream signaling events that control many aspects of stress response including generation of abscisic acid (ABA). ABA in turn, as an endogenous signal starts a second round of perception and downstream signaling to initiate adaptive responses (Zhu, 2002). Arachis cells are driven to quiescence when subjected to both osmotic stress (upstream signal) or ABA treatments (downstream signals) (Fig. 2.3 A) suggesting
Normal Cells | Osmotically stressed

Osmotically balanced

Dephosphorylation of the cytoplasmic component of AhCPK2

Migration of AhCPK2 to the nucleus

Fig 2.10: A working hypothesis on role of AhCPK2 in osmosensory signaling. Details explained in the text.
that the growth inhibition could be a consequence of the downstream signaling events. In the present investigations AhCPK2 dephosphorylation was found to be a direct consequence of change of water potential in the environment which could either be hypoosmotic or hyperosmotic (Fig. 2.8). Presence of ABA on the other hand had no role in determining the state of phosphorylation of AhCPK2 (Fig. 2.8). Dephosphorylation of AhCPK2 thus appears to be involved in the upstream osmosensory signaling pathways. In view of the fact AhCPK2 fails to phosphorylate any known exogenous substrate it is not possible at present to understand the effect of dephosphorylation on its activation. But in other instances dephosphorylation has been documented to have important effects on CDPK activites. For example a soluble phospho-Ser phosphatase from winged bean shoots dephosphorylates an inactivated autophosphorylated winged bean CDPK1 (WbCDPK1) to release the inhibitory effect of autophosphorylation (Ganguly and Singh, 1999). In case of dephosphorylation of AhCPK2 it is possible that any of the four constitutive PP2Cs that act as negative regulators of ABA-responses —are involved in a positive feedback loop in the upstream signaling (Xiong et al., 2002).

From the picture that has emerged from the experiments done in this chapter a simple working model may be hypothesized on the role of AhCPK2 in stress signaling (Fig 2.10). In the normal cells where there is a perfect harmony between the interior of the cell with the exterior environment ahCPK2 remains in phosphorylated state. Upon perception in an imbalance in water potential the cytoplasmic component of AhCPK2 becomes dephosphorylated by a phosphatase that appear to be induced by the immediate signaling action of the osmosensors. The dephosphorylated kinase then migrates to the nucleus to phosphorylate unique transcription factors for initiation of the downstream signaling pathways that possibly involve ABA biosynthesis and its subsequent action.

In the two-component shl-ypd1-Ssk1 osmosensing phosphorelay system (Reiser et al., 2003; Tamas et al., 2000) dephosphorylation of P-Asp from the response regulator Ssk1 (Hawang et al., 2002), the ultimate member of the controls the activation of the Hog1 MAP (mitogen-activated protein) kinase cascade, which regulates adaptive responses (Wurgler-Murphy and Saito, 1997). These relay systems are conserved in plants through dedicated AHK-AHP-ARR cascades (Hwang et al., 2002) and there is ample evidence that MAP kinase cascades are active in Arabidopsis and that they may be involved in the water-deficit response (Mizoguchi, Ichimura and Shinozaki, 1997; Ichimura et al., 2000). The question is what could be the relation between a CDPK mediated osmosensory pathway and the pathway mediated by two component receptors. That a relation do exist between CDPK signaling and the two component osmosensing cascade has been put forward by two lines of evidences. Firstly McCDPK1 from Mesembryanthemum crystallinum phosphorylates CSP1 a two-component
pseudo-response regulator protein, in a Ca\textsuperscript{2+}-dependent manner, that could serve as a transcriptional activator and salt stress induces co-localization of McCDPK1 and CSP1 in the nucleus of ice plants (Patharkar and Cushman, 2000). Secondly CDPK cascade has been found to be antagonistic to MAPK cascade indicating complex feedback and feed forward loops to be present for generating a tailored finely tuned stress response (Ludwig 2005). Thus a balanced interplay between the parallel CDPK and MAPK signal transduction cascades generates specific stress and defense responses.

CDPKs are implicated in abiotic stress response both by their change of expression levels and by their biochemical activation. In most cases they are reported to be transcriptionally induced. For example, MsCK1, ZmCPK1, OsCPK7, VrCPK1, McCPK1, AtCPK10, AtCPK11 NtCDPK2; are CDPKs from alfalfa (Monroy and Dhindsa, 1995), maize (Saijo et al., 1997), rice (Breviario et al., 1995), mung bean (Botella et al., 1996), ice plant (Patharkar and Cushman, 2000) Arabidopsis (Urao et al., 1994) and tobacco (Romeis et al., 2001) respectively that are known to show increased expression levels in response to various abiotic assaults like cold, high salinity or dehydration. Biochemical activation of CDPK activity has been reported in comparatively fewer cases. For example activation of OsCDPK and NtCDPK from rice and tobacco respectively during cold stress (Martin and Busconi, 2001) or osmotic stress (Takahashi et al., 1997). Apart from AhCPK2, NtCDPK2 from tobacco represents only another case where phosphorylation state of a CDPK has been evidenced to change in response to stress (Romeis et al., 2001). While in case of AhCPK2, dephosphorylation of the kinase behaves agonistic to stress perception in case of the NtCDPK2, its activation was accompanied by a phosphorylation-dependent transition from a non-elicited into an elicited enzyme form (Romeis et al., 2001).

Interestingly, these CDPK genes that are induced by similar stresses do not necessarily group in the same CDPK subfamilies (Cheng et al., 2002; Harmon et al., 2001; Hrabak et al., 2003). The \textit{Arachis} CDPK AhCPK2 and the \textit{Arabidopsis} AtCPK10 however share interesting similarities among them. They belong to a unique group of CDPKs where there is an obligatory coupling between the presence of a nuclear localisation sequence in their junction domains and nonconsensus calcium binding EF hands in their respective CaMLDs (Raichaudhuri et al., 2006). None of the kinase shows activity towards any known exogenous substrates (Urao et al., 1994) indicating them to be unique in their choice of substrate. In view of the fact that AhCPK2 migrates to the nucleus in response to stress it is possible that these kinases phosphorylate transcription factors. With the present set of evidences both AhCPK2 and AtCPK10 are now documented to be involved in osmosensory pathways (Urao et al., 1994). Ectopic expression of the catalytic domain of AtCPK10, induced the expression of ABA responsive gene HVA1 (Sheen, 1996). Again HVA1 up-regulation by AtCPK10
mediated stress response pathway was found to be under the negative control of the phosphatase ABA insensitive1 (Sheen, 1996). This observation does show that AtCPK10 works in ABA agonistic manner but does not eliminate the possibility that it works in the upstream signaling like AhCPK2. It should be kept in mind that expression of catalytic domains of kinases ignores the signaling pathway that guides their proper subcellular localization and is unlikely to come up with the actual picture. The results that emerge from this investigation links the function of at least two members of this unique group of kinases directly with osmosensing. Further investigations are needed to understand both the upstream effectors as well as the downstream substrates for these kinases.