

Primary evidence for involvement of IP₃ in heat-shock signal transduction in *Arabidopsis*

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The role of inositol 1,4,5-trisphosphate (IP₃) in transducing heat-shock (HS) signals was examined in *Arabidopsis*. The whole-plant IP₃ level increased within 1 min of HS at 37 °C. After 3 min of HS, the IP₃ 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 IP₃ 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 ([Ca²⁺]_i) increased during HS at 37 °C in suspension-cultured *Arabidopsis* cells expressing apoaequorin. Treatment with U-73122 prevented the increase of [Ca²⁺]_i to some extent. Above results provided primary evidence for the possible involvement of IP₃ in HS signal transduction in higher plants.

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Introduction

The ability to respond to a variety of environmental signals is crucial to plants. A second-messenger Ca²⁺ was found to be involved in the regulation of many responses of plants to environmental signals. Intracellular-free calcium ion concentration ([Ca²⁺]_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 [Ca²⁺]_i in Chinese hamster HA-1 fibroblasts [5] and human epidermoid A-431 cells [6]. In plants, Gong *et al.* [7] observed that [Ca²⁺]_i is significantly elevated during HS. The increase in [Ca²⁺]_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 Ca²⁺ and calmodulin (CaM) are involved in HS signal transduction in higher plants. Heat shock can mediate rapid elevation in [Ca²⁺]_i, and the change in [Ca²⁺]_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 [Ca²⁺]_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⁺/Ca²⁺ exchangers and Ca²⁺ mobilization from the Inositol 1,4,5-trisphosphate (IP₃)-sensitive intracellular Ca²⁺ pool [6, 14].

The intracellular Ca²⁺ 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, Ca²⁺-binding proteins, such as calmodulin, contribute significantly to regulate [Ca²⁺]_i. In addition, cells have monensin-, IP₃-, thapsigargin-, and ionomycin-sensitive Ca²⁺ pools for Ca²⁺ mobilization or Ca²⁺ increases [4, 15]. Microinjection of both IP₃ and cyclic adenosine 5'-diphosphoribose (cADPR) into guard cells has revealed that both compounds have the capacity

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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 °C [14]. Heat shock at 45 °C 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 °C in darkness for 3 days for vernalization. The germinated seeds were then planted in growth chambers at 22 °C day/18 °C 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 °C 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 °C for 20 min, and then the seedlings were subjected to a direct HS by placing them in a temperature-controlled incubator at 37 °C 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 °C 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 °C 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 [3H] 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 °C. 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 [3H] 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 μ l of sample per assay in a total assay volume of 200 μ l. 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 umbelliferone)×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 [Ca²⁺]_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 IP₃ caused by HS

The level of IP₃ in wild *Arabidopsis* seedlings before HS was normalized to 100%. Heat shock at 37 °C caused an increase in concentration of IP₃ in 14-day-old *Arabidopsis* tissue. The initiation of this IP₃ increase occurred within 1 min of HS. After 3 min of HS, the IP₃ level reached a maximum 2.5fold increase (Figure 1A). But IP₃ 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 IP₃ accumulation by a PLC inhibitor during HS suggested that IP₃ accumulation is dependent on PLC activity.

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 IP₃ 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 °C. The appearance of GUS activity in transgenic plants depended on HS temperature. The GUS activity in transgenic *Arabidopsis* seedlings at 22 °C was normalized to zero. The transgenic *Arabidopsis* seedlings showed very low GUS activity after HS at 30 °C for 2 h, whereas the GUS activity was maximal as the seedlings were heat-shocked at 37 °C or 40 °C for 2 h. The GUS activity decreased rapidly after treatment above 40 °C. The seedlings treated at 45 °C for 2 h gave very little GUS activity (Figure 2A). Heat shock at 37 °C was used in the following experiments.

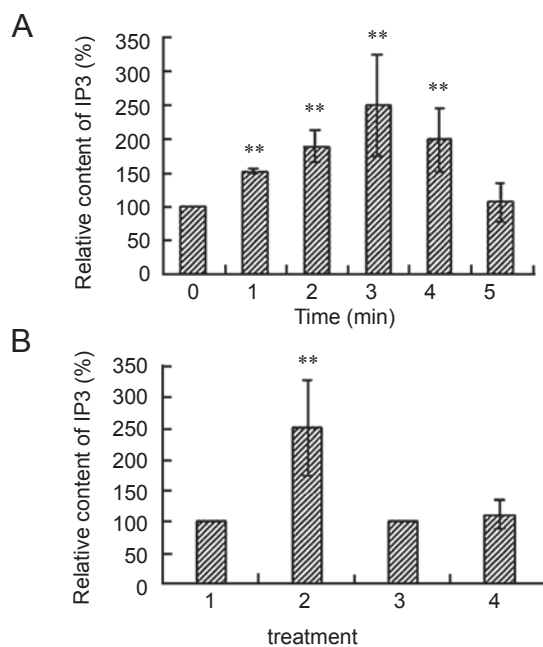


Figure 1 The Change in IP₃ level induced by HS at 37 °C. Samples were extracted from 14-day-old wild *Arabidopsis* seedlings and IP₃ content was assayed by the method as described in Materials and methods. Each point was the mean±SE from five independent experiments. **(A)** The time course of increase in IP₃ level induced by HS at 37 °C. 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 IP₃ induced by HS. 1, 22 °C; 2, HS at 37 °C; 3, treatment with 100 μM U73122 at 22 °C; 4, treatment with 100 μM U73122 before 3 min of HS at 37 °C. ***P*<0.05 vs. 22 °C, 22 °C+U73122 and 37 °C+U73122. There is no significant difference among 22 °C, 22 °C+U73122 and 37 °C+U73122.

The time patterns of the change of GUS activity in transgenic *Arabidopsis* were investigated at 37 °C. Incubation of the seedlings at 37 °C results in a rapid increase in GUS activity during the first 2 h. The GUS activity reached the maximum after HS at 37 °C for 4-6 h (Figure 2B). So, in the following research, the *Arabidopsis* seedlings heat-shocked at 37 °C 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 IP₃, and down-regulation by U-73122

The treatment with different concentration of caged IP₃ could induce GUS activity in the transgenic *Arabidopsis* seedlings under non-HS condition. The GUS activity was zero before IP₃ was added at 22 °C, and increased with increasing concentrations of IP₃. The GUS activity reached almost 20 nM μg protein⁻¹ after the seedlings were treated with 2.5 μM of IP₃ (Figure 3A). It demon-

strated that IP₃ could induce the HSP gene expression instead of HS, showing the possibility of IP₃ involvement in HSP gene expression in *Arabidopsis*. Under HS condition, treatment with different concentrations of IP₃ increased the GUS activity to varying degrees. Of all concentrations of IP₃ used, the effect of 1 μM IP₃ was the most prominent. After the treatment of 1 μM IP₃, the GUS activity reached a maximum 2 fold increase. The GUS activity was not up-regulated by the treatment of over 2.5 μM IP₃ 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 °C for 20 min, and then HS at 37 °C 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 [Ca²⁺]_i induced by HS

The suspension-cultured *Arabidopsis* cells expressing

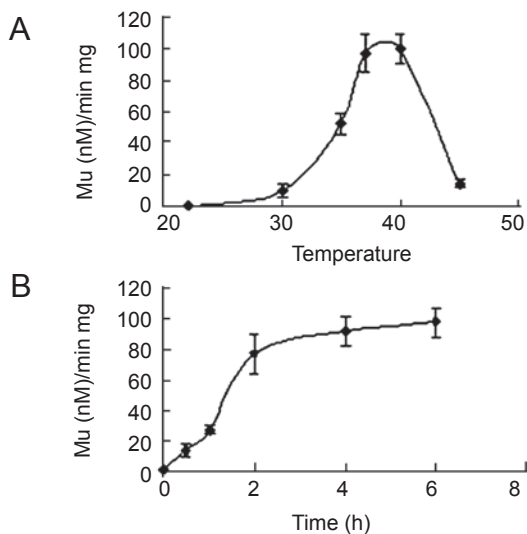


Figure 2 The effects of induced temperature and time on GUS activity in *AtHsp18.2* transgenic *Arabidopsis*. Samples were extracted from 14-day-old transgenic *Arabidopsis* seedlings and GUS activity was assayed by fluorometry as described in Materials and methods. Each data point was the mean±SE of 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 °C. 14-day-old seedlings were heat shocked at 37 °C for different time indicated below.

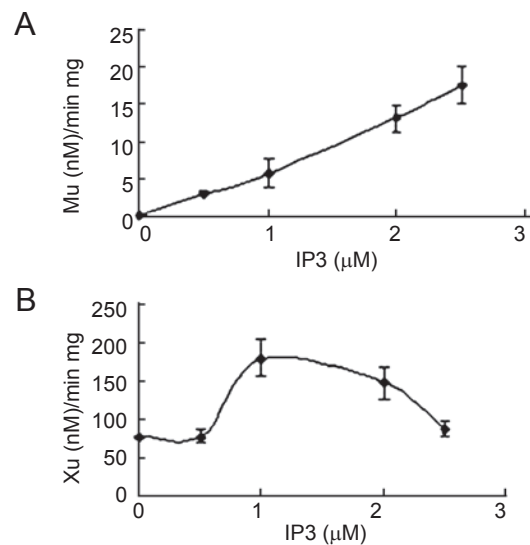


Figure 3 The effects of caged IP₃ on GUS activity in *AtHsp18.2* transgenic *Arabidopsis* at non- HS temperature 22 °C **(A)** and **(B)** HS temperature 37 °C. 14-day-old transgenic *Arabidopsis* seedlings were treated with different concentrations of IP₃ indicated below at 22 °C for 2 h **(A)** and at 37 °C 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.

apoaequorin were employed to investigate the effect of PLC on change in [Ca²⁺]_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 [Ca²⁺]_i in the 4-day-old reconstituted cells kept at 22 °C remained constant in luminescence during the experiment. A significant increase in [Ca²⁺]_i was observed in the cells during HS at 37 °C, but the treatment of cells with 30 μM U-73122 prevented the increase in [Ca²⁺]_i induced by HS to some extent. U-73122 blocked about 40% of the increase in [Ca²⁺]_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 [Ca²⁺]_i during HS, indicating the involvement of PLC in Ca²⁺-CaM pathway of HS signal transduction by affecting elevation in [Ca²⁺]_i.

Discussion

In our recent work, we proposed a new pathway of HS signal transduction: the Ca²⁺-CaM pathway [12, 13]. It shows that Ca²⁺ and CaM play an important role in regulation of HSP gene expression, synthesis of HSPs and the

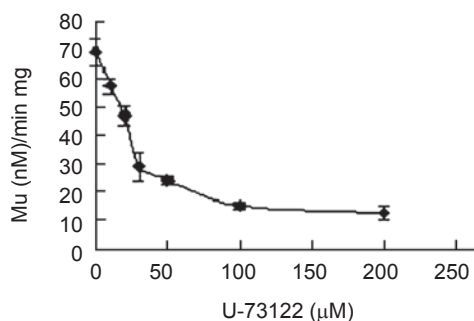


Figure 4 The effects of U-73122 on GUS activity in *AtHsp18.2* transgenic *Arabidopsis* at 37 °C 14-day-old transgenic *Arabidopsis* seedlings were treated with different concentration of U73122 indicated below at 22 °C for 20 min. The seedlings were then heat shocked at 37 °C for 2 h. The samples were 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 IP₃ signaling [20, 29, 30]. Gilroy *et al.* [31] reported that microinjected Ca^{2+} or IP₃ stimulate stomatal closure in *Commelina communis*. Salts and hyperosmotic shock, caused by mannitol, NaCl, or dehydration, induced a rapid and transient increase in IP₃ in *Arabidopsis* [21–23]. Treatment with Aluminum increased the activity of PLC and IP₃ formation up to twofold in

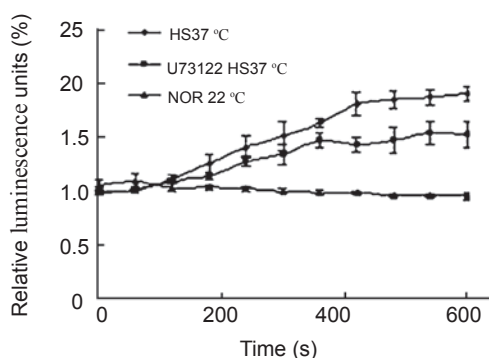


Figure 5 The change in $[Ca^{2+}]_i$ in suspension-culture *Arabidopsis* cells expressing apoaequorin at 22 °C or during HS at 37 °C. Four-day-old suspension-cultured *Arabidopsis* cells expressing apoaequorin were collected to use as experimental material. After *in vivo* reconstitution of the aequorin, 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 IP₃ caused by HS in fibroblasts has been documented. The rise in IP₃ was followed by an increase in $[Ca^{2+}]_i$ [5]. The release of IP₃ is involved in the activation of PLC by HS in fibroblasts [32]. Heat shock induced the increase in IP₃ level in human epidermoid A-431 cells, but U-73122 blocked the increase in IP₃ resulting from HS [14]. This increased production of IP₃ leads to the increased levels of HSP70 mRNA and protein in human A-431 cells. These results suggest that IP₃ is involved in HSP70 production [9, 10]. However, involvement of IP₃ 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 IP₃, 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 IP₃ could up-regulate the expression of HSP genes under both non-HS and HS conditions. This means that increasing IP₃ instead of HS is able to induce the expression of HSP genes (Figure 3).

Increase in IP₃ is induced through the hydrolysis of PtdIns(4,5)P₂ catalyzed by membrane-bound enzyme PLC. PLC activity should play a key role in IP₃ accumulation. Our experimental results showed that IP₃ 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-IP₃ 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 °C. U-73122 causes about 40% decrease in $[Ca^{2+}]_i$ compared with that without U-73122 at 37 °C 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 LaCl₃ 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 $[\text{Ca}^{2+}]_i$ (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.

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