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- Review -

Functional Mechanism of Calmodulin for Cellular Responses in Plants

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Calcium (Ca^{2+}) plays pivotal roles as an intracellular second messenger in response to a variety of stimuli, including light, abiotic- and biotic stresses and hormones. Ca^{2+} sensor is Ca^{2+}-binding protein known to function in transducing signals by activating specific targets and pathways. Among Ca^{2+}-binding proteins, calmodulin (CaM) has been well reported to regulate the activity of down-stream target proteins in plants and animals. Especially plants possess multiple CaM genes and many CaM target proteins, including unique protein kinases and transcription factors. Thus, plants are possible to perceive different signals from their surroundings and adapt to the changing environment. However, the function of most of CaM or CaM-related proteins have been remained uncharacterized and unknown. Hence, a better understanding of the function of these proteins will help in deciphering their roles in plant growth, development and response to environmental stimuli. This review focuses on Ca^{2+}-CaM messenger system, CaM-associated proteins and their role in responses to external stimuli of both abiotic and biotic stresses in plants.

Key words : Calcium, calmodulin, signal transduction, abiotic stress, biotic stress

Introduction

Plants possess tremendous flexibility in modulating their metabolic processes and growth patterns in response to various stimuli, including environmental stresses, pathogenic attack, and hormones. The morphogenic- and developmental plasticity of plants is partially due to the complexity and multiplicity of the Ca^{2+}/CaM messenger system in plants (Fig. 1). Ca^{2+}, a universal second messenger, acts as a mediator of stimulus-response coupling in the regulation of diverse cellular functions. In response to a variety of extracellular stimuli, cytosolic Ca^{2+} concentration in plants is rapidly elevated and transient Ca^{2+} elevation is sensed by several Ca^{2+} sensors or Ca^{2+}-binding proteins [61,68]. According to the reports, Ca^{2+}-dependent protein kinase (CDPK) [12,26], calcineurin B-like protein (CBL) [49], and calmodulin (CaM) [61,85] have been mentioned as Ca^{2+}-related proteins. Among these proteins, CaM is the most conserved Ca^{2+}-binding protein in plants.

CaM has been known as multifunctional regulatory protein and it interacts with other proteins in the cells and regulates their activity. CaM activates numerous target proteins that share very little amino acid sequence homology in their binding sites. The majority of known binding sites is composed of a stretch of 12-30 contiguous amino acids with positively charged amphiphilic characteristics and propensity to form α-helix [69]. If so, how CaMs may differentially regulate diverse target proteins for various responses. It has been suggested that different kinds of CaMs compete for targets *in vivo*, and thus relative abundance, subcellular distribution, and effector-binding affinities of the various CaMs may play important roles in regulating a particular target. In a number of studies, the activities of several enzymes such as protein kinase, NAD-kinase, glutamate decarboxylase and Ca^{2+}- ATPase are especially addressed to be involved in such mechanism [58,69,78,85]. In presentation of Lee and colleagues, SCaM-4 activated mammalian cyclic nucleotide phosphodiesterase, but it was unable to activate the CaM-dependent plant enzyme NAD kinase [13,44]. This means that a single CaM as a modulator could induce reciprocal activation and inhibition of two different enzymes in the regulation of signal transduction pathways. Ca^{2+}-ATPase and glutamate decarboxylase also exhibited a modest CaM isoform-sensitivity and were regulated by CaM [45]. These results suggest a complexity of interactions of CaMs with their targets.

During more than a decade after discovering CaMs in plants, many of CaMs were isolated and characterized and biochemical studies for CaMs were reported. However,

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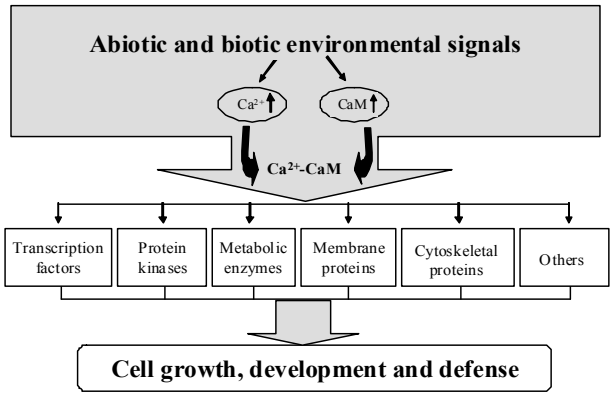

Fig. 1. Ca^{2+}/calmodulin-mediated responses in plants. Ca^{2+} and calmodulin (CaM) are elevated by abiotic and biotic environmental signals and then Ca^{2+}/CaM complex binds to numerous target proteins and modulates their activities. Those target proteins include transcription factors, protein kinases, metabolic enzymes, membrane proteins and cytoskeleton proteins. Finally, the Ca^{2+}/CaM-mediated signal network results in physiological responses such as cell growth, development and defense.

detailed information about CaMs are lacking at present and CaMs could be the good subject of active investigation. In this paper, the characterization and regulatory mechanisms of CaM-binding proteins and protein kinases related to CaM will be reviewed. In addition, this review will describe how CaM and CaM-related proteins could response to abiotic (environmental stresses) and biotic (pathogenic attack and hormones) signals and could function in plant.

Ca^{2+}/CaM-related protein kinases

In plant, the activity of protein kinase was stimulated by Ca^{2+} and associated with CaM [82]. According to reports, protein kinases for this group included Ca^{2+} dependent protein kinase or CaM-like domain protein kinase (CDPK), CaM-dependent protein kinases (CaMKs), $Ca^{2+}$and CaM-dependent protein kinases (CCaMKs), and CDPK-related protein kinases (CRKs). CDPKs contain catalytic-, autoinhibitory-, and Ca^{2+} binding domains and four EF-hand calcium-binding motifs [27,31,85]. CCaMKs contain only three EF-hands, which is similar to visinin (another EF-hand protein) and were activated by CaM. CCaMK binds both Ca^{2+} ions and Ca^{2+}/CaM, but its activation is proposed to occur through the binding of Ca^{2+}/CaM to similar site in autoinhibitory domain of CDPKs [76]. The plant CaMK has not been characterized thoroughly except only one, which is similar in sequence of the CCaMKs and it carries CaM-binding site although lacking EF-hands. CRKs have catalytic domains closely related to those of CDPKs, however their EF-hands are poorly conserved compared to CaM. Although it has not been characterized how these protein kinases are regulated, it was reported that CRK appears to be unresponsive to Ca^{2+} and also is not influenced by the addition of CaM even in the presence of a CaM-binding site [26].

Among four type of protein kinases identified in plant, CDPKs have well been investigated. It was reported that the activation of CDPK is regulated by Ca^{2+}, which promotes the binding of CaM to a region of autoinhibitory sequence [84]. In normal condition, CDPK are kept in a low basal state of activity by an autoinhibitor located in its C-terminal domain. However, the binding of Ca^{2+} somehow disrupts the autoinhibitor and results in the 'release of inhibition'. CDPKs are differently expressed in specific tissues, physiological conditions or developmental stages. They are also related to differences in substrate specificity, subcellular location, Ca^{2+} sensitivity and the specialization of different isoforms with respect to Ca^{2+} binding and their activation. Three CDPK isoforms from soybean show that they are respond differently to different ranges of Ca^{2+} concentrations [46]. Also, the regulation of CDPKs can be influenced by their sensitivity to Ca^{2+} and the type of protein substrate. In the presence of substrates, the sensitivity of Ca^{2+} can be increased tenfold or more. These differences in sensitivity to Ca^{2+} could imply that each isoform of CDPK responds to a specific set of Ca^{2+} signals depending on frequency of oscillation, magnitude and duration of the stimulus [52,67].

CaM binding proteins

In plants, some proteins were identified as being regulated by CaM and the CaM-target proteins have been isolated and their functions have been investigated *in vitro*. According to the reports, CaM-target proteins can be divided into four groups. The first type of CaM-target protein is considered unique to plants including a maize root tip protein [62], TCB60 (a heat shock repressed tobacco protein) [17], and a pollen-specific protein [66]. The second type is proteins resembling CaM-regulated proteins of known functions from other organisms such as Ca^{2+}-ATPases (SCA1) [14], protein kinases (CCaMK) [59], and cyclic nucleotide-gated non-selective cation channel proteins (CNGCs) [1,2,41]. Ca^{2+}-ATPases have properties as ATPase stimulated by

Ca^{2+}/CaM and protein kinases enhance substrate phosphorylation by binding with Ca^{2+}/CaM. CNGCs have been reported from tobacco and Arabidopsis and are permeable for K^+ and Ca^{2+} [47]. Overexpression of a tobacco CNGC (NtCBP4) confers tolerance to Ni^{2+} uptake and hyposensitivity to Pb^{2+} uptake in plants [70]. Mutation of Arabidopsis CNGC (AtCNGC2) abolishes hypersensitive response upon pathogenic infection, suggesting that AtCNGC2 is involved in apoptosis [15]. The third type is proteins of known function in other organisms but regulated by CaM only in plants, such as glutamate decarboxylase (GAD) [71] and apyrase [29,73]. GAD catalyses conversion of glutamate to γ-aminobutyric acid by Ca^{2+}/CaM and stresses. Apyrase functions as nucleotide phosphatase by Ca^{2+}/CaM and has xenobiotic resistance. Especially, disruption of binding of apyrase with CaM inhibited pollen germination in Arabidopsis [73]. The last type is proteins, which initially had not been identified as CaM-binding proteins. These proteins include CGCG proteins [56,63,79,81], PCBP (a nuclear CaM-binding protein) [64], MLO (a modulator of plant defense and cell death) [37], catalase [81], DGK (diacylglycerol kinases) [72], SAUR1 [78], and NtER1 [79].

CGCG family shares the structural features of a CGCG DNA-binding domain in the N-terminus, a CaM-binding domain in the C-terminus, and ankyrin repeats in the central portion. In Arabidopsis, a CGCG protein (AtSR1) exhibits special DNA-binding activity to a novel CGCG box [80]. A transcription activation domain has also been identified in AtSR1 homolog [56]. Furthermore, six Arabidopsis CGCG genes are induced by a variety of environmental- and hormonal signals [80]. Thus, CGCG genes might play an important role in plant stress tolerance. PCBP as nuclear CaM-binding protein has been isolated from potato tubers [64]. Tuberization in potato is controlled by hormonal and environmental signals. Ca^{2+}, an important intracellular messenger, and CaM, one of the primary Ca^{2+} sensors, have been implicated in controlling diverse cellular processes including tuberization in plants [64].

Among proteins binding to CaM, DRL (deformed roots and leaves), a homolog of yeast TOT4 (also known as KTI12) plays a role in meristem activity and organ growth in plants [54]. TOT4/KTI12 combines with elongator, a complex binding to the RNA polymerase II transcription elongator complex [82]. In addition, recessive mutations at the *DRL1* locus caused defective organ formation such as disorganized shoot, inflorescence, flower and root meristems [54]. In barley, MLO was known as a negative regulator of broad-spectrum disease resistance and leaf cell death [82]. Loss of CaM binding to MLO decrease its ability to regulate defense negatively against powdery mildew *in vivo* [38]. It was recently found that Ca^{2+}/CaM binds and activates plant catalases for the regulation of H_2O_2 homeostasis, reducing H_2O_2 by stimulating catalase activity through Ca^{2+}/CaM modulation and producing more NADPH *via* Ca^{2+}/CaM-regulated NAD kinase [25,39,81]. Diacylglycerol kinases (DGKs) catalyze the phosphorylation of diacylglycerol to yield phosphatidic acid (PA), which has been shown to accumulate rapidly in plants in response to stimuli. Tomato DGK (LeCBDGK) associated with membrane is Ca^{2+}/CaM-dependent [82]. SAUR and NtER was identified as CaM-binding proteins [78,79] and they could interact with hormones. SAURs are rapidly induced by auxin (within minutes) and NtER is rapidly responsive to ethylene (within minutes) and abscisic acid, as well as other stress signals [79,81]. Therefore, these genes might be classified as a group of early signal responsive genes.

CaM in plant responses to biotic and abiotic signals

Mechanical, light, and gravity stimuli

Mechanical signals (touch, rain, and wounding), light, and gravity affect on plant morphology such as stem lengthening and thickening, cell wall composition, direction of pollen tube growth, and wrapping of tendrils around supportive surfaces. It was suggested that CaM was associated with the responses mentioned above. In Arabidopsis, CaM-related genes were reported to mediate plant responses according to mechanical stimuli [9]. In transgenic plant overexpressing Mca1, which is induced by touch in Arabidopsis, the CaM-like protein Tch3 or Cml12 were overproduced [54]. CaM also participates in the response to both light and gravity in some plants [19,24,70]. In etiolated seedlings, light-induced opening of the apical hook is a striking example [51]. The most apical part of the hypocotyl goes from positive gravitropism in darkness (to maintain the apical hook) to negative gravitropism as the hook opens in response to light [7,21,51]. Because the gravity response is a light-dependent process mediated by phytochrome, it is possible that cross talk between them involves a CaM-regulated pathway. In phytochrome-deficient mutant tomato, Ca^{2+}/CaM-dependent and -independent pathways

for light-mediated gene activation and chloroplast development were reported [55]. The perception of gravity typically directs root growth downwards, while shoots grow upwards. CaM is also involved in the response of plants to gravity, as an asymmetric growth of cells on opposite sides of an organ [16].

Responses to high/low-temperature stress and salinity stress

It has been reported that adaptive responses to heat shock, cold shock, and salinity stress are mediated by Ca^{2+} ions [21,23,40]. According to the studies, CaM is involved in mediating at least some of these responses in plants [9]. Heat shock induces GABA accumulation and this heat-induced GABA accumulation is abolished if plants are pretreated with CaM antagonists or Ca^{2+} channel blockers [48]. In addition, FKBPs forming complexes with CaM function in protein folding associated with their peptidyl prolyl *cis-trans*-isomerase activity [35,70]. These findings suggest that CaM-related proteins are likely involved in modulating biochemical aspects of plant adaptation to heat stress and thermo-tolerance. There are also accumulating evidences that CaM is involved in mediating responses to cold stress in plants. CaM-related touch-induced genes and CaM-binding CNGCs were implicated in cold stress responses [9,42,60]. Mutating AtCNGC2 abolishes the hypersensitive response upon pathogenic infection, suggesting that AtCNGC2 is involved in apoptosis [15].

Responses to anoxia and metals

Responding to anoxia in plants, CaM was suggested to regulate the transduction of an anaerobic signal [4]. In rice root, the treatment of the Ca^{2+}-channel blockers, ruthenium red (RR) and verapamil and the CaM antagonists *N*-(6-aminohexyl)-5-chloro-1-naphtylenesulfonamide (W-7) and trifluoperazine, induced anoxia: (i) inhibition of amino acids and accumulation of γ-aminobutyric acid (GABA) ; (ii) a decline in the protein contents; (iii) a release of amino acids and K^+ into the growth media. These data indicate that Ca^{2+}/CaM complex is involved in the transduction of an anaerobic signal by activating the Ca^{2+}/CaM-dependent glutamate decarboxylase. CaM is also involved in regulating Ca^{2+} homeostasis by activating plasma- and endomembrane-Ca^{2+}-ATPases, which is induced in response to anoxia [4,71,74].

CaM is a cellular target for various heavy metals such as Pb^{2+}, Sr^{2+} and Cd^{2+}, which could bind at the Ca^{2+}-binding sites of CaM and could inhibit the binding of Ca^{2+} with CaM by mimicking the effect of Ca^{2+} in signal transduction [57]. For example, Pb^{2+} can act as a Ca^{2+} substitute in second messenger system mediated metabolic control [22]. The structural similarity of the protein binding sites for Pb^{2+} and Ca^{2+} is also consistent with reports that the entry of Pb^{2+} into animal and plant cells occurs, at least in part, through Ca^{2+}-permeable channels [32,77]. Consequently, CaM with low affinity to Ca^{2+} exerts different effects on CaM functions. Recent studies mentioned that plasma membrane CaM-binding CNGCs may serve as entry pathway(s) for certain heavy metals [1,3,75]. Indeed, over-expressing of a member of this protein family, tobacco plasma membrane calmodulin-binding transporter, conferred hypersensitivity to Pb^{2+} and enhanced Pb^{2+} accumulation in transgenic plants [1]. By contrast, transgenic Arabidopsis expressing a truncated version of this protein, from which the calmodulin-binding domain and part of the putative cyclic nucleotide-binding domain was removed, displayed attenuated uptake of Pb^{2+} and improved tolerance to this toxic metal [75]. It has been reported that aluminium also has effects on the cytosolic Ca^{2+} homeostasis in root hairs [34]. Aluminium, although not belong to a heavy metal by definition, exerts negative effects on plant growth which could cause major problems in world agricultural industry. Recent study suggests that extracellular CaM might be involved in the effects of aluminium on pollen germination and tube elongation [50].

CaM in plant responses to hormones

It has been reported that CaM is involved in the process of plant responses to hormones, which can trigger changes of the cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) [6,30,36]. CaM acts as Ca^{2+} receptors and activates numerous downstream target proteins and thus it is involved in many physiological responses, such as cell growth or differentiation, stress tolerance or growth arrest, and cell death in plant. Among plant hormones, auxin has been well known to have a regulatory mechanism mentioned above. Auxin induce an elevation of $[Ca^{2+}]_{cyt}$ and then cause the production of TCH3 proteins [5,6]. TCH3, as CaM-related protein, might be involved in tissue reinforcement and cell expansion [6,10]. Ca^{2+}/CaM complex which is induced by auxin leads to the expression of TCH3 and these proteins affect plant growth and development.

In the regulation of plant growth and development, gibberellin (GA_3) and brassinolide (BL) could function as important signaling molecules [20,36]. In plant, GA_3 and BL treatment induced the increased activity of CDPK, which contains a protein kinase catalytic domain, a carboxyl-terminal CaM-like domain with 4 EF-hands as Ca^{2+} binding sites, and a junction domain between the kinase and the CaM-like domain [36,83]. CDPK could function in the cellular signalling processes in plant and the activity of CDPK is inhibited by Ca^{2+} chelator and CaM antagonist [19,36].

According to the reports, abscisic acid (ABA) can induce transient elevations of Ca^{2+} and these signal transduction might be mediated by CaM [8,70,82,86]. CaM is involved in ROS (reactive oxygen species) signaling and thus might induce antioxidant defense [8,11,18,30,43]. Hu and colleagues exhibited that treatment of Ca^{2+} chelator, Ca^{2+} channel blocker or CaM antagonist blocked the activities and the increases in the expression of antioxidant enzymes such as SOD, APX, and GR induced by ABA in plant [30]. This result suggests that Ca^{2+}-CaM is required for ABA-induced antioxidant defense in plant.

CaM in plant defence against pathogens

Harding and his colleagues suggested CaM might be involved in responses to pathogens in plant [25]. They suggested that the CaM-regulated NAD kinase may be a downstream target which, by altering NAD(H)/NADP(H) homeostasis, potentiates active oxygen species production by NADPH oxidase during defense response [25]. In plant responses to pathogens, specific CaMs, which are rapidly induced by pathogens or fungal elicitors, were also isolated from soybean [28]. Transgenic plant overexpressing these CaMs showed that the levels of systemic-acquired-resistance (SAR) genes and pathogenic resistance were increased. In plants, systemic acquired resistance (SAR) is a "whole-plant" resistance response that occurs following an earlier localized exposure to a pathogen. SAR is analogous to the innate immune system in animals, and there is evidence that SAR in plants and innate immunity in animals may be evolutionarily conserved. SAR is important for plants to resist disease, as well as to recover from disease once occurred [65].

In analysis associated with the hypersensitive response to pathogens, Hra32 was reported as hypersensitive reaction associated gene [33]. The hypersensitive response (HR) is inducible plant response associated with disease resistance. It is characterized by rapid, localized cell death at the site of infection and believed to inhibit the spread of invading pathogens. According to the report, the Hra32 is CaM-like protein with four putative EF-hand calcium-binding domains and is induced by *Pseudomonas syringae*. This result indicates that CaM plays a important role in plant defence against pathogens. In another studies for plant-pathogen interactions, a gene designated *DND1* was identified. *DND1* is essential for the hypersensitive response to pathogens and is similar to CNGC, which is CaM-regulated protein [15]. Mutating *Arabidopsis* CNGC (AtCNGC2) abolished the hypersensitive response upon pathogenic infection [15]. This study also suggests that CaM is involved in the plant defence against pathogens.

Future studies

In recent years, Ca^{2+} signaling in plant has received a great deal of attention because of its ability to play important roles in many aspects of plant biology including biotic and abiotic stress responses [82]. Plants possess tremendous flexibility in modulating their metabolic processes and growth patterns in response to environmental stimuli. This is due to Ca^{2+} and Ca^{2+} sensor as key components in the messenger systems evoked during signaling and stimuli response [70]. A remarkably conserved Ca^{2+} sensor has been known as CaM and a complex Ca^{2+}/CaM-mediated signal network affects many aspects of plant growth, development and responses to environmental changes [82]. Plants possess multiple CaM genes encoding virtually identical proteins, a family of closely related CaMs, and an extended family of evolutionarily divergent CaM-like proteins. This suggests that sessile plants evolutionarily have had adapting mechanisms to survive in changing environment. We observed exciting advances in our understanding of the Ca^{2+}/CaM- mediated plant signal network over the past decade. However, much is yet unknown for the functional significance of CaM and CaM-targeting proteins. In addition, it is remained unknown how Ca^{2+}/CaM-mediated network interacts with other signal transduction pathways and how each CaM can perceive and decode the various Ca^{2+} signatures generated from various signals and ultimately lead to a physiological response. If the subjects can be solved, we will be able to provide an encompassing picture of the signalling networks operating within the plant cells.

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초록 : 식물의 세포반응에 대한 칼모듈린의 functional 작용기작 연구

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Ca^{2+}은 다양한 자극과 빛, biotic, abiotic 스트레스, 호르몬 등의 반응에 대한 세포내 2차 신호전달물질로써 중요한 역할을 한다. Ca^{2+}의 반응자들은 특정 물질과 경로를 활성화함으로써 신호전달 기능을 한다고 알려져 있는 Ca^{2+}결합 단백질들이다. 이들 단백질 중, calmidulin (CaM)은 식물과 동물의 특정 단백질의 활성을 조절하는 것으로 잘 알려져 왔다. 특히, 식물은 다양한 CaM 유전자와 특징적인 protein kinase와 전사인자를 포함한 많은 종류의 CaM 관련 단백질들을 가지고 있다. 이로 인해서 식물은 주변의 여러 가지 신호들을 인지할 수 있을 뿐만 아니라 변화된 환경에 적응할 수 있는 것이다. 하지만, 대부분의 CaM이나 이들과 관련된 단백질들의 기능은 최근 활발히 연구되고 있지만 아직 많은 작용 기작이 연구의 대상이 되고 있다. 따라서 CaM의 기능을 좀 더 이해한다면 식물의 환경적 자극에 대한 반응과 식물의 성장과 발달에 있어서 CaM의 역할을 규명하는데 도움을 줄 수 있을 것으로 기대된다. 본 논문은 Ca^{2+}-CaM의 신호전달 시스템과, CaM과 관련된 단백질들, 그리고 식물의 biotic, abiotic 스트레스에 대한 외부 자극의 반응에 있어서 CaM의 작용에 대해 기술하였다.