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Research

MAP Kinase 6-mediated activation of vacuolar processing enzyme modulates heat shock-induced programmed cell death in Arabidopsis

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Summary

- Vacuolar processing enzyme (VPE), a cysteine protease, has been intensively studied in plant hypersensitive cell death, but the role and molecular mechanism of VPEs in response to abiotic stresses remain unclear. This work investigated the involvement of VPEs in Arabidopsis response to heat stress.
- Under heat shock (HS), Arabidopsis VPE activity and the transcript level of γVPE were both upregulated, and γVPE deficiency suppressed vacuolar disruption and delayed caspase-3-like activation in HS-induced programmed cell death (PCD). Moreover, the change of VPE activity generally paralleled the alteration of caspase-1-like activity under HS treatment, indicating that HS-induced VPE activity might exhibit the caspase-1-like activity.
- Further studies showed that MAP Kinase 6 (MPK6) activity was increased after HS treatment, and experiments with inhibitors and mutants suggested that MPK6 was responsible for the γVPE activation after HS treatment. In response to HS stress, reactive oxygen species (ROS) production, increase of cytoplasmic calcium concentration ($[Ca^{2+}]_{cyt}$) and the upregulation of calmodulin 3 (CaM3) transcript level occurred upstream of MPK6 activation.
- Our results suggested that activation of Arabidopsis γVPE was mediated by MPK6 and played an important role in HS-induced Arabidopsis PCD, providing new insight into the mechanistic study of plant VPEs.

Introduction

In plants, genetic programmes for cellular suicide are triggered in response to various environmental biotic and abiotic stresses (Danon *et al.*, 2004; Greenberg & Yao, 2004; Lam, 2004). The term programmed cell death (PCD) in plants includes various forms of cell death composed of a number of orderly processes mediated by intracellular signaling molecules, regardless of the triggers or the hallmarks it exhibits (Zhang & Xing, 2008; Li & Xing, 2011). Heat shock (HS), one of the important environmental stresses, could trigger PCD and several apoptosis-like characters upon exposure to HS have been described in plant cells including DNA ladder, fragmentation of the nucleus and the release of cytochrome C (Vacca *et al.*, 2006; Zuppini *et al.*, 2006). In our previous paper, we investigated the reactive oxygen species (ROS) production, changes of mitochondrial function and morphology, as well as the protective roles of HsfA2 (Zhang *et al.*, 2009a). However, the mechanistic analysis of plant response to HS stress, especially the signaling pathway leading to the execution of PCD, is still lacking in many studies.

Extensive research has provided increasing evidence that PCD in plants and animals shares common events including mitochondrial dysfunction and activation of caspase cascade (Cohen, 1997; Lam & del Pozo, 2000; Lam *et al.*, 2001; Woltering *et al.*, 2002). Moreover, numerous reports indicate that proteases with caspase-like activity exist in plants and mediate processes of cell death in development and stress responses (Woltering *et al.*, 2002; Sanmartin *et al.*, 2005). Our recent studies have detected the caspase-3-like activation in Arabidopsis PCD induced by UV-C and Aluminum toxicity (Zhang *et al.*, 2009b; Li & Xing, 2011).

Previous work has reported the vacuole-localized cysteine proteases called vacuolar processing enzymes (VPEs), which were originally discovered in the maturation of seed storage proteins (Hara-Nishimura *et al.*, 1991). VPEs are endopeptidases with a substrate-specificity towards asparagine residues. They are synthesized as inactive larger pro-protein precursors, from which the C-terminal and N-terminal propeptides are sequentially removed self-catalytically to produce the active mature forms at acidic condition (pH 5.5) (Kuroyanagi *et al.*, 2002). Studies have provided evidence that VPEs are involved in virus-induced hypersensitive cell death in tobacco and exhibit caspase-1-like activity (Hatsugai *et al.*, 2004; Kuroyanagi *et al.*, 2005). Although the two enzymes VPEs and caspase 1 share several structural properties, there is

limited sequence identity between them. Full-genome analysis indicates that there are no caspase-encoding genes in *Arabidopsis* genome; furthermore, the subcellular localizations of the two proteases are different: VPEs are localized in the vacuoles, unlike animal caspases which are localized in the cytosol (Hatsugai *et al.*, 2006).

The *Arabidopsis* genome has four VPE genes: αVPE, βVPE, γVPE and δVPE, which can be separated into two subfamilies: vegetative-type VPEs and seed-type VPEs. αVPE and γVPE are expressed in vegetative organs, whereas βVPE and δVPE are expressed in seeds (Kinoshita *et al.*, 1999; Gruis *et al.*, 2002, 2004). The βVPE is essential for the proper processing of storage proteins (Shimada *et al.*, 2003), and δVPE specifically expressed in the seed coat is associated with cell death (Nakagami *et al.*, 2005). By contrast, the vegetative αVPE and γVPE are upregulated during wounding, senescence and pathogen infection, and may play vital roles in various types of cell death in plants (Kinoshita *et al.*, 1999; Yamada *et al.*, 2004). Recently, VPEs have been identified as plant-specific caspases and a VPE-mediated vacuolar system has been considered as a cellular suicide strategy in plant development and cell death programmes (Hatsugai *et al.*, 2004, 2006). Previous research into plant VPEs has mostly focused on plant senescence, terminal differentiation and pathogen-induced hypersensitive cell death. By contrast, the molecular mechanisms underlying the roles of VPEs in response to abiotic stresses are poorly understood.

Alteration to the phosphorylation state of proteins plays a central role in cellular signal transduction. Mitogen-activated protein kinase (MAPK) cascades are conserved pathways by which extracellular stimuli can be transduced into intracellular responses in all eukaryotic cells (Widmann *et al.*, 1999; Davis, 2000; Kyriakis & Avruch, 2001; Tena *et al.*, 2001; Zhang & Klessig, 2001; Asai *et al.*, 2002; Nakagami *et al.*, 2005). Each MAPK cascade minimally consists of three kinases: MAPKKK, MAPKK and MAPK. In the *Arabidopsis* genome, 20 MAPKs, 10 MAPKKs and 60 MAPKKKs have been identified (Asai *et al.*, 2002; Ichimura *et al.*, 2002). It is well documented that MAPK plays key roles in the regulation of innate immunity and adverse stress responses (Ichimura *et al.*, 2002; Xing *et al.*, 2008). Under HS treatment, MAPK activity has also been detected (Chen *et al.*, 2008), but it remains to establish a specific MAPK cascade which mediates the response to HS stress. A decade ago, MKK1 was first identified as a member of the group of phosphorelay signaling pathway that controls MAPK activation (Morris *et al.*, 1997). Recent investigations have verified a MAPK cascade, extending from MEKK1 through MKK1/2 to MPK4/6 in response to abscissic acid and environmental stresses including cold and high salinity (Teige *et al.*, 2004; Xing *et al.*, 2008). MPK6, as a well-characterized terminal of MAPK cascade in Arabidopsis, can be activated by various environmental stresses and participate in the regulation of several functional proteins including catalase, nitrate reductase, ethylene response factor 104 and so on (Morris *et al.*, 1997; Teige *et al.*, 2004; Xing *et al.*, 2008, 2009; Bethke *et al.*, 2009; Wang *et al.*, 2010).

ROS and calcium (Ca^{2+}) are believed to be the key signaling molecules in plant cells (Fluhr & Bowler, 2000; Romeis *et al.*, 2001), and previous reports have implicated both of them in the activation of MAPK cascades under various stimuli (Xing *et al.*, 2008; Wang *et al.*, 2010). Calmodulin (CaM), an ubiquitous second messenger, acts as the crucial sensor protein in plant signal transduction. In Arabidopsis, CaM has several isoforms and different isoforms can interact with their particular targets upon the different exogenous stimuli. For example, CaM is believed to be necessary for the cellular signaling transduction in Arabidopsis response to cold and heat stresses (Gong *et al.*, 1997; Tahtiharju *et al.*, 1997). Among the isoforms of CaM, CaM3 is considered as a key component of Arabidopsis HS signaling pathway, and the Ca^{2+}-CaM3 cascade participates in the activation of downstream functional proteins under HS treatment (Gong *et al.*, 1997; Xuan *et al.*, 2010).

In this paper, the possible molecular mechanisms underlying the process of VPE-mediated PCD under HS treatment were investigated. Our result indicated that γVPE, among the four Arabidopsis VPEs, was upregulated by HS treatment and played an important role in HS-induced Arabidopsis PCD. Moreover, a MPK6-modulated signaling cascade was demonstrated to be responsible for the activation of γVPE under HS stress.

Materials and Methods

Plant materials and chemical reagents

Plants of wild-type *Arabidopsis thaliana* (L.) Heynh (ecotype Columbia), Arabidopsis VPE-null mutant (*vpe*) and γvpe-1 single mutant (*γvpe*), CaM3-lacking mutant (*cam3*; SALK_001357), as well as MPK6-overexpressing (MPK6-OE) Arabidopsis and several Arabidopsis mutants lacking MAPK genes (*mpk6-2*, SALK_073907; *mpk6-3*, SALK_127507; *mpk3-1*, SALK_151594; *mpk4-1*, NC_003075; purchased from NASC/ABRC) were used. The VPE mutants of Arabidopsis (*vpe* and *γvpe*) were all in the Columbia background, and all the Arabidopsis mutants have been identified using semiquantitative RT-PCR (Supporting Information Fig. S2; Methods S2; Notes S1). All the plants were grown in soil culture in a growth chamber (model E7/2; Conviron, Winnipeg, MB, Canada) with 16 h light photoperiod (120 μmol quanta m^{-2} s^{-1}) and 82% relative humidity at 22°C for 2–4 wk.

BCECF-AM was obtained from Molecular Probes (Eugene, OR, USA). PD98059 was purchased from Sigma-Aldrich (Shanghai, China). All the chemical reagents used in the Supporting Information are listed in Methods S1.

Isolation of Arabidopsis mesophyll protoplasts

The procedures were performed according to our previous study (Zhang *et al.*, 2009b). Small leaf strips (0.5–1 mm) in the enzyme solution including cellulase R10 and macerozyme R10 (Yakult Honsha, Tokyo, Japan) were vacuum-infiltrated for *c.* 30 min and then incubated in darkness for 3 h. After filtration through a 75-μm nylon mesh, the crude protoplast filtrates were sedimented by centrifugation for 3 min at 100 ***g***. The purified protoplasts were suspended in W5 solution (154 mM NaCl,

125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, 1.5 mM Mes-KOH, pH 5.6) and counted in a hemotocytometer.

HS treatment

For the HS treatment of Arabidopsis seedlings, 2-wk-old Arabidopsis seedlings grown in plastic pots were incubated in a 42°C pre-warmed circulating water bath for up to 2 h and then transferred to growth chamber conditions for recovery at 24°C (Moriwaki *et al.*, 1999; Almoguera *et al.*, 2002).

For the HS treatment of Arabidopsis leaves, detached Arabidopsis leaves from 4-wk-old Arabidopsis seedlings were placed on plastic plates and incubated in a 42°C pre-warmed circulating water bath for up to 2 h and then transferred to growth chamber conditions for recovery at 24°C (Moriwaki *et al.*, 1999; Almoguera *et al.*, 2002).

For the HS treatment of isolated protoplasts, newly prepared protoplasts were challenged by a HS treatment in a 40°C pre-warmed circulating water bath for 10 min and allowed to recover at 24°C for different times (Zhang *et al.*, 2009a).

The control samples were not incubated in a water bath for HS treatment, and were maintained in growth chamber conditions at 24°C during the experimental period. Unless stated otherwise, all the controls were the wild type (WT) because there was no difference between WT and mutants under untreated conditions. Plant materials were maintained in the dark for the duration of HS treatment and recovery after HS, and all operations were also performed in the dark to minimize possible oxidative stress caused by light.

Measurement of growth conditions of Arabidopsis seedlings

At indicated times after HS treatment of Arabidopsis seedlings, the leaves from WT, *γvpe* or *vpe* seedlings were collected. The fresh weight was immediately measured, and the dry weight was determined after the material had been oven-dried for 24 h at 105°C. Total leaf area was determined by LI-COR LI-3100C leaf area meter (LI-COR, Lincoln, NE, USA). Each experiment was repeated five times.

Confocal microscopy and *in vivo* imaging of organelles

All microscopic observations were performed using a Zeiss laser confocal scanning microscope (LCSM; LSM510/ConfoCor2, Carl-Zeiss, Jena, Germany). Protoplasts were incubated with BCECF (at a final concentration of 10 μM) for 30 min at room temperature, then treated with HS and subjected to LCSM observation at indicated time of recovery period. The BCECF signal was visualized with excitation at 488 nm and emission at 500–550 nm using a band pass filter, and chloroplast autofluorescence (488 nm excitation) was visualized at 650 nm with a long pass filter.

Protein extraction and caspase-3-like activity assay

Proteins were extracted from HS-treated detached Arabidopsis leaves at the indicated time points after HS according to Zhang *et al.* (2009b). For protein extraction, HS-treated detached leaves were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 15 mM NaCl, 1% Triton X-100, and 100 mg ml^{-1} phenylmethylsulfonyl fluoride) and incubated on ice with gentle shaking using a level shaker for 30 min. Samples were centrifuged for 5 min at 12 000 ***g*** and 4°C, and the supernatants were transferred to new 1.5-ml tubes. Protein concentrations were determined by the method of Bradford. Caspase-3-like activity was measured by determining the cleavage of the fluorogenic caspase-3 substrate Ac-DEVD-pNA (Beyotime, China) using supernatant prepared from cell lysates. The extent of Ac-DEVD-pNA cleavage was measured as the change in A405 resulting from the release of free fluorescent pNA.

VPE enzyme activity assay

Proteins were extracted from HS-treated detached Arabidopsis leaves in 50 mM sodium acetate buffer (pH 5.5) containing 50 mM NaCl, 50 mM dithiothreitol, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. A VPE-specific fluorescent substrate, Ac-ESEN-MCA (Peptide Institute, Inc., Osaka, Japan) was used to measure the activity of VPEs. The extracts were pre-incubated with 100 μM Ac-ESEN-MCA in an acidic buffer (50 mM sodium acetate, pH 5.5, 50 mM dithiothreitol and 0.1 mM EDTA), and then the fluorescence intensity was measured using the TECAN INFINITE M200 ELISA Reader every 5 min during a 1 h reaction. The fluorescence was monitored under an excitation wavelength of 380 nm and emission wavelength of 460 nm.

Treatment with MAPK cascade inhibitor

Before HS treatment, the detached Arabidopsis leaves were pre-incubated in a solution containing a MAPK cascade inhibitor PD98059 (dissolved in DMSO) for 60 min. The inhibitor PD98059 was used at a final concentration of 150 μM to inhibit the activation of MAPK cascade.

Total RNA extraction and quantitative reverse transcript-PCR (qRT-PCR)

Total RNAs were extracted from detached Arabidopsis leaves at indicated times after HS treatment according to manufacturer's instruction using TRI reagent (Sigma). Concentration of RNA was determined by measuring OD at 260 nm. First-strand cDNA was synthesized with the SuperScript II First-Strand Synthesis System for qRT-PCR (Invitrogen). qPCR amplification was carried out using ACTIN as an endogenous control. SYBR Green probes for each gene were used. The primers are listed in Table S2 and the ATG numbers for the cited genes are listed in Table S3. PCR was carried out using 50 ng of cDNA and SYBR PCR master mix (TaKaRa, Dalian, China) following the manufacturer's protocol. Relative quantitation of each single gene expression was performed using comparative threshold cycle method.

Western blot and MAPK activity assay

Proteins were extracted from detached Arabidopsis leaves at the indicated time points after HS. Protein extracts were separated

using SDS-PAGE and then Western blotted. For detection of MAPK proteins, blots were probed with a 1 : 1000 dilution of the polyclonal anti-AtMPK6 antibody (Sigma), anti-AtMPK4 antibody (Sigma) or anti-AtMPK3 antibody (Sigma). For detection of the phosphorylated proportion of MAPKs, blots were probed with anti-ACTIVE MAP kinase polyclonal Ab (pTEpY; Cell Signaling Technology, MA, USA), which recognizes activated MAPKs. Subsequently, blots were washed and incubated with an anti-rabbit horseradish peroxidase secondary antibody. Blots were stained with Ponceau-S and probed with an antibody (Agrisera, Vännäs, Sweden) recognizing the large subunit of Rubisco to confirm even loading and transfer.

Trypan blue staining

At indicated times during the recovery period after HS, HS-treated detached Arabidopsis leaves were stained with a lactophenol/trypan blue solution to observe the cell death.

Results

HS induces growth inhibition and cell death of Arabidopsis

Our results showed that HS treatment effectively inhibited the growth of Arabidopsis seedlings (Fig. 1a). At 6 d post-treatment, leaf area, fresh weight and dry weight all significantly decreased ($P < 0.05$) in HS-treated WT seedlings relative to untreated control sample; subsequently, a significant difference of $P < 0.01$ in seedling size was observed 12 d after HS treatment (Fig. 1b–d). Trypan blue staining of detached WT Arabidopsis leaves revealed cell death at 6 and 12 h after HS treatment (Fig. 1e).

γVPE is involved in HS-induced Arabidopsis death

In order to assess the impact of VPEs on the survival of Arabidopsis, an Arabidopsis mutant that lacks all four VPE genes (VPE-null mutant, *vpe*) was used. Our results showed that in the *vpe* mutant, the inhibition of seedling growth and cell death of detached leaves under HS treatment were efficiently alleviated (Fig. 1), indicating that VPE deficiency suppressed Arabidopsis death induced by HS treatment. To further confirm how VPEs participated in HS-induced Arabidopsis death, VPE protease activity was detected during the recovery time after HS treatment in detached Arabidopsis leaves. Results showed that VPE activity was noticably increased during the 6–12 h recovery period after HS treatment (Fig. 2a). At 6 h into the recovery period a significant increase in VPE activity was detected, which was > 10× ($P < 0.05$) the control level; then the activity leveled off after 9 and 12 h recovery (Fig. 2a). Given the complexity of the expression of different VPE genes in response to adverse stresses,

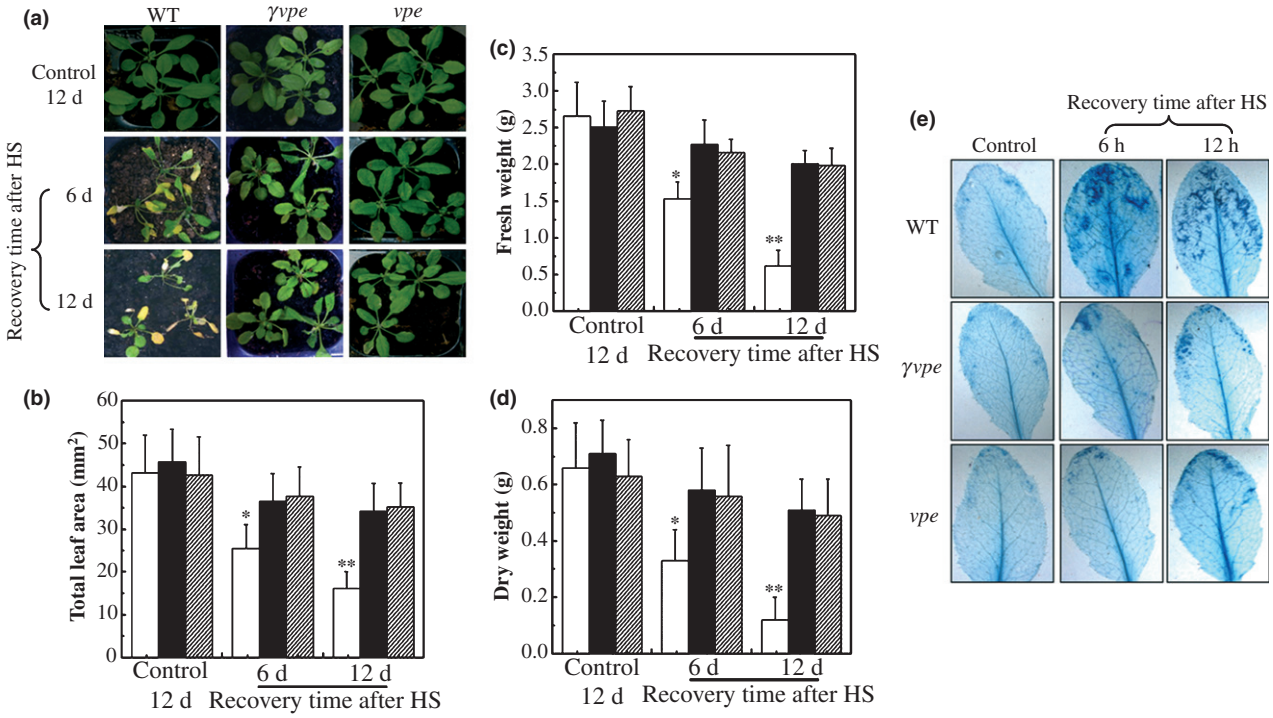

Fig. 1 Growth conditions for *Arabidopsis thaliana* seedlings and trypan blue staining of Arabidopsis leaves under heat shock (HS) stress. (a) Phenotype of wild-type (WT), *γvpe* and *vpe* Arabidopsis seedlings after 6 and 12 d recovery periods after 42°C HS treatment for 2 h. (b) Total leaf area of WT (open bars), *γvpe* (closed bars) and *vpe* (striped bars) Arabidopsis seedlings after 6 and 12 d recovery after 42°C HS treatment for 2 h. (c) Fresh weight of WT, *γvpe* and *vpe* Arabidopsis seedlings after 6 and 12 d recovery after 42°C HS treatment for 2 h. Bars are as in (b). (d) Dry weight of WT, *γvpe* and *vpe* Arabidopsis seedlings after 6 and 12 d recovery after 42°C HS treatment for 2 h. Bars are as in (b). (e) Trypan blue staining of HS-treated detached leaves of WT, *γvpe* and *vpe* after 6 and 12 h recovery after 42°C HS treatment for 2 h. Error bars are ± SD values for five replicates. Statistical analysis was performed with Student's paired *t*-test, asterisks indicate a significant difference from the control: *, $P < 0.05$; **, $P < 0.01$.

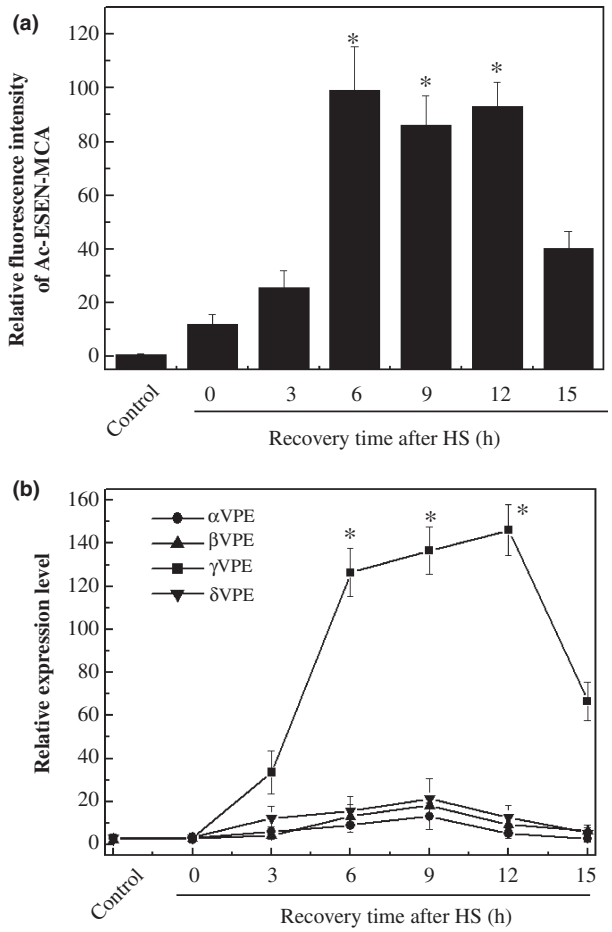

Fig. 2 Changes in vacuolar processing enzyme (VPE) protease activity and VPE gene expression in heat shock (HS)-treated detached *Arabidopsis thaliana* leaves. (a) Changes of VPE protease activity during a 15 h recovery period after HS treatment in the HS-treated detached leaves of wild-type (WT) plants. (b) qRT-PCR analysis of the expression of αVPE, βVPE, γVPE and δVPE during 15 h recovery period after HS treatment in the HS-treated detached leaves of WT. After HS treatment, total RNAs and proteins were extracted from the HS-treated detached leaves at indicated times of the recovery period. A VPE-specific fluorescent substrate, Ac-ESEN-MCA, was used for the measurement of VPE activity. Each data point is the mean ± SD of five replicates. Statistical analysis was performed with Student's paired *t*-test, asterisks indicate a significant difference from the control at *, $P < 0.05$.

changes in transcript proportions of the four types of Arabidopsis VPE genes were investigated in HS-treated detached Arabidopsis leaves. Results of qRT-PCR showed that only γVPE, among the four VPEs, was significantly increased in transcript level after HS treatment in detached Arabidopsis leaves (Fig. 2b). In the HS-treated detached leaves, the transcript level of γVPE was notably increased ($P < 0.05$) after 6–12 h recovery (Fig. 2b), which was consistent with the change in VPE activity. Furthermore, in the detached leaves of γvpe-lacking mutant (*γvpe*), the increase in VPE activity after HS treatment was inhibited (Fig. S1), and HS-induced growth inhibition and cell death were also alleviated (Figs 1, S10; Methods S6; Notes S5). The results given above suggest that γVPE was involved in HS-induced Arabidopsis death.

Lack of γVPE alleviates HS-induced vacuolar rupture and PCD in Arabidopsis

It has been shown that disintegration of vacuolar membranes and activation of caspase-3-like activity are the crucial events in plant cell death (Jones, 2001; Zhang *et al.*, 2009b). Vacuolar collapse in HS-treated Arabidopsis protoplasts was investigated using the fluorescent probe BCECF-AM was used, and diagnosis of vacuolar membrane disintegration was exhibited by BCECF fluorescence. Imaging results demonstrated that protoplasts without HS treatment accumulated BCECF only in the vacuole (Fig. 3a); however, protoplasts that recovered 60 min after HS treatment were separated into three staining types (Fig. 3b–d; Table S1): BCECF-positive with intact vacuolar membrane (26.1 ± 5.6% of the protoplasts); BCECF-negative with disintegrated vacuolar membrane and intact plasma membrane (as shown by distribution of BCECF in the periphery regions inside the cells and the DIC image; 57.2 ± 6.5%); and BCECF-negative with disintegrated vacuolar membrane and plasma membrane (as shown by the DIC image; 17.3 ± 2.3%). The finding suggested that vacuolar collapse occurred under HS treatment.However, protoplasts from the *γvpe* had intact vacuolar and plasma membranes, and accumulated BCECF in the vacuole at 60 min after HS treatment (Fig. 3e,f). Moreover, pretreatment with VPE inhibitor Ac-ESEN-CHO also inhibited HS-induced vacuolar collapse (Fig. S3d; Methods S7; Notes S2).

Subsequently, the caspase-3-like activity in HS-treated detached Arabidopsis leaves was measured using the caspase-3 fluorogenic substrate Ac-DEVD-pNA 15 h into the recovery period after HS. The results show that the induction of caspase-3-like activity was detected after 3 h recovery after HS treatment, reached a peak at 9 h, and then decreased with the increase of recovery time after HS (Fig. 4). The effect of γVPE on HS-induced caspase-3-like activation was also investigated. In HS-treated detached leaves of *γvpe*, the caspase-3-like activation was effectively delayed and reduced after HS treatment (Fig. 4). Moreover, caspase-3-like activation in detached Arabidopsis leaves induced by HS treatment was also delayed and reduced by pretreatment with VPE inhibitor Ac-ESEN-CHO (Fig. S3e; Methods S7; Notes S2). These results indicate that HS induction of vacuolar membrane disintegration and caspase-3-like activation required the involvement of γVPE.

HS-induced γVPE activation can be inhibited by MAPK inhibitor PD98059

In order to investigate whether MAPK cascades affect the HS-induced activation of Arabidopsis γVPE, the common inhibitor of MAPK cascade, PD98059, was used. First, the change in transcript level of γVPE was detected using qRT-PCR. Results showed a significant decrease ($P > 0.05$) in γVPE expression in detached Arabidopsis leaves pretreated with PD98059 at 6 and 12 h after HS treatment, compared to that of the nonpretreated samples (Fig. 5a). The change of VPE activity under HS stress was also analysed. In PD98059-pretreated detached leaves, the increase of VPE activity was effectively inhibited at 6 and

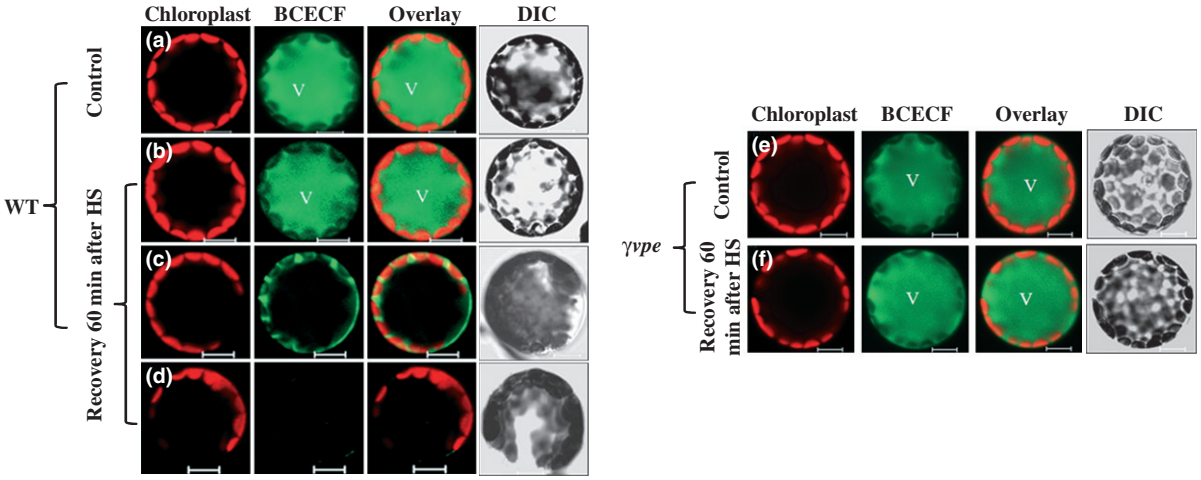

Fig. 3 Vacuolar processing enzyme (VPE) deficiency suppresses heat shock (HS)-induced vacuolar collapse in *Arabidopsis thaliana*. (a) Vacuolar morphology of wild-type (WT) protoplasts without HS treatment. (b, c, d) Morphological changes of vacuole in WT protoplasts at 60 min after HS treatment (42°C, 10 min). (e, f) Vacuolar morphology of *γvpe* protoplasts at 60 min after HS treatment (42°C, 10 min). Protoplasts were incubated with BCECF (at a final concentration of 10 μM) for 30 min at room temperature, and then treated with HS and subjected to LCSM observation at different time points of the recovery period after HS. Bars, 10 μm. V, vacuole; DIC, differential interference contrast.

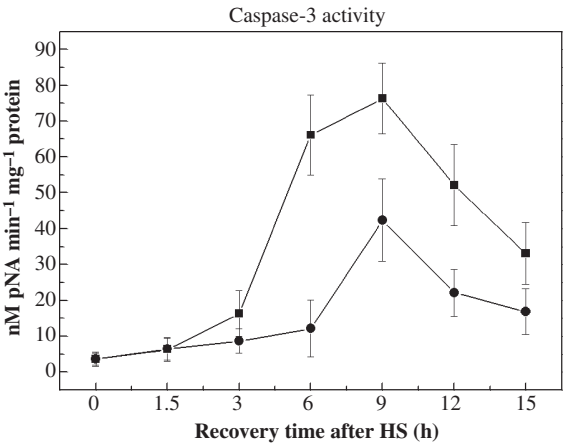

Fig. 4 Caspase-3-like activation in heat shock (HS)-treated detached *Arabidopsis thaliana* leaves. Caspase-3-like activity was tested using an *in vitro* assay using the caspase-3 substrate. The extracts from HS-treated detached leaves of wild-type (squares) or *γvpe* (circles) Arabidopsis at indicated times of the recovery period were used. The samples were incubated with the caspase-3 substrate Ac-DEVD-pNA (200 mM) in an assay buffer containing phenylmethylsulfonyl fluoride and EDTA. Error bars indicate SD values for five replicates.

12 h after HS treatment ($P > 0.05$) (Fig. 5b). The data indicated that MAPK cascades were involved in HS-induced γVPE activation.

MPK6 is activated under HS treatment

The results given above demonstrate that MAPK cascades might be implicated in HS-induced activation of γVPE, but it remains to be established which specific MAPKs mediate this process. MPK3, MPK4 and MPK6 are reported to be involved in Arabidopsis stress response and PCD. Therefore, the expression and activation of MPK3, MPK4 and MPK6 proteins were determined in detached Arabidopsis leaves under HS stress. First, endogenous protein expression of MPK3, MPK4 or MPK6 was immunoprecipitated with specific antibodies against MPK3, MPK4 or MPK6. As shown in Fig. 6(a), specific antibodies for MAPKs detected protein bands of 47, 43 and 43 kD corresponding to MPK6, MPK3 and MPK4, respectively (Fig. 6a). Plant MAPKs have high homology to mammalian ERK1/2 MAPKs, and ERK1/2 antisera that recognize the dually phosphorylated forms (pTEpY) of activated MAPKs can be used to monitor plant MAPK activity (Li *et al.*, 2007). Hence, endogenous kinase activity of MPK3, MPK4 and MPK6 after HS treatment was determined in detached Arabidopsis leaves using anti-ACTIVE MAP kinase polyclonal Ab (pTEpY). As shown in Fig. 6(b), in response to HS stress, while a transient increase in the MPK6 activity (47-kD band) was observed in HS-treated detached WT leaves 15–60 min after HS treatment (Fig. 6b,c; $P < 0.05$), no increase in kinase activity of MPK6 was observed in HS-treated detached leaves of two T-DNA insertion mutants of MPK6 (*mpk6-2* and *mpk6-3*) (Fig. 6b). This clearly suggests that the 47-kD band indicated the activation of MPK6. Our results also showed that HS treatment was unable to transiently activate MPK3 and MPK4 activity (43-kD band) compared to MPK6 (Fig. 6b). This indicates that HS-induced activation of MPK6 occurred by post-translational mechanisms.

Plant growth condition and caspase-3-like activation of MPK6 mutants under HS treatment

In order to test the role of MPK6 in Arabidopsis HS phenotype, two T-DNA insertion mutants of MPK6 (*mpk6-2* and *mpk6-3*) and the MPK6-overexpressing (MPK6-OE) Arabidopsis seedling were used. In comparing growth, *mpk6-2* and *mpk6-3* seedlings showed a lower decrease of fresh weight than that of WT at 6 and 12 d after HS treatment (Fig. 7a); however, the decrease of fresh weight in MPK6-OE seedlings was much higher than that of

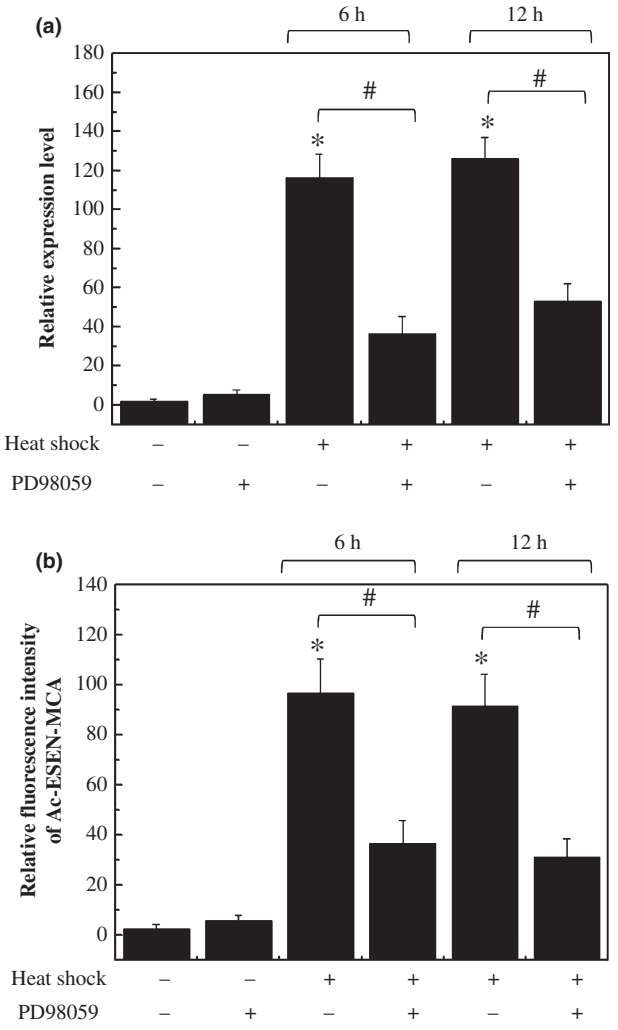

Fig. 5 Effect of MAPK cascade inhibitor (PD98059) on heat shock (HS)-induced γVPE activation in detached *Arabidopsis thaliana* leaves. (a) qRT-PCR analysis of γVPE expression in PD98059-pretreated detached leaves of wild-type (WT) plants after HS. (b) VPE activity analysis in PD98059-pretreated detached leaves of WT after HS. Detached leaves were pre-incubated with or without PD98059 (150 μM) as described in the Materials and Methods section, and then treated with HS (42°C, 2 h). Total RNAs were extracted from the HS-treated detached leaves at the indicated time of the recovery period for the qRT-PCR analysis. The proteins were extracted from the HS-treated detached leaves at the indicated time of the recovery period for the activity assay with the VPE-specific fluorescent substrate, Ac-ESEN-MCA. The control indicates the detached leaves which were dissolved in DMSO solvent but without HS treatment. Each data point is the mean ± SD of five replicates. Statistical analysis was performed with Student's paired *t*-test: *, $P < 0.05$ vs control; #, $P < 0.05$ vs indicated samples.

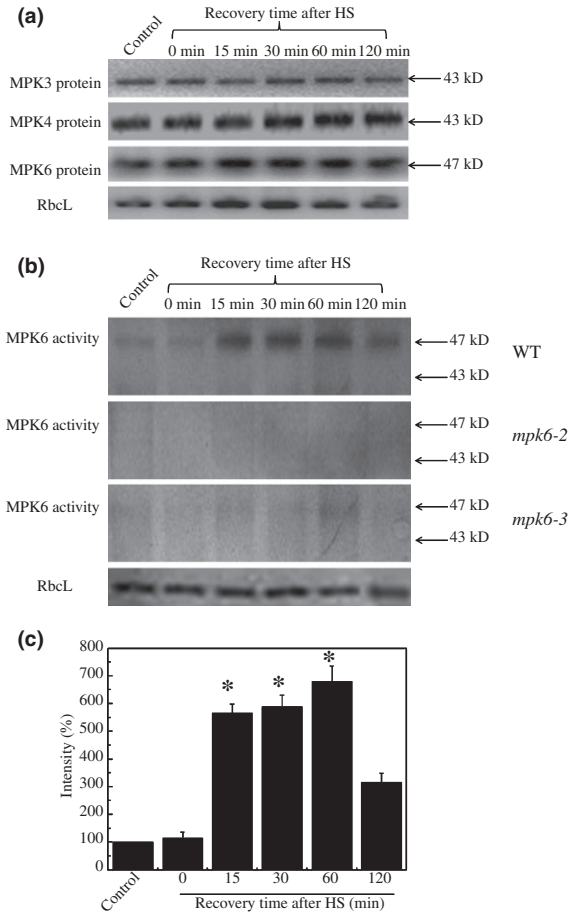

Fig. 6 Activation of MPK6 in the heat shock (HS)-treated detached leaves of *Arabidopsis thaliana*. (a) Immunoprecipitation of MPK3, MPK4 and MPK6 proteins after HS treatment using specific antibodies recognizing MPK3, MPK4 and MPK6, respectively (the specific bands are indicated by arrows). (b) Immunoprecipitation of phosphorylated MPK6 in wild-type (WT) and MPK6 mutants (*mpk6-2*, *mpk6-3*) in response to HS. The phosphorylated MAPK was recognized by the anti-ACTIVE MAP kinase polyclonal Ab (pTEpY), which recognizes activated plant MAPKs (the specific bands are indicated by arrows). Blots were probed with an antibody (Agrisera) recognizing the large subunit of Rubisco as a loading control. (c) Quantitative analysis of MPK6 phosphorylation proportion shown in (b) with Image J software. Control intensity was set as 100%. The proteins were extracted from the HS-treated detached leaves at the indicated time of the recovery period. Data represent mean ± SD of three independent experiments. Statistical analysis was performed with Student's paired *t*-test, asterisks indicate a significant difference from the control at *, $P < 0.05$.

WT 6 and 12 d after HS treatment (Fig. 7a). As a parallel experiment, the caspase-3-like activity in detached leaves of *mpk6-2*, *mpk6-3* and MPK6-OE under HS stress was measured. As shown in Fig. 7(b), a lower increase in caspase-3-like activity was observed in detached leaves of *mpk6-2* and *mpk6-3* compared to that of WT in response to HS treatment after 6, 9 and 12 h recovery; whereas in the MPK6-OE mutant a much greater increase was detected (Fig. 7b).

MPK6 is responsible for γVPE activation under HS treatment

The data presented above indicate that MPK6 was activated by HS treatment, and a lacking mutation in MPK6 (*mpk6-2* and *mpk6-3*) resulted in the alleviated cell death and enhanced HS tolerance of Arabidopsis. Therefore, it would be interesting to know whether MPK6 was implicated in HS-initiated γVPE activation. Analysis by qRT-PCR revealed that, while γVPE transcript level was upregulated in HS-treated detached leaves of WT, there was no obvious change of γVPE mRNA in HS-treated detached leaves of *mpk6-2* and *mpk6-3* 6 and 12 h after HS

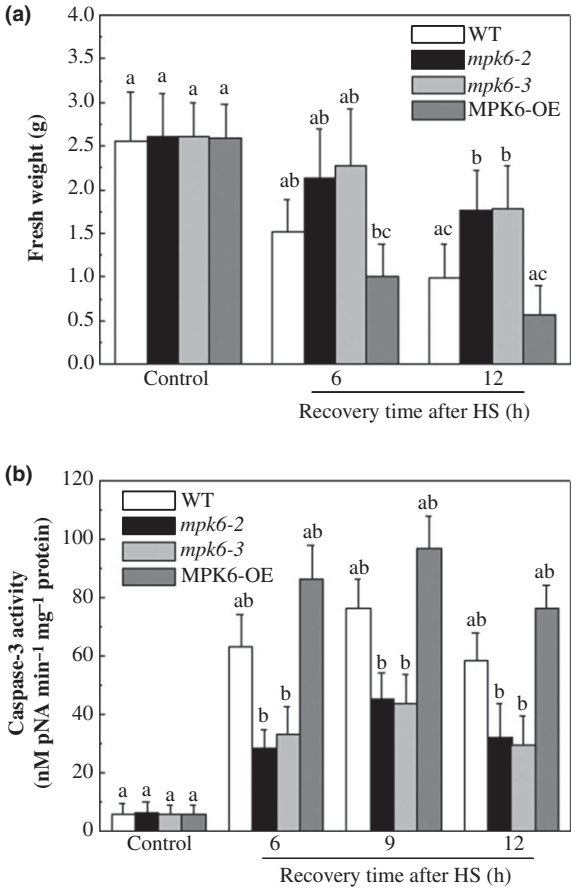

Fig. 7 Plant growth condition and caspase-3-like activation of MPK6 mutants under heat shock (HS) treatment. (a) Fresh weight of WT, *mpk6-2*, *mpk6-3* and MPK6-OE *Arabidopsis thaliana* seedlings after 6 and 12 d recovery periods after HS treatment (42°C, 2 h). (b) Caspase-3-like activity tested using an *in vitro* assay using the caspase-3 substrate in the HS-treated detached leaves of WT, *mpk6-2*, *mpk6-3* and MPK6-OE. The protein extracts from the HS-treated detached leaves at indicated times of the recovery period after HS treatment (42°C, 2 h) were used. The samples were incubated with the caspase-3 substrate Ac-DEVD-pNA (200 mM) in assay buffer containing phenylmethylsulfonyl fluoride and EDTA. Each data point is the mean ± SD of five replicates. Differences among genotypes within each treatment were evaluated by two-way ANOVA (Bonferroni posttest, $P < 0.05$) and are shown by different letters.

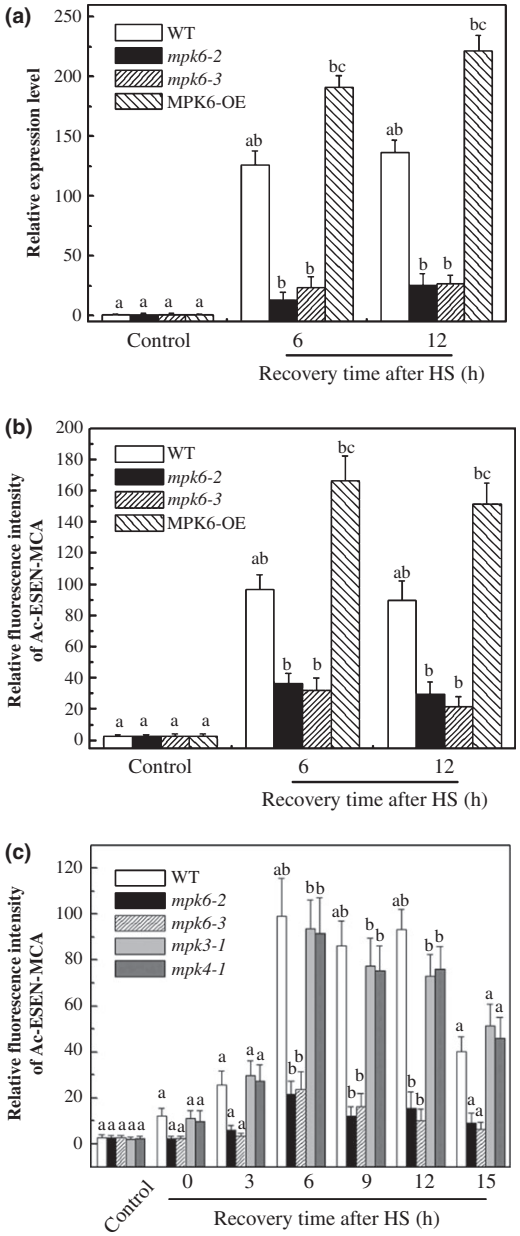

Fig. 8 Role of MPK6 in heat shock (HS)-induced vacuolar processing enzyme (VPE) activation in *Arabidopsis thaliana*. (a) qRT-PCR analysis of γVPE expression in the HS-treated detached leaves of *mpk6-2*, *mpk6-3* and MPK6-OE after HS treatment. Total RNAs were extracted from the detached leaves 6 and 12 h intothe recovery period after HS (42°C, 2 h). (b) Changes in HS-induced VPE activity in the HS-treated detached leaves of *mpk6-2*, *mpk6-3* and MPK6-OE after 6 and 12 h recovery after HS treatment. (c) Changes of HS-induced VPE activity in the HS-treated detached leaves of MPK3/4/6 mutants during 15 h recovery period after HS treatment. The proteins were extracted from the HS-treated detached leaves at indicated time of the recovery period after HS (42°C, 2 h) for the activity assay with the VPE-specific fluorescent substrate, Ac-ESEN-MCA. Each data point is the mean ± SD of five replicates. Differences among genotypes within each treatment were evaluated by two-way ANOVA (Bonferroni posttest, $P < 0.05$) and are shown by different letters.

treatment; instead, the expression level of γVPE gene in HS-treated detached leaves of MPK6-OE was much higher than that of WT (Fig. 8a). As a parallel experiment, the VPE activity in detached leaves of *mpk6-2*, *mpk6-3* and MPK6-OE under HS stress was measured. As shown in Fig. 8(b), a substantial increase in VPE activity was observed in detached leaves of WT in response to HS treatment after 6 and 12 h recovery, whereas no significant increase was observed in HS-treated detached leaves of *mpk6-2* and *mpk6-3*; further, in the MPK6-OE mutant a much greater increase was detected (Fig. 8b). For further confirmation, detached leaves of MPK3 and MPK4 mutants were treated with HS. The data showed that HS-induced increase of VPE activity was not obviously inhibited in detached leaves of MPK3-lacking mutant (*mpk3-1*) and the MPK4-lacking mutant (*mpk4-1*) compared with those of MPK6 mutants (Fig. 8c), suggesting that it was MPK6, not MPK3 and MPK4, that was indispensable for HS-induced γVPE activation.

HS-induced caspase-1-like activation in Arabidopsis

VPEs have been reported to exhibit caspase-1-like activity in tobacco and Arabidopsis (Hatsugai *et al.*, 2004; Nakaune *et al.*, 2005), and our experiment showed that pretreatment with caspase-1 inhibitor Ac-YVAD-CHO noticably alleviated the HS-induced vacuolar collapse and caspase-3-like activation (Fig. S3; Notes S2). Hence, we used caspase-1 fluorogenic substrate Ac-YVAD-pNA to measure the change of caspase-1-like (YVADase) activity in detached Arabidopsis leaves under HS stress (Fig. S4; Methods S3). As shown in Fig. S4(a), caspase-1-like activity was significantly increased in HS-treated detached WT leaves after 6–15 h recovery after HS treatment ($P < 0.05$), and the kinetics of caspase-1-like activity was similar to the change of VPE activity (Fig. 2a). In addition, no change could be detected in HS-treated detached leaves of *γvpe* (Fig. S4a). Furthermore, experiments with detached leaves of *mpk6-2*, *mpk6-3* and MPK6-OE mutants showed that MPK6 activity was required for the HS-induced upregulation of caspase-1-like activity (Fig. S4b).

Discussion

This study is an attempt to understand the possible molecular mechanisms underlying the cellular process of VPE-mediated Arabidopsis PCD induced by HS treatment. The results shown in this work provide evidence for the cellular signaling cascade of the activation and function of VPEs in Arabidopsis response to HS stress.

High temperature is one of the limiting factors for plants growth (Zuppini *et al.*, 2006; Zhang *et al.*, 2009a), and recent studies have reported that HS challenge can cause several apoptosis-like characters and trigger PCD in plant cells (Vacca *et al.*, 2006; Zuppini *et al.*, 2006; Zhang *et al.*, 2009a). In both animal and plant PCD, the caspase-like activation is believed to be the key and final step, and the activation of caspase-3-like protease, which is considered as the major executioner of animal PCD, has been detected under UV-C and aluminum stresses (Zhang *et al.*, 2009b; Li & Xing, 2011). The present study observed the caspase-3-like activation in HS-induced Arabidopsis death, indicating the vital role of caspase-3-like protease in the Arabidopsis HS response (Fig. 4).

The vacuole may be involved in the process of plant development and stress response (Hatsugai *et al.*, 2004), and ultrastructural analysis has shown that the vacuole is the organelle site most sensitive to HS treatment (Jones, 2001). In plants, a vacuole-localized cysteine protease called VPE has been identified and experimental evidence has demonstrated that VPEs exhibit caspase-1-like activity and participate in development, senescence, hypersensitive cell death and hormone signaling (Kinoshita *et al.*, 1999; Hatsugai *et al.*, 2004, 2006; Kuroyanagi *et al.*, 2005). Recent work has further reported that a cellular suicide strategy mediated by the vacuole and VPEs can regulate the development and cell death programmes in plants (Hatsugai *et al.*, 2004). In our work, it was found that HS caused γVPE activation and vacuolar disruption (Figs 2, 3), suggesting the possible involvement of γVPE-mediated vacuolar system in HS-induced Arabidopsis PCD.

In animal apoptosis, there are several kinds of caspases with different functions. Compared with caspase 3, which acts as the executioner of PCD to induce DNA fragmentation and chromatin condensation, several other caspases (such as caspase 8 and caspase 1) can function as mediators activating downstream signaling cascades of PCD (Fan *et al.*, 2005). Our data showed that γVPE might exhibit caspase-1-like activity (Fig. S4) and promote vacuolar disruption and caspase-3-like activation in Arabidopsis PCD induced by HS (Figs 1, 3, 4, S3). The vacuole contains many hydrolases and proteases which can lyse proteins and cellular compartments to regulate the process of plant senescence and death (Hatsugai *et al.*, 2004; Muntz, 2007); notably, in the vacuole-mediated PCD signaling pathway, VPEs function as the key molecules through processing these hydrolases and proteases and disrupting the vacuole (Kuroyanagi *et al.*, 2002; Hatsugai *et al.*, 2004). Hence, it was supposed that γVPE or other vacuolar enzymes released into the cytosol from the vacuole through γVPE-mediated vacuolar disruption, functioned in the activation of a downstream caspase-like pathway in HS-induced Arabidopsis PCD.

In the vacuole-mediated PCD pathway, the upstream signaling cascades of VPE activation are important components but remain unclear. MAPK cascades can be activated by various stimuli and play central roles in the process whereby extracellular stimuli are transduced into intracellular responses (Widmann *et al.*, 1999; Davis, 2000; Kyriakis & Avruch, 2001; Tena *et al.*, 2001; Zhang & Klessig, 2001; Asai *et al.*, 2002; Nakagami *et al.*, 2005). Besides their protective roles, MAPK cascades also can function as negative regulators in plant stress response. For example, MKK7 is shown to negatively regulate polar auxin transport and a mutation in MKK9 results in the enhanced stress tolerance and alleviated senescence in plants (Dai *et al.*, 2006; Alzwiya & Morris, 2007; Zhou *et al.*, 2009). Furthermore, Li *et al.* (2007) report that a 56-KD MAPK protein mediates self-incompatibility-induced PCD by activating caspase-like activity. Our experiment demonstrated that MAPK cascades also participated in the activation of γVPE in HS-induced PCD (Fig. 5). Among various kinds of MAPK proteins, MPK6 is a well-established signaling protein in Arabidopsis, which can be activated by various stimuli, including low temperature, wounding, heavy metals, drought, oxidative stress and plant hormones (Morris *et al.*, 1997; Teige *et al.*, 2004; Xing *et al.*, 2008, 2009; Wang *et al.*, 2010). In HS-treated Arabidopsis, the activation of MPK6 was proved to be responsible for the upregulation of γVPE activity and the subsequent execution of PCD (Figs 6–8).

ROS and Ca^{2+}, as important signal messengers in plant cells, can function in the upstream activation of MAPK cascade under various stimuli (Fluhr & Bowler, 2000; Romeis *et al.*, 2001; Xing *et al.*, 2008; Wang *et al.*, 2010). Under HS stress, ROS production and cytoplasmic calcium concentration ($[Ca^{2+}]_{cyt}$) increase are early events (Gong *et al.*, 1998; Zhang *et al.*, 2009a), and the Ca^{2+}-CaM3 cascade can regulate plant HS response through activating downstream signal transduction (Gong *et al.*, 1997; Xuan *et al.*, 2010). Our data presented a cellular signaling cascade, composed of ROS production, $[Ca^{2+}]_{cyt}$ increase and the upregulation of CaM3 transcript level, which functioned in the upstream activation of MPK6 in response to HS (Figs S5–S9, S11, S12; Methods S4, S5; Notes S3, S4, S6, S7).

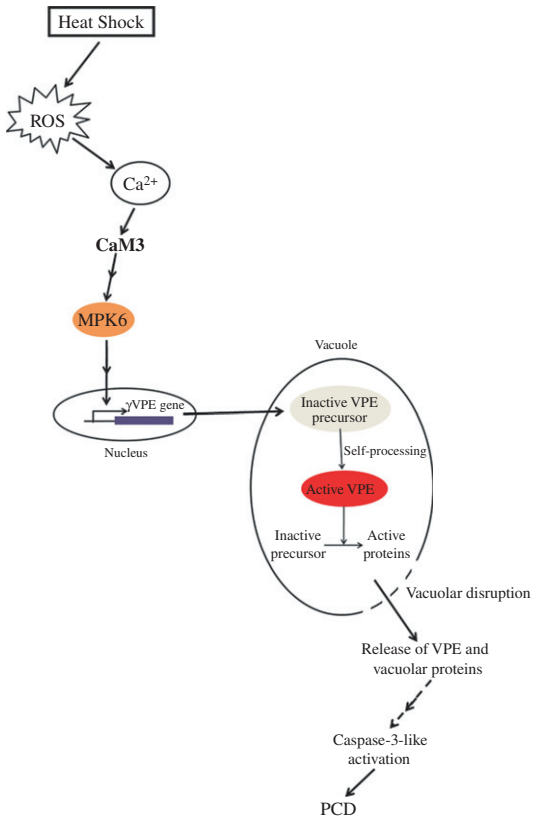

Fig. 9 Proposed working model for MPK6-dependent activation of γVPE in heat shock-induced programmed cell death (PCD). VPE, vacuolar processing enzyme.

In conclusion, our data show that MPK6-mediated activation of Arabidopsis γVPE modulated HS-induced Arabidopsis PCD. According to our experimental results, a potential cascade of cellular events during HS-induced PCD occurred (Fig. 9): HS treatment caused ROS production and increase of $[Ca^{2+}]_{cyt}$, and the Ca^{2+}-CaM3 cascade, in turn, activated MPK6 protein; then the activated MPK6 protein upregulated the transcript level of γVPE, resulting in the accumulation of inactive VPE precursors in the vacuole which can self-process into activated VPEs; subsequently, activated VPEs processed vacuolar hydrolases and proteases and disrupted the vacuole to promote the caspase-3-like activation. These results suggested a possible molecular mechanism underlying the process of HS-induced PCD, and provided a new insight into the cellular signaling cascade of plant VPEs.

Acknowledgements

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References

Almoguera C, Rojas A, Jordano J. 2002. Reversible heat-induced inactivation of chimeric *β*-glucuronidase in transgenic plants. *Plant Physiology* **129**: 333–341.

Alzwiya IA, Morris PC. 2007. A mutation in the Arabidopsis *MAP kinase kinase 9* gene results in enhanced seedling stress tolerance. *Plant Science* **173**: 302–308.

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J. 2002. MAP kinase signaling cascade in *Arabidopsis* innate immunity. *Nature* **415**: 977–983.

Bethke G, Unthan T, Uhrig JF, Poschl Y, Gust AA, Scheel D, Lee J. 2009. Flg22 regulates the release of an ethylene response factor substrate from MAP kinase 6 in *Arabidopsis thaliana* via ethylene signaling. *Proceedings of the National Academy of Sciences, USA* **106**: 8067–8072.

Chen PY, Lee KT, Chi WC, Hirt H, Chang CC, Huang HJ. 2008. Possible involvement of MAP kinase pathways in acquired metal-tolerance induced by heat in plants. *Planta* **228**: 499–509.

Cohen GM. 1997. Caspase: the executioners of apoptosis. *Biochemical Journal* **326**: 1–16.

Dai Y, Wang H, Li B, Huang J, Liu X, Zhou Y, Mou Z, Li J. 2006. Increased expression of MAP KINASE KINASE7 causes deficiency in polar auxin transport and leads to plant architectural abnormality in *Arabidopsis*. *Plant Cell* **18**: 308–320.

Danon A, Rotari VI, Gordon A, Mailhac N, Gallois P. 2004. Ultraviolet-C overexposure induces programmed cell death in *Arabidopsis*, which is mediated by caspase-like activities and which can be suppressed by caspase inhibitors, p53 and *defender against apoptotic death*. *The Journal of Biological Chemistry* **279**: 779–787.

Davis RJ. 2000. Signal transduction by the JNK group of MAP kinases. *Cell* **103**: 239–252.

Fan TJ, Han LH, Cong RS, Liang J. 2005. Caspase family proteases and apoptosis. *Acta Biochimica et Biophysica Sinica* **37**: 719–727.

Fluhr R, Bowler C. 2000. The role of calcium and activated oxygens as signals for controlling cross-tolerance. *Trends in Plant Science* **5**: 241–246.

Gong M, Chen SN, Song YQ, Li ZG. 1997. Effect of calcium and calmodulin on intrinsic heat tolerance in relation to antioxidant systems in maize seedlings. *Australian Journal of Plant Physiology* **24**: 371–379.

Gong M, Van der Luit AH, Knight MR, Trewavas AJ. 1998. *Heat-shock*-induced changes in intracellular Ca^{2+} level in tobacco seedlings in relation to thermotolerance. *Plant Physiology* **116**: 429–437.

Greenberg JT, Yao N. 2004. The role and regulation of programmed cell death in plant–pathogen interactions. *Cell Microbiology* **6**: 201–211.

Gruis DF, Schulze J, Jung R. 2004. Storage protein accumulation in the absence of the vacuolar processing enzyme family of cysteine proteases. *Plant Cell* **16**: 270–290.

Gruis DF, Selinger DA, Curran JM, Jung R. 2002. Redundant proteolytic mechanisms processed storage proteins in the absence of seed-type members of the vacuolar processing enzyme family of cysteine proteases. *Plant Cell* **14**: 2863–2882.

Hara-Nishimura I, Inoue K, Nishimura M. 1991. A unique vacuolar processing enzyme responsible for conversion of several proprotein precursors into the mature forms. *FEBS Letters* **294**: 89–93.

Hatsugai N, Kuroyanagi M, Nishimura M, Hara-Nishimura I. 2006. A cellular suicide strategy of plants: vacuole-mediated cell death. *Apoptosis* **11**: 905–911.

Hatsugai N, Kuroyanagi M, Yamada K, Meshi T, Tsuda S, Kondo M, Nishimura M, Hara-Nishimura I. 2004. A plant vacuolar protease, VPE, mediates virus-induced hypersensitive cell death. *Science* **305**: 855–858.

Ichimura K, Shinozaki K, Tena G, Sheen J, Henry Y, Champion A, Kreis M, Zhang S, Hirt H, Wilson C *et al.* 2002. Mitogen-activated protein kinase cascades in plants: a new nomenclature. *Trends in Plant Science* **7**: 301–308.

Jones AM. 2001. Programmed cell death in development and defense. *Plant Physiology* **125**: 94–97.

Kinoshita T, Yamada K, Hiraiwa N, Nishimura M, Hara-Nishimura I. 1999. Vacuolar processing enzyme is up-regulated in the lytic vacuoles of vegetative tissues during senescence and under various stressed conditions. *Plant Journal* **19**: 43–53.

Kuroyanagi M, Nishimura M, Hara-Nishimura I. 2002. Activation of Arabidopsis vacuolar processing enzyme by self-catalytic removal of an auto-inhibitory domain of the C-terminal propeptide. *Plant Cell and Physiology* **43**: 143–151.

Kuroyanagi M, Yamada K, Hatsugai N, Kondo M, Nishimura M, Hara-Nishimura I. 2005. Vacuolar processing enzyme is essential for mycotoxin-induced cell death in *Arabidopsis thaliana*. *Journal of Biological Chemistry* **280**: 32914–32920.

Kyriakis JM, Avruch J. 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiological Reviews* **81**: 807–869.

Lam E. 2004. Controlled cell death, plant survival and development. *Molecular and Cellular Biology* **5**: 305–315.

Lam E, del Pozo O. 2000. Caspase-like protease involvement in the control of plant cell death. *Plant Molecular Biology* **44**: 417–428.

Lam E, Kato N, Lawton M. 2001. Programmed cell death, mitochondria and the plant hypersensitive response. *Nature* **411**: 848–853.

Li ST, Samaj J, Franklin-Tong VE. 2007. A mitogen-activated protein kinase signals to programmed cell death induced by self-incompatibility in *Papaver* pollen. *Plant Physiology* **145**: 236–245.

Li Z, Xing D. 2011. Mechanistic study of mitochondria-dependent programmed cell death induced by aluminum phytotoxicity using fluorescence techniques. *Journal of Experimental Botany* **62**: 331–343.

Moriwaki M, Yamakawa T, Washino T, Kodama T, Igarashi Y. 1999. Delayed recovery of β-glucuronidase activity driven by an *Arabidopsis* heat shock promoter in heat-stressed transgenic *Nicotiana plumbaginifolia*. *Plant Cell Reports* **19**: 96–100.

Morris PC, Guerrier D, Leung J, Giraudat J. 1997. Cloning and characterization of MEK1, an *Arabidopsis* gene encoding a homologue of MAP kinase kinase. *Plant Molecular Biology* **35**: 1057–1064.

Müntz K. 2007. Protein dynamics and proteolysis in plant vacuoles. *Journal of Experimental Botany* **58**: 2391–2407.

Nakagami H, Pitzschke A, Hirt H. 2005. Emerging MAP kinase pathways in plant stress signaling. *Trends in Plant Science* **10**: 339–346.

Nakaune S, Yamada K, Kondo M, Kato T, Tabata S, Nishimura M, Hara-Nishimura I. 2005. A vacuolar processing enzyme, δVPE, is involved in seed coat formation at the early stage of seed development. *Plant Cell* **17**: 876–887.

Romeis T, Ludwig AA, Martin R, Jones JDG. 2001. Calcium-dependent protein kinases play an essential role in a plant defence response. *EMBO Journal* **20**: 5556–5567.

Sanmartin M, Jaroszewski L, Raikhel NV, Rojo E. 2005. Caspases: regulating death since the origin of life. *Plant Physiology* **137**: 841–847.

Shimada T, Yamada K, Kataoka M, Nakaune S, Koumoto Y, Kuroyanagi M, Tabata S, Kato T, Shinozaki K, Seki M *et al.* 2003. Vacuolar processing enzymes are essential for proper processing of seed storage proteins in *Arabidopsis thaliana*. *Journal of Biological Chemistry* **278**: 32292–32299.

Tahtiharju S, Sangwan V, Monroy AF, Dhindsa RS, Borg M. 1997. The induction of kin genes in cold–acclimating *Arabidopsis thaliana*: evidence of a role for calcium. *Planta* **203**: 442–447.

Teige M, Scheikl E, Eulgem T, Doczi R, Ichimura K, Shinozaki K, Dangl JL, Hirt H. 2004. The MKK2 pathway mediates cold and salt stress signaling in Arabidopsis. *Molecular Cell* **15**: 141–152.

Tena G, Asai T, Chiu WL, Sheen J. 2001. Plant mitogen-activated protein kinase signaling cascades. *Current Opinion in Plant Biology* **4**: 392–400.

Vacca RA, Valenti D, Bobba A, Merafina RS, Passarella S, Marra E. 2006. Cytochrome c is released in a reactive oxygen species-dependent manner and is degraded via caspase-like proteases in tobacco Bright-Yellow 2 cells en route to heat shock-induced cell death. *Plant Physiology* **141**: 208–219.

Wang PC, Du YY, Li Y, Ren DT, Song CP. 2010. Hydrogen peroxide-mediated activation of MAP Kinase 6 modulates nitric oxide biosynthesis and signal transduction in *Arabidopsis*. *Plant Cell* **22**: 2981–2998.

Widmann C, Gibson S, Jarpe MB, Johnson GL. 1999. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiological Reviews* **79**: 143–180.

Woltering EJ, Van der Bent A, Hoeberichts FA. 2002. Do plant caspases exist? *Plant Physiology* **130**: 1764–1769.

Xing Y, Jia WS, Zhang JH. 2008. AtMKK1 mediates ABA-induced CAT1 expression and H_2O_2 production via AtMPK6-coupled signaling in Arabidopsis. *Plant Journal* **54**: 440–451.

Xing Y, Jia WS, Zhang JH. 2009. AtMKK1 and AtMPK6 are involved in abscisic acid and sugar signaling in Arabidopsis seed germination. *Plant Molecular Biology* **70**: 725–736.

Xuan Y, Zhou S, Wang L, Cheng YD, Zhao LQ. 2010. Nitric oxide functions as a signal and acts upstream of AtCaM3 in thermotolerance in Arabidopsis seedlings. *Plant Physiology* **153**: 1895–1906.

Yamada K, Nishimura M, Hara-Nishimura I. 2004. The slow wound-response of VPE is regulated by endogenous salicylic acid in Arabidopsis. *Planta* **218**: 599–605.

Zhang SQ, Klessig DF. 2001. MAPK cascades in plant defense signaling. *Trends in Plant Science* **6**: 520–527.

Zhang LR, Li YS, Xing D, Gao CJ. 2009a. Characterization of mitochondrial dynamics and subcellular localization of ROS reveal that *HsfA2* alleviates oxidative damage caused by heat stress in *Arabidopsis*. *Journal of Experimental Botany* **60**: 2073–2091.

Zhang LR, Xing D. 2008. Methyl jasmonate induced production of reactive oxygen species and alterations in mitochondrial dynamics that precede photosynthetic dysfunction and subsequent cell death. *Plant Cell and Physiology* **49**: 1092–1111.

Zhang LR, Xu QX, Xing D, Gao CJ, Xiong HW. 2009b. Real-time detection of caspase-3-like protease activation *in vivo* using fluorescence resonance energy transfer during plant programmed cell death induced by ultraviolet C overexposure. *Plant Physiology* **150**: 1773–1783.

Zhou CJ, Cai ZH, Guo YF, Gan SS. 2009. An Arabidopsis mitogen-activated protein kinase cascade, MKK9-MPK6, plays a role in leaf senescence. *Plant Physiology* **150**: 167–177.

Zuppini A, Bugno V, Baldan B. 2006. Monitoring programmed cell death triggered by mild heat shock in soybean-cultured cells. *Functional Plant Biology* **33**: 617–627.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Difference in Vacuolar processing enzyme (VPE) activity of detached leaves of WT, *vpe* and *γvpe* at 6 h recovery period after heat shock (HS) treatment.

Fig. S2 Identification of *mpk3-1*, *mpk4-1*, *mpk6-2*, *mpk6-3*, MPK6-OE and *cam3* insertion mutants using semi-quantitative RT-PCR analysis.

Fig. S3 Effects of Vacuolar processing enzyme and caspase-1 inhibitors on the HS-induced vacuolar rupture and caspase-3-like activation in Arabidopsis.

Fig. S4 HS-induced activation of caspase-1-like activity in detached Arabidopsis leaves.

Fig. S5 Imaging of ROS production and cytoplasmic Ca^{2+} content in HS-treated Arabidopsis wild type protoplasts.

Fig. S6 Estimation of HS-induced changes of ROS and Ca^{2+} content by flow cytometry analysis using fluorescence probes H_2DCFDA and Fluo-3 respectively.

Fig. S7 Kinetics of changes in ROS and Ca^{2+} content by flow cytometry analysis after HS treatment.

Fig. S8 Change of CaM3 transcript level in detached Arabidopsis leaves under HS stress.

Fig. S9 Roles of Ca^{2+}-CaM3 in HS-induced activation of MPK6.

Fig. S10 Effect of HS treatment on the growth of Arabidopsis roots.

Fig. S11 Kinetics of changes in ROS level by flow cytometry analysis in *mpk6-2* and *mpk6-3* protoplasts after HS treatment.

Fig. S12 Effect of PD98059 on HS-induced MPK6 activation in detached Arabidopsis leaves.

Table S1 Quantitative analysis of vacuolar state in wild-type protoplasts after HS treatment

Table S2 Primers for several genes

Table S3 The ATG numbers for the cited genes

Methods S1 Chemical reagents.

Methods S2 Identification of Arabidopsis mutants using semi-quantitative RT-PCR.

Methods S3 Detection of caspase-1-like activity.

Methods S4 Confocal microscopy observation.

Methods S5 Flow cytometry analysis.

Methods S6 Phenotypic analysis of root growth.

Methods S7 Treatment with VPE and caspase-1 inhibitors.

Notes S1 Identification of T-DNA insertion mutants.

Notes S2 Effects of VPE and caspase-1 inhibitors on the HS-induced vacuolar rupture and PCD in Arabidopsis.

Notes S3 ROS production and cytoplasmic calcium concentration ($[Ca^{2+}]_{cyt}$) increase in HS-treated protoplasts.

Notes S4 Ca^{2+}-CaM3 cascade functions upstream of MPK6 activation under HS treatment.

Notes S5 Effect of HS treatment on the growth of Arabidopsis roots.

Notes S6 Flow cytometry analysis of the ROS production in *mpk6-2* and *mpk6-3*.

Notes S7 Effect of PD98059 on the HS-induced MPK6 activation.

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