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[Original Paper]

Mechanism for temperature-shift-responsive acute Ca^{2+} uptake in suspension-cultured tobacco and rice cells

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The temperature is one of the key environmental factors surrounding the living organisms in nature. In plant cells, transient increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) occurs as a response to an acute temperature change. Such a rise in $[Ca^{2+}]_c$ reportedly leads to cold acclimatization. Here, we examined the effect of the temperature-shifts from ambient to the lower or higher temperatures, on induction of $[Ca^{2+}]_c$ increases, using suspension-cultured plant cell lines expressing aequorin, a luminescent $[Ca^{2+}]_c$-reporter. The cultures used here include three tobacco (*Nicotiana tabacum* L.) cell lines, namely BY-2, Bel-W3 (O_3-sensitive) and Bel-B (O_3-tolerant), and rice (*Oryza sativa* L., cv. Nipponbare) AQ7 cells. Generally, rice cells and tobacco cells behaved similarly. The levels of $[Ca^{2+}]_c$ spikes induced by cold shock were much greater than those induced by heat shock in all cell lines tested. Effects of inhibitors suggested that the cold shock-induced $[Ca^{2+}]_c$ spike requires the uptake of extracellular Ca^{2+} *via* plasma membrane Ca^{2+} channels, while the heat shock response might be due to channel-independent leakages of extracellular and organelle Ca^{2+} into cytoplasm. (Received Sept.17, 2006; Accepted Nov.17, 2006)

INTRODUCTION

Temperature is one of the key environmental factors drastically affecting the life cycle of living organisms in nature. In recent years, Ca^{2+} was found to be involved as a second messenger in the perception and regulation of many responses of plants to environmental such as cold shock[1], salinity[2], draught[3], and hypoosmotic shock[4]. It has been suggested that the stress-induced changes in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) may be one of the primary signaling events leading to further biochemical cascades and gene expressions required for plant cells' adaptation to environmental stresses.

Regarding cold responses, some reports described that cold shock induces an increase in $[Ca^{2+}]_c$ as a consequence of Ca^{2+} influx across the

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plasma membrane in some plant species examined. Since Ca^{2+} influx into the cytoplasm is an early event in cold acclimatization in plants[1], and blockade of Ca^{2+} influx by Ca^{2+} channel inhibitors results in no cold acclimatization at 4°C[5], the cold-responsive Ca^{2+} uptake mechanisms must be studied in details.

In case of heat shock, Larkindale *et al.*[6] showed that Ca^{2+} might act as s second messenger in possible signaling paths actively limiting the heat-induced oxidative damage and induced cell death.

In the present study which is the first report comparing the $[Ca^{2+}]_c$ signatures in both monocot and dicot cells during acute temperature-shifts, we monitored the $[Ca^{2+}]_c$ changes induced by thermal treatments in 4 plant cell lines cultured *in vitro*, all expressing aequorin, a luminescent $[Ca^{2+}]_c$-reporter. We also analyzed the effects of Ca^{2+} channel inhibitors and Ca^{2+} signaling antagonists on cold shock- and heat shock-induced Ca^{2+} influx. For real-time monitoring of the temperature-shifts in the cell suspensions (micro-domain around the cell surface), we attempted the use of thermo-sensing units equipped with super-fine thermocouples.

MATERIALS AND METHODS

Plant materials

Three aequorin-expressing cell lines of tobacco (*Nicotiana tobacum* L.) namely, BY-2 cells derived from Bright Yellow tobacco, Bel-W3 cells derived from O_3-sensitive Bel-W3 tobacco, and Bel-B cells derived from O_3-tolerant Bel-B tobacco were used. Suspension-cultured cells of rice (*Oryza sativa* L., cv. Nipponbare, cell line AQ7) expressing aequorin was also used. Briefly, the cultures of tobacco were propagated in Murashige-Skoog liquid medium (pH 5.8) containing 2,4-dichlorophenoxy acetic acid (0.2 mg/L). The rice cells were propagated in AA liquid medium. The cultures (30 ml each in a 100 ml conical flask) were kept on gyratory shakers (at 130 rpm) at 23°C in darkness, and subcultured once a week (tobacco, with 4% inocula), or once in two weeks (rice, with 15% inocula). Cells were harvested 6 days (tobacco) or 14 days (rice) after subculturing.

Chemicals

Chemically synthesized coelenterazine[7] was a gift from Profs. M. Isobe and M. Kuse (Nagoya Univ.). Other chemicals used here were of reagent level.

Monitoring of $[Ca^{2+}]_c$

The changes in $[Ca^{2+}]_c$ induced by temperature treatments in aequorin-expressing cells were monitored with the Ca^{2+}-dependent luminescence of aequorin as previously described[8]. The active form of aequorin was reconstituted by adding 1 μM coelenterazine to the apoaequorin-expressing cells 8 h prior to the $[Ca^{2+}]_c$ measurements. The aequorin luminescence was measured with a photometer (CHEM-GLOW, American Instrument, Maryland, USA) and expressed as relative luminescence units (rlu). After each measurement, the amount of remaining aequorin was estimated by luminescence measurements after addition of 1 M $CaCl_2$ in 10% ethanol. $[Ca^{2+}]_c$ was calculated using the equation: $pCa = 0.332558\ (-\log k) + 5.5593$, where k is a rate constant equal to luminescence counts per second divided by total counts[9].

Temperature controls and monitoring

Liquid was removed from the cell suspension (0.2 ml) using micropipettes with fine flat tips, in order to allow immediate changes in extracellular temperature by adding thermo-controlled media (hot or cold, 0.2 ml). The cold- or heat-induced $[Ca^{2+}]_c$ increase was detected with a photometer, and the extracellular temperature was monitored with the thermo-sensing units (Fig. 1). The units consisted

of super-fine thermocouples (KFT-25-200-100, ANBE SMT Co., Japan), an AD/DA 8 channel converter (MR-500, Keyence, Japan), and a PC with a display. The sensory unit has very small heat capacity, thus immediate and accurate measurements are enabled. Since the chains of cells made of 5 to 10 cells suspended in the media are often sized between 20 and 25 μm in diameter, the super-fine thermocouples having 25 μm of diameter (thus having similar surface area per length) may be a good model sensor mimicking the shape of cellular chains. Therefore, the thermo-sensing units are suitable for real-time measurements in order to estimate the cell surface temperature.

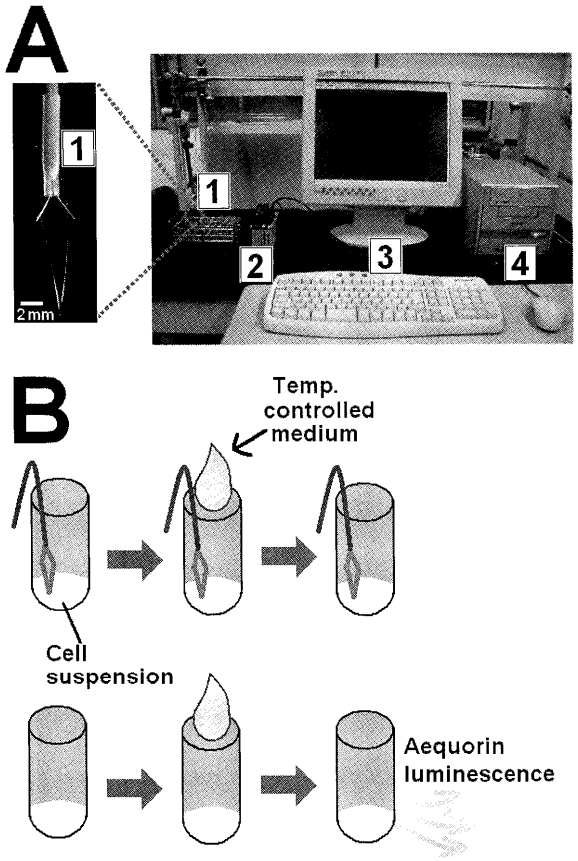

Fig. 1. Thermo-sensing units used to estimate the cell surface temperature during Ca^{2+} movement in plant cells. Plant cells were treated with temperature-controlled media, and temperature in the media was monitored with the thermo-monitoring system. (A) Composition of the units. (1) a super-fine thermocouple, (2) an AD/DA 8-channel converter, (3) display and (4) a PC. (B) Real-time detection of temperature- shifts and induced $[Ca^{2+}]_c$ changes. The induced $[Ca^{2+}]_c$ changes were monitored with aequorin luminescence detected with a photometer, while the changes in temperature in the cultures were measured with the thermo-sensing units.

RESULTS AND DISCUSSION

Temperature shift-induced $[Ca^{2+}]_c$ increases and detection of cell surface temperature

The real-time thermal monitoring in tobacco BY-2 cells revealed that immediate temperature-shifts transiently attained 5.5°C and 73.9°C following cold shock (0°C medium added) and heat shock (90°C medium added), respectively (Fig. 2B). Then the temperature in the media entered the stable phase of gradual thermal slopes (between 10 and 40 sec).

We have monitored the $[Ca^{2+}]_c$ increases induced by acute shifts in cell surface temperature in the tobacco BY-2 cells (Fig 2A). When treated with lowest (5.5°C) and higher temperatures (73.9°C), similar signatures in $[Ca^{2+}]_c$ increases were observed (Fig. 2A, top). $[Ca^{2+}]_c$ started to rise within 3 sec after treatments and attained the peak level *ca.* 7 sec after the temperature-shifts. Then the $[Ca^{2+}]_c$ level returned to the original level spending further 10 to 15 sec.

It is noteworthy that the immediate temperature spikes (0.5 to 10. sec) were detected prior to the cold- and heat-induced $[Ca^{2+}]_c$ transients (3 to 15 sec).

Temperature-dependent induction of $[Ca^{2+}]_c$ increases in four different cell lines

Temperature shifts (from ambient to cold and heat conditions) induced acute increases in $[Ca^{2+}]_c$ in tobacco BY-2, Bel-W3 and Bel-B, and Rice cells (Fig 3). The range of acute cell surface temperature shifts (as a result of medium addition) covered here was between 5.5°C and 73.9°C.

Notably, all cell lines, heat spikes up to 50°C (detected) showed no significant induction of $[Ca^{2+}]_c$ increases (Fig. 3). To date, a number of papers has documented the heat shock-induced $[Ca^{2+}]_c$ increase in various plants such as *Anabaaena* strain sp. (44°C, 60 min), tobacco seedlings (50°C, 120 min), and Arabidopsis seedlings (40°C, 60 min)[6,10,11]. Probably because our heat shock condition employed here gives only a pulse of heat lasting for less than single sec., but requiring just few sec for induction of $[Ca^{2+}]_c$ increase, much higher temperature was required compared to other heat stress conditions reported elsewhere[6,10,11].

In general, the $[Ca^{2+}]_c$ spikes induced by cold shock were more intense compared to heat shock-induced spikes in all cell lines tested. For example, in BY-2 cell, $[Ca^{2+}]_c$ after cold shock (0°C

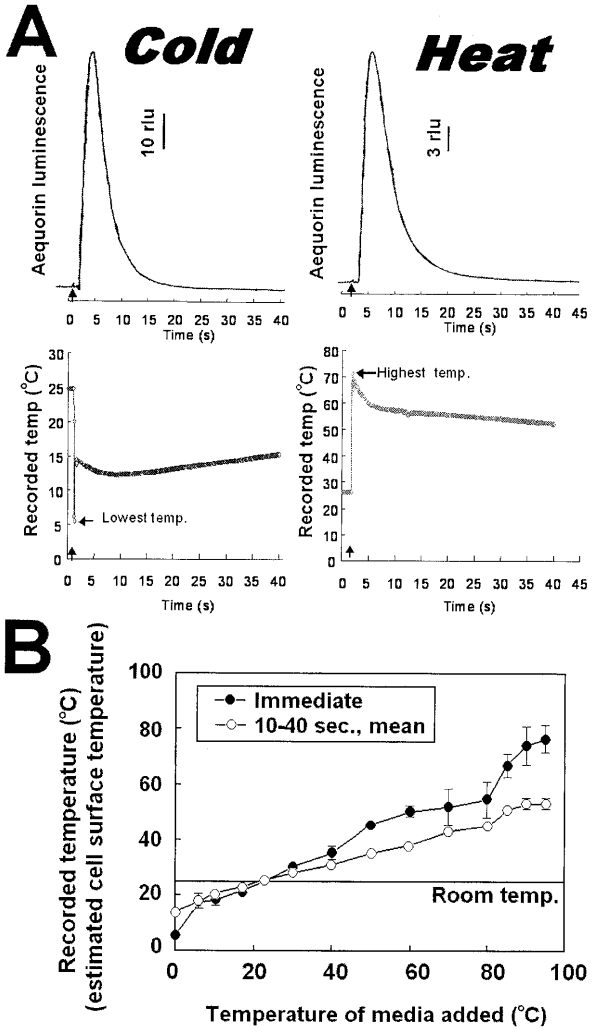

Fig. 2. Induction of $[Ca^{2+}]_c$ increase by acute temperature shifts. (A) Typical records of cold- and heat-responsive $[Ca^{2+}]_c$ increases (top) and records of real-time thermal monitoring (10 counts per sec) (bottom). (B) Effects of medium temperature (added to the medium-removed cells) on the actual changes in extracellular temperature. Closed and open circles represent immediate temperature spikes and the mean temperature between 10 and 40 sec after the treatments, respectively. Vertical bars represent SE (n = 3).

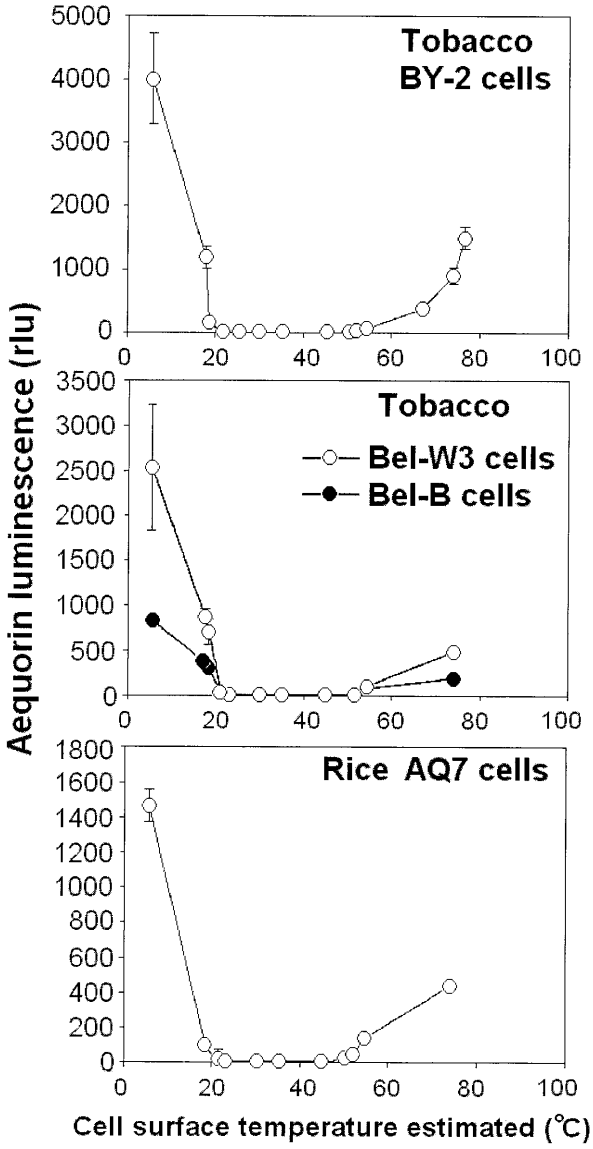

Fig. 3. Effects of temperature-shifts (from ambient to cold or heat conditions) on $[Ca^{2+}]_c$ spikes in 4 plant cell lines, namely tobacco BY-2, Bel-B and Bel-W3 cells, and rice AQ7 cells. Relationships between the extracellular temperature and the induced $[Ca^{2+}]_c$ elevation are shown. Vertical bars, SE (n = 3). rlu, relative luminescence unit.

medium addition) and heat shock (90°C medium addition) attained 2.18×10^{-6} M and 1.57×10^{-6} M, respectively; while at resting condition, $[Ca^{2+}]_c$ was maintained at low level (*ca.* 0.7×10^{-7} M). In Bel-W3 cells, $[Ca^{2+}]_c$ roughly attained *ca.* 2.0×10^{-6} M (in response to cold), and 1.16×10^{-6} M (in response to heat).

In Rice cells, slightly lower level of $[Ca^{2+}]_c$ peak was observed in response to cold shock (1.10×10^{-6} M) and heat shock (0.74×10^{-6} M). Here again, higher sensitivity to cold than heat was confirmed although the actual level of $[Ca^{2+}]_c$ attained by extreme temperature treatments differed from cell line to cell line. These data imply that both monocotyledonous and dicotyledonous cells respond to the cold shock and heat shock in a similar manner.

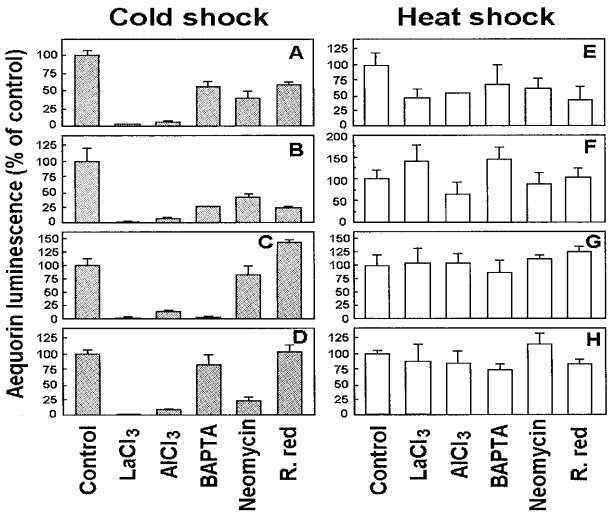

Fig. 4. Effects of various inhibitors on cold- and heat-responsive Ca^{2+} influx in plant cells. Effects of $LaCl_3$ (5 mM), $AlCl_3$ (5 mM), neomycin (0.1 mM), BAPTA (5 mM) and ruthenium red (R. red, 0.02 mM) were examined in Bel-B (A, E), Bel-W3 (B, F), BY-2 (C, G) and AQ7 (D, H) cells. Vertical bars, S.E. (n = 3).

Effects of inhibitors on $[Ca^{2+}]_c$ increase induced by cold shock and heat shock in 4 plant cell lines

To determine the origin of Ca^{2+} mobilized by cold and heat shocks, we tested the effects of ion channel blockers such as La^{3+} (5 mM) and Al^{3+} (5 mM), and Ca^{2+} signaling antagonists such as neomycin (0.1 mM), BAPTA (5 mM) and ruthenium red (0.02 mM) using Bel-B (Fig. 4A, E), Bel-W3 (Fig. 4B, F), BY-2 (Fig. 4C, G) and AQ7 cells (Fig. 4D, H).

In case of cold shock-induced $[Ca^{2+}]_c$ increase, La^{3+} and Al^{3+} showed strong inhibition in all cell lines tested (Fig. 4A-D). According to Lin *et al.*[12], Al^{3+} known as inhibitor of reactive oxygen species (ROS)-responsive Ca^{2+} influx[13,14] also effectively inhibits the ice-stimulated Ca^{2+} influx in tobacco cell suspension. However, Al^{3+} shows no inhibition against mechano-responsive Ca^{2+} influx while La^{3+} non-selectively inhibits the Ca^{2+} influx[13,14]. Thus action of Al^{3+} against the cold shock-responsive $[Ca^{2+}]_c$ increase could be at least partly due to the blockade of ROS-responsive mechanism.

It has been reported cold shock response leading to Ca^{2+} influx is enhanced in the presence of ROS such as H_2O_2[9,15]. Our previous works have shown that there are marked difference between Bel-B and Bel-W3 cells, in the ability to detoxify various ROS not only O_3 but also H_2O_2 and superoxide (unpublished data). Therefore two cell lines differed in sensitivity to ROS-mediated stresses, namely Bel-B and Bel-W3 cells (derived from O_3-tolerant and O_3-sensitive Bel-B tobacco plants and Bel-W3 tobacco plants, respectively) were used for comparison in this study. Our data (Fig. 3 middle) are consistently support the view that cold shock response involves redox events.

In addition, BAPTA a cell impermeable Ca^{2+} chelator showed partial inhibition of the cold- responsive $[Ca^{2+}]_c$ increase depending on the cells used (Fig. 4A-D). Above data suggest that certain portions of Ca^{2+} accumulated in the cytosolic space in response to cold shock may be due to Ca^{2+} influx via plasma membrane-localized Ca^{2+} channels. Our observations are consistent with previous reports by Polisensky *et al.*[16] and Torrecilla *et al.*[10]

Neomycin which is a known inhibitor of IP_3-mediated Ca^{2+} release from the intracellular

Ca^{2+} stores (applicable to plant cells)[17] also showed partial inhibition of the cold-responsive $[Ca^{2+}]_c$ increase (Fig. 4A-D), suggesting that release of Ca^{2+} from the internal Ca^{2+} stores may additively contribute to the cold-shock induced $[Ca^{2+}]_c$ increase.

It has been widely accepted that cold shock stimulates the increase in primary $[Ca^{2+}]_c$ which leads to activated expression of stress-responsive genes through activation of ICE (inducer of CBF expression) that interacts with stress-inducible transcription factors CBF/DREB1[18,19]. At the same time, alternative path enables the activation of IP_3-dependent increase in secondary $[Ca^{2+}]_c$[18].

In rice cell, effect of neomycin in inhibition of the cold-induced $[Ca^{2+}]_c$ increase was most prominent suggesting that IP_3-mediated Ca^{2+} release largely contributes to cold response. Ruthenium red is also known as an inhibitor of Ca^{2+} release from internal stores, shown to be inhibit the cold acclimation-related gene expressions in *Arabidopsis*[20]. Inhibitory action of ruthenium red observed in Bel-W3 cells (Fig. 4B) and Bel-B (to a lesser extent, Fig. 4A) also suggests the involvement of internal Ca^{2+} store in the cold response.

In contrast to cold shock response, $[Ca^{2+}]_c$ increase induced by heat shock (90°C medium added) was very much insensitive to almost all Ca^{2+} channel blockers and Ca^{2+} signaling antagonists especially in tobacco BY-2 cells and rice AQ7 cells (Fig. 4G, H). This indicates that the observed change in $[Ca^{2+}]_c$ after heat treatment (estimated temperature 73.9°C) may be non-biological response in which channel-independent leakage of ions including Ca^{2+} possibly occurred. However, Bel-B cells behaved differently in the presence of inhibitors (Fig. 4E). Such inhibitor-sensitive nature of heat-inducible $[Ca^{2+}]_c$ increase in Bel-B cells (thus maintaining active forms of channels functioning even under heat condition) may be reflecting the ROS-tolerant nature of cell line. It is well known that heat shock treatment damages the membrane via oxidative mechanisms involving rapid generation of ROS during the heat shock-induced programmed cell death[21].

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タバコおよびイネの懸濁培養細胞における温度変化応答性の急激なカルシウムの取り込み：林　村[1]，角野　貴志[1]，鈴木　拓[1]，吉塚　和治[1]，古市　卓也[2]，陽川　憲[3]，河野　智謙[1]（[1]北九州市立大学大学院国際環境工学研究科，[2]名古屋大学大学院医学系研究科，[3]早稲田大学大学院情報生産システム研究科）［キーワード：エクオリン，カルシウムシグナリング，低温ショック，高温ショック］