Cell Research (2006)16: 394-400

www.nature.com/cr

SHORT COMMUNICATION

Primary evidence for involvement of IP_3 in heat-shock signal transduction in *Arabidopsis*

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The role of inositol 1,4,5-trisphosphate (IP_3) in transducing heat-shock (HS) signals was examined in Arabidopsis. The whole-plant IP_3 level increased within 1 min of HS at 37 ºC. After 3 min of HS, the IP_3 level reached a maximum 2.5 fold increase. Using the transgenic *Arabidopsis* plants that have *AtHsp18.2 promoter*-β-*glucuronidase* (GUS) fusion gene, it was found that the level of GUS activity was up-regulated by the addition of caged IP_3 at both non-HS and HS temperatures and was down-regulated by the phospholipase C (PLC) inhibitors {1-[6-((17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione}(U-73122).

The intracellular-free calcium ion concentration ($[Ca^{2+}]_i$) increased during HS at 37 ºC in suspension-cultured *Arabidopsis* cells expressing apoaequorin. Treatment with U-73122 prevented the increase of $[Ca^{2+}]_i$ to some extent. Above results provided primary evidence for the possible involvement of IP_3 in HS signal transduction in higher plants.

Cell Research (2006) **16**:394-400. doi:10.1038/sj.cr.7310051; published online 13 April 2006

Keywords: heat shock, signal transduction, IP_3, PLC, $[Ca^{2+}]_i$, *Arabidopsis*

Introduction

The ability to respond to a variety of environmental signals is crucial to plants. A second-messenger Ca^{2+} was found to be involved in the regulation of many responses of plants to environmental signals. Intracellular-free calcium ion concentration ($[Ca^{2+}]_i$) often shows significant changes in plant cells under the influence of many stress signals such as cold shock, salt, drought, wind and touch [1-4]. Heat shock (HS) induced a large increase in $[Ca^{2+}]_i$ in Chinese hamster HA-1 fibroblasts [5] and human epidermoid A-431 cells [6]. In plants, Gong *et al*. [7] observed that $[Ca^{2+}]_i$ is significantly elevated during HS. The increase in $[Ca^{2+}]_i$ results in promoting the binding activity of the HS transcription factor (HSF) to the HS element (HSE) [8], the heat shock proteins (HSPs) synthesis induced by HS in human epidermoid A-431 cells [9, 10] and sugar beet [11].

We have recently shown that Ca^{2+} and calmodulin (CaM) are involved in HS signal transduction in higher plants. Heat shock can mediate rapid elevation in $[Ca^{2+}]_i$, and the change in $[Ca^{2+}]_i$ is also involved in the binding activity of HSF to HSE, the expression of HSP genes and synthesis of HSPs [12-13]. However in the upstream event of HS signal transduction, the mechanism for increase in $[Ca^{2+}]_i$ induced by HS in plants, is not known yet, whereas in mammalian cells it is known to be mediated by the activation of the reversed mode of Na^+/Ca^{2+}exchangers and Ca^{2+} mobilization from the Inositol 1,4,5-trisphosphate (IP_3)-sensitive intracellular Ca^{2+} pool [6, 14].

The intracellular Ca^{2+} levels are regulated at three levels by calcium influx and efflux, calcium-binding proteins, and intracellular calcium pools. At the cell membrane, antiporters, porters, pumps, and channels are all intensively involved. In the cytoplasm, Ca^{2+}-binding proteins, such as calmodulin, contribute significantly to regulate $[Ca^{2+}]_i$. In addition, cells have monensin-, IP_3-, thapsigargin-, and ionomycin-sensitive Ca^{2+} pools for Ca^{2+} mobilization or Ca^{2+} increases [4, 15]. Microinjection of both IP_3 and cyclic adenosine 5'-diphosphoribose (cADPR) into guard cells has revealed that both compounds have the capacity

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Received 10 Jul 2005; revised 2 Nov 2005; accepted 7 Dec 2005, published online 13 Apr 2006

to elevate $[Ca^{2+}]c$, demonstrating that IP_3 and cADPR-gated channels are functional in Ca^{2+} release in plants [16, 17]. The important role of phosphoinositides in signal transduction in plants and animal cells have been documented [10, 18]. The IP_3 level in human epidermoid A-431 cells increased 237% after HS at 45 ºC [14]. Heat shock at 45 ºC also caused rapid release of IP_3 from the membranes of HA-1 CHO fibroblasts [5]. Acid-induced deflagellation is associated with a rapid accumulation of IP_3 in *Chlamydomonas reinhardtii*. [19]. Light stimulates phosphoinositide turn over in *Samanea saman* pulvini [20]. It has recently been reported that salt and hyperosmotic shock induced a rapid increase in IP_3 in plants. It may play an important role in the processes leading to stress tolerance [21-23]. Treatment with Aluminum increased the activity of phospholipase C (PLC) and IP_3 formation in *coffea arabica* cells [24]. Signals outside the cell can be perceived and amplified in the cell membrane by receptors linked to a variety of signaling pathways, including the IP_3 pathway [25].

IP_3 is generated in many cell types through the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] by membrane-bound PLC activated by plasma membrane receptor responding to extracellular stimuli. The generation of internal calcium signals by IP_3-mediated Ca^{2+} release controls many cellular processes. In this paper, we investigated the accumulation of IP_3 caused by HS in plants, and the possible involvement of IP_3 in upstream events of HS signal transduction in *Arabidopsis*.

Materials and methods

Plant materials

Wild *Arabidopsis thaliana* ecotype Columbia was used for IP_3 measurement.

The suspension-cultured *Arabidopsis* cells expressing apoaequorin were given kindly by Dr MR Knight [1]. The cells expressing apoaequorin were used for measurement of $[Ca^{2+}]_i$.

A β-glucuronidase (GUS) reporter gene under the control of the promoter of the *AtHsp18.2* gene from *Arabidopsis* was introduced into the *Arabidopsis* plant [26]. The transgenic *Arabidopsis* seeds were provided kindly by Dr Takahashi. The transgenic *Arabidopsis* plants were used for other experiments.

Plant growth and treatment

The *Arabidopsis* seeds were surface-sterilized with 75% ethanol for 30 s, and followed by washing with sterilized water for four times. Seeds were then sterilized deeply with 10% sodium hypochlorous acid for 10 min and washed with sterilized water for six times.

The sterilized *Arabidopsis* seeds were sown on Murashige & Skoog (MS) medium (1% sucrose, 0.8% agar, 1×Murashige and Skoog salts and vitamins, pH 5.8) and placed at 4 ºC in darkness for 3 days for vernalization. The germinated seeds were then planted in growth chambers at 22 ºC day/18 ºC night under a fluorescent light with a 16 h photoperiod for another 11 days. The 14-day-old wild-type *Arabidopsis* seedlings were subjected to a direct HS by placing them in a temperature-controlled incubator at 37 ºC for 1, 2, 3, 4, and 5 min. The transgenic *Arabidopsis* seedlings were placed root down in 1 ml of different solutions (distilled water as control, U73122 and caged IP_3 and the concentrations of the compounds are described in the figure legends) at 22 ºC for 20 min, and then the seedlings were subjected to a direct HS by placing them in a temperature-controlled incubator at 37 ºC for 2 h. All treated seedlings were immediately frozen in liquid N_2.

The suspension-cultured *Arabidopsis* cells expressing apoaequorin [1] were grown at 22 ºC in the dark on a shaker at 120 rpm in MS liquid medium supplemented with 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/l 6-benzyladenine (6-BA). The suspension-cultured cells were transferred every 7 days with a 2% inoculum. Heat-shock treatment was performed by transferring the cells directly into a microplate hole kept at 37 ºC in the luminometer (LB960 Microplate Luminometer Centro).

Reagents

Caged IP_3 and 4-methylumbelliferyl-β-D-glucuronide (4-MUG) were obtained from Sigma (St. Louis, USA). {1-[6-((17b-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5- pyrrolidinedione} (U-73122) was from Calbiochem (San Diego, USA). Kit, D-*myo*-IP_3 [^{3}H] Biotrak Assay System, for IP_3 assay was from Amersham Biosciences UK Limited (Buckinghamshire UK). Coelenterazine-h was from Promega (Madison, USA). All other reagents used were of analytical purity and from Sino-American Biotechnology Company (Luoyang, China).

Quantification of IP_3 content

0.2~0.25 g of 14-day-old fresh seedlings were heat-shocked for different time durations and rapidly frozen in liquid N_2. The tissue was ground to a fine powder in liquid N_2 and mixed with 0.4 ml of ice-cold 20% (v/v) perchloric acid. After the mixer was incubated on ice for 20 min, proteins were precipitated by centrifugation at 2000×g for 15 min at 4 ºC. The supernatant was transferred to a new test tube and neutralized to pH 7.5 with ice-cold 1.5 M KOH in 60 mM HEPES buffer. Using a [^{3}H] IP_3 receptor binding assay kit, the neutralized samples were assayed for IP_3 content. Assays were carried out according to the manufacturer's instructions by using 50 μl of sample per assay in a total assay volume of 200 μl. The IP_3 content of each sample was determined by interpolation from a standard curve generated with commercial IP_3. Each point is the mean±SE of five data from five independent experiments.

Assay of GUS activity

The 100 mg seedlings frozen in liquid N_2 were ground, then 1 ml GUS extraction buffer (0.1% Triton X-100, 0.1% sarcosyl, 10 mM EDTA, 10 mM 2-mercaptoethanol, 50 mM potassium phosphate buffer pH 7.0) was added; this was followed by centrifugation at 13000 *g* for 10 min at 4°C. The supernatant was used as crude extracts. The GUS activity in crude extracts was measured by the method described by Jefferson *et al*. [27] with 4-MUG as substrate. Protein concentration was determined by the Bradford method using bovine serum albumin as the standard. The GUS activity is given as nM Mu (4-methyl unbelliferone)×min^{-1}×mg $protein^{-1}$. Each GUS activity presented here is mean±SE of multiple determinations from three to five independent experiments.

In vivo reconstitution of aequorin and measurement of $[Ca^{2+}]_i$

Four-day-old suspension-cultured cells were collected by filtration, washed with medium and re-suspended in 1 ml of fresh medium. *In vivo* reconstitution of the aequorin was performed by adding coelenterazine-h into the liquid medium containing cells to 2.5 μM of final concentration, and by incubating the cells in the dark at room temperature on a shaker at 120 rpm for at least 4 h. One hundred ml of reconstituted cells were transferred to the microplate hole for measuring luminescence. The luminescence of the cells was recorded every 5 s using a digital luminometer (LB960 Microplate Luminometer Centro) during all the experiment [28]. The emitted light was expressed as RLUs (relative luminescence units) [1]. All measurements were performed in the dark. Data were analyzed with Software wakrowin version 4.31. Each datum is the mean±SE from over five independent experiments.

The above-mentioned data were analyzed to indicate significant difference at the P=0.05 level using t test.

Results

Rapid accumulation of IP_3 *caused by HS*

The level of IP_3 in wild Arabidopsis seedlings before HS was normalized to 100%. Heat shock at 37 ºC caused an increase in concentration of IP_3 in 14-day-old *Arabidopsis* tissue. The initiation of this IP_3 increase occurred within 1 min of HS. After 3 min of HS, the IP_3 level reached a maximum 2.5fold increase (Figure 1A). But IP_3 level increased only 13% in the Arabidopsis seedlings treated with 100 μM U73122, a PLC inhibitor, after 3 min of HS (Figure 1B). The prevention of IP_3 accumulation by a PLC inhibitor during HS suggested that IP_3 accumulation is dependent on PLC activity.

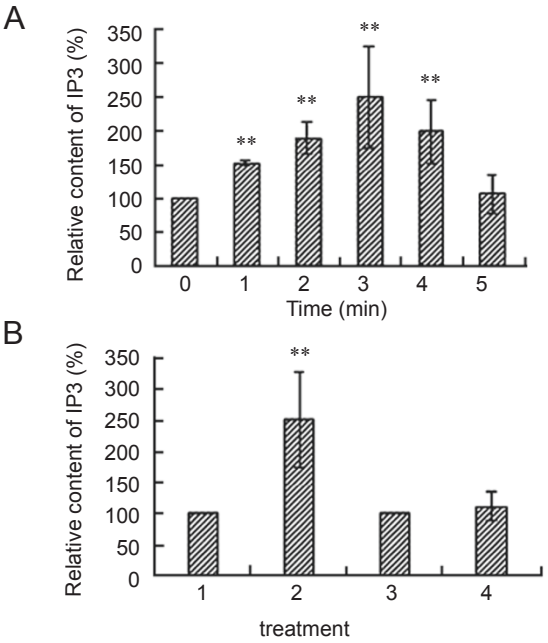

Figure 1 The Change in IP_3 level induced by HS at 37 ºC. Samples were extracted from 14-day-old wild *Arabidopsis* seedlings and IP_3 content was assayed by the method as described in Materials and methods. Each point was the mean±SE from five independent experiments. **(A)** The time course of increase in IP_3 level induced by HS at 37 ºC. 0, before HS; 1, 1 min after HS; 2, 2 min after HS; 3, 3 min after HS; 4, 4 min after HS; 5, 5 min after HS. ***P*<0.05 vs. before HS. **(B)** U73122, a phospholipase C inhibitor, blocks the increase in IP_3 induced by HS. 1, 22 ºC; 2, HS at 37 ºC; 3, treatment with 100 μM U73122 at 22 ºC; 4, treatment with 100 mM U73122 before 3 min of HS at 37 ºC. ***P*<0.05 vs. 22 ºC, 22 ºC+U73122 and 37 ºC+U73122. There is no significant difference among 22 ºC, 22 ºC+U73122 and 37 ºC+U73122.

Induction of GUS activity by HS in AtHsp18.2 transgenic Arabidopsis

A transgenic *Arabidopsis* plant with *AtHsp18.2 promoter-GUS* fusion was used as material in the experiment, in order to investigate the role of IP_3 in the expression of HSP genes. The GUS expression was under control of the *AtHsp18.2* promoter in transgenic Arabidopsis, so the expression of *AtHsp18.2* was determined by monitoring GUS activity. All the transgenic plants used in this research were grown at 22 ºC. The appearance of GUS activity in transgenic plants depended on HS temperature. The GUS activity in transgenic Arabidopsis seedlings at 22 ºC was normalized to zero. The transgenic *Arabidopsis* seedlings showed very low GUS activity after HS at 30 ºC for 2 h, whereas the GUS activity was maximal as the seedlings were heat-shocked at 37 ºC or 40 ºC for 2 h. The GUS activity decreased rapidly after treatment above 40 ºC. The seedlings treated at 45 ºC for 2 h gave very little GUS activity (Figure 2A). Heat shock at 37 ºC was used in the following experiments.

The time patterns of the change of GUS activity in transgenic *Arabidopsis* were investigated at 37 ºC. Incubation of the seedlings at 37 ºC results in a rapid increase in GUS activity during the first 2 h. The GUS activity reached the maximum after HS at 37 ºC for 4-6 h (Figure 2B). So, in the following research, the *Arabidopsis* seedlings heat-shocked at 37 ºC for 2 h were used as experimental material for the assay of GUS activity.

Up-regulation of HSP gene expression induced by the addition of caged IP_3*, and down-regulation by U-73122*

The treatment with different concentration of caged IP_3 could induce GUS activity in the transgenic *Arabidopsis* seedlings under non-HS condition. The GUS activity was zero before IP_3 was added at 22 ºC, and increased with increasing concentrations of IP_3. The GUS activity reached almost 20 nM Mu min^{-1} $mg\ protein^{-1}$ after the seedlings were treated with 2.5 μM of IP_3 (Figure 3A). It demon-

strated that IP_3 could induce the HSP gene expression instead of HS, showing the possibility of IP_3 involvement in HSP gene expression in *Arabidopsis*. Under HS condition, treatment with different concentrations of IP_3 increased the GUS activity to varying degrees. Of all concentrations of IP_3 used, the effect of 1 μM IP_3 was the most prominent. After the treatment of 1 μM IP_3, the GUS activity reached a maximum 2 fold increase. The GUS activity was not up-regulated by the treatment of over 2.5 μM IP_3 at HS temperature (Figure 3B).

A PLC inhibitor, U-73122, was used to investigate involvement of PLC in HSP gene expression. The transgenic Arabidopsis seedlings were treated with different concentrations of U-73122 at 22 ºC for 20 min, and then HS at 37 ºC for 2 h. Treatment with different concentrations of PLC inhibitor U-73122 decreased the GUS activity quickly and obviously (Figure 4). The down-regulation of the HSP gene expression by U-73122 demonstrated that PLC might be involved in HS signal transduction.

Involvement of PLC in increase of $[Ca^{2+}]_i$ induced by HS

The suspension-cultured *Arabidopsis* cells expressing

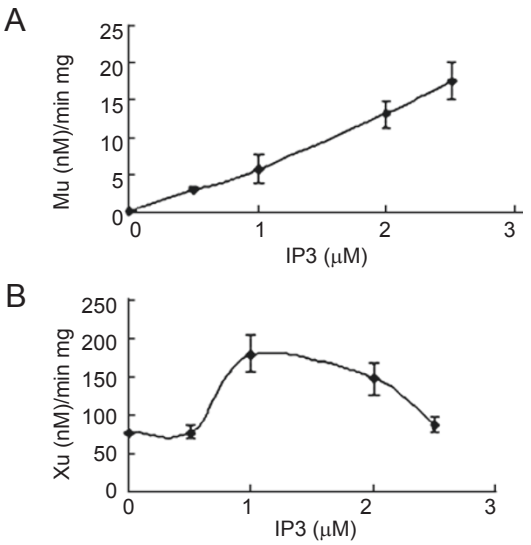

Figure 3 The effects of caged IP_3 on GUS activity in *AtHsp18.2* transgenic *Arabidopsis* at non- HS temperature 22 ºC **(A)** and **(B)** HS temperature 37 ºC.14-day-old transgenic Arabidopsis seedlings were treated with different concentrations of IP_3 indicated below at 22 ºC for 2 h **(A)** and at 37 ºC for 2 h **(B)** The samples were the extracted and GUS activity was assayed. Each datum is presented as mean±SE from three independent experiments.

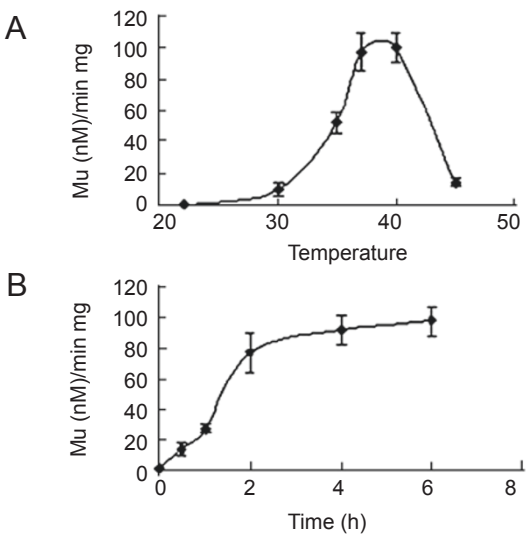

Figure 2 The effects of induced temperature and time on GUS activity in *AtHsp18.2* transgenic *Arabidopsis*. Samples were extracted from 14-day-old transgenic *Arabidopsis* seedlings and GUS activity was assayed by fluorometry as described in Materials and methods. Each data point was the mean±SE of over three repeats. **(A)** GUS activity in transgenic *Arabidopsis* as a function of assay temperature. 14-day-old seedlings were incubated for 2 h at the temperatures indicated below. **(B)** Time course of appearance of GUS activity in transgenic *Arabidopsis* plants at 37 ºC. 14-day-old seedlings were heat shocked at 37 ºC for different time indicated below.

apoaequorin were employed to investigate the effect of PLC on change in $[Ca^{2+}]_i$ induced by HS. Aequorin is a bioluminescent protein from the coelenterate *Aequorea Victoria*. This protein binds to calcium ions and emits a finite amount of blue light. The changes in luminescence in the cells expressing apoaequorin will directly reflect changes in cytosolic calcium concentration. The $[Ca^{2+}]_i$ in the 4-day-old reconsitituted cells kept at 22 ºC remained constant in luminescence during the experiment. A significant increase in $[Ca^{2+}]_i$ was observed in the cells during HS at 37 ºC, but 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 (Figure 5). The result showed that the down-regulation of PLC inhibitor 30 μM U-73122 on the expression of HSP gene was due to inhibition of the increase in $[Ca^{2+}]_i$ during HS, indicating the involvement of PLC in Ca^{2+}-CaM pathway of HS signal transduction by affecting elevation in $[Ca^{2+}]_i$.

Discussion

In our recent work, we proposed a new pathway of HS signal transduction: the Ca^{2+}-CaM pathway [12, 13]. It shows that Ca^{2+} and CaM play an important role in regulation of HSP gene expression, synthesis of HSPs and the

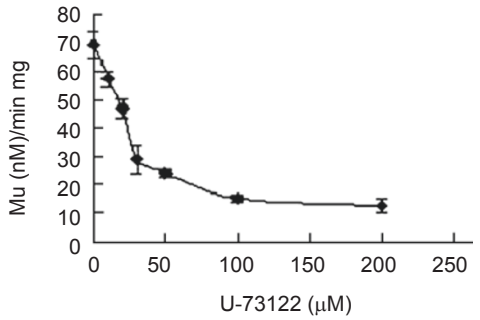

Figure 4 The effects of U-73122 on GUS activity in *AtHsp18.2* transgenic *Arabidopsis* at 37 ºC 14-day-old transgenic *Arabidopsis* seedlings were treated with different concentration of U73122 indicated below at 22 ºC for 20 min. The seedlings were then heat shocked at 37 ºC for 2 h. The samples were extracted and GUS activity was assayed. Each data point was the mean±SE from three independent experiments.

DNA-binding activity of HSF. But how HS induces increase in $[Ca^{2+}]_i$ in plants largely remains a mystery. What is the upstream event of increase in $[Ca^{2+}]_i$ in the Ca^{2+}-CaM pathway of HS signal transduction?

In plants, there is the evidence that signals such as light and abscisic acid (ABA) are relayed via IP_3 signaling [20, 29, 30]. Gilroy *et al*. [31] reported that microinjected Ca^{2+} or IP_3 stimulate stomatal closure in *Commelina communis*. Salts and hyperosmotic shock, caused by mannitol, NaCl, or dehydration, induced a rapid and transient increase in IP_3 in Arabidopsis [21-23]. Treatment with Aluminum increased the activity of PLC and IP_3 formation up to twofold in

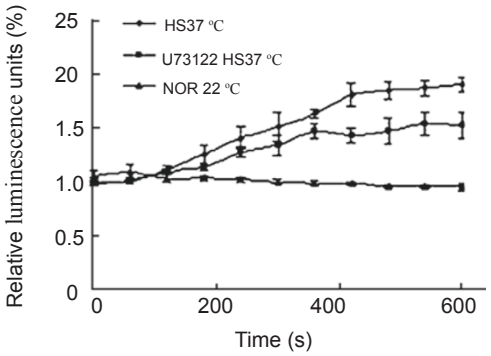

Figure 5 The change in $[Ca^{2+}]_i$ in suspension-culture *Arabidopsis* cells expressing apoaequorin at 22 ºC or during HS at 37 ºC. Four-day-old suspension-cultured *Arabidopsis* cells expressing apoaequorin were collected to use as experimental material. After *in vivo* reconstitution of the aequorin, the reconstituted cells were measured for luminescence by using a digital luminometer. Luminescence was measured by the method as described in Materials and methods. Each datum is presented as mean±SE from over five independent experiments.

Coffea arabica cells [24]. These observations suggest that different organisms utilize unique phosphoinositide-signaling pathways to elicit the cellular adaptations following an environmental change. A rapid release of IP_3 caused by HS in fibroblasts has been documented. The rise in IP_3 was followed by an increase in $[Ca^{2+}]_i$ [5]. The release of IP_3 is involved in the activation of PLC by HS in fibroblasts [32]. Heat shock induced the increase in IP_3 level in human epidermoid A-431 cells, but U-73122 blocked the increase in IP_3 resulting from HS [14]. This increased production of IP_3 leads to the increased levels of HSP70 mRNA and protein in human A-431 cells. These results suggest that IP_3 is involved in HSP70 production [9, 10]. However, involvement of IP_3 in HS signal transduction in higher plants has not been reported.

Herein the experimental data support the suggestion that higher plants respond to heat stress also by utilizing phosphoinositide-signaling pathways. Heat stress caused a rapid and transient rise of IP_3, and a PLC inhibitor, U-73122, blocked the accumulation (Figure 1). Further experiments using a transgenic *Arabidopsis* plant with *AtHsp18.2 promoter-GUS* fusion showed that the treatment with IP_3 could up-regulate the expression of HSP genes under both non-HS and HS conditions. This means that increasing IP_3 instead of HS is able to induce the expression of HSP genes (Figure 3).

Increase in IP_3 is induced through the hydrolysis of PtdIns(4,5)P_2 catalyzed by membrane-bound enzyme PLC. PLC activity should play a key role in IP_3 accumulation. Our experimental results showed that IP_3 accumulation caused by HS was blocked by PLC inhibitor U-73122 (Figure 1B), and the $[Ca^{2+}]_i$ in suspension-culture *Arabidopsis* cells (Figure 5) and the GUS activity in heat-shocked transgenic Arabidopsis seedlings were down-regulated by U-73122 (Figure 4). It suggested that PLC-IP_3 is responsible for the expression of HSP genes induced by HS.

Using suspension-culture *Arabidopsis* cells expressing apoaequorin, we showed that HS causes rapid increase in $[Ca^{2+}]_i$ in *Arabidopsis* cells (Figure 5). This work further supports our previous result in wheat [12]. The result presented here also indicated that a PLC inhibitor, U-73122, blocks in part, not all, the increase in $[Ca^{2+}]_i$ induced by HS. During HS, the $[Ca^{2+}]_i$ in cell treated with U-73122 is lower than that without treatment with U-73122, but higher than that in the cells maintained at 22 ºC. U-73122 causes about 40% decrease in $[Ca^{2+}]_i$ compared with that without U-73122 at 37 ºC for 10 min. Influx of Ca^{2+} into cytoplasm during HS may come not only from intracellular Ca^{2+} pools but also from extracellular sources. Our previous work also shows that treatment with cytoplasmic membrane calcium ion channel blocker verapamil or $LaCl_3$ depressed the increase in $[Ca^{2+}]_i$ (Data not shown) and the GUS activity

in the transgenic Arabidopsis seedlings [33]. All results suggest that HS mobilizes Ca^{2+} from not only intracellular but also extracellular sources.

The IP_3 level peaked 3 min after HS (Figure 1A), and it takes 7 min of HS to reach a maximal $[Ca^{2+}]_i$(Figure 5). Our previous work showed that the *AtHsp18.2* expression began to appear 10 min after HS [33]. These results could define the order of the signal transduction steps during HS. The calcium ion release is located downstream of IP_3 accumulation in the Ca^{2+}-CaM pathway of HS signal transduction. Heat shock may activate PLC activity and causes accumulation of IP_3, and then the calcium release pathway gated by IP_3 causes Ca^{2+} mobilization and the expression of HSP genes finally. The research herein about early events of Ca^{2+} mobilization in HS signal transduction is only the beginning, and further studies are ongoing.

Acknowledgements

We are grateful to Professor MR Knight (Department of Plant Sciences, University of Oxford, Oxford, UK) for the suspension-culture *Arabidopsis* cells expressing apoaequorin. We thank Dr Takahashi and Professor Yoshibumi Komeda (Molecular Genetics Research Laboratory, The University of Tokyo, Tokyo, Japan) for the transgenic *Arabidopsis* seeds. This work was supported by the National Natural Science Foundation of China (No. 30270796) and Natural Science Foundation of Hebei Province, China (No. C2005000171).

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Edited by Lu Liang