**Research Paper**

**RBOH1-dependent H$_2$O$_2$ production and subsequent activation of MPK1/2 play an important role in acclimation-induced cross-tolerance in tomato**

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**Abstract**

H$_2$O$_2$ and mitogen-activated protein kinase (MAPK) cascades play important functions in plant stress responses, but their roles in acclimation response remain unclear. This study examined the functions of H$_2$O$_2$ and MPK1/2 in acclimation-induced cross-tolerance in tomato plants. Mild cold, paraquat, and drought as acclimation stimuli enhanced tolerance to more severe subsequent chilling, photooxidative, and drought stresses. Acclimation-induced cross-tolerance was associated with increased transcript levels of RBOH1 and stress- and defence-related genes, elevated apoplastic H$_2$O$_2$ accumulation, increased activity of NADPH oxidase and antioxidant enzymes, reduced glutathione redox state, and activation of MPK1/2 in tomato. Virus-induced gene silencing of RBOH1, MPK1, and MPK2 or MPK1/2 all compromised acclimation-induced cross-tolerance and associated stress responses. Taken together, these results strongly suggest that acclimation-induced cross-tolerance is largely attributed to RBOH1-dependent H$_2$O$_2$ production at the apoplast, which may subsequently activate MPK1/2 to induce stress responses.

**Key words:** Cross-tolerance, hydrogen peroxide, mitogen-activated protein kinase, reactive oxygen species, Respiratory burst oxidase homologue 1, signal transduction, Solanum lycopersicum.

**Introduction**

Plants are often exposed to various unfavourable environmental stresses (i.e. extreme temperatures, drought, salt, fungi, bacteria, and herbicides) throughout their life cycles. To survive against stresses, plants have intricate defence mechanisms to increase their tolerance. Acclimation is the process in which an individual organism adjusts to a gradual change in its environment (such as a change in temperature, humidity, or photoperiod), allowing it to maintain performance across a range of environmental conditions. Recent studies have also revealed that there exists a kind of adaptation mechanism called cross-tolerance, whereby plants tolerant to one stress are often tolerant to a range of other stresses (Pastori and Foyer, 2002; Capiati et al., 2006; Suzuki et al., 2012). Increased anoxia tolerance was reported in Arabidopsis plants in response to heat (Banti et al., 2008), while NaCl and wounding induced resistance to UV-B in barley and salt tolerance in tomato plants (Capiati et al., 2006; Carkirlar et al., 2008). While the capacity to acclimate to novel environments has been well documented in different plant species, very little is still known about the in-depth mechanisms of such acclimation in plants.

Cold acclimation has been most studied in terms of the physiological and molecular mechanisms in plants. At the metabolic level, acclimation induced accumulation of osmolytes,
cryoprotectants, and abscisic acid (ABA) and production of compatible solutes (e.g. proline, raffinose, and glycine betaine) to stresses such as low non-freezing temperatures and moderate light (Browse and Xin, 2001). Acclimation is also able to stabilize proteins and cellular structures and to maintain cell turgy by osmotic adjustment and cellular redox balance (Janska et al., 2010). At the molecular level, secondary messengers such as cytosolic Ca2+, nitric oxide (NO), ABA, and reactive oxygen species (ROS) such as hydrogen peroxide (H2O2) are found to be involved in the perception and transduction of low temperature signal to trigger cold acclimation-induced changes in physiological processes (Zhao et al., 2009; Janska et al., 2010; Zhou et al., 2012). Foliar application of these chemicals increased the tolerance to an array of stresses such as drought, salt, and extreme temperature. For example, H2O2 enhanced the transcription of a subset of stress-responsive genes and the antioxidant capacity of cells by increasing the activities of antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR), and the biosynthesis of non-enzymic antioxidants such as ascorbic acid and glutathione with an increase in the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) (Thannickal and Fanburg, 2000; Jiang et al., 2012). Maintaining the redox homeostasis is a prerequisite for the development of tolerance against both biotic and abiotic stresses (Foyer et al., 1997; Mou et al., 2003; Jiang et al., 2012). ROS, especially H2O2 generated by NADPH oxidases encoded by Respiratory Burst Oxidase Homologue (RBOH) genes play important roles in plant responses to biotic and abiotic stresses (Torres et al., 2002; Kwak et al., 2003; Yoshioka et al., 2003; Torres and Dangl, 2005; Marino et al., 2012). In Arabidopsis, there are increased transcript levels of rbohD and rbohE and ROS accumulation in response to infection with virulent Pseudomonas syringae pv. tomato DC3000, and these responses were greatly compromised in rbohD and rbohE mutants (Torres et al., 2002; Kwak et al., 2003). Similarly, silencing RBOH4 and RBOH5 in Nicotiana benthamiana plants reduced ROS production and compromised resistance to Phytophthora infestans (Yoshioka et al., 2003). Meanwhile, ROS, NO, cytosolic Ca2+, and plant hormones such as ABA and brassinosteroids (BRs) cross-talk in stress responses (Dempsey and Klessig, 1995; Desikan et al., 2004; Wendehenne et al., 2004; Xia et al., 2009; Cui et al., 2011). For example, H2O2 cooperates with NO in plant HR/cell death and abiotic stresses (Wendehenne et al., 2004; Cui et al., 2011) and plays a critical role in BR-induced stress tolerance (Xia et al., 2009). Expression of RBOHs is also regulated by plant hormones such as ABA and BRs. Elevation of ABA and BR levels resulted in increased production of H2O2 via RBOHs together with increased tolerance against a subset of stresses (Xia et al., 2009; Zhang et al., 2009). A major contributor to induced ROS production for RBOHs may act as converging regulators in the orchestration of plant adaptation to environmental stresses (Marino et al., 2012). However, there has been no genetic evidence to show that RBOHs are involved in acclimation-induced cross-tolerance.

The mitogen-activated protein kinase (MAPK) cascade, minimally composed of a MAPK kinase, MAPK kinase, and a MAPK, is one of the major pathways by which extracellular stimuli are transduced into intracellular signals in plant stress responses (Tena et al., 2001; Zhang and Klessig, 2001). For example, wounding induced increased activation of MAPKs and systemic response to insect attack in tomato leaves (Stratmann and Ryan, 1997). For its involvement in plant signal transduction in response to biotic and abiotic stresses, MAPK signalling also interacts with ROS, NO, and ABA signalling pathways (Lu et al., 2002; Samuel and Ellis, 2002; Mittler et al., 2004; Pitzschke et al., 2009). BR- and ABA-induced apoplastic H2O2 could activate MAPKs in plants, leading to enhanced antioxidant defence system in leaves of tomato and maize (Lin et al., 2009; Nie et al., 2013). On the other hand, NO and NADPH oxidase-dependent oxidative bursts are also regulated by MAPK signals in N. benthamiana and tomato plants (Yoshioka et al., 2003; Asai et al., 2008; Nie et al., 2013). It is, therefore, quite plausible that the MAPK cascade is also involved in acclimation-induced cross-responses to multiple stresses.

Previously, Zhou et al. (2012) reported the potential role of H2O2 elevation in cold acclimation in tomato plants. To obtain insights into the signalling events in acclimation-induced abiotic stress cross-tolerance in tomato, the current study investigated whether pretreatment of one type of mild stress induces acclimation to multiple types of subsequent, more severe, stress treatments and if so, how the cross-acclimation is related to apoplastic H2O2 accumulation and expression of RBOH1, the tomato homologue of AtRbohf and NrRbohA, which play a critical role in both stress and adaptation responses in Arabidopsis and tobacco, respectively (Kwak et al., 2003; Yoshioka et al., 2003). This work also examined how cross-acclimation and the associated stress response were affected by silencing of RBOHI and MPK1 and 2. The results have provided strong evidence that apoplastic H2O2 and RBOHI-mediated MPK1/2 activation plays a critical role in induction of cross-acclimation to abiotic stresses in tomato plants.

**Materials and methods**

**Plant materials, virus-induced gene silencing constructs, and Agrobacterium-mediated virus infection**

**Tomato** (*Solanum lycopersicum* L. cv. Condine Red) seeds were germinated in a growth medium filled with a mixture of peat and vermiculite (7:3, v/v) in trays in a growth chamber. When the first true leaf was fully expanded, seedlings were transplanted into plastic pots (15 cm diameter and 15 cm depth, one seedling per pot) containing the same medium and were watered daily with Hoagland's nutrient solution. The growth conditions were as follows: a 14/10 light/dark cycle, 25/20 °C, and photosynthetic photon flux density (PPFD) 600 μmol m−2 s−1.

The tobacco rattle virus (pTRV) virus-induced gene silencing (VIGS) constructs used for silencing of tomato RBOHI genes was generated by cloning a 311-bp RBOHI cDNA fragment, which was amplified using the forward (5′-ATACCGGAGCTCAAGAATTGTTGGTTGATTTG-3′) and reverse (5′-ATACCCGGGTACCTGGTATTACCTT-3′) primers according to Liu et al. (2002). The amplified fragment was digested with SacI and XhoI and ligated into the same sites of pTRV2. The resulting plasmid was transformed into Agrobacterium.
Cladophora glomerata were exposed to 4 °C and 400 μmol m⁻² s⁻¹ PPFD for 30 d before they were used. Leaflets in the middle of the fifth fully expanded leaves, which showed 20–30% transcript levels of control plants, were used. The expression of MPK1 and MPK2 in pTRV-MPK1, pTRV-MPK2, and pTRV-MPK1/2 plants is shown in Supplementary Fig. S1A (available at JXB online). Each replicate had 12 plants.

Acclimations and tolerance analysis

To investigate acclimation-induced cross-tolerance to abiotic stresses, tomato seedlings at the five-leaf stage were transferred to a growth chamber for pretreatment with cold (8 °C, 400 μmol m⁻² s⁻¹ PPFD, 2 d), or drought (in growth medium with 20% moisture, 3 d), paraquat (PQ; 10 μg/ml, 2012). Chlorophyll fluorescence was measured using an Imaging-PAM Chlorophyll Fluorometer equipped with a computer-operated PAM-control unit (IMAG-MAXI, Heinz Walz, Effeltrich, Germany). Seedlings were kept in the dark for approximately 30 min before measurements were taken. The intensities of the actinic light saturation and saturating light settings were 280 μmol mol⁻¹ s⁻¹ and 2500 μmol mol⁻² s⁻¹ photosynthetically active radiation, respectively. The maximum quantum yield of PSII (Fv/Fm) was measured and calculated as previously described (Zhou et al., 2012).

Analysis of H₂O₂

Histochemical staining of H₂O₂ was performed as previously described (Thordal-Christensen et al., 1997), with minor modifications as described previously (Xia et al., 2009). Leaf discs were vacuum infiltrated with 1 mg ml⁻¹ 3,3'-diaminobenzidine (DAB) in 50 mM TRIS-acetate buffer (pH 3.8) and incubated at 25 °C in the dark for 6 h. Leaf discs were rinsed in 80% (v/v) ethanol for 10 min at 70 °C and mounted in lactic acid/phenol/water (1:1:1, v/v/v), and H₂O₂ accumulation was detected by an Olympus motorized system microscope (BX61, Olympus, Tokyo, Japan). H₂O₂ was visualized at the subcellular level using CeCl₃ for localization (Zhou et al., 2012). Sections were examined using a transmission electron microscope (H7650, Hitachi, Tokyo, Japan) at an accelerating voltage of 70 kV. Electron-dense CeCl₃ deposits which are formed in the presence of H₂O₂ are visible by transmission electron microscopy (Bestwick et al., 1997). H₂O₂ in leaf tissue was extracted and analysed as previously described (Willekens et al., 1997).

Antioxidant assays

For antioxidant enzyme assays, leaf tissues (0.3 g) were ground with 2 ml ice-cold buffer containing 50 mM PBS (pH 7.8), 0.2 mM EDTA, 2 mM AsA, and 2% (w/v) polyvinylpyrrolidone. Homogenates were centrifuged at 12 000 g for 20 min, and the resulting supernatants were used for the determination of enzyme activity. All steps were performed at 4 °C. An aliquot of the extract was used to determine the protein content, following the method as previously described (Bradford, 1976), using bovine serum albumin as a standard. The activity of CAT, APX, SOD, and GR were measured following the protocol used as previously described (Xia et al., 2009). All spectrophotometric analyses were conducted on a SHIMADZU UV-2100PC spectrophotometer (Shimadzu, Kyoto, Japan). For the measurement of GSH and GSSG, plant leaf tissue (0.3 g) was homogenized in 2 ml of 2% metaphosphoric acid containing 2 mM EDTA and centrifuged at 4 °C for 10 min at 14 000 g. Total and oxidized glutathione (GSH+GSSG and GSSG, respectively) levels were determined as previously described (Griffith, 1980).

Isolation of plasma membranes and determination of NADPH oxidase activity

Leaf plasma membranes were isolated using a two-phase aqueous polymer partition system (Xia et al., 2009). The NADPH-dependent O₂-generating activity in isolated plasma membrane vesicles was determined by following the protocol used as previously described (Zhou et al., 2012).

Total RNA extraction and gene expression analysis

Total RNA was isolated from tomato leaves using Trizol reagent (Sangon, China), according to the manufacturer’s recommendations. Genomic DNA was removed using a RNAasy Mini Kit (Qiagen, Germany). Total RNA (1 μg) was reverse transcribed using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan), following the manufacturer’s instructions. Gene-specific quantitative real-time PCR (qRT-PCR) primers were designed based on their cDNA sequences and are listed in Supplemental Table S1. These 10 genes encode MAPKs, defence-regulatory or -related proteins and antioxidant enzymes: MPK1 (encoding mitogen-activated protein kinase 1), MPK2 (encoding mitogen-activated protein kinase 2), NPR1 (encoding non-expressor of PR 1), NPR1.1 (encoding non-expressor of PR 1.1), PRI (encoding pathogen-las-related 1), Fe-SOD (encoding Fe-SOD), Cu/Zn-SOD (encoding Cu/Zn-SOD), catP (encoding cytosolic ascorbate peroxidase), CAT1 (encoding catalase 1), and GRI (encoding glutathione reductase 1). qRT-PCR was performed using the iCycleri Q real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Each reaction (25 μl) consisted of 12.5 μl SYBR Green PCR Master Mix (Takara, Chiga, Japan), 1 μl diluted cDNA, and 0.1 μmol forward and reverse primers. The cycling conditions were as follows: 95 °C for 3 min, and 40 cycles of 95 °C for 10 s and 58 °C for 45 s. Tomato Actin was used as an internal control. Relative gene expression was calculated as previously described (Livak and Schmittgen, 2001).

MPK1 and 2 activation assay

Tomato leaves were collected after cross-acclimation, ground in liquid nitrogen, and homogenized in an extraction buffer (100 mM HEPES pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na₃VO₄, 10 mM NaF, 50 mM β-glycerophosphate, 1 mM phenylmethylsulphonyl fluoride, 5 μg/ml antipain, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 10% glycerol, and 7.5% polyvinylpyrrolidone). Homogenates were centrifuged at 16 000 g, extracted proteins were separated by SDS-PAGE, and MPK1 and MPK2 activation was detected by protein blotting using phospho-p44/42 MAPK (ERK1/2, Thr202/Tyr204) monoclonal antibody (Cell Signalling Technology, Danvers, MA, USA; Faulkner et al., 2013; Nie et al., 2013). ERK1/2 could specifically detect activated MPK1/2 with a molecular mass of 46 kD in tomato plants (Nie et al., 2013).

Statistical analysis

A completely randomized block design with four blocks was applied in each experiment with 10 plants as a replicate. Measurements were replicated four times and randomly arranged in each block. An
analysis of variance was carried out according to the general linear model procedure of statistical analysis system (SAS). Differences between treatment means were separated by the Tukey’s test at $P < 0.05$.

**Results**

**Acclimation-induced cross-tolerance is associated with increased H$_2$O$_2$ accumulation at the apoplast**

To determine whether cold (CA), drought (DA), and paraquat (PA) acclimation could induce cross-acclimation in tomato, tomato plants were first unacclimated or acclimated to cold at 8 °C, 10 $\mu$M PQ, or drought soil moisture at 20% and then exposed to chilling (4 °C) for 3 d, high concentration of PQ (50 $\mu$M) for 1 d, and extreme drought (moisture less than 15%) for 3 d. Light-saturated Asat decreased 19–28% while the maximal quantum efficiency of PSII ($F_v/F_m$) decreased slightly after these acclimations (Fig. 1). However, Asat of unacclimated plants were decreased by 80, 56, and 72% while $F_v/F_m$ were reduced 45, 32, and 29% after exposure to chilling, PQ, and extreme drought stresses, respectively (Fig. 1). Importantly, CA, PA, and DA all significantly alleviated the stress-induced decreases in Asat and $F_v/F_m$, as indicated from their increases of 28–136% for Asat and 18–29% for $F_v/F_m$ as compared to unacclimated plants (Fig. 1). All these results suggested that these acclimations could induce cross-tolerance in tomato plants.

A previous study (Zhou et al., 2012) found that cold acclimation induced H$_2$O$_2$ accumulation in tomato leaves. To determine whether acclimations other than cold acclimation could also induce H$_2$O$_2$ accumulation at the apoplast by triggering NADPH oxidase activity, this study analysed $RBOH1$ expression, NADPH oxidase activity, and H$_2$O$_2$ accumulation in CA, PA, and DA plants. As shown in Fig. 2, the three types of acclimation all resulted in significant increases in H$_2$O$_2$ accumulation, and the increase was accompanied by significant increases in both $RBOH1$ expression and plasma membrane NADPH oxidase activity. $RBOH1$ expression increased by 1.1-fold, 0.9-fold, and 2.5-fold after CA, PA, and DA, respectively. Similarly, NADPH oxidase activity increased by 96, 66, and 81%, and H$_2$O$_2$ concentration increased by 1.3-fold, 98%, and 86% after CA, PA, and DA, respectively (Fig. 2A–C).

The in situ detection of H$_2$O$_2$ using DAB staining revealed increased H$_2$O$_2$ accumulation in acclimated leaves, and this was especially apparent in CA plants (Fig. 2D). Using a CeCl$_3$-based procedure, it was found that all these acclimations induced H$_2$O$_2$ accumulation in the cell walls of mesophyll cells that face intercellular spaces (Fig. 2E). These results suggest a potential role of NADPH oxidases in these acclimated plants.

The role of $RBOH1$ in acclimation-induced cross-tolerance

To determine whether acclimation-induced H$_2$O$_2$ accumulation at the apoplast was associated with cross-tolerance, this study compared tolerance against chilling, PQ, and drought stresses in pTRV plants and in $RBOH1$-silenced plants (pTRV-$RBOH1$). pTRV-$RBOH1$ plants showed similar Asat and $F_v/F_m$ values to those of pTRV plants when they were grown under clement environments (Fig. 3). Chilling, PQ, and drought stresses all resulted in significant decreases in Asat and $F_v/F_m$ in pTRV plants and pTRV-$RBOH1$ plants. Meanwhile, pTRV-$RBOH1$ plants wilted earlier than pTRV plants after exposure to chilling and drought stress and showed more severe leaf bleaching after PQ stress. Importantly, decreases in Asat and $F_v/F_m$ were largely alleviated by CA, PA, and DA in pTRV plants but not in pTRV-$RBOH1$ plants. Accordingly, $RBOH1$ functioned as an important role in acclimation-induced cross-tolerance in tomato plants.

$RBOH1$ transcript levels, NADPH oxidase activity, and H$_2$O$_2$ accumulation were significantly lower in pTRV-$RBOH1$ plants as compared to pTRV plants under normal conditions.
H2O2 and MAPKs in stress tolerance

Similarly to that observed in normal plants, CA, PA, and DA resulted in significant increases in \( RBOH1 \) transcript levels, NADPH oxidase activity, and H\(_2\)O\(_2\) accumulation in the leaves of pTRV plants. Such increases in \( RBOH1 \) transcript levels, NADPH oxidase activity, and H\(_2\)O\(_2\) accumulation, however, were not observed in pTRV-\( RBOH1 \) plants after CA, PA, and DA (Fig. 4, Supplementary Fig. S2), suggesting that \( RBOH1 \) is necessary for CA-, PA-, and DA-induced increases in NADPH oxidase activity and H\(_2\)O\(_2\) content.

To get insight into the mechanism for cross-acclimation-induced H\(_2\)O\(_2\) accumulation and enhanced stress tolerance, this study examined changes in the transcript levels of 10 stress-responsive and defence-related genes in VIGS plants in response to different acclimation and chilling stresses (Fig. 5). These genes analysed are \( MPK1 \), \( MPK2 \), \( NPR1 \), \( NPR1.1 \), \( PRI \), \( Fe-SOD \), \( Cu/Zn-SOD \), \( cAPX \), \( CAT1 \), and \( GR1 \). Silencing of \( RBOH1 \) resulted in significant decreases in the transcript levels of all these genes (Fig. 5). In contrast, all acclimation treatments induced increased transcript levels of these genes, ranging from 2- to 8-fold in pTRV plants. Importantly, silencing of \( RBOH1 \) compromised CA-, PA-, DA-, and chilling-induced upregulation of all 10 genes (Fig. 5). Similarly, chilling also induced transcription of these stress-responsive and defence-related genes, and the increases were more significant in PA plants (Fig. 5). However, PA- and chilling-induced transcription was again compromised in pTRV-\( RBOH1 \) plants.

This study then analysed changes in the activities of antioxidant enzymes (SOD, APX, CAT, and GR) and glutathione levels (GSH and GSSG) in pTRV and pTRV-\( RBOH1 \) plants after different acclimation and chilling treatments. Similarly to the changes in their transcript levels, silencing of \( RBOH1 \) resulted in significant decreases in the activities of all these antioxidant enzymes (Fig. 6A). On the other hand, silencing of \( RBOH1 \) resulted in significant decreases in GSH content and increased GSSG content, leading to a substantial reduction in both overall GSH+GSSG content and GSH/GSSG.
Ratio (Fig. 6A). In contrast, chilling induced increased GSH and GSSG accumulation and decreased GSH/GSSG ratios. Interestingly, CA, PA, and DA all significantly induced GSH accumulation and reduced GSSG content in pTRV plants, causing sharp increases in total GSH+GSSG and GSH/GSSG ratio (Fig. 6A). Importantly, silencing of RBOH1 abolished acclimation- and chill-induced increases in GSH content but busted GSSG accumulation, leading to significant decreases in GSH/GSSG ratios (Fig. 6A).

In agreement with the increase in transcript levels of MPK1 and MPK2, MPK1/2 activation was also induced in pTRV plants after CA, PA, and DA. Silencing of RBOH1 led to a 70% reduction in MPK1/2 activation when compared to that of pTRV plants. Significantly, CA, PA, and DA treatments all failed to induce MPK1/2 activation in pTRV-RBOH1 plants (Fig. 6B), suggesting that H2O2 at the apoplast is essential for activation of MPK1/2.

Role of MAPK1 and 2 activation in acclimation-induced cross-tolerance

To determine the role of MPK1 and 2 in H2O2-mediated cross-tolerance, this study compared PQ-induced tolerance in plants silenced with MPK1 (pTRV-MPK1), MPK2 (pTRV-MPK2), and MPK1/2 co-silenced (pTRV-MPK1/2) plants with that of pTRV plants for their differences in Asat and Fv/Fm after chilling at 4 °C. pTRV-MPK1, pTRV-MPK2, and pTRV-MPK1/2 plants grew weaker than pTRV plants, and Asat values of those plants were also lower than that of pTRV plants, but Fv/Fm values were similar to that of pTRV

Fig. 3. Changes in light-saturated CO2 assimilation rate (Asat) and maximum quantum yield of PSII (Fv/Fm) in response to chilling (4 °C, 3 d), paraquat (50 μM, 1 d), and drought (<15% moisture, 3d) in RBOH1-silenced plants after cross-acclimation pretreatment. Leaflets in the middle fifth leaf were used. Data are mean±SD of four biological replicates. Different letters above the bars indicate values that are significantly different (P < 0.05) according to Tukey’s test. —, no acclimation; CA, cold acclimation; PA, paraquat acclimation; DA, drought acclimation.

Fig. 4. Changes in RBOH1 transcript levels (A), NADPH oxidase activity (B), and H2O2 accumulation (C) in RBOH1-silenced tomato plants after acclimation to cold (8 °C, 3 d), paraquat (10 μM, 2 d), or drought (<15% moisture, 3 d). Leaflets in the middle fifth leaf were used. Data are mean±SD of four biological replicates. Different letters above the bars indicate values that are significantly different (P < 0.05) according to Tukey’s test. Control, no acclimation; CA, cold acclimation; PA, paraquat acclimation; DA, drought acclimation; FW, fresh weight.
plants when they were grown under the normal environment (Fig. 7A, Supplementary S1B). However, pTRV-MPK1, pTRV-MPK2, and pTRV-MPK1/2 plants showed lower Asat and $F_v/F_m$ values than pTRV plants after exposure to a chilling at 4 °C for 3 d. Importantly, silencing of $MPK1$, $MPK2$, and $MPK1/2$ all compromised PA-induced chilling tolerance, as indicated by decreases in both Asat and $F_v/F_m$.

This study then examined the transcript levels of antioxidant-related genes in PQ-acclimated pTRV-MPK1, pTRV-MPK2, and pTRV-MPK1/2 plants after chilling. Silencing of $MPK1$ and $MPK2$, or $MPK1/2$ all resulted in decreased transcript levels of Cu/Zn-SOD, cAPX, GR1, and CAT1 in the normal environment, and blocked CA- and PA-induced increases in the transcript levels (Fig. 7B). Furthermore,
cosilencing of MPK1 and 2 resulted in substantially lower transcript levels of these genes than silencing of either MPK1 or MPK2 (Fig. 7B). All these results suggested that MPK1 and MPK2 are necessary components for acclimation-induced stress tolerance.

Discussion

There have been many reports about acclimation-induced stress tolerance in plants. For example, wounding acclimation enhanced salt tolerance in tomato while heat and salt
acclimation increased stress response against anoxia in Arabidopsis and UV in barley (Capiati et al., 2006; Banti et al., 2008; Carkirlar et al., 2008). The current study found that cold acclimation not only increased tolerance to chilling but also to drought and photooxidative stress, and it is also true for other acclimation, suggesting that pretreatment of different types of mild abiotic stresses could induce a common effect on tolerance to a spectrum of stresses.

This work found that RBOH1-dependent H$_2$O$_2$ production plays a critical role in acclimation-induced stress tolerance in tomato plants. ROS, especially H$_2$O$_2$, produced at the apoplast play an indispensable role in signal recognition and transduction in plant growth, development, and stress response. Hormones such as ABA and BRs could trigger apoplastic H$_2$O$_2$ generation while exogenous H$_2$O$_2$ significantly increases tolerance against chilling, paraquat, and high light in plants such as potato and cucumber (Wu et al., 1995; Kwak et al., 2003; Xia et al., 2009). Meanwhile, cold-acclimation-induced cold tolerance is associated with increased H$_2$O$_2$ accumulation, and loss of function of RBOHs resulted in reduced tolerance against biotic and abiotic stresses (Dat et al., 2003; Zhou et al., 2012). The current study found that not only CA but also DA and PA induced RBOH1 transcription, NADPH oxidase activity, and H$_2$O$_2$ accumulation at the apoplast. In tomato, RBOH1 is involved in responsiveness to wounding (Sagi et al., 2004). The current study found that silencing of RBOH1 compromised acclimation-induced tolerance, transcription of stress-responsive and defence-related genes, and activation of MPK1/2. Taken together, these findings indicate that H$_2$O$_2$ is a universal signalling molecule in acclimation and that RBOH1-dependent H$_2$O$_2$ generation plays a critical role in acclimation-induced cross-tolerance in tomato plants.

The responses of plants to acclimation and stress are frequently associated with changes in the cellular redox state (Foyer and Noctor, 2005). The current study found that acclimation leads to increases in both the activity of antioxidant...
enzymes and GSH accumulation and, ultimately, to an increase in the GSH/GSSG ratio while silencing of RBOH1 abolished induction of such changes (Fig. 6). H2O2 could influence the expression of stress-responsive and defense-related genes and the activity of antioxidant enzymes, GSH biosynthesis, and defense responses in plants (Desikan et al., 2001; Vandenabeele et al., 2003; Xie et al., 2009). In agreement with these studies, these acclimations induced significant changes in the antioxidant-related gene transcript levels, enzyme activities, and redox homeostasis, and this effect was highly dependent on accumulation of RBOH1-induced H2O2 after acclimation in this study (Figs. 5 and 6). Importantly, acclimated plants exhibited higher GSH/GSSG ratios than those of unacclimated plants during acclimation and chilling. Glutathione homeostasis could influence plant metabolism and stress response by modifying the activity of redox-sensitive enzymes such as Rubisco activase through reduction/oxidation of disulphide bridges/sulphydryl groups and glutathionylation of sulphydryl groups (Szalai et al., 2009; Jiang et al., 2012). Accordingly, acclimation-induced changes in glutathione homeostasis may partially contribute to increased CO2 assimilation in acclimated plants (Fig. 6A). There is also evidence that the glutathione redox state is involved in the regulation of the transcription and stability of defence-related genes and proteins (Baena-Gonzalez and Aro, 2002; Mou et al., 2003). Interestingly, there were significant increases in the transcript levels of NPR1 and PRI, which are essential regulators for the onset of systemic acquired resistance in acclimated plants, and the increases were again abolished in RBOH1-silenced plants (Fig. 5). Recently, this study group reported that BRs enhance tolerance against chilling/PQ stresses and CO2 assimilation by H2O2-dependent change of the glutathione redox state (Xia et al., 2009; Jiang et al., 2012). Therefore, acclimation-induced stress tolerance appears to involve a conserved stress-responsive and defense-related mechanism that is activated by the RBOHs-H2O2-GSH/GSSG-dependent signalling pathway. It will be of great interest to study whether these acclimations could induce resistance against biotic stress.

MAPK cascades are known to mediate the transduction of environmental and developmental signals into intracellular responses (Mizoguchi et al., 1996; Teige et al., 2004). Tomato MPK1/2 are orthologues of Arabidopsis MPK6, which is involved in plant responses to pathogens (Menke et al., 2004; Schikora et al., 2011). Several studies revealed that MPK1/2 function in host-specific Avr Pto-dependent resistance to the bacterial pathogen P. syringae (Ekenren et al., 2003; Pedley and Martin, 2004) and in Mi-1-mediated resistance to aphids and herbivorous insects in tomato plants (Li et al., 2006; Kandoth et al., 2007). The current study observed that pTRV-MPK1, pTRV-MPK2, and pTRV-MPK1/2 plants showed decreased tolerance against chilling, extending an earlier observation that SIMPK1 and SIMPK2 are not only involved in plant pest resistance but also in plant tolerance to abiotic stresses (Nie et al., 2013). Significantly, all acclimations increased MPK1/2 activation while silencing of MPK1 and MPK2 abolished PA-induced transcription in antioxidant genes and chilling tolerance, as observed in BR-induced stress response and MPK1/2 activation (Fig. 7; Nie et al., 2013). All these results indicate that MPK1/2 play an important role in acclimation-induced stress response.

Studies have revealed that there is an interesting relationship between NADPH oxidase-produced ROS and MAPK activation in plants exposed to various stresses or stimuli (Yoshioka et al., 2003; Pitzschke and Hirt, 2006; Nie et al., 2013). Several plant hormones such as ABA and BRs as well as ABA- and BR-induced H2O2 are known to activate MAPKs, which are involved in ABA- and BR-induced antioxidant defence responses (Zhang et al., 2006; Lin et al., 2009). There are also evidences that MAPKs are involved in regulation of RBOHs in plants (Yoshioka et al., 2003; Asai et al., 2008; Zhang et al., 2010). The current study found that acclimation induced H2O2 accumulation and activation of MPK1/2, while silencing of RBOH1 resulted in reduced MPK1/2 activation and abolished acclimation-induced activation of MPK1/2, suggesting that NADPH oxidase-produced H2O2 regulated MPK1/2 activation during acclimation (Fig. 6B). Very recently, (Nie et al., 2013) found that there exists a positive feedback circuit between RBOH1 and MPK1/2 in BRs signalling cascade. It is, therefore, plausible that H2O2-activated MAPKs are also important in maintaining H2O2 generation by NADPH oxidase during acclimation.

Cross-tolerance could be generated by signalling cross-talk by means of shared components that interrelate the signalling cascades triggered by each type of stress and, as a result, one type of stress can activate responses that lead to tolerance to other types of stresses (Capiati et al., 2006). It is not surprising that other signalling molecules such as NO and Ca2+ could also be involved in acclimation and cross-tolerance. In tomato, CDPK1, a Ca2+-dependent protein kinase, participates in responses to wounding and salt stress (Capiati et al., 2006). More recently, it has been found that nitrate reductase-dependent NO generation participates in cold-induced chilling tolerance (Zhao et al., 2009). ROS such as H2O2 are also involved in regulation of the generation or the levels of these signals, which may contribute to the critical role of ROS in acclimation and subsequent cross-tolerance.

In conclusion, this work demonstrated that mild cold, PQ, or drought pretreatment can result in enhanced tolerance to multiple abiotic stresses by triggering a significant increase in endogenous H2O2 level at the apoplast, which is associated with upregulation of RBOH1 transcription. The elevated H2O2 induced transcription of a subset of stress- and defence-related genes, activity of antioxidant enzymes, reduced cellular redox status, and activation of MPK1/2. Silencing of RBOH1 and MPK1/2 both compromised acclimation-induced stress tolerance and associated changes in gene transcription. All these findings support the involvement of RBOH1-dependent activation of MPK1/2 in acclimation-induced cross-tolerance in plants.

Supplementary material

Supplementary data are available at JXB online.

Supplementary Fig. S1. Relative mRNA abundance of MPK1 and MPK2 and phenotypes in VIGS plants.
Supplementary Fig. S2. Cross-acclimation-induced ROS accumulation in pTRV and pTRV-RBOH1 plants. Supplementary Table S1. Primers for qRT-PCR.

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