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Over-expression of mitochondrial heat shock protein 70 suppresses programmed cell death in rice

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ABSTRACT

In this study, we identified and functionally characterized the mitochondrial heat shock protein 70 (mtHsp70). Over-expression of mtHsp70 suppressed heat- and H_2O_2-induced programmed cell death (PCD) in rice protoplasts, as reflected by higher cell viability, decreased DNA laddering and chromatin condensation. Mitochondrial membrane potential ($\Delta\psi_m$) after heat shock was destroyed gradually in protoplasts, but mtHsp70 over-expression showed higher $\Delta\psi_m$ relative to the vector control cells, and partially inhibited cytochrome *c* release from mitochondria to cytosol. Heat treatment also significantly increased reactive oxygen species (ROS) generation, a phenomenon not observed in protoplasts over-expressing mtHsp70. Together, these results suggest that mtHsp70 may suppress PCD in rice protoplasts by maintaining mitochondrial $\Delta\psi_m$ and inhibiting the amplification of ROS.

1. Introduction

Plant programmed cell death (PCD) shares similar biochemical and biological hallmarks with apoptosis in animal cells, such as cell shrinkage, chromatin condensation, DNA fragmentation [1]. However, certain features of plant PCD differ from animal cell apoptosis. First, reactive oxygen species (ROS) sources are more complex in plant cells. Besides plasma membrane NADPH oxidase [2] and mitochondria [3], chloroplast may also be involved in ROS bursts during plant PCD [4]. Second, although mitochondria are known to play a central role in regulating animal cell apoptosis, their function in plant PCD has yet to be determined. Many studies have indicated that mitochondrial membrane potential ($\Delta\psi_m$), which is controlled by the permeability transition pore (PTP) in plants, decreases prior to PCD and that maintaining $\Delta\psi_m$ may inhibit PCD [5,6]. Thus, several studies have focused on the regulation of mitochondrial $\Delta\psi_m$. For example, the voltage-dependent anion channel (VDAC), a PTP component, is up-regulated during the process of PCD [7]. In tobacco, mitochondrial-associated hexokinases have been identified as negative regulators of PCD that control the interaction of PTP subunits [8]. Third, although no caspase homolog has been identified in plants, many studies have found that a specific caspase inhibitor can suppress plant PCD effectively, thus indicating the presence of caspase-like activity in plant cells [9]. Moreover, the activity of the cysteine protease VPE in vacuoles may be similar to that of caspase-1 in animal cells [10]. Very recently, phytaspase, a novel PCD-related subtilisin-like protease in tobacco and rice, was found to possess caspase-like specificity [11].

In our previous study, we identified a mitochondrial heat shock protein 70 (mtHsp70) that is up-regulated during salt-induced PCD in rice root-tip cells [12]. In animal cells, Hsp70s play an anti-apoptotic role via two pathways. In one, Hsp70 binds directly to Apaf-1 and inhibits the formation of apoptosomes [13]. In the other, Hsp70 interacts with members of the Bcl-2 family, protects $\Delta\psi_m$, and inhibits the release of cytochrome *c*, endonucleases, and apoptosis-inducing factor from mitochondria to the cytosol [14,15]. Usually, mtHsp70 is required for polypeptide translocation across the mitochondrial inner membrane and subsequent protein-folding reactions in the matrix. In animal cells, the loss of mtHsp70 protein content in the marginal zone may lead to decreased functional mitochondria, whereas mtHsp70 over-expression preserves mitochondrial function, lessens oxidative stress and subsequent apoptosis [16], and inhibits cytochrome *c* release [17]. Although the anti-apoptotic role of Hsp70 in animals is well-defined, little information about a similar role in plants has been reported. Cronjé et al. demonstrated that Hsp70/Hsc70 accumulation was correlated negatively with PCD in tobacco protoplasts [18]. Using a

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; $\Delta\psi_m$, mitochondrial membrane potential; H_2DCFDA, 2′,7′-dichlorodihydrofluoresceindiacetate; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide; mtHsp70, mitochondrial heat shock protein 70; PCD, programmed cell death; ROS, reactive oxygen species

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maize respiratory-deficient NCS mutant, Kuzmin et al. showed that a decrease in $\Delta\psi_m$ triggers signaling to express cytosolic and mitochondrial heat shock proteins that have a protective effect on cells and, particularly, dysfunctional mitochondria [19]. Hsp101, Hsp70, and small Hsp induced by mild heat shock may inhibit the execution of PCD upstream and downstream of cytochrome *c* release in *Arabidopsis thaliana* suspension cells [20]. However, direct experimental evidence about the roles of mitochondrial Hsp70 in plant PCD is still lacking.

In this study, we demonstrated that mtHsp70 over-expression in transgenic rice protoplasts influenced heat- and H_2O_2-induced PCD, mitochondrial membrane potential, and ROS generation. Our results indicate that mtHsp70 may suppress PCD by maintaining $\Delta\psi_m$ and inhibiting cellular ROS bursts in rice protoplasts.

2. Materials and methods

2.1. Rice transformation

Total RNA was isolated from rice (*Oryza sativa* L. cv. Nipponbare) roots using the Trizol reagent (Invitrogen, USA). Reverse transcription polymerase chain reaction (RT-PCR) was used to obtain full-length mtHsp70 cDNA (Os02g0774300). This product was then cloned into the pGEM T-easy vector (Promega, USA) for DNA sequence analysis. The RT-PCR primers were as follows: forward primer 5′-TAT*AGATCT*CATGGCGGCGTCGCTGCT-3′ (*Bgl*II site is shown in italics) and reverse primer 5′-GCG*ACTAGT*TCACTTCTTGACC TCCTC-3′ (*Spe*I site is shown in italics), based on sequence information in the U.S. National Center for Biotechnology Information (NCBI) database. The PCR-amplified products were double-digested with *Bgl*II and *Spe*I, and then directionally cloned into the plant expression vector pCAMBIA1304. This 35S over-expression construct (over-expressing, OX) and empty 35S vector (vector control, VC) were introduced respectively into *Agrobacterium tumefaciens* strain EHA105 electronically. *Agrobacterium*-mediated transformation of rice callus (*O. sativa* L. cv. Nipponbare) was performed according to the protocol of Nishimura et al. [21]. Transformed calli, selected by hygromycin resistance, were used to generate suspension cell lines.

2.2. Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qPCR) was conducted using an iCycler iQ fluorescent quantitative PCR apparatus (Bio-Rad, Hercules, CA, USA). Total RNA was extracted from VC and OX suspension cells using the Trizol reagent (Invitrogen). qPCR was carried out as follows: 10 μl SYBR Premix Ex Taq™ (Takara, Otsu, Japan), 0.4 μl upstream and downstream primer, respectively, 2 μl cDNA templates, and 7.2 μl ddH_2O were mixed together. 18S rRNA (GenBank accession no. X00755) was used as an internal control. Each sample was run in four independent experiments. The reaction primers were as follows:

qPCR of mtHsp70, upstream 5′-GTGAGATGGCGACAGACAACAAG-3′ and downstream 5′-GATGGTGATTTGCTGCTCCTTCC-3′; qPCR of 18s rRNA, upstream 5′-CCTATCAACTTTCGATGGTAGGATA-3′ and downstream 5′-CGTTAAGGGATTTAGATTGTACTCATT-3′.

2.3. Cell culture and protoplast preparation

Rice cell suspensions, including VC and OX lines, were routinely propagated and cultured at 28 °C. Briefly, transformed calli mentioned above were used to establish the cell suspension culture. The small, loose, and dry cell clusters was placed in MS liquid medium (pH 5.8) and grown in darkness in a shaking incubator at 120 rpm at 28 °C for about 2 weeks for the first subculture. Later 10 ml of inocula were transferred to new media every 7 days. Protoplasts were prepared as described by Maas et al. [22]. Briefly, protoplast were isolated from 3 to 4 d suspension cells. The suspension cell aggregates were transferred into a sterile 100 mm × 20 mm petri dish, and treated with 20 ml of CPW-9 M enzyme solution (1.5% cellulase RS, 0.5% macerozyme R-10, pH 5.8). The digestion mixture was incubated in the dark at 28 °C for 3 h, and washed with CPW-9 M enzyme buffer. The protoplasts were collected through a 35 μm nylon mesh filter, followed by centrifugation at 600 rpm for 5 min.

2.4. Heat shock and H_2O_2 treatment of protoplasts

Heat shock at 48 °C was induced by transferring rice protoplasts into a water bath at 48 °C for 15 min. After heat shock, the protoplasts were returned to 28 °C for recovery. Suspended rice protoplasts were also treated with 100 μM H_2O_2 in the dark. At the indicated time, aliquots of protoplasts were collected by filtration for analysis.

2.5. Cell viability

Protoplasts treated by heat stress or H_2O_2 at different time points were incubated in 0.05% Evans blue solution for 10 min at room temperature. Hundred protoplasts were counted under light microscope, among these protoplasts, blue cells were scored as dead cells.

2.6. DNA laddering analysis

Low molecular weight DNA ladder fragments were selectively extracted from protoplasts by using DNA ladder detection kit from Applygen Technologies Inc. (Beijing, China). DNase-free RNase A was then used to digest existing RNA. Finally, 10 μg DNA from each sample was subjected to electrophoresis on 2% agarose gel.

2.7. In situ detection of DNA fragmentation using TUNEL assay

Protoplasts were treated at 48 °C for 15 min, recovered at 28 °C for different time periods, and then detected using an In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Shanghai, China) according to the manufacturer's protocol. After staining, the protoplasts were transferred to slides and observed using Zeiss Axio Imager A1 fluorescence microscopy with a filter set for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) detection: Green 38, Excitation BP450–490, Beam plitter FT495, Emission BP500–550. The total number of protoplasts was counted at each time point in black-and-white images. At the same time, TUNEL-positive protoplasts (green fluorescence) was also counted in fluorescent images. Taken together, about 100 protoplasts were counted in black-and-white images, and the percentage of TUNEL-positive protoplasts were calculated as: (the number of TUNEL-positive protoplasts/the total number of protoplasts) × 100%.

2.8. Fluorescent staining of nuclei using 4′,6-diamidino-2-phenylindole (DAPI)

Protoplasts were stained with 50 μg/ml DAPI (Sigma–Aldrich, Shanghai, China) for 10 min at 37 °C, washed with PBS buffer three times, and then examined using Zeiss Axio Imager A1 fluorescence microscopy. Filter set parameter: Blue 49, Excitation G365, Beam plitter FT395, Emission BP420–470. Every sample was repeated three times, and every image was taken continuously more than 10 pictures. Software for fluorescence intensity analysis is

Image-Pro Plus 6.0, background parameter is 220. In total, 100 cells from each sample were analyzed for DAPI positivity.

2.9. Mitochondrial membrane potential assay

A stock solution of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylimidacarbocyanine iodide (JC-1) (Molecular Probes, Eugene, OR, USA) was prepared at a concentration of 1 mg/ml in dimethylsulfoxide (DMSO). Fresh staining solution (5 μg/ml) was prepared before each use by diluting stock solution with staining buffer (10 mM HEPES, pH 7.2, 0.6 M sorbitol, 1 mM $CaCl_2$, 1 mM $MgCl_2$). Suspensions of protoplasts at different recovery times were incubated with JC-1 staining solution for 20 min in the dark at 37 °C. After washing with staining buffer three times, cells were imaged with Zeiss Axio Imager A1 fluorescence microscopy, using red (Red 20, Excitation BP540–552, Beam plitter FT560, Emission BP575–640) or green (Green 38, Excitation BP450–490, Beam plitter FT495, Emission BP500–550) filters, or the fluorescence intensity was measured using a fluorescent microplate reader (GENios Plus; Tecan, Männedorf, Switzerland). Protoplasts without heat treatment were incubated in JC-1 staining solution and measured as a negative control. For the positive control, protoplasts were incubated with 10 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP) for 20 min at room temperature, and then stained with JC-1. Fluorescence measurements were repeated five times per sample, and $\Delta\psi_m$ was evaluated as the red/green ratio.

2.10. Western-blot analysis

Protoplasts were treated at 48 °C for 15 min and recovered at 28 °C for different time periods. For cytochrome *c* detection, the cytosolic fractions were prepared via ultra-centrifugation (100,000×*g*, 1 h), and the supernatants were collected. Western-blotting was carried out according to methods described previously [13], using an anti-cytochrome *c* antibody (7H8.2C12; BD Pharmingen, San Diego, CA, USA).

2.11. Detection of reactive oxygen species

Intracellular generation of ROS was measured using 2′,7′-dichlorodihydrofluoresceindiacetate (H_2DCFDA) (Molecular Probes Europe, Leiden, Netherlands). Protoplasts with or without 48 °C treatment/28 °C recovery were incubated with 0.5 μmol/l H_2DCFDA for 30 min at 37 °C in darkness. After washing three times with HEPES buffer, cells were observed immediately with Zeiss Axio Imager A1 fluorescence microscopy, filter set parameter: Green 38, Excitation BP450–490, Beam plitter FT495, Emission BP500–550. Every sample was repeated three times, and every image was taken continuously more than 10 pictures. Software for fluorescence intensity analysis is Image-Pro Plus 6.0, background parameter is 220. In total, 100 cells from each sample were analyzed for DCF positivity.

2.12. Bioinformatics analysis

Phylogenetic trees were constructed from the multiple alignments by the neighbor-joining method, using the DNAMAN software (Lynnon Biosoft, Pointe-Claire, Québec, Canada). Reference amino acid sequences used in tree construction were obtained from the NCBI protein database. Signal peptide and localization analysis was carried out with SignalP and TargetP software.

2.13. Statistical analysis

Statistical analysis was done with SPSS software (SPSS Inc., Chicago, IL, USA). Data are mean ± S.D. of three independent experiments. Statistical significance was defined as '∗' (Student's *t*-test with $P < 0.05$) or '∗∗' (Student's *t*-test with $P < 0.01$).

3. Results

3.1. The product of Os02g0774300 has two homologs in rice and was putatively identified as a mitochondrial heat shock protein 70

In our previous proteomic study, the expression of a putative mitochondrial heat shock protein 70 (Os02g0774300) was up-regulated after salt-induced PCD in rice root-tip cells [12]. Bioinformatics analysis indicated that Os02g0774300 was on chromosome 2 in rice, and the product of another *hsp70* (Os03g0113700) on chromosome 3 was also targeted in mitochondria (data not shown). These two genes share 92% identity and high sequence similarity with mtHsp70 in other plant species (Fig. 1A). Analysis on signal peptide indicated that signal peptide probability in Os02g0774300 sequence was 0.961, cleavage site was between Ser31 and Ala32. TargetP analysis indicated that the signal peptide was mitochondrial transit peptide with probability of 0.908.

3.2. Over-expression of mtHsp70 in rice suspension cells

To assess the effects of mtHsp70 on PCD in vivo, we over-expressed mtHsp70 in rice suspension cells under the control of the 35S promoter (see Section 2). mtHsp70 expression was detected both in vector control (VC) and over-expressing (OX) rice protoplasts. As shown in Fig. 1B, OX protoplasts displayed high levels of mtHsp70 expression under normal growth condition. Under moderate heat shock condition (treated at 48 °C for 15 min and recovered at 28 °C) we used in the study, the expression of mtHsp70 was partially inhibited both in VC and OX protoplasts, but the expression level in OX protoplasts was still higher (about 2.7-times) than that in VC (Fig. 1C).

3.3. mtHsp70 over-expression suppresses heat- and H_2O_2-induced PCD in rice protoplasts

To determine whether the PCD process could be influenced by the over-expression of mtHsp70, we compared heat- and H_2O_2-induced PCD processes between VC and mtHsp70 OX protoplasts. Cell viability was determined via Evans blue staining assays, as shown in Fig. 2A: after heat shock treatment, the cell viability of both VC and OX protoplasts gradually decreased. However, over-expression of mtHsp70 effectively reduced the percentage of dead cells (39.8% after 6 h recovery) compared with VC (63.2% after 6 h recovery).

DNA fragmentation is a specific hallmark of PCD. To confirm that heat-induced cell death in rice protoplasts was PCD, we detected DNA fragmentation using agarose gel electrophoresis (DNA laddering). As shown in Fig. 2B, heat treatment induced significant fragmentation of chromosomes in VC protoplasts (occurred at 2 h after treatment, and became significant at 4 or 6 h), whereas the occurrence of DNA ladder was delayed (occurred at 4 h after treatment) in OX line. We also examined TUNEL-positive staining resulting from 15 min of heat shock, followed by different recovery periods at 28 °C. As shown in Supplementary Fig. S1, protoplasts over-expressing mtHsp70 showed fewer TUNEL-positive nuclei (about 70% after 24 h) than did VC (>90% after 24 h) at every recovery time.

We further analyzed nuclear morphology and chromatin condensation in heat-treated VC and OX protoplasts. Following incubation at 48 °C for 15 min, the protoplasts were stained with DAPI at different times after treatment. As shown in Fig. 2C and D, DAPI fluorescence signal in vector control protoplasts (VC) is

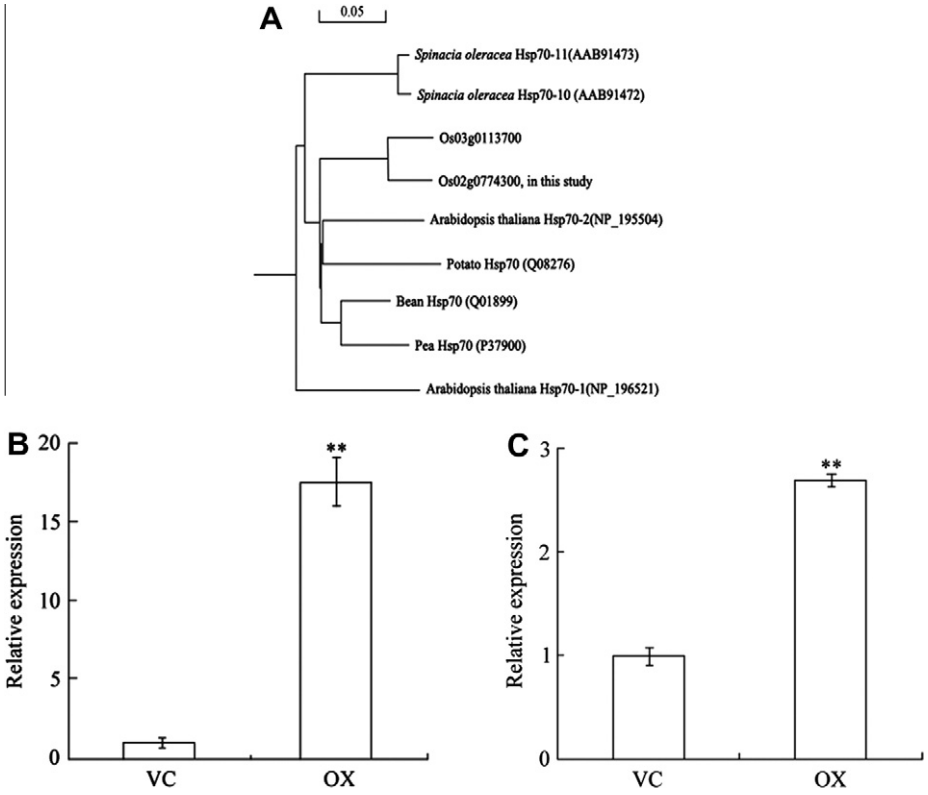

Fig. 1. mtHSP70 sequence analysis and its expression level in transgenic rice cell. (A) The phylogenetic relationship of Os02g0774300 protein with eight other mtHSP70s from plant species. Vertical distances are arbitrary and horizontal distances reflect the number of differences between branch nodes. (B) Expression of mtHSP70 in vector control (VC) and transgenic (OX) rice protoplasts, using real-time PCR. 18S rRNA was used as an internal control. (C) Expression of mtHSP70 in vector control (VC) and transgenic (OX) rice protoplasts in response to heat treatment. Before total RNA extraction, protoplasts were treated at 48 °C for 15 min, and allowed to recover at 28 °C for 2 h. Data are mean ± S.D. of three independent experiments.

much bright than that in mtHSP70 over-expression protoplasts (OX) at every recovery time within 6 h period, which reflected that chromatin condensation and nucleus compaction in OX protoplasts were significantly lower than that in VC.

It has been reported that H_2O_2 could induce PCD in different types of plant cells. As shown in Fig. 3A, 100 μM H_2O_2 treatment led to significant cell death in VC protoplasts (91% after 6 h treatment), which was suppressed in OX line (43% after 6 h treatment). H_2O_2 also induced DNA ladder in VC protoplasts after 30 min treatment, which could not be detected in OX line before 2 h treatment (Fig. 3B). Taken together, these results indicated that over-expression of mtHsp70 inhibited PCD induced by different stimuli in rice protoplasts.

3.4. mtHsp70 suppresses heat-induced PCD by preserving mitochondrial membrane potential and reducing ROS generation

5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) has been used as a reporter molecule to analyze mitochondrial trans-membrane potential in a variety of intact cells, as well as isolated mitochondria in vitro. In living cells, JC-1 molecules exist as monomers at depolarized $\Delta\psi_m$ and exhibit green fluorescence after excitation at 490 nm (Fig. 4A, when $\Delta\psi_m$ was destroyed by CCCP). At hyperpolarized potential, JC-1 accumulates in the mitochondrial matrix, with subsequent formation of J-aggregates characterized by red fluorescence (Fig. 4A, CO). Because the formation and maintenance of J-aggregates in mitochondria depend upon the existence of intact $\Delta\psi_m$, the red/green ratio reflects $\Delta\psi_m$ changes (Fig. 4A, HS). After heat shock at 48 °C, $\Delta\psi_m$ of both VC and OX protoplasts decreased during the recovery time, although transgenic protoplast $\Delta\psi_m$ was significantly higher than that of VC (Fig. 4B). Consistent with these $\Delta\psi_m$ changes, the over-expression of mtHsp70 partially inhibited the release of cytochrome *c* from mitochondria to the cytosol (Western-blot analysis was repeated three times, and a representing result was shown in Fig. 4C. More results were provided in Supplementary Figs. S2 and S3). These results indicate that mitochondrial $\Delta\psi_m$ changes and cytochrome *c* release are early events during heat-induced PCD, compared with proteolysis and nuclear morphological changes, and mtHsp70 over-expression preserves mitochondrial $\Delta\psi_m$ during heat-induced PCD in rice protoplasts.

An ROS burst is an early PCD event in both animal and plant cells. In this study, heat shock at 48 °C elicited a significant increase in VC ROS content (Fig. 5A: 0 and 0.5 h), with a second increase 2.5 h after heat shock. Similar phenomena were also observed in OX protoplasts, but the ROS level at each recovery time was significantly lower than that of VC (Fig. 5B). These results also indicate that mitochondrial $\Delta\psi_m$ change is related to ROS generation during heat-induced PCD in rice protoplasts.

4. Discussion

Hsp70 is a widely studied Hsp family member. *A. thaliana* contains 14 *Hsp70* genes, which are localized in the cytoplasm, endoplasmic reticulum, mitochondria, and chloroplasts [23]. Our bioinformatics analysis indicated that, in rice, products of two Hsp70 genes are targeted to mitochondria (Fig. 1A). Although the function of mtHsp70 as a molecular chaperone is well studied, few studies have addressed the relationship between mtHsp70 and plant PCD. The current study provides direct experimental evidence about the effects of mtHsp70 on PCD in rice protoplasts.

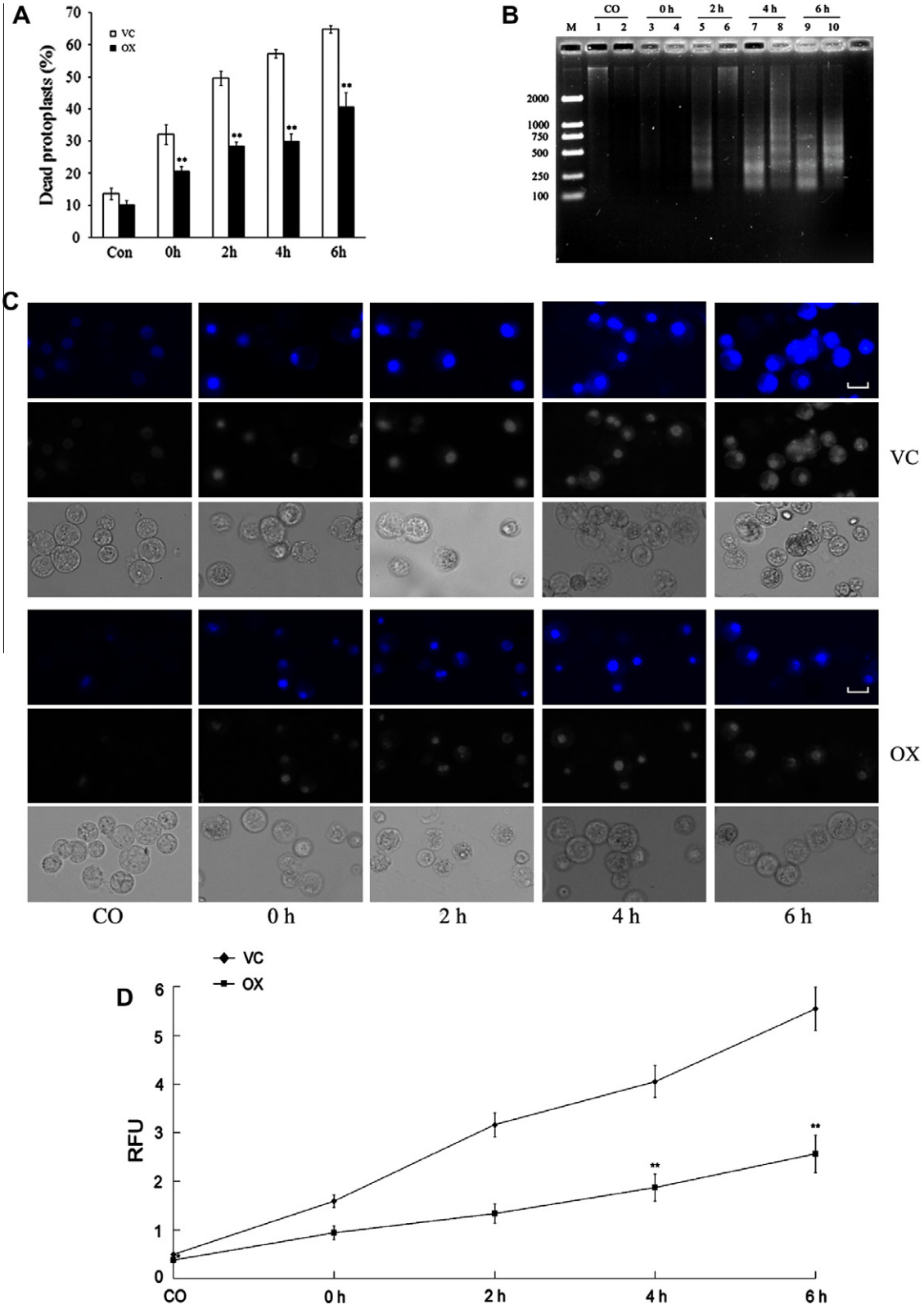

Fig. 2. Over-expression of mtHSP70 suppressed heat-induced PCD in rice protoplasts. (A) Rice protoplasts viability. Vector control (VC) or transgenic (OX) protoplasts were treated at 28 °C for 2 h (Con) or 48 °C for 15 min and allowed to recover at 28 °C for 0, 2, 4, or 6 h. Protoplast viability was detected using Evans blue staining. The 100% value corresponds to heat treatment at 90 °C for 15 min. Data are mean ± S.D. of three independent experiments. (B) DNA fragmentation in rice protoplasts after heat treatment detected by agarose electrophoresis. Protoplasts were treated at 28 °C for 2 h (CO) or 48 °C for 15 min and allowed to recover at 28 °C for 0, 2, 4, or 6 h. Lane M, molecular weight marker; Lane 1, 3, 5, 7, 9, vector control (VC) protoplasts; and Lane 2, 4, 6, 8, 10, transgenic (OX) protoplasts. (C) Nuclear condensation in rice protoplasts after heat shock. Vector control (VC) or transgenic (OX) protoplasts were treated at 28 °C for 2 h (CO) or 48 °C for 15 min and allowed to recover at 28 °C for 0, 2, 4, or 6 h. Samples were stained with DAPI and observed using fluorescence microscopy. Images represent typical examples. *Bar* represents 10 μm. (D) Quantification of DAPI-staining in (C). Software for fluorescence intensity analysis is Image-Pro Plus 6.0, background parameter is 220. In total, 100 cells from each sample were analyzed for DAPI positivity. Data are mean ± S.D. of three independent experiments.

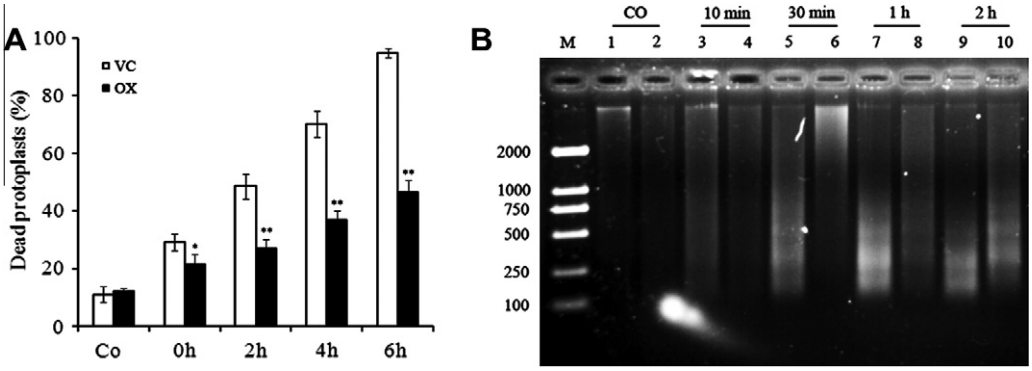

Fig. 3. Over-expression of mtHSP70 suppressed H_2O_2-induced PCD in rice protoplasts. (A) Rice protoplasts viability. Vector control (VC) or transgenic (OX) protoplasts were grown at 28 °C (Con) or treated with 100 μM H_2O_2 in the dark for 0, 2, 4, or 6 h. Protoplast viability was detected using Evans blue staining. The 100% value corresponds to heat treatment at 90 °C for 15 min. Data are mean ± S.D. of three independent experiments. (B) DNA fragmentation in rice protoplasts after 100 μM H_2O_2 treatment detected by agarose electrophoresis. Protoplasts were grown at 28 °C or treated with 100 μM H_2O_2 in the dark for 10, 30 min, 1 and 2 h. Lane M, molecular weight marker; Lane 1, 3, 5, 7, 9, vector control (VC) protoplasts; and Lane 2, 4, 6, 8, 10, transgenic (OX) protoplasts.

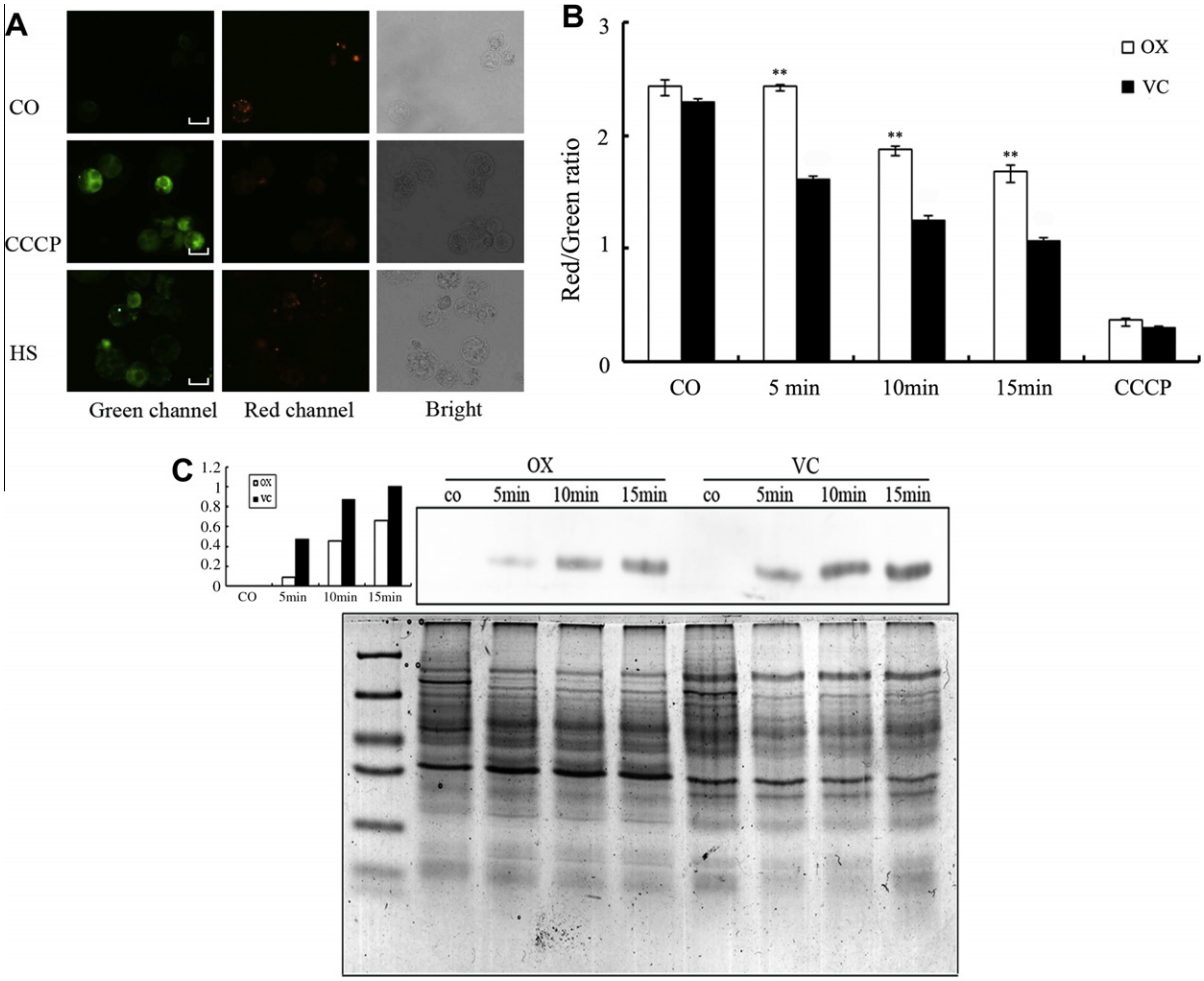

Fig. 4. Assay of mitochondrial membrane potential in rice protoplasts. (A) Calibration of JC-1 with respect to membrane potential. Vector control protoplasts were treated at 28 °C for 2 h (CO) or 48 °C for 15 min and allowed to recover at 28 °C for 5 min (HS). Protoplasts treated with carbonylcyanide *m*-chlorophenylhydrazone were used as positive control (CCCP). Protoplasts were incubated with JC-1 staining solution for 20 min. After staining, protoplasts were imaged with a fluorescence microscopy. For visualizing the JC-1 monomer signal, a green filter was used. For detecting the J-aggregates signal, a red filter was used. *Bar* represents 10 μm. (B) Vector control (VC) or transgenic (OX) protoplasts (4×10^5/100 μl) were treated at 28 °C for 2 h (CO) or 48 °C for 15 min and allowed to recover at 28 °C for 5, 10, or 15 min. Protoplasts were incubated with JC-1 staining solution for 20 min, and fluorescence was measured in a fluorescent microplate reader. Mitochondrial membrane potential was evaluated as the ratio of the red signal (RFU at 590 nm) to the green signal (RFU at 530 nm). For the positive control, protoplasts were incubated with CCCP for 20 min, and then stained with JC-1. Data are mean ± S.D. of five independent experiments. (C) Measurement of the release of cytochrome *c* to the cytosol of rice protoplasts upon heat shock. Vector control (VC) or transgenic (OX) protoplasts were treated at 28 °C for 2 h (CO) or 48 °C for 15 min and allowed to recover at 28 °C for 5, 10, or 15 min. Cytosolic fractions were prepared by ultra-centrifugation and analyzed by immunoblot with an antibody against cytochrome *c*. The lower panel shows SDS–PAGE, revealing the amount of sample loaded. To quantify the blotting signal in the upper left panel, the relative intensity of VC sample protoplasts treated at 48 °C and recovered at 28 °C for 15 min was set as 100%.

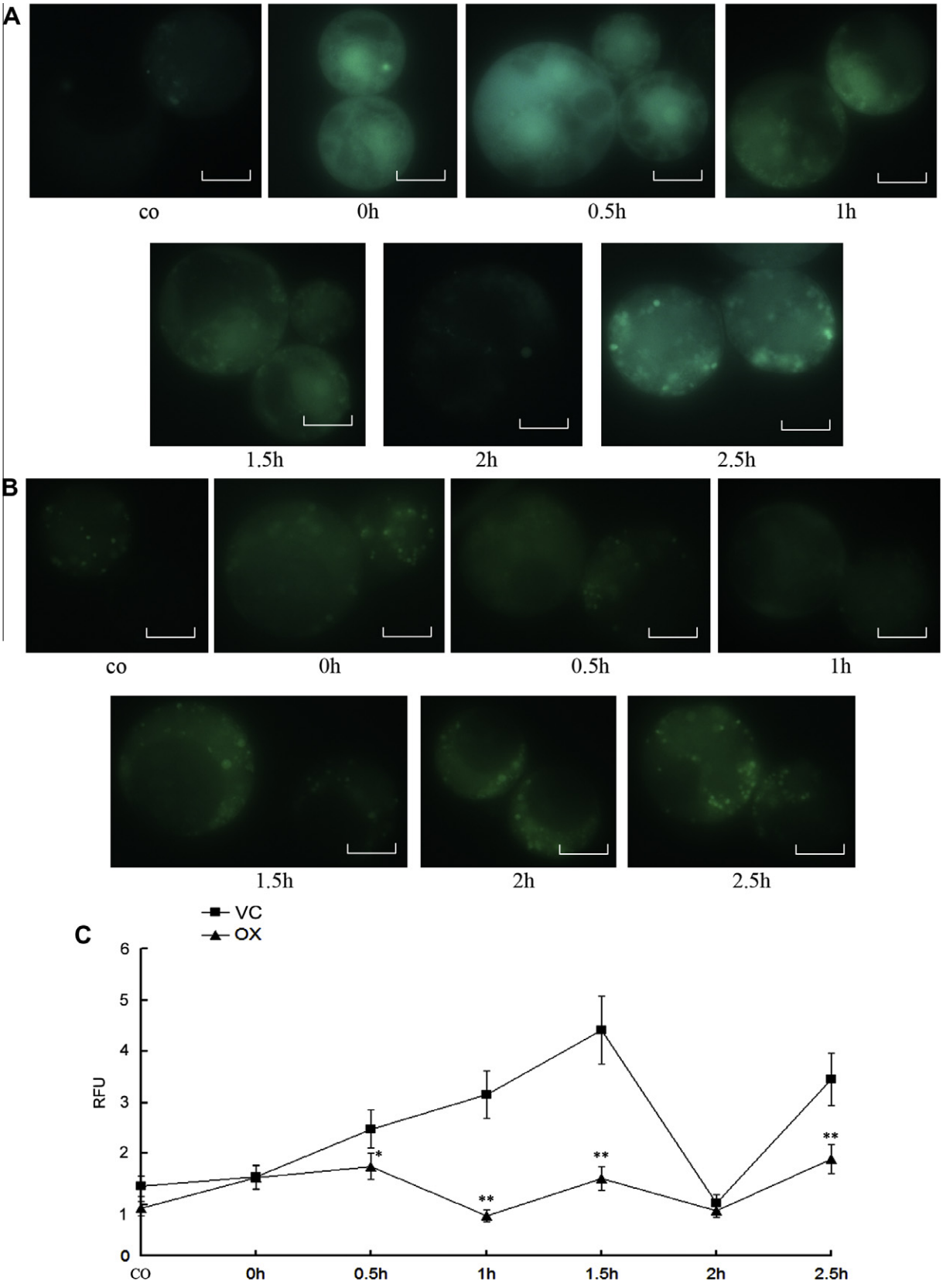

Fig. 5. Generation of reactive oxygen species (ROS) in rice protoplasts. Protoplasts were treated at 28 °C for 2 h (CO) or 48 °C for 15 min and allowed to recover at 28 °C for 0, 0.5, 1, 1.5, 2, or 2.5 h, and then stained with H_2DCFDA. ROS production was visualized by fluorescent microscopy. (A) Vector control protoplasts and (B) transgenic protoplasts. Photographs show typical examples. *Bar* represents 10 μm. (C) Quantification of ROS-staining patterns in (A) and (B). Software for fluorescence intensity analysis is Image-Pro Plus 6.0, background parameter is 220. In total, 100 cells from each sample were analyzed for DCF positivity. Data are mean ± S.D. of three independent experiments.

Specific heat shock, usually between 45 and 55 °C, is a convenient trigger to induce PCD in plant cell culture systems [24]. High temperature is also the main factor inducing Hsp70 expression, with most plant *Hsp70* genes induced by heat treatments at 37–45 °C. In *Arabidopsis* cell culture, Hsp70 synthesis is induced by heat shock at 37 °C and suppressed at 50 °C, with mild heat shock at 37 °C significantly increasing cell viability and inhibiting the execution of PCD upstream and downstream of cytochrome *c* release, which suggested the regulatory role of 37 °C-induced Hsp70 in 50 °C-induced PCD [20]. Although moderate heat shock conditions (48 °C for protoplasts) in our study inhibited mtHsp70 expression, transgenic protoplasts exhibited significantly higher expression level than VC (Fig. 1C), which mimic the accumulation of Hsp70 at 37 °C. Based on these observations, we studied the effects of mtHsp70 over-expression on heat- and H_2O_2-induced PCD in rice protoplasts. As shown in Fig. 2, cell death (Fig 2A), DNA fragmentation (Fig 2B), and chromatin condensation (Fig 2C) were significantly decreased in mtHsp70 over-expressing cells, with chromatin condensation and nuclear changes delayed. mtHSP70 over-expression also inhibited H_2O_2-induced PCD (Fig. 3), which supported the idea that mtHSP70 is a universal, rather than heat specific negative factor in rice protoplast PCD.

Mitochondrial $\Delta\psi_m$ and subsequent cytochrome *c* release play a central role in animal apoptosis, because released cytochrome *c* facilitates the formation of the Apaf-1 complex [1]. Although cytochrome *c* release has been observed in many types of plant PCD, it does not directly activate the PCD process, because the addition of purified cytochrome *c* has no effect on DNA fragmentation [25]. The significance of $\Delta\psi_m$ reduction may rely on two aspects of plant PCD: first, the release of other mitochondrial molecules, such as DNase, can cause DNA fragmentation [25]. Second, cytochrome *c* release disrupts the electron transport chain, resulting in ROS generation, increasing the cellular ROS level threshold, and activating downstream proteolytic machinery [4]. In our study, mtHsp70 protected $\Delta\psi_m$, as evaluated by the transition of JC-1 monomer and aggregates (Fig. 4A and B). Consistent with this, cytochrome *c* release from mitochondria was inhibited partially (Fig. 4C). Although the precise mechanism must still be determined, these results indicate that mtHsp70 inhibits heat-induced PCD by maintaining mitochondrial $\Delta\psi_m$. As an effector and mediator, the involvement of ROS has been well-characterized in plant PCD. In this study, we observed a significant increase in ROS formation in VC rice protoplasts after heat treatment, as well as its attenuation by mtHsp70 over-expression (Fig. 5). The effects of mtHsp70 on $\Delta\psi_m$ and ROS production support the idea that the rate of ROS generation is $\Delta\psi_m$-dependent [26], and mitochondria can amplify the ROS level inside the cell [27], an phenomena which was demonstrated by pharmacological study that cyclosporin A (CsA) protected $\Delta\psi_m$ and indeed inhibited ROS formation during PCD process [28,29]. In short, mtHsp70 over-expression inhibits mitochondrial membrane permeabilization and cytochrome *c* release. As a result, ROS signals could not be amplified to the PCD threshold, and the subsequent PCD execution events were inhibited. Thus, arguably, heat-induced PCD was partially inhibited by mtHsp70 over-expression. In plant cells, ROS production (e.g., NADPH oxidase, mitochondria, peroxisomes, plastids phytohormones) and scavenging (e.g., enzymes, antioxidants) networks are very complex [2], and ROS production by different compartments can be integrated to trigger PCD [27]. Not surprisingly, other organelles also play important regulatory roles in PCD through the ROS signaling pathway.

In conclusion, to our knowledge, this is the first report of direct experimental evidence that mitochondrial heat shock protein 70 suppresses programmed cell death in rice protoplasts, by maintaining $\Delta\psi_m$ and preventing ROS signal amplification.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.11.051.

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