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Progress in Natural Science 19 (2009) 1201–1208
Progress in Natural Science
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Review

Progress in the participation of Ca^{2+}–calmodulin in heat shock signal transduction

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Received 1 December 2008; received in revised form 10 December 2008; accepted 11 December 2008

Abstract

A novel heat shock (HS) signal transduction pathway in plants for the participation of Ca^{2+}–calmodulin (CaM) in HS signal transduction was identified. HS induces a rapid increase in intracellular free calcium ion levels ($[Ca^{2+}]_i$), and the involvement of phospholipase C-inositol 1,4,5-trisphosphate is one of the factors leading to elevation in $[Ca^{2+}]_i$ induced by HS. HS also increases the expression of the CaM gene and the accumulation of the CaM protein. The CaM isoform, AtCaM3, in *Arabidopsis* is a key member in the HS signal transduction pathway. AtCaM3 regulates the activity of CaM-binding protein kinase (AtCBK3) or protein phosphatase (AtPP7), promoting the activation of the HS transcription factor, AtHSFA1a, by phosphorylation/dephosphorylation and the expression of heat shock protein genes, then improving heat tolerance in plants.

Keywords: Ca^{2+}; Calmodulin; Protein kinase; Heat shock transcription factor; Signal transduction

1. Introduction

Global warming due to human activities has become a common understanding of meteorologists in the world. The Intergovernmental Panel on Climate Change (IPCC) sent out a strong warning on climate crisis due to global warming in the IPCC Fourth Assessment Report. This report pointed out that warming of the climate system is unequivocal; the increase in the global average air temperature will reach 1.1–6.4 °C during the 21st century. It is predicted that the production of the main cereal crops will decrease by 5–10% during the next 20 years due to global warming. The study of thermotolerance in plants becomes important and imperative in this situation. The study of critical genes controlling thermotolerance in plants is the basis for understanding the mechanism of adaptation to elevated temperature. How cells perceive and transduce outside stimuli, the changes in physiological reaction and gene expression caused by the stimuli, and the molecular pathway of modulation and transduction are main interest points in cell signal transduction. The study of the heat shock signal transduction pathway is one of the ways by which we are able to find genes related to thermotolerance in plants. The mechanism by which plants withstand environmental stresses remains largely unknown. The investigation in this field is currently at the preliminary stage.

Plants are both sessile and poikilothermic; they can neither move to avoid environmental stresses nor adjust their core temperature to withstand them. As a result, they have to evolve an elaborate stress response network and a wide array of mechanisms for adapting to stressful

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1002-0071/$ - see front matter © 2009 National Natural Science Foundation of China and Chinese Academy of Sciences. Published by Elsevier Limited and Science in China Press. All rights reserved.
doi:10.1016/j.pnsc.2008.12.011

environments. This may be the crux of survival for plants under changing environments. One of the best characterized responses to environmental stresses is the induction of heat shock proteins (Hsps). The heat shock (HS) response has been observed in every organism studied to date. Hsps function as molecular chaperones, which are essential for cellular processes including protein folding, subcellular localization, assembly and signal transductions. Under stress conditions, the synthesis of molecular chaperones allows cellular proteins to avoid and/or recover from stress-induced protein aggregation [1–4]. The functional analysis for individual Hsp has been documented. The members of the Hsp100 family play a critical role in heat tolerance [5,6]. The role of small Hsps in elevating thermotolerance in tobacco (*Nicotiana plumbaginifolia*) seedlings has been reported [7,8]. Charng et al. [9] reported that a novel heat-stress-associated 32-kD protein (Hsa32) accumulated in *Arabidopsis thaliana* seedlings after HS, which is required not for induction but rather maintenance of acquired thermotolerance. The results in our laboratory showed that Hsp genes *atDjA2* and *atDjA3* play roles in improving thermotolerance [10]. In addition to Hsps, heat shock transcription factors (HSF), which mediate the transcriptional activation of Hsp genes, have a unique function of increasing thermotolerance in *Arabidopsis* and tomato (*Lycopersicon esculentum*). Among at least 17 members in the tomato HSF family, LpHsfA1 has a unique role as master regulator of thermotolerance [11]. The suppression of *AtHsfA1a* activity impaired basal and acquired thermotolerance in *Arabidopsis* [12]. Li et al. [13] provided the proof for regulation of *AtHsfA2* on expression of heat stress genes and thermotolerance in *Arabidopsis*. Charng et al. reported that the *AtHsfA2* knockout mutant showed more sensitivity to severe heat stress than the wild type [14]. The role of *AtHsfA3* regulated by upstream transcription factor DREB2A in improving thermotolerance in *Arabidopsis* has been documented recently [15,16].

However, the pathways by which HS signals are perceived and transduced to activate Hsp gene expression and induce thermotolerance remain unknown. Only a few models have been offered previously. Ananthan et al. [17] proposed that the accumulation of stress-denatured proteins could be the signal for increased expression of Hsp genes. The involvement of membrane fluidity and mitogen-activated protein kinases (MAPKs) in HS signal transduction was proposed [18]. Recent evidence shows that multiple signaling molecules, including H_2O_2, ethylene, abscisic acid (ABA) and salicylic acid (SA), are involved in HS response. The generation of reactive oxygen species (ROS) can induce Hsp synthesis, suggesting an intimate connection between HS response and oxidative stress [19–22]. Our results indicated that HS induced a rapid increase in intracellular free calcium ion levels ($[Ca^{2+}]_i$) of wheat (*Triticum aestivum* L.) seedlings and *Arabidopsis* suspension cells. The increase in $[Ca^{2+}]_i$ regulates expression of Hsp genes and synthesis of Hsps through the following signaling molecules: calmodulin (CaM) → CaM-binding protein kinase or CaM-binding protein phosphatase → the increase in binding activity of HSF to the heat shock element (HSE) → induction of Hsp genes → accumulation of Hsps, then improving heat tolerance in plants. Based on our findings, we propose a pathway for the participation of Ca^{2+}–CaM in HS signal transduction. Herein, we discuss the signal molecules involved in the novel HS signal transduction pathway, combining our study with the research progress in the field worldwide.

2. Calcium initial response of heat shock signal transduction

Ca^{2+} has firmly been established as a primary intracellular second messenger in plants and is widely employed by eukaryotic organisms to regulate a variety of cellular processes directly or indirectly [23–26]. It was reported that HS induced a large increase in $[Ca^{2+}]_i$ in *Drosophila melanogaster*, Chinese hamster and HeLa cells [27]. Heat shock also induced a 4-fold increase in the $[Ca^{2+}]_i$ in the protoplast of pea (*Pisum sativum*) leaves [28]. Gong et al. [29] observed that HS caused a rapid and transient increase in $[Ca^{2+}]_i$ in tobacco transformed with the Ca^{2+}-sensitive, luminescent protein aequorin. We also observed that the initiation of this $[Ca^{2+}]_i$ increase occurred within 1 min of HS in wheat tissue. After 4 min of HS, the $[Ca^{2+}]_i$ reached a maximum 3-fold increase [30]. In suspension-cultured *Arabidopsis* cells expressing aequorin, $[Ca^{2+}]_i$ increased rapidly after HS and reached a maximum after 10 min of HS [31]. Further results demonstrated that the expression of Hsp genes was up-regulated by the addition of $CaCl_2$ and down-regulated by the calcium ion chelator EGTA, the calcium ion channel blockers $LaCl_3$ and verapamil, during HS at 37 °C. Moreover, Ca^{2+} is also involved in the synthesis of Hsps and HS-induced thermotolerance in wheat [30,32,33]. The rapid elevation in $[Ca^{2+}]_i$ caused by HS indicates that the change in $[Ca^{2+}]_i$ is an initial response of HS signal transduction. However, the molecular mechanism for rapid elevation of $[Ca^{2+}]_i$ induced by HS is not well understood.

Ca^{2+} ion channel genes in response to heat and cold sensors have been cloned from the dorsal root or trigeminal ganglia in mammals, which belong to the mammalian transient receptor potential (TRP) family of ion channels. Cold and thermal stimuli can be perceived by TRP, activating and turning on the TRP ion channel, allowing Ca^{2+} into neurons, resulting in a series of neural responses [34–36]. However, there has been no sequence data so far that could confirm the existence of the homologue of the TRP ion channel family in plants.

Major routes for influx of Ca^{2+} into cytoplasm from extracellular sources and intracellular Ca^{2+} pools are Ca^{2+} channels in the plasma membrane and endomembrane systems. The mechanisms by which Ca^{2+} channels are regulated are quite complicated [37]. The phosphoinositide-signaling pathway (PLC-IP_3) is one of the probable pathways by which Ca^{2+} entry into the cytoplasm from intracellular Ca^{2+} pools, causes the elevation in $[Ca^{2+}]_i$.

It was reported in animals that HS induced a rapid release of IP_3 from the membranes of HA-1 CHO fibroblasts. The release of IP_3 was involved in the activation of PLC induced by HS [38]. Heat shock also induced an increase in the IP_3 level in human epidermoid A-431 cells. This increased production of IP_3 led to the increased level of Hsp70 mRNA [39]. In addition, activation of PLC-γ1 enhanced mouse embryonic fibroblasts survival during the cellular response to heat stress [40].

Our work also provided primary evidence for the possible involvement of the PLC-IP_3 signal system in the elevation of $[Ca^{2+}]_i$ and the expression of Hsp genes induced by HS in higher plants. The IP_3 level in wild type (WT) *Arabidopsis* seedlings increased within 1 min of HS at 37 °C. After 3 min of HS, the IP_3 level reached a maximum 2.5-fold increase. However, the IP_3 level changed very little after 3 min of HS at 37 °C in the *Arabidopsis* seedlings treated with 100 μM U-73122, a PLC inhibitor, suggesting that IP_3 accumulation is dependent on PLC activity. Using the transgenic *Arabidopsis* seedlings that have the *AtHsp18.2 promoter-b-glucuronidase* (GUS) fusion gene, we observed that the level of GUS activity was up-regulated obviously by the addition of caged IP_3 at non-HS temperature, and with increasing concentration of IP_3, indicating that IP_3 could induce the Hsp gene expression instead of HS. Heat stress at 37 °C could increase the GUS activity in the transgenic *Arabidopsis* seedlings but not U-73122. The above results demonstrated that PLC-IP_3 might be involved in HS signal transduction. Using suspension-cultured *Arabidopsis* cells expressing apoaequorin, we observed a significant increase in $[Ca^{2+}]_i$ during HS at 37 °C. However, the treatment of cells with 30 μM U-73122 prevented the increase in $[Ca^{2+}]_i$ induced by HS to some extent. U-73122 blocked about 40% of the increase in $[Ca^{2+}]_i$ after 10 min of HS compared to that without U-73122 [31].

Our recent work showed that HS mobilizes Ca^{2+} likely through multiple pathways. Ca^{2+} channels in the plasma membrane are also involved in Ca^{2+} mobilization besides the PLC/IP_3 signal system. In other words, HS mobilizes Ca^{2+} from not only intracellular Ca^{2+} pools but also from extracellular sources.

3. Involvement of CaM in HS signal transduction

The regulation of calcium signatures on a number of plant cellular physiological processes are mediated by many different calcium sensors, among which CaM is the most important one [26,41]. Unlike yeast and mammalian cells, higher plant cells encode and express a variety of CaM isoforms. The *Arabidopsis* genome harbors seven CaM and 50 CaM-like (CML) genes that encode potential calcium sensors [26]. The physiological functions of so many CaMs and CMLs have not been well understood so far. Differential expression is evident among the distinct CaM genes in response to many different stimuli, providing a molecular basis for a great diversity of Ca^{2+} signal transductions. It was reported that the distinct CaM isoforms were located in various subcellular organs, expressed in different developmental stages and activated different target proteins [41,42].

Gong et al. [43] observed that the level of CaM protein was up-regulated clearly by HS in maize (*Zea mays* L.) seedlings. Our study showed that the mRNA level of *CaM1-2* increased after HS at 37 °C for only 10 min, the expression of *CaM1-2* reached its maximum after 20 min of HS in wheat. The concentration of CaM protein in wheat tissue also showed an obvious increase during HS at 37 °C, and reached a maximum 2-fold increase after 90 min of HS. We also observed an increase in the mRNA level of *AtCaM3* after HS at 37 °C for only 5 min in *Arabidopsis*. The expression level of the *AtCaM3* gene reached a maximum 3.5-fold increase after 20 min of HS. The expression kinetics of CaM and Hsp genes showed that the elevation in expression of the *CaM1-2* gene in wheat and the *AtCaM3* gene in *Arabidopsis* preceded that of *Hsp70*, *Hsp26* and *AtHsp18.2* genes. The different temporal expression between CaM and Hsp genes indicates that CaM is probably involved in an early step and located upstream in the HS signal transduction pathway. Further results demonstrated that the expression of Hsp genes, *Hsp26*, *Hsp70* and *Hsp18.2*, was down-regulated by the CaM antagonists N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) and chlorpromazine (CPZ) during HS at 37 °C. Moreover, the synthesis of Hsps and HS-induced thermotolerance in wheat was also inhibited by treatment with the CaM antagonists CPZ and TFP [30,32,33]. The connection between the Ca^{2+}–CaM signal system of upstream and the expression of Hsp genes of downstream in the HS signal transduction confirms involvement of the Ca^{2+}–CaM signal system in HS response.

To determine which CaM isoforms in *Arabidopsis* are involved in the Ca^{2+}–CaM pathway of HS signal transduction, we investigated the expression of eight different CaM genes in *Arabidopsis* (*AtCaM1–7* and *CML8*) before and after HS by real-time quantitative PCR. The result showed that HS at 37 °C up-regulated the expression of *AtCaM3*. The further evidence that activation of the *AtCaM3* gene preceded the increase in expression of the *Athsp18.2* gene suggested that *AtCaM3* is probably involved in HS signal transduction [32].

Molecular and genetic analysis for AtCaM3 confirmed its role in HS signal transduction. The mutant of the *AtCaM3* gene was obtained by T-DNA insertion. The thermotolerance of the mutant *cam3* was much weaker than that of WT, but the thermotolerance of the mutants of other CaM isoforms (such as *cam2* and *cam4*) was not impaired (Fig. 1(a)). However, the complementing transgenic lines, when the AtCaM3 gene was transformed into and expressed in the *cam3* mutant, were obtained and exhibited increased or similar thermotolerance compared to WT (Fig. 1(b)). Moreover, the thermotolerance of overexpression of *AtCaM3* gene lines was much higher than that of WT (Fig. 1(c)). The above results provide functional evidence for involvement of AtCaM3 in HS signal transduction [44].

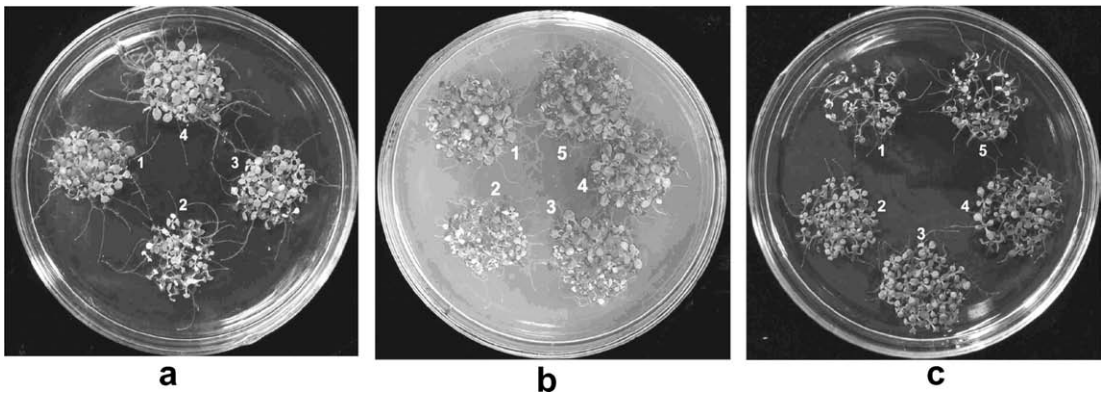

Fig. 1. The thermotolerance of the mutants of *AtCaM3* gene *cam3*, WT, the complementation transgenic lines (*cam3*/*CaM3*) and overexpression of *AtCaM3* gene lines. (a) Knockout of *AtCaM3* impairs the thermotolerance of *Arabidopsis* seedlings. 1, WT; 2, *AtCaM3* mutant *cam3*; 3, *AtCaM4* mutant *cam4*; 4, *AtCaM2* mutant *cam2*. Six-day-old seedlings grown at 22 °C were shifted to 45 °C for 50 min, and then returned to 22 °C for 6 d. (b) The effects of complemented knockout on the thermotolerance of *Arabidopsis* seedlings. 1, WT; 2, *cam3*; 3–5, complemented *cam3*/*CaM3* lines 1-2, 7-4 and 43-1, respectively. The growth condition and heat treatment for seedlings were the same as (a). (c) Overexpression of *AtCaM3* improves thermotolerance of *Arabidopsis* seedlings. 1, WT; 2–4, transgenic *AtCaM3*-overexpressing lines 3-22, 3-21 and 3-7; 5, empty vector control 1300. Six-day-old seedlings grown at 22 °C were shifted to 45 °C for 70 min, and then returned to 22 °C for 6 d.

4. CaM-dependent protein reversible phosphorylation in HS signal transduction

It has been documented in the above sections that HS induces rapid increase in $[Ca^{2+}]_i$ and CaM, the expression of Hsp genes was promoted by Ca^{2+} and CaM. How does CaM play a role in this HS signal transduction pathway? What are the downstream molecules of CaM?

Many physiological processes are regulated by protein reversible phosphorylation catalyzed by protein kinases/phosphatase, including the activation of HSF [45]. Many investigations on the phosphorylation regulation of HSF activity in mammalians have been reported, but few in plants. It was reported that *Arabidopsis* HSF1 is phosphorylated at some Sers by a cyclin-dependent kinase, causing a decrease in the DNA-binding activity of the HSF1 [46]. Holmberg et al. reported that overexpression of *CaMKII* enhanced both the level of *in vivo* Ser230 phosphorylation and the transactivation of HSF1 [47]. Watillon et al. isolated the first plant homologue of human *CaMKII*, *CB1* from the apple [48]. After that many homologous genes of *CB1* were isolated from maize, rice, *Arabidopsis* and tobacco in Lu's laboratory. These genes were named calmodulin-binding protein kinases (CBKs). The CBKs are involved in the response to interior and environmental changes [49,50]. Three CBK genes, *AtCBK1*, *AtCBK2* and *AtCBK3*, were found in *Arabidopsis*. Using the yeast two-hybrid (Y2H) assay and fluorescence resonance energy transfer (FRET) measurement, we found that AtCBK3 interacts with AtHSFA1a. The results *in vitro* indicated that AtHSFA1a was phosphorylated specifically by AtCBK3 in the presence of both calcium and CaM. *AtCBK3* was used as a target gene in following experiments.

Compared to the WT, the *AtCBK3* T-DNA insertional mutant *cbk3* plants were clearly impaired in basal thermotolerance. The complemented transgenic lines were obtained by transformation of the *AtCBK3* gene into mutant *cbk3*, and exhibited a similar level to WT plants in thermotolerance (Fig. 2(a)). The results of real-time PCR showed that the complemented transgenic lines recovered the expression of the *AtCBK3* mRNA at a level comparable to or higher than that of the WT plants, indicating that rescue of thermotolerance in complemented transgenic lines was due to recovered expression of the *AtCBK3* gene. Moreover, the thermotolerance of overexpression of *AtCBK3* gene lines was much higher than that of WT (Fig. 2(b)). We found that the transgenic lines in which thermotolerance was improved exhibited 3- to 12-fold increase in the expression of the *AtCBK3*, comparable with that of WT plants. The relationship between thermotolerance and overexpression of the *AtCBK3* gene in a dose-dependent fashion indicated that *AtCBK3* is an important gene relative to plant thermotolerance [51].

Protein phosphatases function not only by counterbalancing the protein kinases, but also by taking a leading role in many signaling events [52]. Andreeva et al. firstly obtained *PP7* cDNA that encodes a novel protein Ser/Thr phosphatase from *Arabidopsis* [53]. *Arabidopsis* PP7 possesses a CaM-binding site. It is the first protein phosphatase that is able to specifically interact with CaM in a Ca^{2+}-dependent manner found in the plant kingdom [54]. The study of the specific function of AtPP7 by molecular and genetic approaches found that the T-DNA insertion AtPP7 knockout line *pp7* impaired the thermotolerance of *Arabidopsis* seedlings, while the overexpression of *AtPP7* resulted in plants with increased thermotolerance, showing the relationship between the expression of *AtPP7* and thermotolerance [55]. AtPP7 is also a component downstream of CaM in HS signal transduction. The function of AtPP7 in HS signal transduction provides evidence for positive regulation of protein phosphatases.

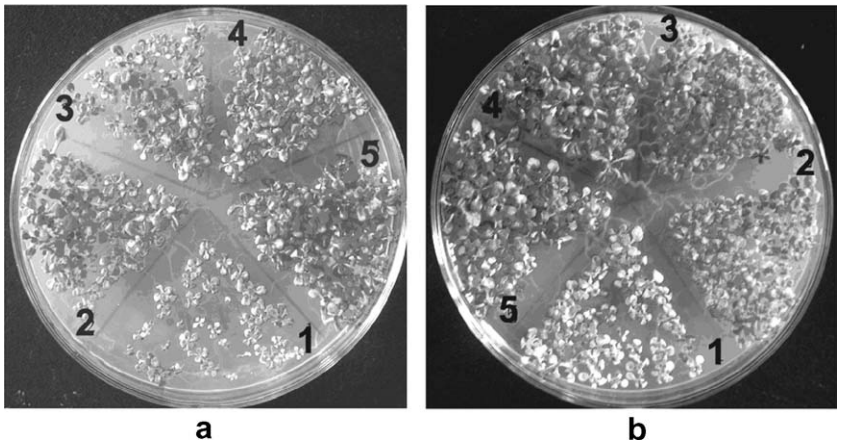

Fig. 2. The thermotolerance of the mutants of the *AtCBK3* gene *cbk3*, WT, the complementation transgenic lines (*cbk3*/*CBK3*) and overexpression of *AtCBK3* gene lines. (a) The comparison of *AtCaM3* knockout mutant with the complemented transgenic lines in the basal thermotolerance of *Arabidopsis* seedlings. 1, At*CBK3* mutant *cbk3*; 2, WT; 3–5, complemented transgenic lines 12-2, 5-4, 24-3, respectively. Ten-day-old seedlings grown at 22 °C were shifted to 45 °C for 30 min, and then returned to 22 °C. Plants were photographed 7 d after the return to 22 °C. (b) Overexpression of *AtCBK3* improves thermotolerance of *Arabidopsis* seedlings. 1, WT; 2–5, transgenic *AtCBK3*-overexpressing lines 11-6, 60-2, 34-3 and 76-2. Ten-day-old seedlings of WT and transgenic lines grown at 22 °C were shifted to 45 °C for 45 min, and then returned to 22 °C for 7 d.

The results mentioned above demonstrated that the overexpression of *AtCBK3* or *AtPP7* is able to improve the thermotolerance of *Arabidopsis* seedlings. Although the function of protein kinases and phosphatases is reversible, the study of phosphorylation of HSF1 in mammalian cells indicated that the activity of HSFs is not simply activated or depressed by phosphorylation or dephosphorylation of HSFs. The reversible phosphorylation of HSFs involves distinct protein kinases/phosphatases and multiple phosphorylation sites. The activity of HSFs can be activated by phosphorylation in some sites, or depressed by phosphorylation in other sites [47,56,57]. The HSF gene family in the plant kingdom is more complex than that in mammalians [58], so the phosphorylation regulation of HSFs in plants is complicated. The mechanism of how reversible phosphorylation regulates activity of HSFs remains to be solved and is the subject of ongoing studies.

5. Regulation of binding activity of HSF to HSE in HS signal transduction

The expression of Hsp genes induced by HS is mediated by HSFs. The regulation of activity of the HSF is a central mechanism of transcriptional regulation for Hsp gene expression [12,58,59]. Under the normal growth condition, the HSF proteins are present in the cytoplasm as inactive monomers. During HS, the HSF proteins are converted from transcriptional inactive monomers to active trimmer followed by transport into the nucleus, where HSF binds to the HSE in the promoter region of Hsp genes. Binding of HSF to the HSE activates transcription of the Hsp genes [59–61].

Mosser [62] reported that *in vitro* DNA-binding activity of the HSF could be induced by Ca^{2+} in HeLa cells. The results in our laboratory showed that the binding activity of the HSF to HSE was promoted not only by Ca^{2+} but also by CaM. Heat shock at 44 °C increased binding of HSF to HSE in maize cell extract; however, at non-HS temperature (27 °C), 1–10 mM $CaCl_2$ can activate HSF in place of HS treatment. The addition of Ca^{2+} chelant EGTA decreased the activity of HSF significantly at 44 °C, but addition of 1 or 5 mM $CaCl_2$ to the extract pretreated by 5 mM EGTA partly restored the binding activity of HSF to HSE. The binding activity of HSF to HSE in maize cell extract was inhibited by CaM antagonists W7 and CPZ or CaM antiserum at 44 °C. Re-addition of 10^{-8} or 10^{-7} M CaM to the sample pretreated with CaM antiserum restored the ability of the HSF to bind to HSE. CaM could promote activity of HSF at both non-HS (27 °C) and HS (44 °C) temperatures, but other proteins, for example BSA, could not [63]. Similar results were obtained in wheat and tomato [64]. Based on the above results, we proposed that CaM induced the expression of Hsp genes and increased plant thermotolerance through activating the DNA-binding activity of HSF.

The components upstream of HSF in the HS signal transduction pathway, AtCaM3, AtCBK3 and AtPP7, were all defined to be able to promote the binding of HSF to HSE, activate the expression of Hsp genes and then improve plant thermotolerance.

The different genetic background lines, including WT, *AtCaM3* knockout mutant *cam3* and *AtCaM3*-overexpressing lines, were used for the following studies. We compared the DNA-binding activity of the HSF in different genetic background lines. The result showed that DNA-binding activity of the HSF in WT was higher than that in mutant *cam3*, lower than that in overexpression of *AtCaM3* gene lines, indicating that AtCaM3 affected the thermotolerance of *Arabidopsis* seedlings through regulating the binding activity of HSF to HSE. We detected that the expression of *Athsp18.2*, *AtHsp25.3* and *AtHsp83* after HS at 37 °C for 1 h in mutant *cam3* was only 1/3–1/

2, and in overexpression of *AtCaM3* gene lines was 1.5- to 2.5-fold of that in WT. In addition, the accumulation of HSP18.2 protein after HS at 37 °C for 2 h in WT was higher than that in the mutant *cam3*, and lower than that in overexpression of *AtCaM3* gene lines. Accordingly, the expression of the *AtCaM3* gene indeed regulates the activity of HSF, the expression of Hsp genes and affects the thermotolerance of plants [44].

To understand how expression of the *AtCBK3* and *AtPP7* genes affects thermotolerance of *Arabidopsis* seedlings, the lines with different genetic background on *AtCBK3* and *AtPP7* genes were used to analyze the binding activity of HSF to HSE, expression of Hsp genes and accumulation of Hsps. The results showed that the DNA-binding activity of the HSF in WT was higher than that in mutant *cbk3*, and lower than that in overexpression of *AtCBK3* gene lines, indicating that AtCBK3 affected the thermotolerance through regulating the binding activity of HSF to HSE. After HS at 37 °C for 1 h, the mRNA level of *AtHsp18.2*, *AtHsp25.3* and *AtHsp83* in the *cbk3* mutant seedlings was only half of that in WT seedlings, and in the different *AtCBK3*-overexpressing lines was 1- to 3-fold higher than that in WT plants. The immunoblotting analysis showed HSP18.2 and HSP25.3 were not expressed at 22 °C, but induced by HS at 37 °C for 2 h. The synthesis of HSP18.2 protein in mutant *cbk3* was lower than that in WT plants, while the accumulation of HSP18.2 and HSP25.3 proteins in the different *AtCBK3*-overexpressing lines was higher than that in WT seedlings under the HS condition. We proposed that during HS, the activity of AtHsfA1a was promoted by phosphorylation catalyzed by AtCBK3, and then expression of Hsps was activated, which subsequently improved the thermotolerance of seedlings. The results mentioned above indicated that AtCBK3 is an important component downstream of CaM in the HS signal transduction [51]. AtPP7 is another component downstream of CaM. The expression of *AtHsp70* and *AtHsp101* genes, and the HSP70 protein levels were up-regulated in *AtPP7* overexpression lines after HS at 37 °C. The results suggest that the AtPP7 enhances thermotolerance through up-regulating Hsp genes [55].

6. Discussion and prospect

Attempts to increase thermotolerance by overexpression of a single HSF or Hsp gene have only limited impact, as the response of plants to heat stress is a complex process. The investigation of the HS signal transduction pathway is an effective way to understand the mechanism of tolerance to heat in plants. We propose a pathway for the participation of Ca^{2+}–CaM in HS signal transduction based on the experimental results in our laboratory. The HS signal is perceived by an unknown receptor in the plasma membrane. Receptor activation is closely followed by an increase in $[Ca^{2+}]_i$ through the opening of Ca^{2+} channels in the plasma membrane or endomembrane of the intracellular Ca^{2+} pool. However, the receptors sensing high temperature and Ca^{2+} channels in the plant plasma membrane are unknown. A possible cause leading to elevation of $[Ca^{2+}]_i$ is involvement of the PLC-IP_3 pathway. Heat shock activates PLC activity and causes accumulation of IP_3, then Ca^{2+} channels gated by IP_3 in the endomembranes cause Ca^{2+} mobilization. This elevated level of $[Ca^{2+}]_i$ directly activates CaM and promotes the expression and accumulation of CaM. Activated CaM regulates the activity of CaM-binding protein kinases or phosphatase, promoting activation of HSF by phosphorylation/dephosphorylation. The activation of HSF initiates the transcription and translation of Hsp genes, then enhancing thermotolerance (Fig. 3). The CaM isoform AtCaM3 [44], CaM-binding protein kinases AtCBK3 [51] and CaM-binding protein phosphatase AtPP7 [55] are important members in the pathway for the participation of Ca^{2+}–CaM in HS signal transduction. The functional analysis for these genes showed that they play a major role in controlling and regulating plant thermotolerance.

One of the important features for cell signal transduction is that it is a network, as is HS signal transduction. It is possible to assume that several pathways exist for the regulation of CaM on activity of HSF in HS signal transduction. In lower eukaryotes it has been proposed

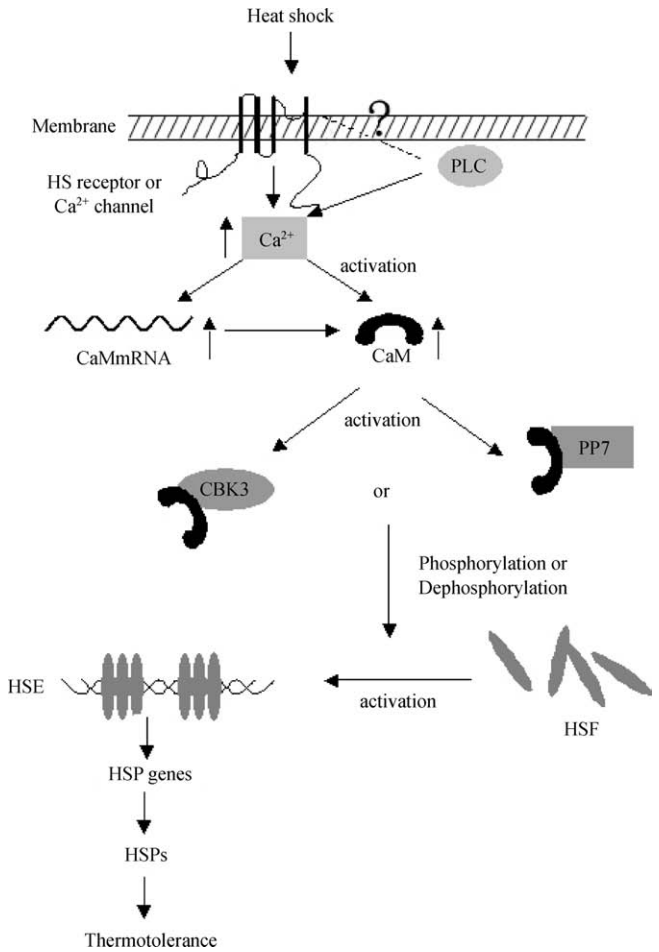

Fig. 3. Model for the Ca^{2+}–CaM pathway in HS signal transduction.

that Hsp70 intervenes directly in regulating the heat-induced conformational change in HSF. The feedback modulation of Hsp70, Hsp90 and Hsp40 to human HSF1 activity [65–68] and/or activity of AtHSF1 in *Arabidopsis* has been reported [69]. Using co-immunoprecipitation and Y2H techniques, the binding of Hsp70 to HSF in maize has been shown [70]. In addition, we also proved the binding of Hsp70 to CaM employing gel-overlay, co-immunoprecipitation and Y2H techniques [70,71]. We proposed another branched pathway for the regulation of CaM to the activity of HSF. The branch involves the binding of activated-CaM by HS directly to cytoplasmic Hsp70, causing the HSF-Hsp70 complex to release HSF, which activates transcription of Hsp genes [71]. It is necessary to provide more evidence for interaction among CaM, Hsp70 and HSF *in vitro* and *in vivo* to define this branched pathway.

The receptor sensing HS signal and the mechanism for increase in $[Ca^{2+}]_i$ in upstream events of the Ca^{2+}–CaM pathway of HS signal transduction are unclear to date and remain to be solved. It needs direct proof to further confirm the involvement of PLC-IP_3 and Ca^{2+} channels in the plasma membrane in elevation of $[Ca^{2+}]_i$ due to HS, although we provided primary evidence for the participation of PLC-IP_3 in HS signal transduction.

Acknowledgements

This work was supported by the National Key Basic Special Funds of China (Grant Nos. G1999011702, 2006 CB100101), the National Natural Science Foundation of China (Grant Nos. 3977075, 30270796), and the Natural Science Foundation of Hebei Province, China (Grant No. C2005000171). We thank Dr. Maskit Maymon (Department of Molecular, Cellular and Developmental Biology, University of California, Los Angeles, USA) for modifying the translation of the English version of this paper.

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