Extracellular calmodulin: A polypeptide signal in plants?

SUN Daye (孙大业), TANG Wenqiang (唐文强) & MA Ligeng (马力耕)

Institute of Molecular and Cell Biology, College of Life Science, Hebei Normal University, Shijiazhuang 050016, China

Correspondence should be addressed to Sun Daye (email: dysun@public.sj.he.cn)

Received April 23, 2001

Abstract Traditionally, calmodulin (CaM) was thought to be a multi-functional receptor for intracellular Ca^{2+} signals. But in the last ten years, it was found that CaM also exists and acts extracellularly in animal and plant cells to regulate many important physiological functions. Laboratory studies by the authors showed that extracellular CaM in plant cells can stimulate the proliferation of suspension cultured cell and protoplast; regulate pollen germination and pollen tube elongation, and stimulate the light-independent gene expression of Rubisco small subunit (rbcS). Furthermore, we defined the trans-membrane and intracellular signal transduction pathways for extracellular CaM by using a pollen system. The components in this pathway include heterotrimeric G-protein, phospholipase C, IP_3, calcium signal and protein phosphorylation etc. Based on our findings, we suggest that extracellular CaM is a polypeptide signal in plants. This idea strongly argues against the traditional concept that there is no intercellular polypeptide signal in plants.

Keywords: signal transduction, extracellular calmodulin, polypeptide signal.

For many years, people thought there were no polypeptide signals in plants. Since it was discovered in the late 1960s, calmodulin (CaM) has been described as a multi-functional receptor for intracellular Ca^{2+} in regulating protein activity as well as gene expression[1]. In the early 1980s, CaM was found outside of animal and plant cells (extracellularly, also referred to as apoplast in plant), and has extracellularly regulated multiple important biological functions. With the unveiling of its transmembrane and intracellular signaling transduction mechanism, we propose that extracellular CaM is a universal polypeptide signal in living organisms.

1 Discovery of extracellular CaM and its universal existence

In the early 1980s, Boynton et al.[2] found that in the T51B rat liver cells, DNA synthesis during proliferation can be inhibited by CaM antagonist, and this inhibition can be reversed by exogenous CaM. Gorbacherskaya et al.[3] found that exogenous CaM could stimulate the proliferation of human lymphocytes. Later, Wong et al.[4] also reported that exogenous CaM could enhance human platelet secreted thromboxane. In those papers, the authors neither indicated whether the action site of CaM was inside or outside the cell, nor found CaM from outside of the cell. In 1984, while studying the growth of B16 melanoma cells, Mac Neil et al.[5] began to speculate that exogenous CaM functioned outside of the cell and raised the concept of "extracellular CaM". In 1988, Crocker et al.[6] first reported in the animal field the detection of extracellular CaM from

serum-free culture medium of human, rabbit and rat culture cells. By using membrane impermeable CaM antagonist and CaM antibody, they convinced people that CaM actually worked outside of the cell to stimulate the proliferation of K563 leukemia lymphocyte.

In plants, Biro et al.[8] in 1984 first reported the detection of CaM from cell wall, which was even earlier than the report of extracellular CaM in animal system. The finding was totally accidental. After CaM was found in plant cells in 1980, Roux's lab started to localize and isolate CaM from different organelles of oat coleoptile cells. At that time, Dr. Terry[9], who developed the centrifugation method to extract cell wall solutions, came to visit the lab. They ran a test for CaM with extracted extracellular solution as well as other isolated organelles. CaM was detected unexpectedly from cell wall extractions and the concentration was even higher than that in different organelles. Soon after, Josefina[10] also reported the detection of CaM in cell wall of hypocotyls cells in *Cice aricium* L. Unfortunately these results did not get much attention. Later on, by using immuno-fluorescence to localize CaM in garden pea seedlings, Dauwalder et al.[11] failed to detect any CaM signals from the cell wall, so she denied Biro's work.

In 1987, our lab started reinvestigating the question of extracellular CaM. Using wheat coleoptiles as material, we first extracted the soluble proteins of cell wall by vacuum infiltration-centrifugation. Then we used Birek's method to isolate salt soluble proteins of cell wall. From these protein fractions, we detected both the soluble and bonded forms of CaM in cell wall by phosphate diesterase (PDE) activity assay, which is the traditional method for testing CaM activity[12]. After successfully raising plant CaM antibody, we were able to use immuno-gold electron microscopy to localize CaM in the cell wall of maize root tip[13, 14]. Additionally we also partially purified cell wall salt soluble CaM from wheat coleoptiles by using hydrophobic-affinity chromatography. Characteristic analysis, including hydrophobicity, heat resistance, UV absorbance spectrum, molecular weight, gel shifting in the presence or absence of Ca^{2+}, Ca^{2+} dependent activation of PDE etc. proved that extracellular CaM and intracellular CaM are similar[15]. To date, we have used serial methods such as radioimmunoassay, ELISA, PDE assay, NAD kinase assay and immuno-electron microscopy etc. to detect the extracellular calmodulin in many plant species. Cell wall CaM seems to exist in all the species we have chosen including oat, wheat, maize, cauliflower, carrot, tobacco, tomato, *Angelia dahurica* etc.[8, 12—15, 31, 34]. These results provided robust evidence that the presence of extracellular calmodulin is common in the plant kingdom and implied that it might have significant functions.

Based on our work with *Angelia dahurica*[23], tobacco, tomato (unpublished) and the published data from Crocker et al. on different cultured cell lines, the concentration of extracellular CaM is estimated at around 10^{-9} to 10^{-8} mol/L. This concentration may vary significantly depending on organism and cell type. For example, Crocker et al. reported that, in human and other animal cultured cell lines, extracellular CaM concentration could vary from 10^{-10} to 10^{-7} mol/L[6]. Additionally, extracellular CaM concentration changes dramatically at the different growth stages. In tobacco suspension cultured cells, extracellular CaM concentration changes several fold when

the cells go from initial growth stage to logarithmic stage. Similar result can also be found in human K562 cultured cells[6]. There is no doubt that CaM can be secreted out of the cell although the mechanism is still a mystery. Remgard et al.[16] observed that when treating frog sciatic nerves with ^{3}H-Thymine, the new synthesized CaM could be secreted by the none-nerve cell in the outgrowth region of the nerve. Zhang et al.[17] also reported that in rabbit pancreas cells, large amounts of CaM existed in the secretion granules. This indicates that CaM can be exported out as a secretion protein, but its exact way is unclear.

2 The biological functions of extracellular CaM

If the presence of extracellular CaM is a common phenomenon, and it is secreted out of the cell, the next question we asked is what is the function of extracellular calmodulin?

In animal system, as discussed before, exogenous CaM (extracellular) was first found to be able to stimulate the proliferation of rat liver cells, human lymphocyte, B_{16} melanoma as well as X*enopus* egg cell division and enhance platelet cell secrete thromboxane. Later, other reports showed that extracellular CaM could stimulate the proliferation of normal human umbilical vein endothelial cell[18] and keranocyte[19]. Extracellular CaM is also required for the development of pre-implanted human embryos[20]. Remgard et al.[16] found that treating frog sciatic nerve with 30 pM exogenous CaM *in vitro* can partially inhibit the growth and proliferation of axon outgrowth and non-neuronal cell, and this effect can be relieved by CaM antibody. These results also implied that endogenous extracellular CaM in nerve cells might be already in the optimal concentration. Houston et al.[21] reported that human umbilical vein endothelial cells can inhibit the secretion of tumor necrosis factor (TNF_α) from human whole blood and mononuclear cells but promote the secretion of elatase from neutopils. When they looked for the protein factor that mediates this effect from the endothelial cell culture medium, it was unexpectly found to be CaM.

As shown in table 1, the functional studies of extracellular CaM in plants are similar to animal systems, which have been mainly focused on cell proliferation and division. We tried to demonstrate that CaM could function at extracellular site to stimulate cell proliferation and division by using exogenous CaM, CaM antibodies and CaM antagonists W7-agarose etc. As a protein, CaM and its antibody are normally considered to be unable to diffuse freely through plasma membrane. As for W7-agarose, a CaM antagonist covalently linked to agarose beads which is even larger than a cell, there is no way that it can get into the cell. Obviously, the experimental results obtained by using these reagents strongly indicated that CaM could work outside of the cell.

After the discovery of the function of extracellular CaM in suspension cultured cells and protoplasts[22—25, 31], we began to consider that some biological phenomena that still have no satisfaction explanation might be mediated by extracellular CaM. This made us reconsider that extracellular CaM might act as a polypeptide signal. The idea gave us a wider and brighter vision for the study of extracellular CaM.

Table 1 The biological functions of extracellular CaM

Cell type	Biological function	Reference
Human and mammalian cells		
B16 melanoma cells	stimulate proliferation	[5]
K562 leukemia lymphocyte cells	stimulate proliferation	[6]
Normal human umbilical vein endothelial cell	stimulate proliferation	[18]
Keranocyte cells	stimulate proliferation	[19]
Human embryo cells	promote the development of pre-implanted embryo	[20]
axon outgrowth and non-neuronal cell	stimulate the growth and proliferation	[16]
Human whole blood and mononuclear cells	inhibit the secretion of TNFα	[21]
Neutrophils	increase the secretion of elatase	[21]
Plant cells		
Angelia dahurica suspension cells	stimulate cell proliferation	[22, 23]
	stimulate regeneration of protoplast cell wall and cell division	[24, 25]
Pollens from various plants	regulate pollen germination and pollen tube elongation	[26, 27]
Carrot suspension cells	stimulate cell proliferation	[28]
Maize root protoplasts	stimulate cross plasma membrane redox reaction	[29]
Lily pollen	regulate the inhibition effect of Al^{3+} on pollen germination and pollen tube elongation	[30]
Tobacco suspension cells	induce the dark expression of *rbcS* genes	[38]

For example, it has been shown that exogenous CaM could promote the germination of pollen, while CaM antagonist, TFP, could inhibit pollen germination[7, 32]. But in those papers, the authors did not pay attention to the site of CaM action. The CaM antagonists used in their experiments were all membrane permeable, so it is very hard to tell if CaM worked inside or outside of the cell, or both. We repeated their experiment first by using *Hippeastrum rutilum poleen* pollen, and observed that exogenous CaM could promote pollen germination and pollen tube elongation. When we use membrane impermeable CaM antibody and W7-agarose, the germination rate and the elongation of pollen tubes reduced significantly. The inhibition could be relieved by exogenous CaM[26, 27]. These experiments indicated that exogenous CaM indeed function outside of the plasma membrane. The data also implied that endogenous extracellular CaM is important in regulating pollen germination and pollen tube elongation[27]. Afterwards, we tested pollen from more than ten other plant species including tobacco, lily, and the results consistently showed that extracellular CaM is important in regulating pollen germination and pollen tube elongation. Furthermore, we treated tobacco stigmas with CaM and its antibody, and microinjected CaM antibody into the transmitting tissue of the style. The results showed that extracellular CaM could affect pollen germination and pollen tube elongation along the transmitting tissue. This provides semi- *in vivo* evidence for the regulation of pollen germination and pollen tube elongation by extracellular CaM[34].

In 1994, Prof. Zhu and his colleagues at Beijing University made an interesting experiment. They found that microinjection of activated CaM to the hypocotyls of etiolated *rbcS GUS* trans-

formed tobacco seeding could induce GUS expression[33]. More interestingly, the induction was not limited to the injecting site, but along the whole vascular tissue. We wondered if the injected CaM could travel along the vascular tissue and work from outside the cells to induce GUS expression. We shared this idea with Prof. Zhu, and decided to collaborate to test this hypothesis. First, we generated suspension cultured cells using the *rbcS GUS* transformed tobacco. By using this system, we showed that the expression of *rbcS-GUS* in the dark underwent a dynamic change from low to high then decreased again. Similarly, the concentration of extracellular CaM in the medium had such a dynamic pattern, but the peak of CaM concentration was 24 hours before the expression peak of *rbcS-GUS*. When we added exogenous CaM to the culture medium, the expression of *rbcS-GUS* increased after a 24-hour delay. The addition of CaM antagonist W_7-agarose and CaM antibody inhibited the expression of *rbcS-GUS*. These results indicated that extracellular CaM is also involved in regulating *rbcS-GUS* expression[38].

As we know, there is an *rbcS* gene family. The expression of some members of this family is dependent on light, and these are called "light dependent *rbcS* genes" such as *rbcS-3B* and *rbcS-3C* in tomato cells[36]. Other members are called "light independent *rbcS* genes", which means they can be expressed without light, such as *rbcS-1*, *rbcS-2* and *rbcS-3A* in tomato[36]. The expression level of light independent *rbcS* genes all shows a dynamic change from low to high then back to low again during development. Obviously, the inducer for the expression of light dependent *rbcS* members is light, but the mechanism responsible for light independent *rbcS* genes' expression is still unknown[37]. Sawbridge et al. pointed out that the regulation factor responsible for light independent *rbcS* expression should fit into three criteria. (1) This factor should have a similar dynamic pattern to the expression of *rbcS* genes. (2) The concentration and dynamic pattern of this factor should not be regulated by light. (3) This factor only regulates the expression of light independent *rbcS* genes, and should not have any effect on the light dependent *rbcS* genes. To test the idea that CaM is this regulation factor, we used different probes, which specifically recognize different subtypes of tomato *rbcS* gene family members, and did Northern blot analysis. We were able to show that extracellular CaM could up regulate the transcription of *rbcS-3A,* which is a light independent *rbcS* gene, but had no effect on the transcription of light dependent *rbcS-3C* gene[38]. At the same time, we also observed that there was no obvious change for extracellular CaM concentration either under light or dark condition. Extracellular CaM satisfied all of the criteria. In conclusion, we suggest that extracellular CaM is the key factor responsible for the light independent regulation of *rbcS* gene expression during developmental process.

Some acid soil contains high levels of Al^{3+}, which is highly toxic for plants. The toxic effect of Al^{3+} on plant cells can be observed after several seconds or minutes. But the time for Al^{3+} to get into the cell may take even hours. So people suspected that the original functional site for Al^{3+} might be outside the plasma membrane. We observed that Al^{3+} could inhibit pollen germination and pollen tube elongation, and this inhibition could be fully relieved by exogenous CaM. This recovery effect cannot be observed by applying the same concentration of S-100 protein, which is

structurally similar to CaM but has no CaM activity. An Al^{3+} chelating reagent, citric acid, can neither recover the inhibitory effect of Al^{3+} on pollen germination and pollen tube elongation, which indicates that the recovery effect of exogenous CaM is not due to the reduction of free Al^{3+} by CaM. The recovery effect of exogenous CaM can be inhibited by CaM antibody or EGTA. Because CaM, CaM antibody and EGTA are thought to be membrane impermeable, these data show that extracellular CaM could be one of the original Al^{3+} targets outside the cell. We considered that at least one mechanism responsible for the toxicity of Al^{3+} to plant growth is the inhibition of extracellular CaM activity[30]. Recently, we also have direct evidence that the stimulating effect of La^{3+} on plant growth is related to extracellular CaM (unpublished data).

3 Signal transduction mechanism for extracellular CaM

After we discovered the extracellular CaM functions described above, we started to investigate how the cell transduces the extracellular CaM signal into the cell. Since CaM is a 17 ku acidic protein, its chance of free diffusing cross the plasma membrane is small. If CaM really can function outside the cell, a signal transduction mechanism must exist.

3.1 Cell surface receptor

In animal systems, after discovering that extracellular CaM could mimic endothelial cell to inhibit the secretion of TNFα from mononuclear cells, Houston et al. further investigated the binding site or potential receptor on the cell with ^{125}I labeled CaM. They found that there were two kinds of CaM binding sites on the membrane of mononuclear cells: a high affinity binding site with a K_d around 1.8 nmol/L, a low affinity binding site with a K_d of about 30 nmol/L. Treating the cell with trypsin causes a 75% to 90% loss of those binding sites, which indicates those binding sites are protease sensitive. After that, they detected two CaM receptor-like proteins (110 ku and 44 ku) from the plasma membrane of mononuclear cells by a cross-linking method[21].

In our lab, after we demonstrated the site that exogenous CaM acts outside the cell, we began to search for the potential CaM receptor on the cell surface, using biotin and ^{125}I labeled CaM as the probe. We found that there were cell surface CaM binding sites on *Angelia dahurica* suspension cultured cells and protoplasts generated from lily pollen. The binding between these sites and labeled CaM is dose and time saturable and can be partially competitively inhibited by unlabeled CaM. Furthermore, we also detected proteins that can specifically bind to CaM from the outside of the pollen plasma membrane by using a chemical cross-linking method (unpublished data).

Based on the above work and the results from heterotrimeric G protein and PLC_β studies (see below), we believe that there are potential CaM receptors on the cell surface. But we do not know if these receptors bind directly or indirectly to extracellular CaM and transduce the signal into the cell.

3.2 G-protein

In animal systems, it is well known that heterotrimeric G-proteins are very important in signal transduction pathways. There is not much evidence supporting that heterotrimeric G-proteins participate in plant cell signal transduction, even though genome-sequencing studies show that there are heterotrimeric G proteins like subunit genes in plants. By using various experimental approaches including Western blot, pertussis toxin induced ADP-ribosylation, and GTPase activity assay, we first demonstrated the presence of heterotrimeric G-protein in pollen. Microinjection with anti-animal Gα antibody and membrane impermeable G-protein antagonists and agonists showed that heterotrimeric G-protein might participate in the pollen germination and pollen tube elongation[40]. Furthermore we detected heterotrimeric G-protein specific GTPase activity in outside-out plasma membrane vesicles, and found that this activity can be stimulated by exogenous CaM in a dose dependant manner. Taken together, our work demonstrates that heterotrimeric G-protein participates in the extracellular CaM signal transduction pathway in pollen[40]. Also our work implies that if there is a CaM receptor on the membrane, it should be G protein coupled.

Later on, we used the same techniques and further proved that heterotrimeric G protein also mediates the regulation of extracellular CaM to light independent expression of rbcS gene[41]. These data indicates that heterotrimeric G protein signal transduction pathways may work somewhat commonly in plants.

3.3 Intracellular signal transduction components

With regard to intracellular signal transduction components for extracellular CaM, our results showed that Ca^{2+} channel antagonist could inhibit the stimulating effect of pollen germination and pollen tube elongation by extracellular CaM and G-protein agonists. Meanwhile Ca^{2+} channel agonists and Ca^{2+} carriers could relieve the inhibitory effect of CaM antibodies and G-protein antagonists on pollen germination and pollen tube elongation. This pharmacological evidence showed plasma membrane Ca^{2+} channels could be downstream player of G protein in mediating extracellular CaM signal[42]. In addition, we also showed that when using Fluo-3 as a Ca^{2+} probe, extracellular CaM did increase cytoplasmic Ca^{2+} concentration[39]. Other pharmacological experiments together with microinjection of animal PLC_β antibody, IP_3 and IP_3 receptor antibodies showed the PLC/IP_3/IP_3R signal transduction pathway may also participate in the signal transduction pathway of extracellular CaM regulating of pollen germination and pollen tube elongation[43]. We then detected a PLC_β like protein from pollen plasma membranes by Western blot. Also we were able to show that exogenous CaM could stimulate PLC_β-like activity in pollen tube sub-protoplasts, while the G-protein specific inhibitor PTX and agonist CTX could inhibit or stimulate this PLC_β-like activity. These data further showed that PLC_β might participate in the extracellular CaM signal transduction pathway (unpublished data). In summary, our evidence shows that, in the numerous signal transduction pathways inside the cell, there are at least two, the Ca^{2+} and PLC-IP_3-IP_3R pathways, that might be involved in the extracellular CaM signal trans-

duction. More recently we have also found that extracellular CaM can enhance the protein phosphorylation level in the cytoplasm and nucleus of tobacco suspension cells[48].

Based on our evidence in the pollen system, we propose a model for the signal transduction mechanism of extracellular CaM (fig. 1). In this model, activated extracellular CaM binds to its receptor and activates the heterotrimeric G-protein by some unknown coupling mechanism. Active G-protein can activate two downstream pathways: the opening of plasma membrane Ca^{2+} channel to allow the influx of extracellular Ca^{2+} and the activation of PLC_{β}, which will produce the secondary messengers IP_3 and DAG. Once IP_3 binds to its receptor, Ca^{2+} will be released into the cytoplasm from intracellular Ca^{2+} stores such as the ER. Although we still do not know much about the order of these two Ca^{2+} related signaling events and their cross-talk pattern, the increased cytoplasm Ca^{2+} concentration might activate protein kinases to regulate physiological reactions as well as gene expression.

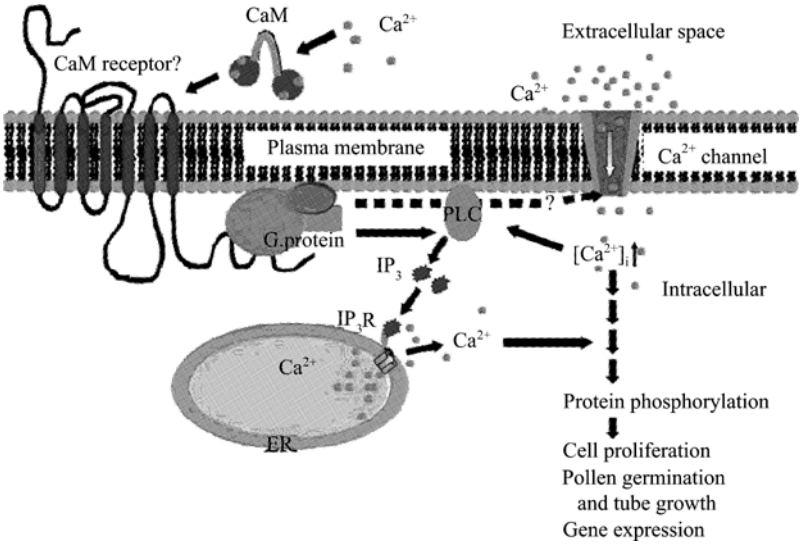

Fig. 1. A hypothetic model of extracellular calmodulin signal transduction pathway.

Still, more evidence is needed to confirm this model. Although the model we proposed above is based only on a pollen system, it might also be applicable to other plant systems. We do not exclude the possibility that the other signal transduction passway is also included in the transmembrane mechanism of extacellular calmodulin.

4 Discussion and perspective

Due to the common presence and the regulation of multiple functions of extracellular CaM in animal and plant cells, and the existence of the transmembrane and intracellular signal transduction pathway, we believe that extracellular CaM is a universal, multifunctional polypeptide first messenger[31, 45,46]. In *Dictyostlium discoidem* and some animal cells, cAMP and Ca^{2+} can act both

a component of intracellular signal transduction pathways as well as an extracellular messenger. We called this kind of signal molecule "compatible function messengers"[45], which might play an important role in regulating cell-cell communication. Compared with those "compatible function messengers", extracellular CaM is more popular and regulates more physiological responses.

As it was shown above, the study of plant extracellular CaM is much faster and more detailed than those in animal system. The reason could be that it is more significant in plants. In animal systems, the function of extracellular CaM was masked by the flood of all kinds of other extracellular polypeptide signals, such as hormones, growth factors, and neuron transmitters, which are all important in regulating cell growth and development. But in plants, most plant growth regulators (phytohormone, salisalic acid, brassinosteroid etc.) are small molecules and the family members are limited. Because plant cells have cell wall, many people believe polypeptides or proteins are too big to pass through the cell wall and contact the plasma membrane freely. Therefore, for quite a long time, our work on the extracellular CaM was not well accepted, and it was hard to make rapid progress. In the last several years, it was finally shown that polypeptide signals could also exist in plants. For example, systemin and ENDO40, which are involved in pathogen-plant interactions, were the two first discovered plant polypeptide signals[47]. Recently, it was found that polypeptide signals such as AGPs, expansin, CLV1, and SCR[46] are all important for plant developmental regulation. Thus, plant extracellular signal polypeptides open a whole new field for plant biologists to examine. More than 10 years of study in our lab has given us a clearer vision on the existence, function and signal transduction mechanisms of extracellular CaM. Raising the idea that CaM can function as an extracellular polypeptide signal will no doubt contribute highly to the study on this field.

For a long time, because of the limited understanding of extracellular polypeptide signals and their physiological functions, people could not answer some biological questions very well. The discovery that extracellular CaM could regulate pollen germination, pollen tube elongation and light independent expression of rbcS gene both made significant steps toward fully understanding these basic biological questions. The studies of Al^{3+} and La^{3+} elements, which was collaborated work requested by another lab after they recognized the importance of extracellular CaM, also shed lights in understanding how Al^{3+} and La^{3+} work in plants. We believe that studies of cell wall polypeptide signals can help us understand more about plant growth and development process as well as their regulation mechanism.

Study of extracellular polypeptide signals in plant, their signal transduction pathways and their functions in regulating cell growth and development process is just starting. Although people have already suggested that systemin and ENDO40 are plant polypeptide signals, their signal transduction mechanisms still need to be explored. The discovery of some receptor kinases and their polypeptide ligands in plant developmental biology makes them perfect candidates to be in the family of extracellular polypeptide signals, but they are mostly involved in highly specific regulatory process like meristem determination and pollen recognition. Cell wall component stud-

ies indicate the existence of regulatory polypeptides, their nature and the regulatory mechanisms still need to be clarified. In comparison, extracellular CaM carries a signal that could regulate multiple physiological processes, and we already have a preliminary blueprint of its working mechanism. However, there are still many questions that need to be answered. For example, there is no signal peptide sequence on CaM, how does it get into apoplast space? Some evidence has shown that aside from the regular secreted pathway mediated by signal peptides, there might be signal peptide independent ways to export proteins out of the cell[16]. The secretion of CaM might belong to one of those ways. However more evidence is needed. Given that there is high concentration of Ca^{2+} in the cell wall, it is still poorly understood how apoplast CaM activity is regulated. Additionally, it will be very important to find the potential CaM receptor on the membrane to fill the gap of CaM perception mechanism. The regulatory role of extracellular CaM in plant developmental processes is also very important and needs to be explored. Those are the major questions we are focusing on at present.

Acknowledgements We sincerely thank Yu Sun (University of Texas at Austin) for modifying the translation of the English version of this paper. We thank Sun Ying, Guo Yi, Cui Sujuan, Mao Guhong, Zhong Suqiao and Zhou Junli and many other people in our lab for helpful discussion and the development of ideas. This work was supported by the National Natural Science Foundation of China (Grant Nos. 39730230, 39870365), the National Key Basic Research Special Funds of China (Grant No. G19990117) and the National Outstanding Young Scientist Fellowship of China (Grant No. 30025024).

References

1. Cheng, W. Y., Cyclic 3', 5'-nucleotide phosphodiestrase: demonstration of an activator, Biochm. Biophys. Res. Commun., 1970, 38: 533—538.
2. Boynton, A. L., Whitfield, J. F., MacManus, J. P., Calmodulin stimulates DNA synthesis by rat liver cells，BBRC, 1980,95(2): 745—749.
3. Gorbacherskaya, L. V., Borovkova, T. V., Rybin, U. O. et al., Effect of exogenous calmodulin on lymphocyte proliferation in normal subjects, Bull Exp. Med.Biol., 1983, 95: 361—363.
4. Wong, P. Y.-K., Lee, W. H., Chao, PH.-W., The role of calmodulin in prostaglandin metabolism, Ann. NY Acad. Sci., 1980, 356: 179—189.
5. Mac Neil, S., Dawson, R. A., Crocker, G. et al., Effects of extracellular calmodulin and calmodulin antagonists on B16 melanoma cell growth, J. Invest. Dermatol., 1984, 83: 15—19.
6. Crocker, D. G., Dawson, R. A., Mac Neil, S. et al., An extracellular role for calmodulin-like activity in cell proliferation, Biochem. J., 1988, 253: 877—884.
7. Polito, V. S., Calmodulin and calmodulin inhibitors: effect on pollen germination and tube growth, in Pollen: Biology and Implications for Plant Breeding (eds. Mulvshy, D. L., Ottaviaro, E.), New York: Elsevier, 1983. 53—60.
8. Biro, R. L., Sun, D. Y., Roux, S. J.et al., Characterization of oat calmodulin and radioimmunoassay of its subcellular distribution, Plant Physiol., 1984,75: 382—386.
9. Terry, M. E., Bonner, B. A., An examination of centrifugation as a method of extracting an extracellular solution from peas, and its use for the study of IAA-induced growth, Plant Physiol., 1980, 66: 321—325.
10. Josefina, H. N., Aldasars, J. J., Rodriguez, D., Localization of calmodulin on embryonic *Cice aricium* L, in Molecular and Cellular Aspects of Calcium in Plant Development (ed. Trewavas, A. J.), New York, London: Plenum Press, 1985, 313.
11. Dauwalder, M., Roux, S. J., Hardison, L., Distribution of calmodulin in pea seedling: immunocytochemical localization in plumules and root apices, Planta, 1986, 168: 461—470.
12. Ye, Z. H., Sun, D. Y., Guo, J. F., Preliminary study on wheat cell wall calmodulin, Chin. Sci. Bull. (in Chinese), 1988, 33(8): 624—626.

13. Li, J. X., Liu, J. W., Sun, D. Y., Immunoelectron microscopic localization of calmodulin in maize root cell, Cell Res., 1993, 3: 11—19.
14. Li, J. X., Sun, D. Y., Comparative studies on immunoreactivity of antibodies against plant and animal calmodulin, Acta Botanica Sinica (in Chinese), 1992, 34(4): 257—263.
15. Ye, Z. H., Guo, J. F., Sun, D. Y., Studies on the cell wall calmodulin and calmodulin-binding protein of wheat etiolated coleoptiles, Acta Phytophysiologica Sinica (in Chinese), 1989, 15(3): 223—229.
16. Remgard, P., Ekstrom, P. A. R., Ekstrom, A. et al., Calmodulin and in vitro regenerating frog sciatic herves: release and extracellular effects, European J. Neuroscience, 1995, 7: 1386—1392.
17. Cheung, M. Z., Duo, H. Y., Cheung, G. I., Localization of calmodulin in rabbit pancreas, Chinese J. of Experimental and Clinical Immunology (in Chinese), 1992, 4(6): 13—15.
18. Dawson, R. A., Mac Neil, S., Mitogenis role for extracellular calmodulin-like activity in normal human umbilical vein endothelial cells, Br. J. Haematol., 1992, 82: 151—160.
19. Goberdhan, N. J., Dawson, R. A., Freedlander, E. et al., Calmodulin-like protein as an extracellular mitogen for the keranocyte, Br. J. Dermatol., 1993, 129: 678—688.
20. Woodward, B. J., Lenton, E. A., Mac Neil, S., Requirement of preimplantation human embryos for extracellular calmodulin for development, Human Repro, 1993, 8(2): 272—276.
21. Houston, D. S., Carson, C., Esmon, C. T., Endothelial cell and extracellular calmodulin inhibited monocyte tumor necrosis factor release and augment neutrophil elastase, The J. of Biol. Chem., 1997, 272(18): 11778—11785.
22. Li, H. B., Cheng, G., Sun, D. Y., The effects of extracellular calmodulin on the cell proliferation of suspension cultured cell, Chin. Sci. Bull. (in Chinese), 1992, 37(19): 1804—1808.
23. Sun, D. Y., Li, H. B., Cheng, G., Extracellular calmodulin accelerates the proliferation of suspension cultured cells of *Anglica dahurica*, Plant Science, 1994, 99: 1—8.
24. Bian, Y. Q., Sun, D. Y., The effects of exogenous calmodulin on cell wall regeneration and cell division of protoplasts, Acta Phytophysiologica Sinica (in Chinese), 1994, 20(3): 293—297.
25. Sun, D. Y., Bian, Y. Q., Zhao, B. H. et al., The effects of extracellular calmodulin on cell wall regeneration and cell division of protoplasts, Plant & Cell Physiology, 1995, 36(1): 133—138.
26. Ma, L. G., Sun, D. Y., The studies of extracellular action side of calmodulin on initiation and promoting pollen germination and tube growth of *Hippeastrum rutilum*, Prog. in Nat. Sci. (in Chinese), 1996, 6(4): 505—506.
27. Ma, L. G., Sun, D. Y., The effects of extracellular calmodulin on initiation of *Hippeastrum rutilum* pollen germination and tube growth, Planta, 1997, 202: 336—340.
28. Zhao, H. J., Zhu, Y. X., The effect of extracellular calmodulin on plant cell division and proliferation, Chi. Bioche. J., 1996, 12(6): 413—417.
29. Sun Yu, Chen, J., Sun, D. Y., Extracellular calmodulin stimulates the transplasma membrane redox reaction of root protoplasma in Zea mays, Acta Botanica Sinica (in Chinese), 1998, 40(5): 437—441.
30. Ma, L. G., Fan, Q. S., Yu, Z. Q. et al., Does aluminum inhibit pollen germination via extracellular calmodulin? Plant Cell Physiol., 2000, 41(3): 372—376.
31. Sun, D. Y., Tang, J., Li, H. B., The presence and biological significance of extracellular calmodulin in cell, Chin. Sci. Bull. (in Chinese), 1995, 40(14): 1153—1159.
32. Gong, M., Yang, Z., Cao, Z., Involvement of calmodulin in pollen germination and pollen tube growth, Acta Phytophysiologica Sinica, 1994, 20: 240—247.
33. Zhu, Y. X., Gu, X. S., Zhao, H. J. et al., Extracellular calmodulin stimulates RbcS-GUS expression of etiolated transgenic tobacco plants in full darkness, Plant Growth Regulation, 1998, 25: 23—28.
34. Cui, S. J., Wang, H. H., Ma, L. G. et al., The effects of extracellular calmodulin of style and pollen on pollen germination and pollen tube growth, Acta Phytophysiologica Sinica (in Chinese), 1998, 42: 320—326.
35. Ma, L. G., Xu, X. D., Cui, S. J. et al., Effects of extracellular calmodulin on pollen germination and tube growth, Chin. Sci. Bull. (in Chinese), 1998, 43(2): 143—146.
36. Wanner, L. A., Gruissem, W., Expression dynamics of the tomato rbcS gene family during development, Plant Cell, 1991,

3: 1289—1303.

37. Sawbridge, T. I., Knight, M. R., Jenkins, G. I., Ontogenetic regulation and photoregulation of members of the *Phaseolus vulgaris* L. rbcS gene family, Planta, 1996, 198: 31—38.
38. Zhang, S. Q., Ma, L. G., Sun, D. Y., The effect of extracellular calmodulin on rbcS-3A expression in suspension-cultured tomato cells, Acta Botanica Sinica (in Chinese), 2000, 42(6): 653—655.
39. Shang, Z. L., Ma, L. G., Wang, X. C. et al., Effect of extracellular calmodulin on the cytosolic Ca^{2+} concentration in lily pollen grains, Acta Botanica Sinica (in Chinese), 2001, 43(1): 12—17.
40. Ma, L. G., Xu, X. D., Cui, S. J. et al., The presence of a heterotrimeric G protein and its role in signal transduction of extracellular calmodulin in pollen germination and tube growth, Plant Cell, 1999, 11: 1351—1363.
41. Guo, Y., Ma, L. G., Zhang, L. et al., The involvement of hetrotrimeric G protein in signal transduction of extracellular calmodulin in regulating rbcS expression, Chin. Sci. Bull., 2000, 45(20): 2195—2200.
42. Ma, L. G., Xu, X. D., Cui, S. J. et al., The involvement of phosphoinositide signaling pathway in the initiatory effects of extracellular calmodulin on pollen germination and tube growth, Acta Phytophysiologica Sinica (in Chinese), 1998, 24: 196—200.
43. Wang, X., Cui, S. J., Ma, L. G. et al., The involvement of PLC-IP3 pathway in pollen tube growth by microinjection study, Acta Botanica Sinica (in Chinese), 2000, 42(1): 697—702.
44. Franklin-Tong, V. E., Signaling and the modulation of pollen tube growth, Plant Cell, 1999, 11: 727—738.
45. Sun, D. Y., The universality and biological significance of signal molecules with intracellular-extracellular compatible functions, Chin. Sci. Bull. (in Chinese), 1999，44(19): 1729—1734.
46. Sun, D. Y., Apoplast——The important signal source for fate decision of cell development, Acta Botanica Sinica (in Chinese), 2000, 42(5): 441—445.
47. Marx, J., Plants, like animals, may make use of peptide signals, Science, 1996, 237(6): 1338—1339.
48. Zhang, L. Q., Li, F., Sun, D. Y., The effects of extracellular calmodulin on protein phospherylation in cytoplasmic fraction from suspension-cultured tobacco cells, Acta Phytophysiologica Sinica (in Chinese), 2001, 27(3): 201—206.