Plant, Cell & Environment

Plant, Cell and Environment (2012) **35**, 1543–1557 doi: 10.1111/j.1365-3040.2012.02508.x

Heat shock-induced biphasic Ca^{2+} signature and OsCaM1-1 nuclear localization mediate downstream signalling in acquisition of thermotolerance in rice (*Oryza sativa* L.)

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ABSTRACT

We investigated heat shock (HS)-triggered Ca^{2+} signalling transduced by a Ca^{2+} sensor, calmodulin (CaM), linked to early transcriptome changes of HS-responsive genes in rice. We observed a biphasic $[Ca^{2+}]_{cyt}$ signature in root cells that was distinct from that in epicotyl and leaf cells, which showed a monophasic response after HS. Treatment with Ca^{2+} and A23187 generated an intense and sustained increase in $[Ca^{2+}]_{cyt}$ in response to HS. Conversely, treatment with Ca^{2+} chelator, L-type Ca^{2+} channel blocker and CaM antagonist, but not intracellular Ca^{2+} release inhibitor, strongly inhibited the increased $[Ca^{2+}]_{cyt}$. HS combined with Ca^{2+} and A23187 accelerated the expression of *OsCaM1-1* and *sHSPC/N* genes, which suggests that the HS-induced apoplastic Ca^{2+} influx is responsible for the $[Ca^{2+}]_{cyt}$ response and downstream HS signalling. In addition, the biphasic response of OsCaM1-1 in the nucleus followed the Ca^{2+} signature, which may provide the information necessary to direct HS-related gene expression. Overexpression of *OsCaM1-1* induced the expression of Ca^{2+}/HS-related *AtCBK3*, *AtPP7*, *AtHSF* and *AtHSP* at a non-inducing temperature and enhanced intrinsic thermotolerance in transgenic *Arabidopsis*. Therefore, HS-triggered rapid increases in $[Ca^{2+}]_{cyt}$, together with *OsCaM1-1* expression and its nuclear localization, are important in mediating downstream HS-related gene expression for the acquisition of thermotolerance in rice.

Key-words: apoplastic Ca^{2+}; calmodulin; Ca^{2+} signalling; heat shock protein; heat shock signalling.

INTRODUCTION

Heat shock (HS) causes a broad spectrum of cellular damage, by extensive denaturation and aggregation of proteins, modifying membrane fluidity and permeability, and subsequently disrupting the balance of metabolic processes (Larkindale & Knight 2002). The HS response is a conserved cellular defence mechanism to against elevated temperatures induces HS proteins (HSPs) accumulation (Lindquist & Craig 1988; Vierling 1991). The expression of *HSP* genes is regulated at the transcriptional level by HS transcription factors (HSFs), which recognize the conserved HS elements (HSEs) in the promoter regions of *HSP* genes (Schöffl, Prändl & Reindl 1998; Nover *et al*. 2001; Baniwal *et al*. 2004). Arabidopsis (*Arabidopsis thaliana*) HSF binding protein AtHSBP interacts with HSF to reduce HSF transactivation as a negative regulator during the attenuation of HS response (Hsu & Jinn 2010; Hsu, Lai & Jinn 2010). In addition, many HSFs and HSPs are involved in improving plant thermotolerance (Queitsch *et al*. 2000; Mishra *et al*. 2002; Lohmann *et al*. 2004; Nishizawa *et al*. 2006; Charng *et al*. 2007; Yoshida *et al*. 2008; Ikeda, Mitsuda & Ohme-Takagi 2011).

HSPs comprise several evolutionarily conserved protein families containing high-molecular mass proteins such as HSP100, HSP90, HSP70/DnaK, HSP60/GroEL, and small-molecular mass proteins (sHSPs) of 16 to 42 kDa (Vierling 1991; Krishna & Kanelakis 2003; Sarkar, Kim & Grover 2009). Plant sHSPs are probably the most abundant and unusually complex heat-inducible proteins and are classified into different subfamilies according to amino acid sequence similarity and distinct subcellular compartments. sHSPs have been divided into seven (Siddique *et al*. 2008) to nine (Sarkar *et al*. 2009) subclasses (i.e. Class I to XI subclasses) in the model plants *Arabidopsis* and rice. In specific situations of HS and recovery, some nucleoplasmic sHSPs (sHSPC/N; Classes I, II and III) relocate in the nucleus and cytoplasm in a time- and temperature-dependent fashion, which may be essential for acquired thermotolerance (Lin, Roberts & Key 1984; Jinn *et al*. 1997).

The role of Ca^{2+} as an ubiquitous second messenger in animal and plant cells is well established. An increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) was early implicated as responding to a diverse range of biotic and abiotic stimuli and plant hormone signals (McAinsh & Pittman 2009); in addition, recent cell wall remodelling data suggested its role in conferring thermotolerance (Wu & Jinn 2010; Wu *et al*. 2010). HS response critically depended on a preceding Ca^{2+} transient through the plasma membrane; hence, the HS response is regulated by the transient entry of apoplastic Ca^{2+} (Saidi *et al*. 2009; Saidi, Finka & Goloubinoff 2011). Complex signal-specific Ca^{2+} signatures are decoded by

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Ca^{2+} binding proteins such as calmodulin (CaM) that function as Ca^{2+} sensors and affect other downstream cellular effectors (Rudd & Franklin-Tong 2001; Luan *et al*. 2002).

CaM is the major Ca^{2+} sensor and has highly conserved Ca^{2+} binding structures known as EF-hands for sensing changes in $[Ca^{2+}]_{cyt}$ and regulating downstream signalling events. The involvement of elevated Ca^{2+} signal coupled with CaM in the HS transduction pathway in plants was suggested as early as 1998 by measuring the Ca^{2+}-dependent protein aequorin luminescence in tobacco (*Nicotiana plumbaginifolia*) in response to HS (Gong *et al*. 1998). In wheat (*Triticum aestivum*), HS induced a threefold increase in $[Ca^{2+}]_{cyt}$ coupled with induced *TaCaM1*-1 expression (Liu *et al*. 2003). In transgenic *Arabidopsis* plants, *UidA* reporter gene expression directed by *AtHSP18.2* promoter was affected by Ca^{2+} and CaM inhibitors (Liu, Sun & Zhou 2005). In addition, elevated $[Ca^{2+}]_{cyt}$ and CaM levels were involved in the expression of *HSP* genes likely by regulating the activity of the HSF (Mosser *et al*. 1990; Li *et al*. 2004). HS induced a Ca^{2+}-dependent CaM level in *Arabidopsis* and also regulated a CaM-binding protein kinase, which promoted the expression of *HSF* and *HSP* genes (Liu *et al*. 2008). Knocking out or overexpressing specific CaM isoforms in *Arabidopsis* significantly reduced or increased thermotolerance, respectively, which suggests that CaM is a key component in HS signal transduction (Zhang *et al*. 2009). In the monocot rice, a role of Ca^{2+}/CaM in the HS transduction pathway and expression of HS-related genes has not been well documented.

Our lab has been involved in analysing both the abundance and complexity of rice s*HSPC/N* genes (Tseng *et al*. 1993; Lee *et al*. 1995; Guan *et al*. 2004). In this study, we aimed to identify components of Ca^{2+}/CaM in HS signalling in rice. Our data demonstrated that HS-induced $[Ca^{2+}]_{cyt}$ transients had monophasic and biphasic shapes in different tissues; peculiarly, root cells displayed a biphasic response. The effects of various pharmacological agents on HS-induced increase in $[Ca^{2+}]_{cyt}$ indicates that HS may induce an influx of apoplastic Ca^{2+} through plasma membrane. We identified the rice CaM1-1 isoform, whose expression resembles the biphasic $[Ca^{2+}]_{cyt}$ signal, also driving the downstream *sHSPC/N* genes up-regulation, perhaps through its nuclear localization. We further used transgenic *Arabidopsis* to show that OsCaM1-1 is an important component of the HS signal transduction pathway.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of rice (*Oryza sativa* L. cv. Tainung no. 67) were sterilized in 10% chlorine, then germinated on moist paper towel in a 28 °C dark growth chamber (Lin *et al*. 1984). Three-day-old etiolated rice seedlings without endosperms were incubated in Milli-Q purified water (Millipore, Billerica, MA, USA) under constant shaking at various temperature regimes (Jinn *et al*. 1993). Seeds of *A. thaliana* (Col-0) were incubated at 4 °C for 3 d before sowing, then plants were grown in a growth chamber at 22 °C with 16 h light at 60 to 100 μmol $m^{-2\,s-1}$ (Hsu *et al*. 2010).

Study of the kinetic changes in Ca^{2+} oscillation

The change $[Ca^{2+}]_{cyt}$ level was measured by use of Fluo-3 pentaacetoxymethyl ester (Fluo-3/AM; Merck, Rahway, NJ, USA) as described (Liu *et al*. 2003), with slight modification. One-centimetre segments of root tips collected from 3-day-old etiolated rice seedlings were pre-loaded with 1 μM Fluo-3/AM for 1 h at room temperature in the dark and rinsed with Milli-Q purified water for 1 h. Root tips were mounted in different solutions of pharmacological chemicals or inhibitors (Supporting Information Table S1) then submitted to HS treatment. The fluorescence intensity was quantified every 30 s under excitation filter (488 ± 10 nm) and emission filter (530 ± 40 nm) in XY-T scan mode by use of a Leica TCS SP2 laser scanning confocal microscope (LSCM; Leica Lasertechnik GmbH, Heidelberg, Germany). Time series for time-lapse scanning was performed with the Leica TCS SP2 imaging system. An automatic temperature controller (TC-324B; Warner Instruments, Hamden, CT, USA) was used to maintain temperature settings for all corresponding treatments. All pharmacological chemicals and inhibitors used here and in other studies of various plant materials are summarized in Supporting Information Table S1.

RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from 3-day-old etiolated rice seedlings without endosperm, and from 6-day-old *Arabidopsis* seedlings on 1/2 MS medium by use of Trizol reagent (Invitrogen, Carlsbad, CA, USA) and the TURBO DNA-free Kit (Applied Biosystems, Foster City, CA, USA). RT-PCR analysis involved use of ready-to-go RT-PCR beads (GE Healthcare, Buckinghamshire, UK). Primers used to amplify CaMs and HS-responsive genes (i.e. HSPs and HSFs) are in Supporting Information Tables S2 and S3. The rice 18S rRNA (Ambion, Austin, TX, USA) and *Arabidopsis* 18S rRNA (Fwd, 5′-GGTAATTCCAGCTCCAA TAGC-3′ and Rev, 5′-GTCAATTCCTTTAAGTTTCAG CCTTGC-3′) were used as internal standards for semi-quantitative RT-PCR analysis as described (Guan *et al*. 2004). PCR products were analysed by agarose gel electrophoresis and quantified by use of ImageQuant (Molecular Dynamics, Sunnyvale, CA, USA).

Subcellular localization of OsCaMs-GFP fusion proteins

The corresponding open reading frames (ORFs) of *OsCaM1-1* and *OsCaM2* were amplified with the primers for OsCaM1-1, Fwd, 5′-TCTCCATGGCGGACCAGCT CACCGACGAC-3′ and Rev, 5′-TCTCCATGGCCTTG GCCATCATGACCTT-3′; and OsCaM2, Fwd, 5′-TCTCC CCATGGCGGACCAGCTCACCGACGAGCAG-3′ and

Rev, 5′-TCTCCATGGTCACTTGGCCATCATGACCTT-3′, then subcloned into pRTL2-GFP (Carrington & Freed 1990) to generate OsCaM1-1-GFP and OsCaM2-GFP fusion proteins. *Arabidopsis* protoplast isolation and transfection were as described (Yoo, Cho & Sheen 2007). The pSAT4A-mCherry-N1 (The Arabidopsis Biological Resource Center, Stock # CD3-1081) was included in each transfection as a control for nuclear localization. About 10^6 protoplasts were transfected with 100 to 300 μg plasmid DNA for each construct and incubated at 26 °C in the light for 16 to 24 h, then imaging was examined by LSCM with the following excitation wavelengths: GFP excited at 488 nm and captured at 505 to 525 nm; chlorophyll autofluorescence emitted at 630 to 680 nm; mCherry excited at 543 nm and captured at 587 to 610 nm. Results were from four independent experiments with 10 to 20 cells analysed for each transformation event.

Production of *OsCaM1-1*-overexpressing lines in *Arabidopsis*

The OsCaM1-1-GFP clone was excised from the pRTL2.OsCaM1-1-GFP vector then subcloned into pCambia3300 (CAMBIA, Canberra, Australia). *Agrobacterium tumefaciens*-mediated genetic transformation of *Arabidopsis* involved the floral dip method (Clough & Bent 1998). Three OsCaM1-1-overexpressing plants (OsCaM1-1.OE1, OE2 and OE3) were screened by using 0.1% Basta herbicide and validated by RT-PCR with the OsCaM1-1 and pRTL2-GFP-specific primers 5′-TCTCCATGGCGGA CCAGCTCACCGACGAC-3′ and 5′-GCTTGATTGCTT GAGATTCG-3′, respectively.

Thermotolerance tests

Thermotolerance tests were performed as described (Charng *et al*. 2006; Hsu *et al*. 2010). *Arabidopsis* OsCaM1-1.OEs and wild-type plants were grown under exactly comparable conditions for seed collection. T3 seeds underwent thermotolerance treatments as follows. The plate was sealed with plastic electric tape and submerged in a water bath at the indicated temperature, then allowed to grow at 22 °C for 3 d for testing thermotolerance in seed germination. For hypocotyl elongation measurements, seedlings were grown for 2.5 d in the dark before undergoing heat treatment, then grown for another 2.5 d in the dark. For root elongation measurements, 4-day-old plantlets were grown vertically in the light, underwent heat treatment as described previously and were then returned to the growth incubator for an additional 5 d. The *AtHSP101* (At1g74310) T-DNA knockout plants (*hot1*) with an HS-sensitive phenotype (Hong & Vierling 2001) were analysed in parallel as a control for validation of the HS treatment.

Cellular ion leakage analysis

HS-induced ion leakage was measured in 10-day-old plants. Pre-weighed 100 mg seedlings were briefly rinsed with Milli-Q purified water and immediately placed in a tube with Milli-Q purified water. Tubes underwent continuous, soft shaking in a water bath at the indicated temperature. Electrolyte leakage was measured with use of a conductivity meter (SUNTEX SC170, Taipei, Taiwan) as described (Wu *et al*. 2010).

SDS–PAGE and Western blot analysis

Fifty micrograms of total proteins from 10-day-old seedlings were extracted with buffer containing 60 mM Tris-HCl (pH 8.0), 2% (w/v) SDS, 15% (w/v) sucrose, 60 mM DTT and 1 mM PMSF, then separated by 12.5% (w/v) sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli 1970). The polyclonal antibody against rice sHSP was used at 1:2000 (v/v) dilution as described (Jinn *et al*. 1993). Protein signals were identified by use of alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA, USA) and quantified by use of LAS-3000 (Fujifilm, Tokyo, Japan) and ImageQuant.

Statistical analysis

All experiments were independently repeated at least three times. Statistical analysis involved Student's *t*-test (two-tailed, unpaired). $P < 0.05$ was considered statistically significant.

RESULTS

HS induced biphasic $[Ca^{2+}]_{cyt}$ signal in rice root cells

To analyse whether 37 °C HS treatment modifies $[Ca^{2+}]_{cyt}$ level in rice, we treated root cells of 3-day-old etiolated rice seedlings with Fluo-3/AM, a Ca^{2+}-sensitive fluorescent probe able to detect oscillation in $[Ca^{2+}]_{cyt}$ (Liu *et al*. 2003). At 28 °C-control temperature, untreated root cells did not show strong modification of $[Ca^{2+}]_{cyt}$ response (Fig. 1a). We detected a biphasic elevation in $[Ca^{2+}]_{cyt}$ with 37 °C HS for 30 min, with a first peak after 10 min and a second peak after 26 min with higher peak amplitude (Fig. 1a). As compared with a monophasic increase in $[Ca^{2+}]_{cyt}$ response in epicotyl and leaf cells (Fig. 1b,c), that of root cells was distinctively biphasic.

By progressively shifting the temperature from 28 to 34 °C at 2 °C increments every 5 min before 37 °C for 30 min, the amplitude in $[Ca^{2+}]_{cyt}$ oscillation was similar to that produced by a single 37 °C-HS treatment (Fig. 1d). These results were consistent with a previous study showing HS response associated with the intensity of temperature-induced $[Ca^{2+}]_{cyt}$ oscillation (Saidi *et al*. 2009).

HS-induced apoplastic Ca^{2+} entry into the cytosol

Previous studies have shown that Ca^{2+} enters plant cells through Ca^{2+}-permeable ion channels within plasma

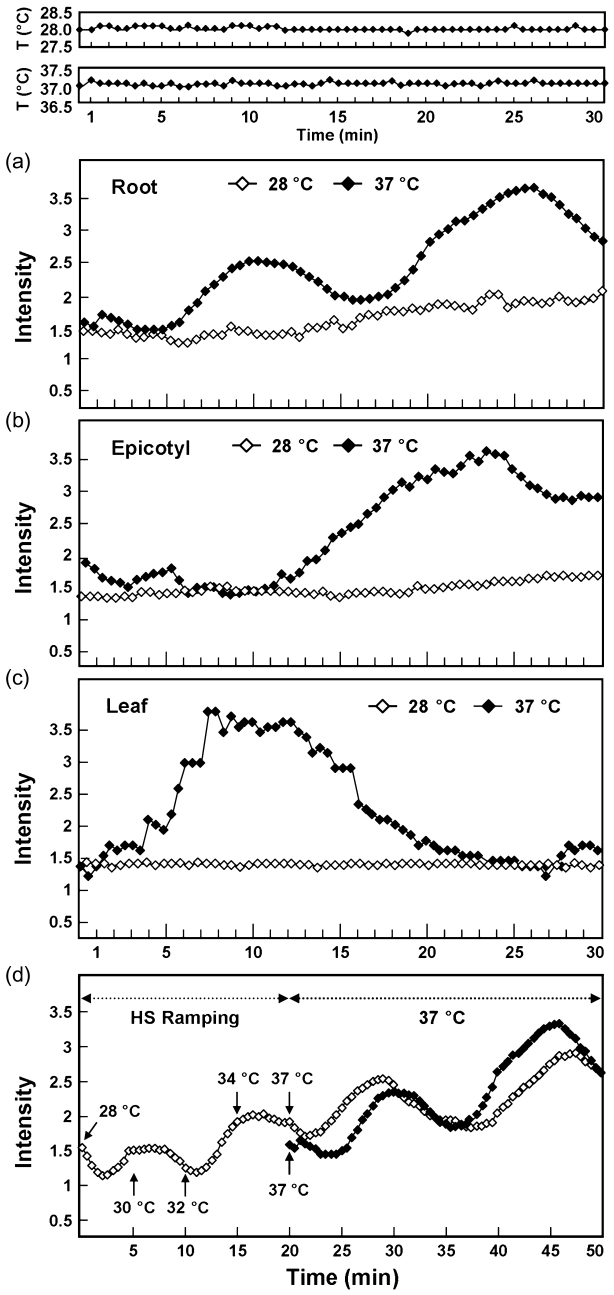

Figure 1. Heat shock (HS) triggered a biphasic $[Ca^{2+}]_{cyt}$ signature in root cells. Rice tissues were pretreated with Fluo-3/AM for fluorescence intensity measurement by laser scanning confocal microscopy. A biphasic $[Ca^{2+}]_{cyt}$ signature was induced by 37 °C-HS for 30 min in root cells (a) but induced a monophasic $[Ca^{2+}]_{cyt}$ response in epicotyl (b) and leaf (c) cells. Temperature shifts from 28 to 34 °C with a 2 °C increment every 5 min before 37 °C enhanced $[Ca^{2+}]_{cyt}$ responses progressively in root cells (d). Fluorescence intensity was measured by scanning 10 to 20 cells per assay in four independent replicates, which all showed similar profiles. The treatment at 28 or 37 °C was recorded, with ± 0.1 °C variation, as shown at the top of panel a.

membranes (White 2000; Saidi *et al*. 2009). Application of Ca^{2+} during 37 °C-HS accelerated $[Ca^{2+}]_{cyt}$ oscillation, which resulted in high-frequency $[Ca^{2+}]_{cyt}$ peaks in root cells (Fig. 2). Exogenous Ca^{2+} had no effect at 28 °C and even produced a small decrease in $[Ca^{2+}]_{cyt}$. Thus, Ca^{2+} application at a normal temperature may not mimic the HS-inducible response in elevating $[Ca^{2+}]_{cyt}$, and unnecessary excess of Ca^{2+} in the cell environment at a normal temperature may provoke a small leakage of $[Ca^{2+}]_{cyt}$.

The Ca^{2+} ionophore A23187 (Poutrain *et al*. 2009) at 28 °C-control temperature caused a unique and tardy transient increase in $[Ca^{2+}]_{cyt}$ after 12 min, which then decreased steadily back to the resting level (Fig. 3a). This increase could be attributed to free Ca^{2+} from apoplasts. The addition of A23187 at 37 °C-HS accelerated and reinforced the effectiveness of HS in elevating $[Ca^{2+}]_{cyt}$ (Fig. 3a), as shown by the 1.5-fold increase in fluorescent intensity. This result suggested an intrinsic apoplastic Ca^{2+} transport overdriven by the A23187 during 37 °C-HS.

To validate the hypothesis that HS induces Ca^{2+} influx from apoplasts, we used chemicals interfering with HS-triggered apoplastic Ca^{2+} transport, such as the Ca^{2+} chelator EGTA and L-type plasma membrane Ca^{2+} channel blockers $LaCl_3$ and verapamil, which have been used in other studies of various plant materials (Supporting Information Table S1). EGTA inhibited $[Ca^{2+}]_{cyt}$ oscillation at 37 °C-HS (Fig. 3b), as did $LaCl_3$ and verapamil (Fig. 3c,d), which even decreased $[Ca^{2+}]_{cyt}$.

Alternatively, Ca^{2+} may be released from subcellular organelles where it is stored. $[Ca^{2+}]_{cyt}$ response under HS was largely unaffected by other organelle Ca^{2+} release blockers such as LiCl, neomycin, caffeine and ruthenium red (Supporting Information Fig. S1).

CaM was involved in HS-induced Ca^{2+} entry into the cytosol

Ca^{2+}-CaM complexes have been described as active downstream signalling components of stress events. In wheat and

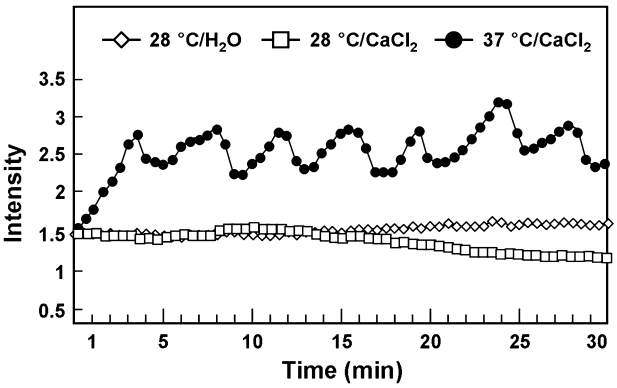

Figure 2. $CaCl_2$ treatment accelerated $[Ca^{2+}]_{cyt}$ oscillations during 37 °C-HS. Rice root cells were treated with 10 mM $CaCl_2$ at 28 or 37 °C. H_2O treatment was used as a reference. The fluorescence intensity was measured as described in Fig. 1. HS, heat shock.

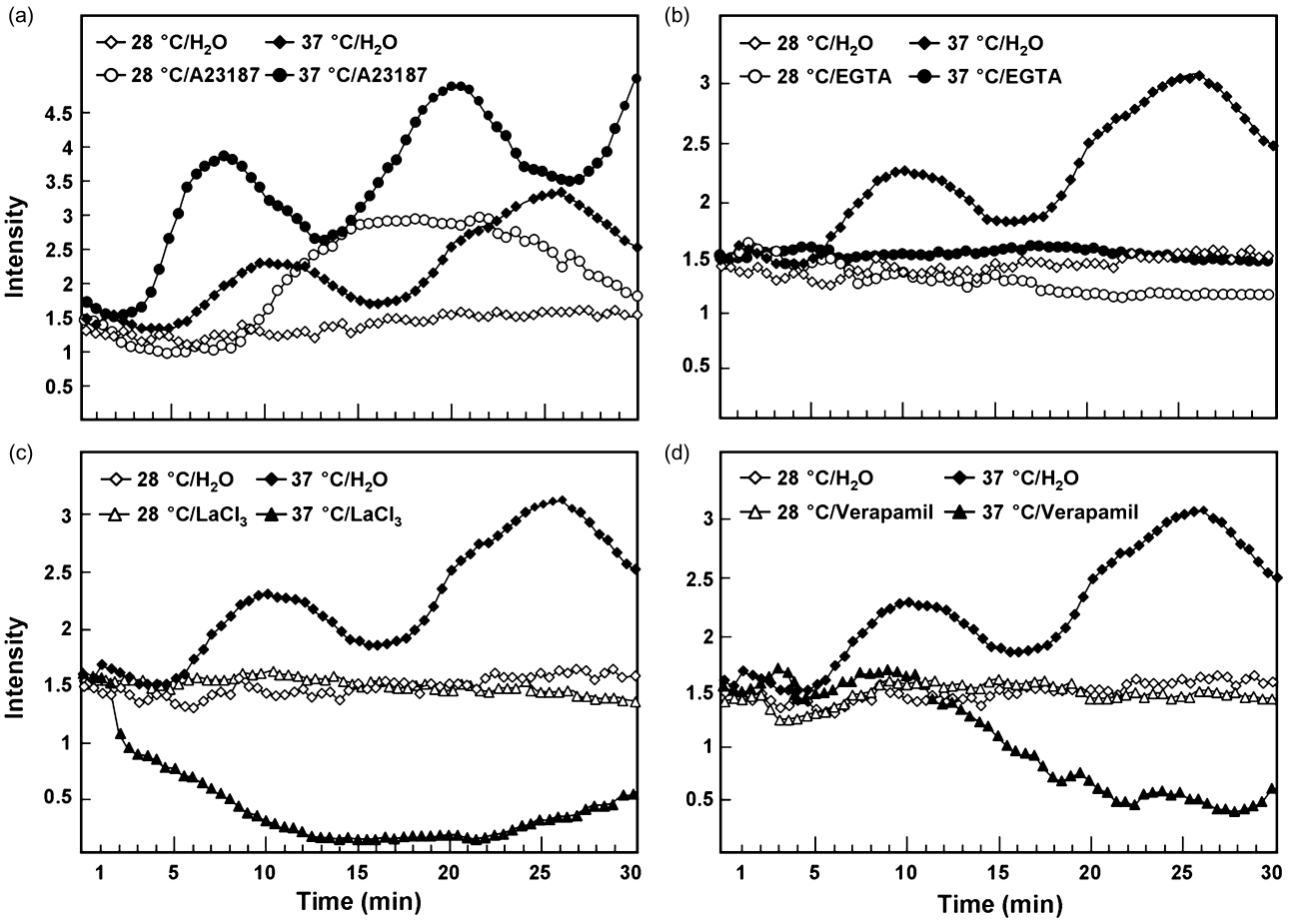

Figure 3. Heat shock (HS) induced entry of apoplastic Ca^{2+} into the cytosol. Rice root cells were treated with Ca^{2+} ionophore A23187 at 10 μM (a) or with apoplastic Ca^{2+} transport blockers such as 5 mM EGTA (b), 1 mM $LaCl_3$ (c) and 100 μM verapamil (d) at 28 or 37 °C. H_2O treatment was used as a reference. The fluorescence intensity was measured as described in Fig. 1.

Arabidopsis, TaCaM1-2 (Liu *et al*. 2003) and AtCaM3 (Liu *et al*. 2005; Zhang *et al*. 2009) play important roles in HS signalling. To examine a possible CaM requirement in $[Ca^{2+}]_{cyt}$ oscillation in the HS response, we tested the effect of the CaM antagonists chlorpromazine (CPZ) and trifluoperazine (TFP). Both compounds abolished the efficiency of HS on $[Ca^{2+}]_{cyt}$ response (Fig. 4a,b).

CaM genes were differentially regulated during early HS response

Our results suggest a link between Ca^{2+} signalling and CaM function during HS. However, in rice, regulation of *OsCaM* genes induction during HS is still not well understood. The rice genome contains five potential *OsCaM* genes, including three *OsCaM1* genes (*OsCaM1-1*, *–2* and *–3*), *OsCaM2* and *OsCaM3*, and up to 32 *OsCaM*-like genes (Boonburapong & Buaboocha 2007). Here, we focused on the canonical *OsCaM* gene expression in response to HS.

OsCaM1-1 and *OsCaM1-2* expression was low in seedlings at 28 °C-control temperature (Fig. 5). The expression of *OsCaM1-3* and *OsCaM3* was barely detected, and that of *OsCaM2* was absent. Under 41 °C-HS, all five genes were immediately induced but with different intensities. Expression of the highly induced *OsCaM1-1* strongly oscillated with time under HS and peaked as soon as 10 and 20 min, but that of the other four *OsCaM* genes did not highly oscillate. Therefore, the expression of *OsCaM* genes was differentially regulated by HS.

sHSPC/N genes are differentially regulated during early HS response

Previous works in wheat and *Arabidopsis* indicated that an increase in mRNA level of *CaM* during HS is followed by *HSP* genes induction (Liu *et al*. 2003, 2005; Zhang *et al*. 2009). To determine whether the expression pattern of rice *sHSPC/N* genes is concomitant with that of $[Ca^{2+}]_{cyt}$ signal-associated *OsCaM1-1*, we analysed the gene expression of *sHSPC/N* isoforms of different classes (see Supporting Information Table S2). By scanning mRNA expression profiles every 5 min under 41 °C-HS (Fig. 6a), we found that *OsHSP17.4-CI*, *OsHSP17.7-CI* and *OsHSP17.9A-CI* expression oscillated with time similar to that of *OsCaM1-1*,

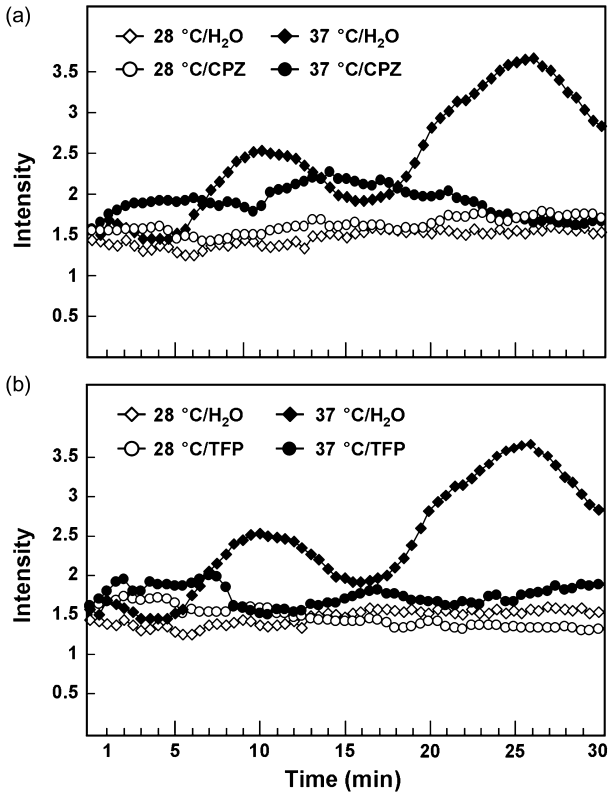

Figure 4. Calmodulin (CaM) participated in heat shock (HS)-induced $[Ca^{2+}]_{cyt}$ response. Rice root cells were treated with CaM antagonist chlorpromazine at 100 μM (a; CPZ) and trifluoperazine at 150 μM (b; TFP) at 28 or 37 °C. H_2O treatment was used as a reference. The fluorescence intensity was measured as described in Fig. 1.

with a short delay in mRNA induction as compared with *OsCaM1-1* expression. Of note, *OsHSP16* and *OsHSP18* genes expression did not oscillate (Fig. 6b) but rather was gradually induced by HS.

Apoplastic Ca^{2+} as a mediator of *OsCaM1-1* and *sHSPC/N* genes induction during the early HS response

We showed that Ca^{2+} influx was induced by exogenously supplied Ca^{2+} and A23187 during HS (Figs 2 & 3a) and that HS differentially induced *CaM* and *sHSPC/N* genes expression, with *OsCaM1-1* and some *OsHSP17* genes showing oscillation expression patterns resembling that of the $[Ca^{2+}]_{cyt}$ signal (Figs 5 & 6a). At 28 °C-control temperature, application of $CaCl_2$ up to 10 mM did not modify the expression profile of *OsCaM1-1* and *sHSPC/N* genes (Supporting Information Fig. S2a). However, the expression of *OsCaM1-1* was slightly enhanced but remained lower than that at 41 °C-HS (Supporting Information Fig. S2a). We obtained a similar result with 10 μM A23187 treatment at 28 °C (Supporting Information Fig. S2b). A23187 concentrations up to 50 μM remained insufficient to promote *sHSPC/N* genes expression (data not shown).

At 41 °C-HS with or without Ca^{2+} (Fig. 7), Ca^{2+} enhanced the effect of HS on temporal expression of *OsCaM1-1* and *OsHSP17* genes with oscillating expression patterns under HS (panel B). Similar to the expression of *OsCaM1-1*, that of *OsHSP17* genes was lower without Ca^{2+} supplied (panel A). Like treatment with Ca^{2+}, HS with A23187 reinforced the expression of *OsCaM1-1* and *OsHSP17* genes (panel C), with a prolonged effect over time. Pre-incubation with Ca^{2+} at 28 °C for 1 h before 41 °C-HS also shortened the induction time and intensified the increase in expression of *OsHSP17* genes (panel D); the induction of *OsHSP17* genes was shortened from 20 to 10 min and that of *OsCaM1-1* was highest in the initial 5 min. Meanwhile, the expression of *OsHSP16* and *OsHSP18* genes was induced by both Ca^{2+} and A23187 treatment under HS, also with a delay in comparison with *OsCaM1-1* expression (Supporting Information Fig. S3). As compared with the expression of *OsHSP17* genes, that of *OsHSP16* and *OsHSP18* genes did not decrease with Ca^{2+} treatment at 30-min HS (except for *OsHSP18.0-CII*).

The Ca^{2+} chelator EGTA remarkably diminished HS-induced *OsCaM1-1* and *OsHSP17* genes expression (Fig. 8a), which could be reversed by exogenously supplied Ca^{2+} (right of the panel). Similarly, *OsCaM1-1* and *OsHSP17* genes expression decreased with $LaCl_3$ and verapamil treatment (Fig. 8b). Treatment with CaM antagonists

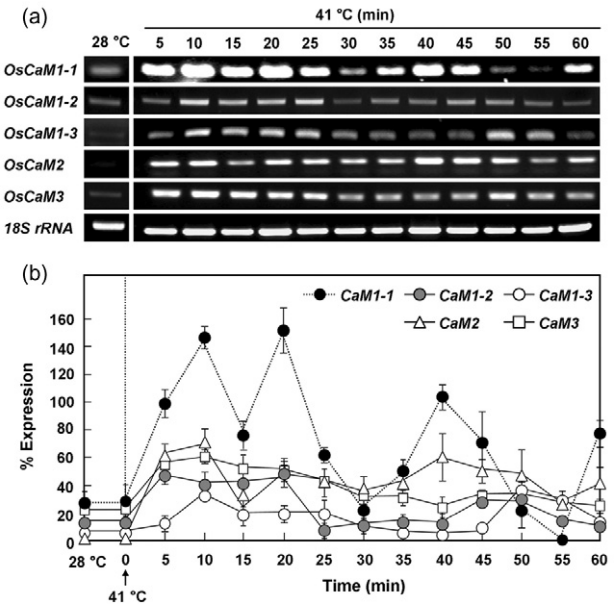

Figure 5. Differential expression of *OsCaM* genes during heat shock (HS). Rice seedlings were subjected to 41 °C-HS for 60 min, then the expression of *OsCaM* genes at the indicated times was analysed by RT-PCR. (a) Expression of five canonical *OsCaM* genes. (b) Quantification of ethidium bromide-stained PCR products by use of ImageQuant. Expression of *OsCaM* genes was normalized to that of 18S rRNA. *OsCaM1-1* expression at 40 min HS was set to 100%. Results are means ± SD of three independent replicates, with three technical repeats for each treatment. A set of representative gels is shown in panel (a).

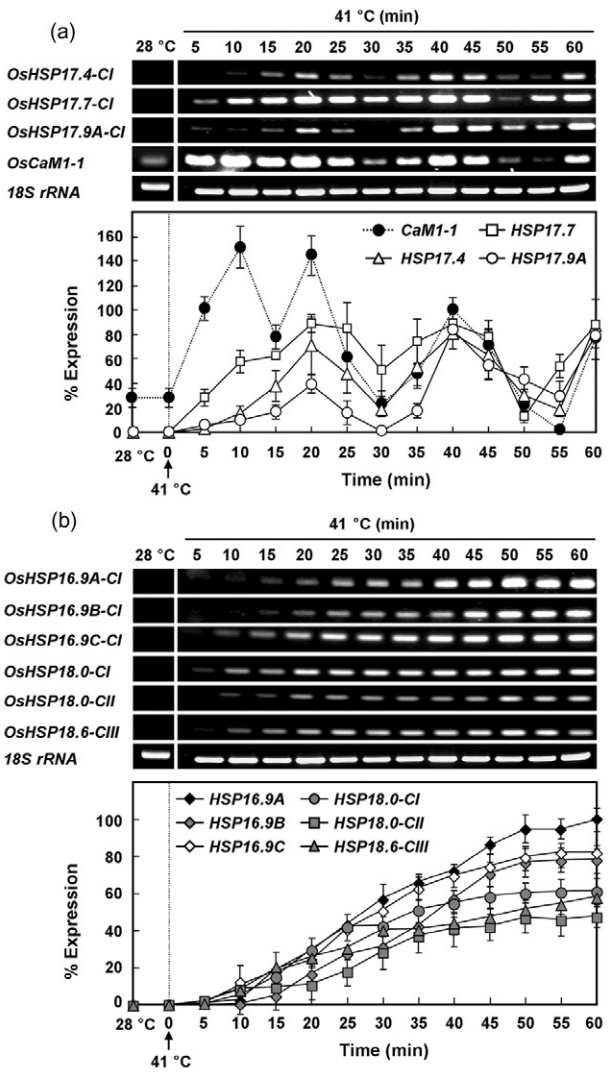

Figure 6. Oscillation versus linear increase of *sHSPC/N* mRNA during heat shock (HS). (a) Expression of *OsHSP17* genes similar to that of *OsCaM1-1* under 41 °C HS. (b) Expression of *OsHSP16* and *OsHSP18* genes was induced linearly. The expression levels were analysed and shown as in Fig. 5.

CPZ and TFP also blocked the *OsCaM1-1* and *OsHSP17* genes expression (Fig. 8c). Conversely, the use of the intracellular Ca^{2+} inhibitors LiCl, neomycin, caffeine and ruthenium red had no effect on *OsCaM1-1* and *OsHSP17* genes expression during HS (Fig. 8d and the bottom panel of Supporting Information Fig. S4d). The expression of *OsHSP16* and *OsHSP18* genes was also affected by the Ca^{2+} influx inhibitors but not intracellular release inhibitors (Supporting Information Fig. S4).

HS induced a biphasic signal of OsCaM1-1 in the nucleus

To analyse the relationship between HS-responsive function and subcellular localization of OsCaM proteins, *OsCaM* genes fused with *GFP* were transiently overexpressed in *Arabidopsis* protoplasts. GFP, as well as the GFP-tagged OsCaM1-1 (*OsCaM1-1*, *–2* and *–3* genes are identical, so only *OsCaM1-1* was tested) and OsCaM2, were stably detected in the cytoplasm and nucleus during 30 min treatment at 28 °C-control (Supporting Information Fig. S5).

Notably, 37 °C-HS induced a biphasic signal of nuclear-localized OsCaM1-1 (Fig. 9a and Supporting Information Fig. S6A), with an early peak at 8 min and a second peak at 24 min. GFP and OsCaM2-GFP nuclear location remained unaffected with 37 °C-HS (Supporting Information Fig. S7). *OsCaM1-1-GFP*-transformed protoplasts treated with Ca^{2+} or A23187 during 37 °C-HS activated an advanced biphasic signal of the nuclear-localized OsCaM1-1-GFP (Fig. 9b,c; Supporting Information Fig. S6b and c), and shortened the first peak from 8 to 2 min. The fluorescent intensity in the nucleus during HS and HS combined with Ca^{2+} and A23187 was increased ~1.6-fold higher than with 28 °C-control temperature; however, the localization profile of GFP and OsCaM2-GFP was not modified with similar treatments (data not shown).

Overexpression of *OsCaM1-1* induced expression of HS-related genes and enhanced thermotolerance in *Arabidopsis*

To dissect the function of OsCaM1-1, we generated three *Arabidopsis* transgenic lines by overexpressing *OsCaM1-1-GFP* (Fig. 10a; OsCaM1-1.OE1, OE2 and OE3), with confirmation by herbicide selection (top) and RT-PCR (bottom) analyses. OsCaM1-1-OE lines and wild-type (WT) plants showed no phenotypic change under normal growth conditions (Fig. 10b, Untreated). Basal and acquired thermotolerance analyses both revealed an increased HS-resistant phenotype as compared with the WT in seed germination and 3-day-old seedling growth of the OsCaM1-1.OE lines (Fig. 10b, Basal and Acquired). The reference *hot1* mutant, affected in the *AtHSP101* gene (Hong & Vierling 2001), showed a HS-sensitive phenotype in our assays. In acquired thermotolerance analysis, hypocotyl and root elongation rates were significantly higher in plants overexpressing *OsCaM1-1* than in the WT (Fig. 10c,d). As well, OsCaM1-1.OE lines showed a substantial decrease in ion leakage with HS (Fig. 10e). Therefore, OsCaM1-1.OE lines showed better thermotolerance than the WT. In *Arabidopsis* and other plants, thermotolerance is characterized by an accumulation of HSP (Kotak *et al.* 2007). Consistent with this observation, we found *Arabidopsis* OsCaM1-1-OE lines with increased accumulation of sHSP (Fig. 10f). A vector control line carrying *35S*::*GFP* and WT plants did not differ in phenotype under normal conditions or in thermotolerance analysis (Supporting Information Fig. S8).

To identify genes targeted by *OsCaM1-1* overexpression, we investigated candidate genes coding for HS-responsive proteins. In *Arabidopsis*, AtCaM3 regulates

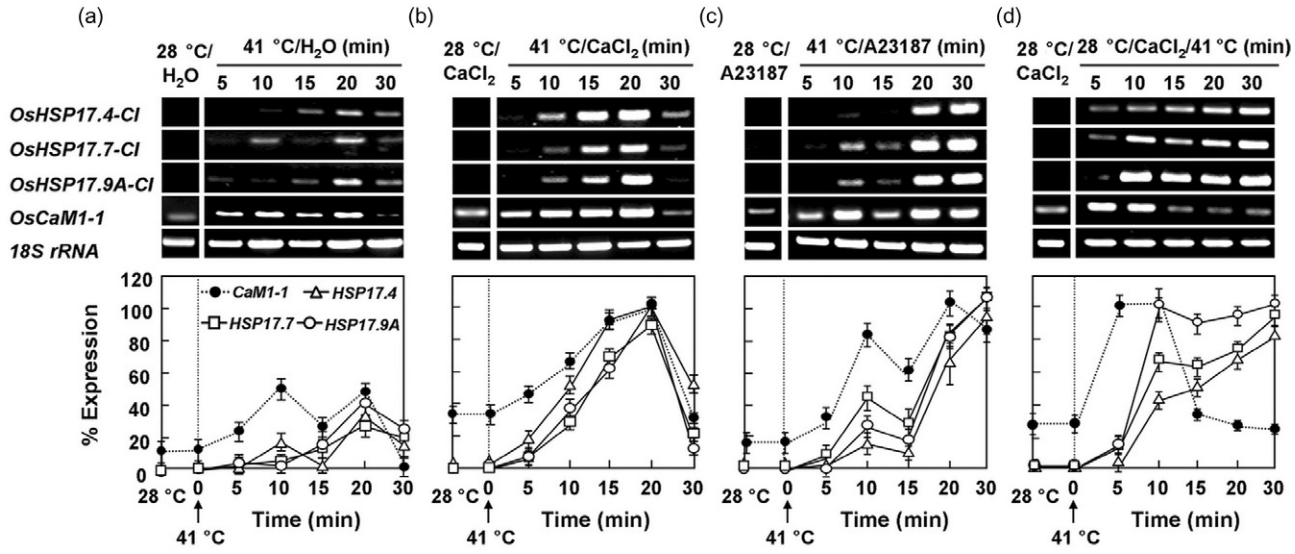

Figure 7. Application of Ca^{2+} and A23187 during heat shock (HS) promoted early and enhanced expression of *OsCaM1-1* and *sHSPC/N* genes. Rice seedlings were treated at 41 °C HS for 30 min in H_2O (a), with 5 mM $CaCl_2$ (b), 10 μM A23187 (c) or pre-incubation with 5 mM $CaCl_2$ at 28 °C for 1 h (d). Expression of *OsCaM1-1* and *OsHSP17* genes at the indicated times was analysed by RT-PCR. *OsCaM1-1* expression with 20 min HS in the presence of $CaCl_2$ was set to 100%. The expression levels were analysed and shown as in Fig. 5.

the activity of AtCBK3 or AtPP7 participating with Ca^{2+}/CaM in HS signal transduction by controlling the HSE binding activity of AtHSFA1a through its phosphorylation status (Liu *et al.* 2007, 2008). The expression of *AtCBK3* and *AtPP7* was induced in the OsCaM1-1.OE lines (Fig. 11a). Seven *AtHSF* isoforms of Class-A HSF and six isoforms of *sHSPC/N* were up-regulated (Fig. 11b,c), which suggests that overexpression of *OsCaM1-1* could mimic the HS response to promote the expression of HS-responsive factors and genes.

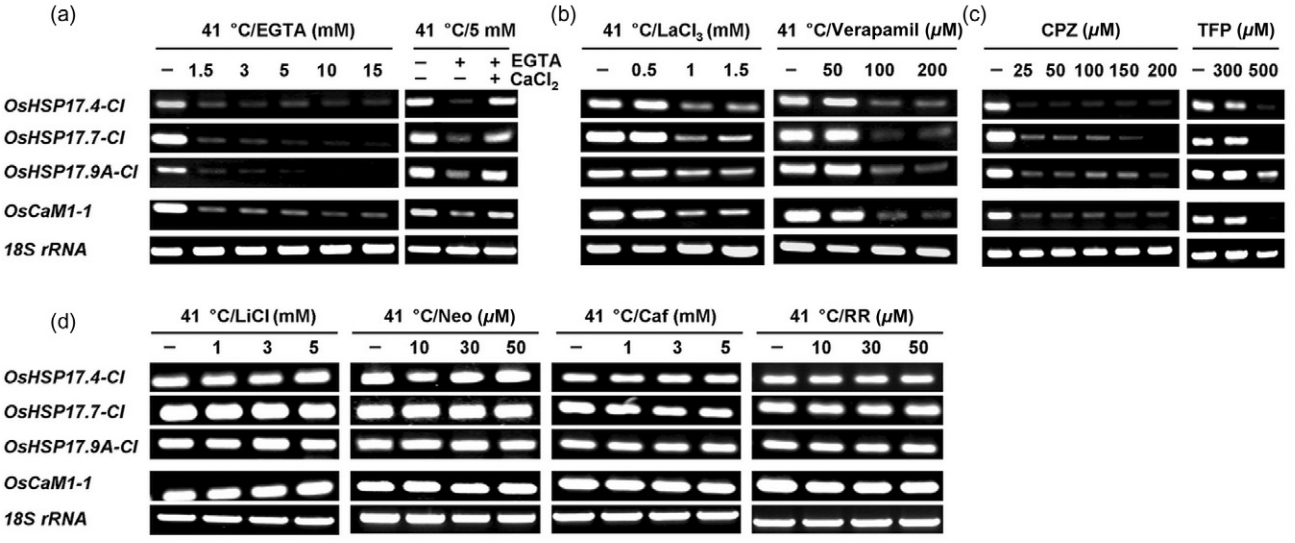

Figure 8. Apoplastic Ca^{2+} entry blockers and Calmodulin (CaM) inhibitors diminished expression of *OsCaM-1* and *sHSPC/N* genes under heat shock (HS) but were not significantly affected by intracellular Ca^{2+} release inhibitors. Expression of *OsCaM1-1* and *OsHSP17* genes in rice seedlings treated at 41 °C HS for 1 h with chemicals at different concentrations. Shows application of Ca^{2+} transport blockers EGTA (a); $LaCl_3$, verapamil (b); CaM antagonists of chlorpromazine (CPZ) and trifluoperazine (TFP) (c); and intracellular Ca^{2+} release blockers, LiCl, neomycin (Neo), caffeine (Caf) and ruthenium red (RR) (d). The addition of $CaCl_2$ counteracting the EGTA effect is shown in the right panel of (a).

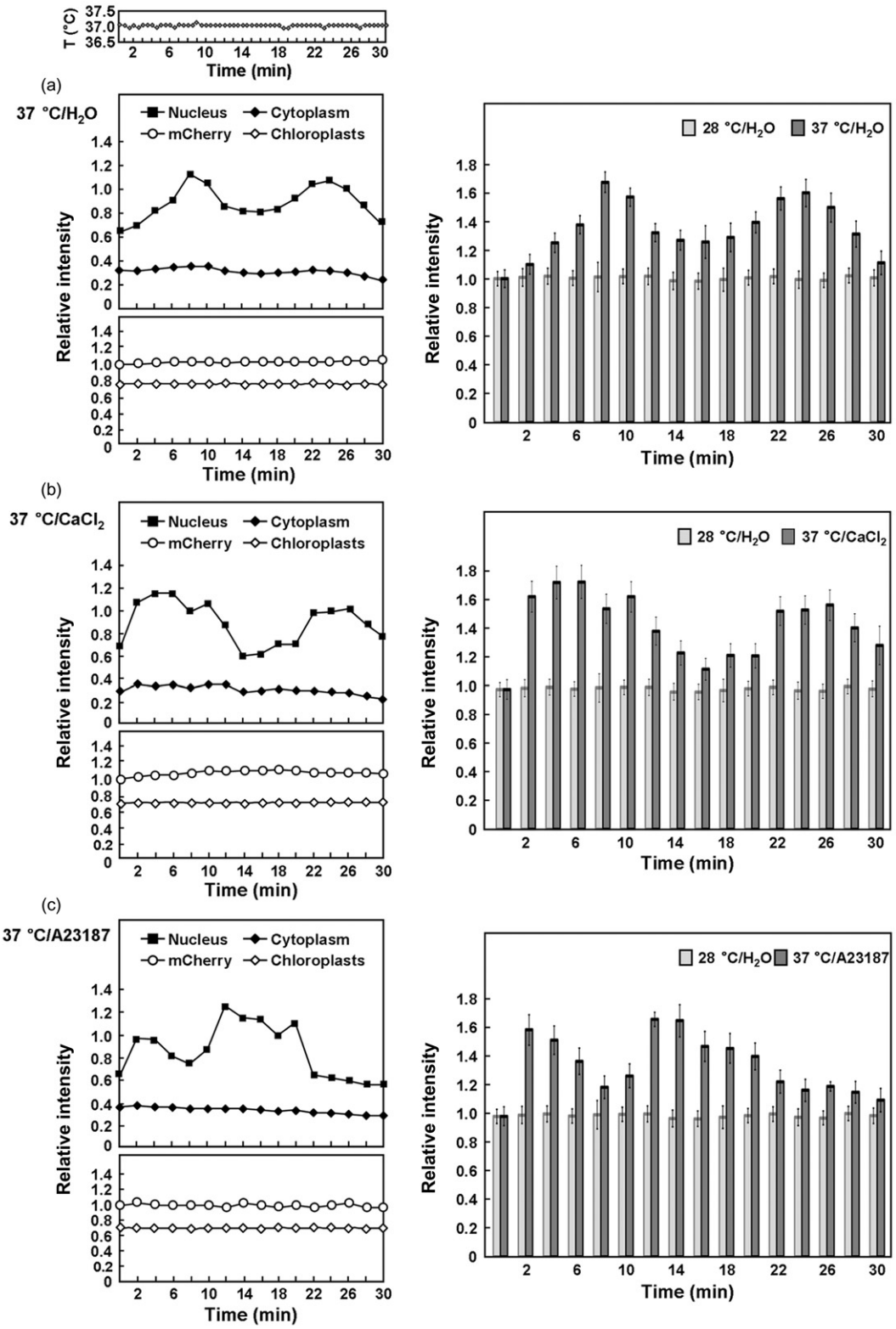

Figure 9. Biphasic signal of the nuclear-localized OsCaM1-1 during heat shock (HS) and Ca^{2+} effect. *Arabidopsis* protoplasts were transfected with *OsCaM1-1-GFP* and a nucleus marker of mCherry, then analysed by laser scanning confocal microscopy. Fluorescence intensity of nucleus, cytoplasm and chloroplasts was measured every 2 min at HS. (a–c) 37 °C-HS treatment with H_2O, 5 mM $CaCl_2$ or 10 μM A23187, respectively. A set of experimental data from three independent replicates is shown (left panels; the initial signal of mCherry was set to 100%), which all showed similar profiles. The relative fluorescence intensity of the nucleus was normalized to that with 28 °C/H_2O-control treatment (right panels). Results are means ± SD of three independent replicates.

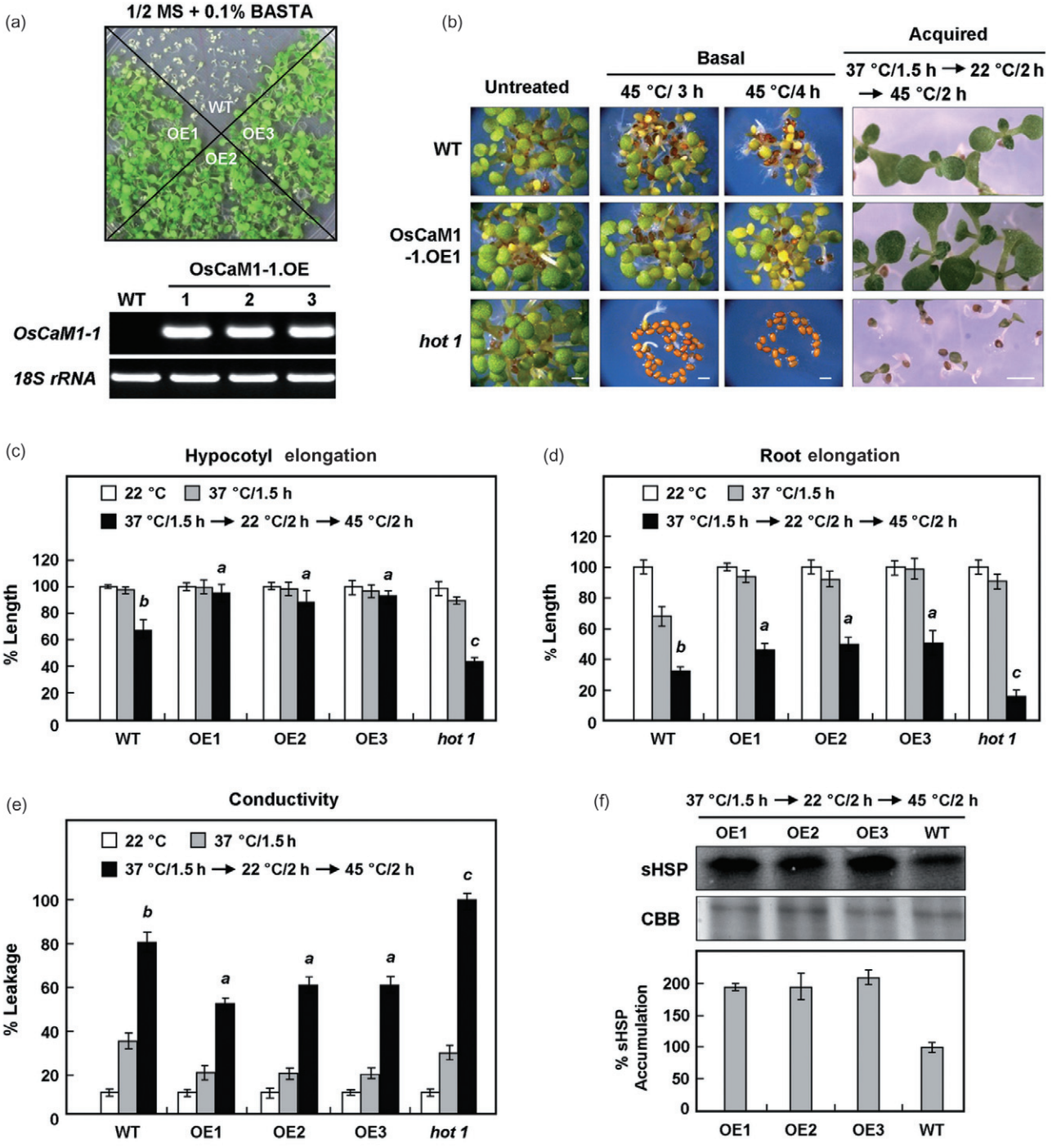

Figure 10. Overexpression of *OsCaM1-1* enhanced thermotolerance in *Arabidopsis*. T-DNA-homozygote seeds in T3 were analysed. (a) Three independent transgenic lines constitutively overexpressing *OsCaM1-1-GFP* (OsCaM1-1.OE1 to OE3) were confirmed by selection with the 0.1% herbicide BASTA and RT-PCR analysis. (b) Basal and acquired thermotolerance assays of the wild type (WT), *hot1* mutant and OsCaM1-1.OE1 lines. Bars = 2 mm. (c,d) Hypocotyl and root elongation, respectively, in response to HS. Results are mean ± SD of three independent replicates ($n = 30$ seedlings). *a*, *b* and *c* represent significantly different values ($P < 0.05$). (e) Ion leakage measured in 100 mg of 10-day-old seedlings by electrolyte conductivity. The highest value was set to 100%. (f) Small HSP (sHSP) was detected with anti-sHSP antibodies, and a loading control was obtained with Coomassie Brilliant Blue (CBB) staining. Calibration of sHSP level is given as histograms in the bottom panel.

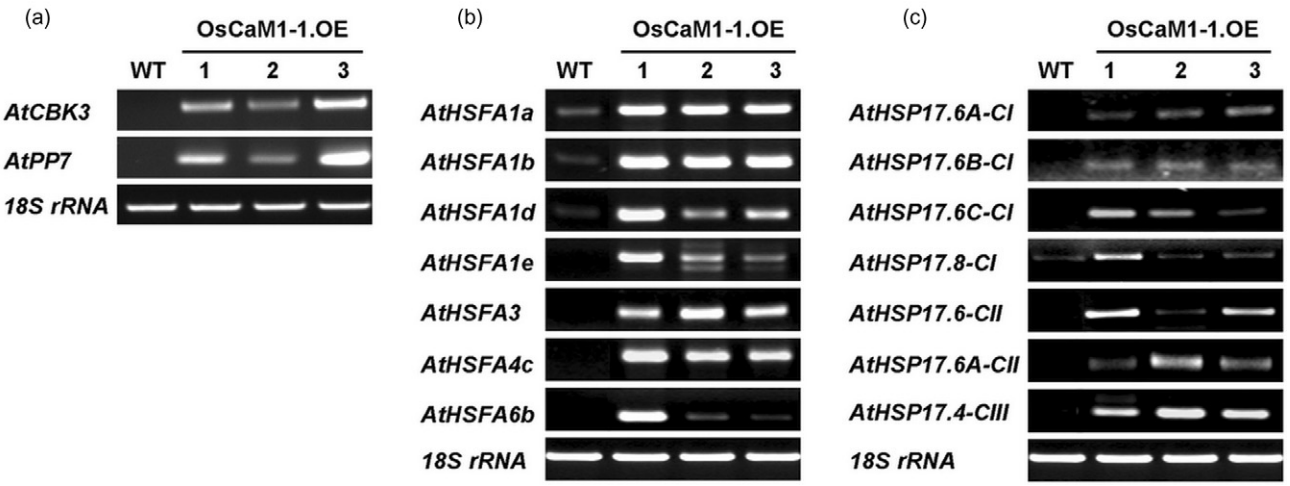

Figure 11. Overexpression of *OsCaM1-1* promoted heat shock (HS)-responsive genes induction in *Arabidopsis*. Wild type (WT) and *OsCaM1-1-GFP*-overexpression (OsCaM1-1.OE1 to OE3) lines were grown at 22 °C. Expression of *CBK3* and *PP7* (a), Class A *HSF* (b) and *sHSPC/N* (c) genes was analysed by RT-PCR. 18S rRNA was used as a loading control.

DISCUSSION

The HS-triggered $[Ca^{2+}]_{cyt}$ response may correspond to the induction of the expression of Ca^{2+}/HS-related genes

In this investigation, the challenge of rice seedlings with HS provoked a monophasic $[Ca^{2+}]_{cyt}$ signal located in epicotyls and leaves and a biphasic $[Ca^{2+}]_{cyt}$ signal in roots (Fig. 1). Previous work has shown that H_2O_2 triggered a biphasic Ca^{2+} elevation in *Arabidopsis*, the early Ca^{2+} peak localized to the cotyledon, whereas the late Ca^{2+} rise was restricted to the root (Rentel & Knight 2004). However, aequorin-expressing tobacco cell cultures also displayed a biphasic elevation in $[Ca^{2+}]_{cyt}$ transient in response to H_2O_2 (Lecourieux *et al*. 2002). Thus, different tissues of rice may use different HS signal transduction/perception mechanisms through their own Ca^{2+} signature; nevertheless, the basis for such oscillatory behaviours in plants remains to be investigated.

The H_2O_2-triggered $[Ca^{2+}]_{cyt}$ elevation was associated with the level of a detoxification gene *glutathione-S-transferase* (*GST*) induction (Rentel & Knight 2004); the ozone-induced biphasic $[Ca^{2+}]_{cyt}$ signal indicated that the first Ca^{2+} peak was insufficient for *GST* gene induction, but the second Ca^{2+} peak was required for the ozone-induced *GST* increase (Clayton *et al*. 1999). In rice, A23187 treatment at 28 °C caused a transient, first spike-like Ca^{2+} signal and slightly enhanced the expression of *OsCaM1-1* (Fig. 3a), but was insufficient to induce the expression of *sHSPC/N* genes (Supporting Information Fig. S2b). Thus, the HS-induced biphasic Ca^{2+} signature might have a corresponding effect on the expression of *sHSPC/N* genes.

By use of pharmacological inhibitors, we confirmed that the main measurable Ca^{2+} influx into the cytosol arises from the apoplastic space, which agrees with our recently published studies (Wu & Jinn 2010; Wu *et al*. 2010). Seedlings treated with the inhibitors did not show cell damage (Supporting Information Fig. S9); therefore, the modulation of $[Ca^{2+}]_{cyt}$ response was not part of a cytotoxic response. We also found that a voltage-gated Ca^{2+} channel blocker reduced the amplitude of the $[Ca^{2+}]_{cyt}$ peak (Fig. 3) and concomitantly inhibited the expression of *sHSPC/N* genes (Fig. 8). Other voltage-gated Ca^{2+} channel blockers such as nifedipine and mibefradil also lowered the HS-triggered $[Ca^{2+}]_{cyt}$ responses and expression of *OsCaM1-1* and *OsHSP17* genes (data not shown). Membrane fluidity of the cell changes with HS (Lehel *et al*. 1993; Horváth *et al*. 1998); hence, the administration of the membrane fluidizer benzyl alcohol (Cooper & Meddings 1991) indeed induced the expression of *OsHSP17* genes and sHSP protein accumulation at a non-inducing temperature (Supporting Information Fig. S10). Simultaneously, the addition of EGTA counteracted the benzyl alcohol effect, which suggests that regulation of the HS response depended on apoplastic Ca^{2+} entry through the plasma membrane.

OsCaM1-1 is an important mediator of the Ca^{2+}-mediated HS signal transduction pathway

The rice genome contains 243 *CaM* and *CaM-like* genes that possibly encode for EF-hand motif proteins, among which only five (*CaM1-1, CaM1-2, CaM1-3, CaM2* and *CaM3*) encode for canonical CaM isoforms (Boonburapong & Buaboocha 2007). We show here that CaM antagonists greatly impaired the HS-induced Ca^{2+} signature and expression of *sHSPC/N* genes (Figs 4 & 8), then suggested the *OsCaM* genes as an important component in the HS response. The rice canonical *OsCaM* genes encode highly conserved proteins ($\geq$ 98.7%), but most *OsCaM* isoforms did not show similar magnitude and kinetics of mRNA induction by HS in our experiments (Fig. 5). *OsCaM1-1*, *–2* and *–3* in rice and *AtCaM2*, *3* and *5* in *Arabidopsis* are encode-identical proteins, which are also found in three human CaM-encoding loci (Friedberg & Taliaferro

2005). Maintaining different genes to encode the identical CaM protein indicates the non-redundancy and highlights the promoter regions carrying the determinant elements that are responsible for gene maintenance and selective expression in response to different stimuli. In *Arabidopsis*, Zhang *et al*. (2009) confirmed that AtCaM2 and AtCaM4 (96.6%) have a high amino acid sequence identity with AtCaM3; however, the defective mutants of *AtCaM2* and *AtCaM4* did not show differences in thermotolerance as compared with WT plants. The authors proposed that different *CaM* genes are differentially regulated by the distinct *cis*-elements under differing stress conditions. Our observation also supports this suggestion; we found that the promoter regions of *OsCaM* genes also exhibit different regulation elements (data not shown). Probably all of the three rice OsCaM1 isoforms are to ensure identical biological functions but may be differentially induced depending on the physiological process, as we demonstrated for the OsCaM1-1 in Ca^{2+}-mediated HS signal transduction. This suggestion resembles that in *Arabidopsis*, in which among three identical members of the *AtCaM* family, only *AtCaM3* was involved in HS response (Liu *et al*. 2005).

The biphasic nuclear localization of OsCaM1-1 may be involved in Ca^{2+}-mediated HS signalling

CaM has been described as a preferential cytosolic protein but has also been found in other compartments such as nucleus, peroxisomes and extracellular matrix (Ma *et al*. 1999; van der Luit *et al*. 1999; Yang & Poovaiah 2003; Kim *et al*. 2009) depending on the environmental stimuli and cytosolic Ca^{2+} oscillations (Teruel *et al*. 2000). Thorogate & Török (2004) indicated that the translocation from the cytosol to the nucleus is a major response by CaM to stimulation of cells by Ca^{2+}. Consistent with the role of the OsCaM1-1 in Ca^{2+}-mediated HS signalling, OsCaM1-1 signal transiently oscillated in the nucleus of *Arabidopsis* protoplasts (Fig. 9) and onion epidermal cells (data not shown) with HS. The OsCaM1-1 nuclear localization suggested an additional function of this protein in signal transduction to downstream factors possibly located in the nucleus, and indeed, CaM-related proteins were found required for phosphorylation status of transcription factors and concomitant transcriptional activation (Bouche *et al*. 2005). The A23187 promotes the nuclear translocation of several human proteins including CaM (Raynal *et al*. 1996; Craske *et al*. 1999). In this study, HS-induced OsCaM1-1 nuclear localization was also enhanced by Ca^{2+} and A23187 combined with HS. This biphasic nuclear signal of OsCaM1-1 might be coordinately regulated by the biphasic Ca^{2+} signature and thereby functions as an important mediator of downstream HS signalling.

OsCaM1-1 plays an important role in HS signal transduction and acquisition of thermotolerance

To identify Ca^{2+}-responsive genes in plants, Kaplan *et al*. (2006) generated specific $[Ca^{2+}]_{cyt}$ transients in *Arabidopsis* and linked them to early transcriptome changes. Our previous studies (Guan *et al*. 2004) identified and characterized that clusters of canonical HSE modules were varied in the promoters of *OsHSP17* genes (cluster on chromosome 3) and *OsHSP16* genes (cluster on chromosome 1), which explained why genes on chromosome 3 were induced rapidly at 32 and 41 °C and by various chemical inducers. Here we identified three *OsHSP17* genes (Fig. 6a) whose expression pattern with HS showed a similar pattern as that for both the Ca^{2+} signature and *OsCaM1-1*, in a time- and temperature-dependent manner (Figs 1 & 5–7). The other *sHSPC/N* genes analysed were induced linearly in a non-oscillating mode (Fig. 6b). We suggest that rapid transcriptome changes of *OsCaM1-1* and *OsHSP17* genes were induced by HS-triggered Ca^{2+} signature, showing that these Ca^{2+}-responsive genes reached their maximal expression levels rapidly, within 30 min following the stimulus treatment, which resembled the observation of Kaplan *et al*. (2006). Our results also support that different mechanisms may be involved in the selective induction of *sHSPC/N* genes by HS (Guan *et al*. 2004).

The wheat *TaCaM1-2* mRNA was quickly induced in 10 min after HS, but the increase in expression of *HSP* genes was detected 20 min later, which indicates that *TaCaM1-2* is functional upstream of the induction of HS-related genes (Liu *et al*. 2003). Analysis of temporal changes in the expression of *AtCaM3* and *AtHSP18.2* confirmed that up-regulation of *AtCaM3* expression was earlier than that of *AtHSP18.2* (Liu *et al*. 2005). Here, the expression of *OsHSP17* genes was also delayed as compared with that of *OsCaM1-1*, which suggests that *OsCaM1-1* functions before *OsHSP17* genes. Thus, the different temporal expression of *OsCaM1-1* and *OsHSP17* indicates that *OsCaM1-1* is probably involved in an early step in modulating expression of downstream *HSP* genes.

Shou *et al*. (2004) showed that overexpression of a tobacco MAPKKK (*Nicotiana* PK1) can mimic H_2O_2 signalling to induce the expression of *GST*, *HSP17.8* and *PR1* genes under normal growth conditions, which leads to enhanced freezing tolerance in maize (*Zea mays*). We demonstrated that overexpression of *OsCaM1-1* in *Arabidopsis* enhanced intrinsic thermotolerance (Fig. 10), and *AtHSF* and *AtHSP* gene expression was induced in OsCaM1-1.OE lines under normal growth conditions (Fig. 11b,c). *AtHSFA1a*, *AtHSFA1b* and *AtHSFA3* are important for the initial phase of the induction of HS-responsive genes (Lohmann *et al*. 2004; Busch, Wunderlich & Schöffl 2005; Yoshida *et al*. 2008), and *AtHSP17* genes respond to HS (Schmid *et al*. 2005; Swindell, Huebner & Weber 2007). The expression of *AtCBK3* and *AtPP7* genes was also promoted (Fig. 11a), as demonstrated by overexpression of *AtCaM3* inducing the expression of *AtCBK3* and *AtPP7* and then modulating the phosphorylation status of AtHSFA1a, which in turn regulated the expression of *AtHSP* genes in *Arabidopsis* (Liu *et al*. 2007, 2008). Thus, constitutively activated *OsCaM1-1* expression may mimic the HS signal and induce the expression of HS-responsive gene cascades in Arabidopsis. In the present study, we did not obtain all the

overexpression lines of the *OsCaM* genes; therefore, we cannot conclude that only OsCaM1-1 is involved in HS signal transduction. Nevertheless, our data showed that OsCaM1-1 is a major component participating in the HS signal transduction in rice.

Additionally, protein kinase inhibitors such as staurosporine and protein phosphatase inhibitors such as Okadaic acid and NaF have been frequently used to study the regulation of signal transduction processes in plants (Tamaoki 1991). We also found that the significant modification of HS effects by these inhibitors in rice seedlings was through reduced expression of *OsCaM1-1* and *sHSPC/N* genes (data not shown). We suggest that in rice, Ca^{2+}-CaM regulates gene expression by modulating the phosphorylation status of HSFs, which in turn regulates the expression of *HSP* genes for HS signal transduction as that was reported in *Arabidopsis* (Liu *et al*. 2007, 2008).

In conclusion, our data indicate a prerequisite for the biphasic Ca^{2+} signature in rice by linking *OsCaM1-1* perception with subsequent induced expression of HS-related genes in response to early HS. Here we propose a model of the HS transduction pathway, with OsCaM1-1 as a major decoder of Ca^{2+} influxes for downstream multicomponent transcriptional machinery (Supporting Information Fig. S11).

ACKNOWLEDGMENTS

This work was supported by grants from National Taiwan University (NTU; 10R80917-3) and partially by the National Science Council, Taiwan (98–2311-B-002–007-MY3) to T-L.J.. We thank Drs Chu-Yung Lin and Tsan-Piao Lin (NTU) for critically reading the manuscript and giving comments. H-C.W. was supported by NTU, French CNOUS Joseph Fourier PhD fellowships and a financial award (Aide à la Mobilité Internationale) by Ecole Doctorale SIBAGHE (Université de Montpellier II, France). We appreciate the reviewers and editors for their exceptionally helpful comments about the manuscript.

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Received 12 April 2011; accepted for publication 6 March 2012

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. HS-triggered $[Ca^{2+}]_{cyt}$ responses were not significantly affected by intracellular Ca^{2+} release inhibitors in rice root cells.

Figure S2. Expression of rice sHSPC/N genes did not respond to exogenous-supplied Ca2+ and A23187 in the absence of HS.

Figure S3. Exogenous-supplied Ca2+ and A23187 during HS induced early and enhanced expression of OsCaM1-1 and sHSPC/N genes.

Figure S4. Apoplastic Ca2+ entry blockers and CaM inhibitors diminished OsCaM-1 and sHSPC/N genes induction under HS but were not significantly affected by intracellular Ca2+ release inhibitors.

Figure S5. OsCaMs were detected in cytoplasm and nucleus.

Figure S6. Biphasic signal of the nuclear-localized OsCaM1-1 during HS and Ca2+ effect.

Figure S7. Localization of GFP and OsCaM2 remained unaffected during HS.

Figure S8. Overexpression of *OsCaM1-1* enhanced intrinsic thermotolerance in Arabidopsis.

Figure S9. Root elongation was not affected by the chemicals tested in this study.

Figure S10. HS-like response induced by benzyl alcohol, a membrane fluidizer, at a non-HS temperature and inhibited by EGTA.

Figure S11. The hypothetical model of molecular mechanism implicated in the HS-induced Ca2+ signature is likely interpreted by OsCaM1-1 association with the multicomponent transcriptional machinery.

Table S1. Chemicals used in this study and in other studies of various plant materials and their effects on [Ca2+]cyt response and on OsCaM and sHSPC/N genes induction under HS in rice.

Table S2. Specific primers for rice OsCaM and sHSPC/N genes analysed.

Table S3. Specific primers for Arabidopsis HS-responsive genes analysed.

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