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A Role of Hexokinases in Plant Resistance to Oxidative Stress and Pathogen Infection

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Previously, we reported that mitochondria-associated hexokinases are active in controlling programmed cell death in plants (Plant Cell 18, 2341-2355). Here, we investigated their role under abiotic- and biotic-stress conditions. Expression of *NbHxk1,* a *Nicotiana benthamiana* hexokinase gene, was stimulated by treatment with salicylic acid or methyl viologen (MV), and was also up-regulated by pathogen infection. In response to MV-induced oxidative stress, *NbHxk1*-silenced plants exhibited increased susceptibility, while the *HXK1*- and *HXK2*-overexpressing *Arabidopsis* plants had enhanced tolerance. Moreover, those overexpressing plants showed greater resistance to the necrotrophic fungal pathogen *Alternaria brassicicola*. *HXK*-overexpression also mildly protected plants against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000, a response that was accompanied by increased H_2O_2 production and elevated *PR1* gene expression. These results demonstrate that higher levels of hexokinase confer improved resistance to MV-induced oxidative stress and pathogen infection.

Keywords: ion leakage/ fungus infection/ hexokinase overexpression/ methyl viologen/ virus-induced gene silencing

Plants are often exposed to severe environmental conditions that adversely affect their growth and development. Reactive oxygen species (ROS) are major damaging agents that commonly form in response to high light intensity, drought, air pollutants (e.g., ozone or sulfur dioxide), UV, pathogen infection, and herbicides such as paraquat (Baker and Orlandi, 1995; Alscher et al., 1997; Park and Paek, 2006; Pitzschke et al., 2006). Even under normal conditions, higher plants produce ROS during metabolic processes in the chloroplasts, mitochondria, and peroxisomes (Neill et al., 2002). Excess amounts of ROS result in oxidative damage to the membrane and the cellular macromolecules, frequently leading to necrosis and death. This toxicity explains the evolution of complex non-enzymatic and enzymatic detoxification mechanisms in plants (Pitzschke et al., 2006).

Sugars serve fundamental roles as metabolic nutrients and structural components for most organisms. In plants, they regulate many vital developmental and metabolic processes, including germination, growth, photosynthesis, metabolism of carbon and nitrogen, flowering, stress responses, and senescence (Rolland and Sheen, 2005). However, mechanisms of signal transduction via sugars and the integration of sugar signals to modulate plant growth and development are still largely unknown. Hexokinase is a major regulatory enzyme in sugar metabolism and sugar-sensing in animals and plants (Stülke and Hillen, 1999; Rolland et al., 2001; Rolland and Sheen, 2005). Mitochondria-associated hexokinase has a pivotal role in the control of apoptosis in animal cells (Downward, 2003; Birnbaum, 2004; Majewski et al., 2004). Hexokinase is an integral component of the permeability transition (PT) pore through its interaction with porin or the VDAC (voltage-dependent anion channel) (Wilson, 2003); its binding to the VDAC interferes with the opening of the PT pore, thereby inhibiting cytochrome *c* release and apoptosis (Pastorino et al., 2002; Azoulay-Zohar et al., 2003). Thus, dissociation of hexokinases from the mitochondria strengthens, while its overexpression inhibits, mitochondrial dysfunctioning and cell death that are induced by various agents (Gottlob et al., 2001; Bryson et al., 2002; Majewski et al., 2004).

Previously, we reported that mitochondria-associated hexokinases play a critical role in the control of programmed cell death (PCD) in plants (Kim et al., 2006). There, virus-induced gene silencing of *NbHxk1,* a *Nicotiana benthamiana* hexokinase gene, induced PCD in the leaves. NbHxk1 is associated with the mitochondria, using the N-terminal membrane anchor. Conversely, overexpression of two mitochondria-associated *Arabidopsis* hexokinases, HXK1 and HXK2, confers enhanced resistance to H_2O_2- and α-picolinic acid-induced cell death based on our FACS analyses. Furthermore, the exogenous addition of recombinant NbHxk1 inhibits H_2O_2/clotrimazole–induced cytochrome *c* release from the mitochondria, suggesting a direct link between plant hexokinases and the PCD process. In this current study, we further analyzed the effects of cellular hexokinase levels on plant responses to methyl viologen and pathogen infection, both of which cause oxidative stress.

MATERIALS AND METHODS

Plant Materials

Fully developed leaves from four-week-old *Nicotiana benthamiana* plants were treated with 5 mM salicylic acid (SA), 100 μM methyl jasmonate, 50 μM methyl viologen, *Pseudomonas syringae* pv. *tomato* T1 (OD_{600}=0.1), or *P. syringae* pv. *tabaci* 11528 (OD_{600}=0.3) for 1, 3, or 6 h. Samples treated only with H_2O for 1 h served as the control. For the methyl jasmonate treatment, leaves were sprayed with a MeJa solution (100 μM) and sealed tightly in a plastic bag

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for the time indicated. Plant materials were then frozen in liquid nitrogen and stored at –70°C for RNA extraction. Seeds of *HXK1*- and *HXK2*-overexpressing transgenic *Arabidopsis* plants (Jang et al., 1997; Xiao et al., 2000) were obtained from Dr. Jyan-Chyun Jang (Ohio State University, Columbus, OH, USA).

RNA Isolation and Semi-Quantitative RT-PCR Analysis

Total RNA (1 μg) was reverse-transcribed using 0.5 μg of oligo (dT) and 200 units of SuperScriptII (Invitrogen, USA). An appropriate number of PCR cycles (15 to 35) was determined empirically for each template-primer pair so that amplification was in the exponential range and had not reached a plateau. *NbHxk1* transcripts were detected with HXK-C primers (Kim et al., 2006).

Measurement of Oxidative Stress Tolerance

Methyl viologen (MV) was dissolved in Tween 20 (0.1%) to 50 μM. Leaf discs of TRV and *NbHxk1*-silenced plants were incubated with MV (50 μM) at 26°C for 9 h under continuous light prior to measuring their chlorophyll contents. Five leaf discs from each of five independent plants of TRV and NbHxk1 VIGS were analyzed. The degree of tolerance to MV-mediated oxidative stress was also assessed at the whole-plant level. To observe the development of necrotic lesions, 20 mL of MV (50 μM) was sprayed onto TRV and NbHxk1 VIGS plants.

Measurement of Ion Leakage

Ion leakage was measured as previously described (Kim et al., 2003). After treatment, leaf discs were floated on 0.4 M sorbitol, then incubated in the dark for 12 h before the solution was analyzed for sample conductivity. After autoclaving, the solution was measured for subtotal conductivity. Membrane leakage was defined by relative conductivity, which was calculated as sample conductivity divided by total conductivity (the sum of sample conductivity and subtotal conductivity). Conductivity was measured with a conductivity meter (Model 162; Thermo Orion, Beverly, MA, USA).

Pathogen Inoculation

Leaves were infected with *Pseudomonas syringae* pv. *tomato* DC3000, as described by Alvarez et al. (1998). The strain was first grown overnight in King's B medium containing 100 μg mL^{-1} rifampicin, then washed twice and re-suspended in 10 mM $MgCl_2$. Four-week-old plants were infiltrated with the suspension (10^4 colony-forming units mL^{-1}). Other plants were infected with the fungal pathogen *Alternaria brassicicola*, as described by Oh et al. (2006). This inoculation was performed by applying a 10 μL drop of spore suspension (5×10^5 spores mL^{-1}) to leaves detached from four-week-old plants. Inoculated leaves were kept at 100% relative humidity in a Petri dish for the indicated times. Cell death was observed by lactophenol-aniline blue staining.

H_2O_2 Detection by DAB Staining

To visualize H_2O_2 at the pathogen inoculation site, 3,3′-diaminobenzidine (DAB) staining was performed as described by Thordal-Christensen et al. (1997).

RESULTS

Expression of *NbHxk1* in Response to Biotic and Abiotic Stresses

To investigate *NbHxk1* expression in response to biotic and abiotic stresses, we performed semiquantitative RT-PCR analyses (Fig. 1). Transcript levels in the *Nicotiana* leaves were slightly higher after infection with the non-host pathogen *Pseudomonas syringae* pv. *tomato* T_1 (PstT1), while those levels remained the same in response to infection by a host pathogen, *P. syringae* pv. *tabaci* 11528 (Pstab 11528). Interestingly, expression increased upon methyl viologen (MV) treatment compared with only small elevations due to exposure to salicylic acid (SA) or methyl jasmonate (MeJA). As our control, actin expression remained constant. We previously reported that *NbHxk1* mRNA levels rise after treatment with H_2O_2, heat, or thapsigargin (an inhibitor of Ca^{2+}-ATPase) (Kim et al., 2006), all of which activate PCD in plant cells (Balk et al., 1999; Houot et al., 2001; Ordenes et al., 2002). We also examined the expression patterns of the *Arabidopsis HXK1* (hexokinase-1) and *HXK2* (hexokinase-2) genes, using the AtGenExpress Visualization Tool (AVT) (http://jsp.weigelworld.org/expviz/expviz.jsp) and a TAIR Microarray Expression Search (http://www.arabidopsis.org/). *HXK1* mRNA expression moderately increased in response to abiotic stresses, including cold and high salt, as well as after infection with either the bacterium *P. syringae* pv. *tomato* DC3000 (Pst DC3000) or the fungus *Phytophthora infestans*. There, the accumulation of *HXK2* transcripts was strongly stimulated by osmotic stresses (mannitol) and high salt, while cold and powdery mildew infection only moderately increased levels. Taken together, these results demonstrate that expressions of *NbHxk1*, *HXK1*, and *HXK2* all are induced in response to various biotic and abiotic stresses.

Increased Susceptibility of NbHxk1 VIGS Plants to Methyl Viologen

Because *NbHxk1* gene expression was induced upon MV

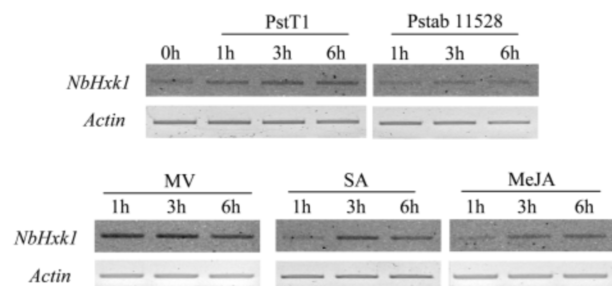

Figure 1. Expression of *NbHxk1* in response to biotic and abiotic stimuli.
Three-week-old *Nicotiana benthamiana* seedlings were treated with *Pseudomonas syringae* pv. *tomato* T1 (PstT1), *P. syringae* pv. *tabaci* 11528 (Pstab 11528), methyl viologen (MV; 50 μM), salicylic acid (SA; 5 mM), or methyl jasmonate (MeJA; 100 μM) prior to semiquantitative RT-PCR analysis. Transcript level of actin was measured as control.

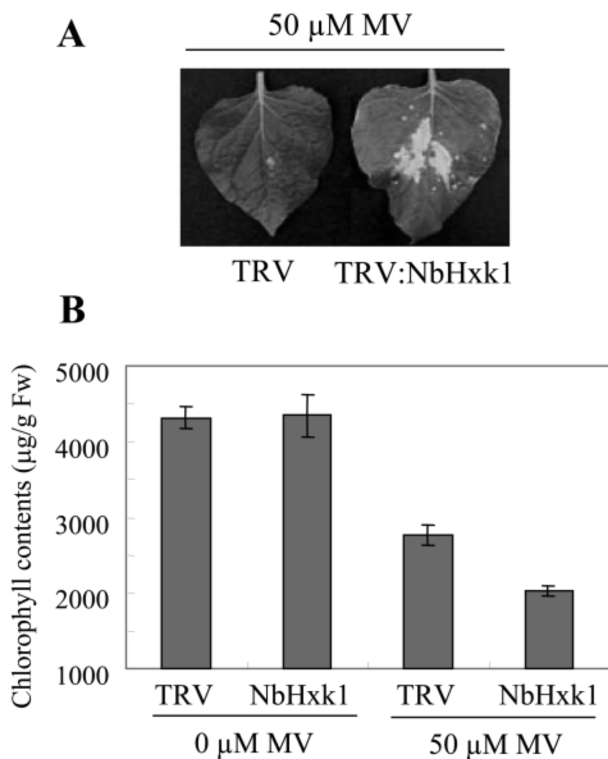

Figure 2. Reduced tolerance of NbHxk1 VIGS plants against MV. **A**, Cell death and bleaching phenotype after MV (50 μM) treatment. **B**, Total chlorophyll contents of leaf discs after MV (50 μM) treatment.

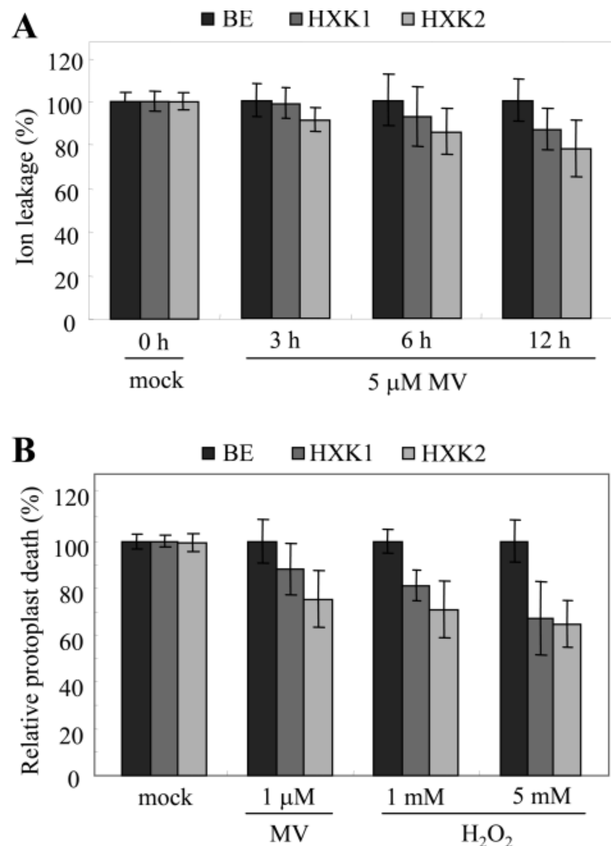

Figure 3. Enhanced resistance of *Arabidopsis* hexokinase-OE plants against methyl viologen. **A**, Leaf discs of control and hexokinase-OE plants were incubated with MV (5 μM) for 3, 6, or 12 h before measuring relative electrolyte leakage (%). Mock indicates relative electrolyte leakage without treatment at 0 h. Data points are means ± SD for 3 experiments. **B**, Leaf protoplasts were incubated with MV (1 μM) or H_2O_2 (1 or 5 mM) for 6 h, then stained with Evans blue (to visualize dead cells) prior to counting stained and intact cells (>300 protoplasts per sample) to calculate relative protoplast death (%). Mock indicates relative protoplast death without treatment at 0 h. Data points are means ± SD for 3 experiments.

treatment, we examined the susceptibility of *NbHxk1*-silenced plants to that agent (Fig. 2). When 50 ìM MV was sprayed onto leaves of the TRV control and TRV:NbHxk1 VIGS lines at 10 days after infiltration (DAI) before the transgenic plants exhibited any PCD phenotypes, we observed the development of necrotic lesions after 3 d (Fig. 2A). Following MV exposure, the NbHxk1 VIGS leaves manifested bleaching and HR-like cell death, whereas those of the TRV control developed only minor yellowing symptoms. We next used leaf discs to test MV susceptibility (Fig. 2B). After they were isolated from the TRV and TRV:NbHxk1 lines, they were incubated with 50 ìM MV for 9 h under lights. Chlorophyll contents of NbHxk1 VIGS leaves were reduced to ~73% of the TRV control level (Fig. 2B). These results demonstrate that NbHxk1-deficiency renders the plants more susceptible to MV.

Overexpression of *Arabidopsis HXK1* and *HXK2* Confers Enhanced Resistance against MV

We tested whether hexokinase overexpression suppresses MV-induced cell death, using transgenic *Arabidopsis* plants that over-express two *Arabidopsis* hexokinase genes, *HXK1* or *HXK2* (Figure 3). Both have N-terminal membrane anchors and are localized primarily in the mitochondria (Balasubramanian et al., 2007; Moore and Sheen, unpublished data, cited in Rolland and Sheen, 2005). Overexpression of these genes results in altered sugar responses during seedling development and in gene expression within *Arabidopsis* (Jang et al., 1997; Xiao et al., 2000). Furthermore, their elevated activities partially protect the protoplasts from H_2O_2- and α-picolinic acid-induced programmed cell death, based on flow cytometry (Kim et al., 2006). Here, we prepared leaf discs from control *Arabidopsis* plants (Bensheim. ecotype: BE), and HXK1- and HXK2-overexpressing (OE) transgenics, then incubated them with 5 μM MV for 3, 6, or 12 h under lights. Relative ion leakage was used as an indicator of plasma membrane damage (Fig. 3A). The hexokinase-OE lines exhibited less leakage than the control; in particular, HXK2 overexpression decreased ion leakage to ~80% of that measured in the control.

The effect of MV was further tested on three-week-old control and transgenic plants. Their isolated protoplasts were treated with MV or H_2O_2 for 6 h, then stained with Evans blue to visualize dead protoplasts. We compared the ratios of stained protoplasts to total intact protoplasts in the HXK1- and HXK2-OE lines with those from the control BE protoplasts to calculate relative protoplast death (%). After treatment with either agent, the hexokinase-OE lines had significantly less death than the control protoplasts, indicat-

ing increased resistance to MV and H_2O_2 (Figure 3B). This enhanced survival of hexokinase-OE protoplasts in response to H_2O_2 is consistent with previously reported results that were based on flow cytometry (Kim et al., 2006). Interestingly, we repeatedly observed that *HXK2* expression conferred better protection against MV than *HXK1* expression.

Overexpression of *HXK1* and *HXK2* Leads to Enhanced Resistance to Pathogens

Because ROS production is involved in plant pathogen defense and HR cell death, we investigated whether HXK-OE plants show altered responses to infection by the necrotrophic fungal pathogen *Alternaria brassicicola* or the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Pst DC3000) (Figs. 4 and 5). Four-week-old control or hexokinase-OE plants were inoculated by spore suspensions of *A. brassicicola*. In that infection cycle, spore germination is followed by the extension of the germ tube over the leaf surface. At the tips of the hyphae, the fungi develop appressoria for direct penetration into the host, eventually colonizing the tissue (McRoberts and Lennard, 1996). At 2 days post-inoculation (dpi), prominent necrotic regions with yellowing symptoms appeared on the control leaves, whereas such development was retarded in the hexokinase-OE lines, particularly the HXK2-OE plants (Fig. 4A). For example, lactophenol-aniline blue staining of the infected area at 4 dpi revealed significantly reduced necrotic areas (< 50% of the control) in both OE lines. Microscopic observation of those stained areas confirmed that the spreading lesions on the control leaves were more heavily colonized by hyphae than on the OE plants, indicating reduced fungal growth in the hexokinase-OE lines (Fig. 4B).

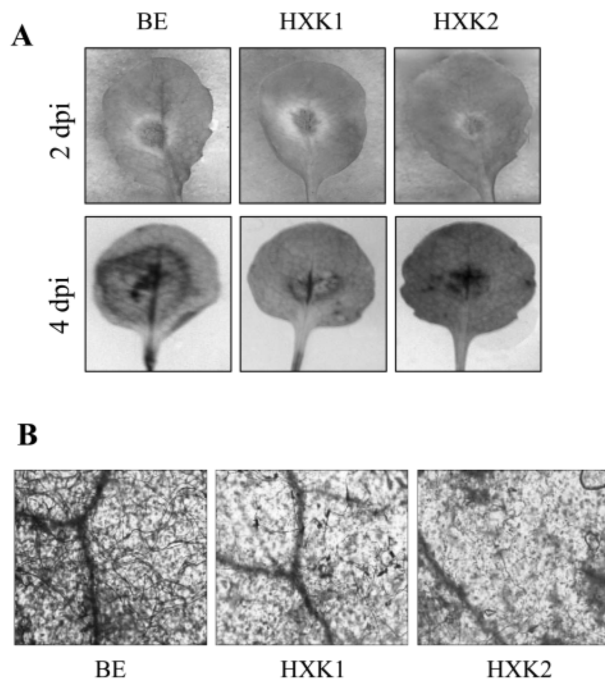

Figure 4. Disease development after inoculation with *Alternaria brassicicola*.
Four-week-old plants were challenged with 10 μL of fungal spore suspensions (5×10^5 spores mL^{-1}). Experiment was repeated 3 times, with similar results. **A**, Necrotic lesion phenotypes of leaves at 2 days post-inoculation (dpi), and staining of necrotic lesions with lactophenol–aniline blue at 4 dpi. **B**, Fungal mycelium on leaves, visualized by lactophenol–aniline blue staining, was examined by microscopy.

Next, we investigated whether hexokinase overexpression influences the plant response to a bacterial pathogen. After infiltration of the Pst DC3000 suspension (10^4 colony-

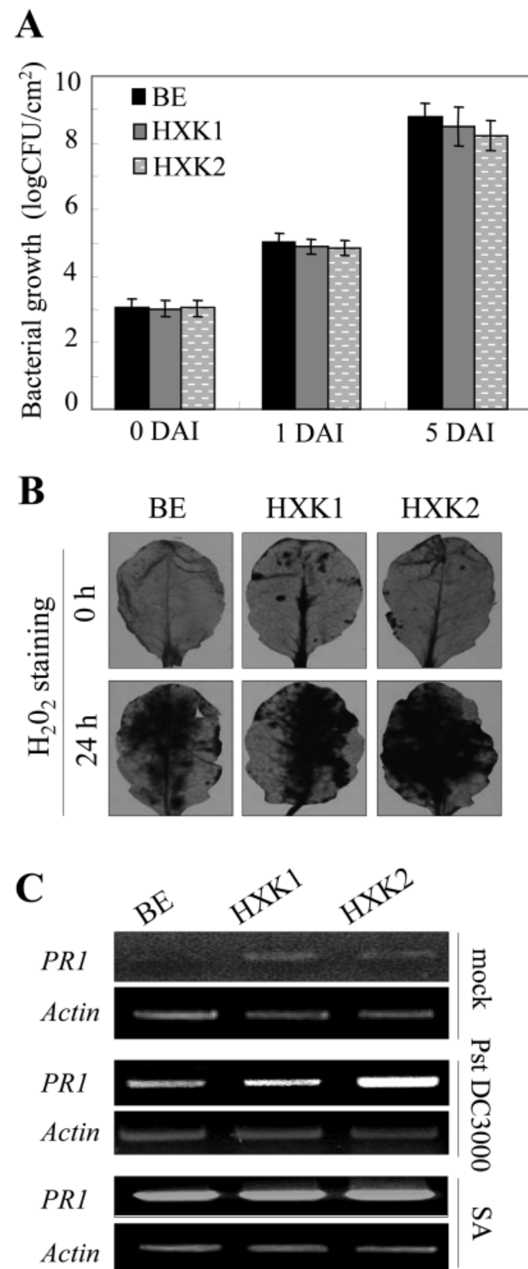

Figure 5. Disease development after inoculation with *Pseudomonas syringae* pv. tomato DC3000. Four-week-old plants were challenged with 20 μL of bacterial suspensions (10^4 colony-forming units mL^{-1}). **A**, Bacterial colony numbers in control and hexokinase-OE lines were counted at 1 and 5 days post-inoculation (dpi). Data points are means ± SD for 3 replicate plants. **B**, Inoculated leaves were stained with 3,3′-diaminobenzidine (DAB) to determine H_2O_2 production. **C**, Semiquantitative RT-PCR for transcript levels of *PR1*. Total RNA was extracted from untreated leaves (mock), and from leaves at 24 h after treatment with Pst DC3000 or salicylic acid (5 mM; SA). As control for RNA amounts, actin mRNA levels were examined.

forming units mL^{-1}), bacterial growth on the HXK-OE leaves at 5 dpi was ~3-fold less than on the control (Fig. 5A). These results indicate that hexokinase overexpression increases disease resistance, albeit only weakly. Because H_2O_2 plays a central role in eliciting cell death and activating a defense signaling pathway (Neill et al., 2002), we examined H_2O_2 production via DAB staining at 24 h post-inoculation (Fig. 5B). Hexokinase-OE plants, particularly from the HXK2-OE lines, showed greater H_2O_2 staining than the control. This implies that the slightly increased resistance by hexokinase-OE plants is related to elevated production of H_2O_2 upon pathogen infection. We also evaluated *PR1* gene expression in hexokinase-OE plants after Pst DC3000 inoculation (Fig. 5C), and found that it was more strongly stimulated in the HXK2-OE lines than in either the HXK1-OE or control plants. Interestingly, a low level of PR1 transcript was detected in the hexokinase-OE lines before pathogen inoculation (mock). Accumulation of those transcripts upon SA treatment did not differ significantly between the control and the hexokinase-OE lines (Figure 5C). As a control, actin expression remained constant. Therefore, we conclude that the mild resistance by HXK-OE plants against Pst DC3000 is correlated with elevated H_2O_2 production and defense-gene expression.

DISCUSSION

In this study, we further investigated the functioning of mitochondria-associated hexokinases during oxidative stress and pathogen infection. Diminished hexokinase levels via NbHxk1 VIGS or increased hexokinase levels by HXK1/HXK2 overexpression respectively reduced or enhanced plant tolerance to methyl viologen. MV is a compound that destroys the tissues of green plants by interfering with their photosynthesis (Timbrell, 1996). Its herbicidal property depends on the transformation of bipyridil residues to mono-cation radicals. The radical reacts with oxygen to render a superoxide anion, which can lead to the formation of H_2O_2 and hydroxyl radicals. Both products cause oxidative damage to cellular components, including protein inactivation, DNA damage, and lipid peroxidation (Timbrell, 2000; Suntres, 2002).

In mammals, mitochondria-associated hexokinases play a role in regulating apoptosis (Downward, 2003; Birnbaum, 2004; Majewski et al., 2004). Mammalian hexokinases I and II interact with VDAC, a critical component of the mitochondrial permeability transition pore. Hexokinase binding to VDAC suppresses the release of intermembrane-space proteins that both initiate the execution phase of apoptosis and inhibit apoptosis (Pastorino et al., 2002). Thus, targeted disruption of that mitochondria–hexokinase interaction induces cytochrome *c* release and apoptosis, while greater hexokinase activity protects animal cells against oxidant-induced apoptosis (Bryson et al., 2002; Pastorino et al., 2002; Majewski et al., 2004). Accordingly, in plants, silencing of *NbHxk1* activates PCD, whereas *Arabidopsis* plants over-expressing HXK1 and HXK2 have enhanced resistance to H_2O_2-and α-picolinic acid-induced PCD (Kim et al., 2006). Because MV generates H_2O_2 within a cell, the reduced/increased tolerance of hexokinase-depleted/hexokinase-OE plants to MV (Figs. 2 and 3) seems to involve similar mechanisms to those employed against H_2O_2. Based on results reported by Kim et al. (2006), this hexokinase association with mitochondria is apparently critical to maintaining the integrity of the outer mitochondrial membrane against oxidative stresses in plants, i.e., by regulating the release of cytochrome *c* into the cytosol. Thus, an elevation in mitochondria-associated hexokinase levels would protect cells against oxidative stresses such as MV, and conversely, a dissociation or decreased levels of such hexokinases might reduce the ability of a cell to maintain mitochondrial integrity under MV stress, leading to PCD. However, the underlying mechanism for this anti-apoptotic effect by hexokinases remains to be determined.

We repeatedly observed that *Arabidopsis* HXK2 protects plant cells from PCD more efficiently than HXK1 does when they are overexpressed (Figs. 3-5). Multiple isoforms of hexokinases are found in various sub-cellular compartments (Frommer et al., 2003). Like NbHxk1, the *Arabidopsis* HXK1 and HXK2 have N-terminal membrane anchors, and are localized primarily in the mitochondria (Balasubramanian et al., 2007; Moore and Sheen, unpublished data, cited in Rolland and Sheen, 2005). Interestingly, based on proteomics analyses of *Arabidopsis* mitochondrial proteins, HXK2, but not HXK1, is associated with the outside of the mitochondria as a glycolytic pathway complex (Giegé et al., 2003). It is possible that the mitochondria-associated form of HXK2 may be more abundant than that of HXK1, thus contributing more than HXK1 in maintaining mitochondrial integrity under stress conditions.

Our HXK1- and HXK2-OE plants exhibited enhanced resistance to a fungus, *A. brassicicola* (Fig. 4). Although the HXK-OE plants showed only weak resistance against the bacterium Pst DC3000, increased H_2O_2 production and *PR1* gene expression were evident after the pathogen challenge (Fig. 5). Furthermore, *PR1* was constitutively expressed in the HXK-OE plants, albeit at low levels. Based on these characteristics, HXK-OE plants may be more efficient than the control plants in activating their diverse defense reactions against that fungus. ROS are produced during pathogen defense, and H_2O_2 can diffuse into plant cells and activate certain reactions, including defense gene expression and PCD (Baker and Orlandi, 1995; Neill et al., 2002). During incompatible reactions, a rapid burst of H_2O_2 production occurs, followed by its prolonged accumulation. However, during compatible reactions, only the first peak of H_2O_2 production is detected, and the pathogen can infect the host plant. Furthermore, the oxidative burst that follows a pathogen infection leads to systemic immunity in distal parts of the plant (Alvarez et al., 1998). Despite the correlation between enhanced resistance and increased H_2O_2 accumulation in our HXK-OE plants, the precise mechanism underlying the protective effect of hexokinases against pathogens remains to be determined. Altered sugar responses and gene expression in HXK-OE plants (Jang et al., 1997; Xiao et al., 2000) may indirectly contribute to this phenomenon.

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