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Molecules and Cells

Pathogen Inducible Voltage-Dependent Anion Channel (AtVDAC) Isoforms Are Localized to Mitochondria Membrane in *Arabidopsis*

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Voltage-dependent anion channels (VDACs) are reported to be porin-type, β-barrel diffusion pores. They are prominently localized in the outer mitochondrial membrane and are involved in metabolite exchange between the organelle and the cytosol. In this study, we have investigated a family of VDAC isoforms in *Arabidopsis thaliana* (AtVDAC). We have shown that the heterologous expression of AtVDAC proteins can functionally complement a yeast mutant lacking the endogenous mitochondrial *VDAC* gene. AtVDACs tagged with GFP were localized to mitochondria in both yeast and plant cells. We also looked at the response of *AtVDACs* to biotic and abiotic stresses and found that four *AtVDAC* transcripts were rapidly upregulated in response to a bacterial pathogen.

INTRODUCTION

Mitochondrial functions are essential for cell physiology, playing central roles in cell survival and apoptotic death. One of the major functions of mitochondria in cell survival is oxidative phosphorylation, which results in the production of metabolic energy in the form of ATP. Mitochondria also play key roles in the mechanisms of apoptotic cell death through the release of apoptosis-inducing factors and proteins such as cytochrome *c*. A flux of solutes between the cytosol and mitochondria can be regulated through channels in the mitochondrial membranes. Porin-type proteins such as voltage-dependent anion channels (VDACs) are mainly involved in mediating the molecular traffic across the outer mitochondrial membrane (OMM) (Lee and Wei, 2007; Martinou, 2001; Zamzami and Kroemer, 2001).

VDAC is known to be a highly conserved OMM protein found in all eukaryotes and is the most abundant component of the OMM that is involved in the flow of metabolites and anions (Blachly-Dyson and Forte, 2001). Although the amino acid sequence homology of VDACs among animals, fungi and higher plants is relatively low, the physical properties and mechanisms are similar (Blachly-Dyson and Forte, 2001; Young et al., 2007). VDACs mediate the exchange of ions and small molecules such as NADH and ATP that are important for mitochondrial function (Blachly-Dyson and Forte, 2001; Rostovtseva and Bezrukov, 1998). It has also been reported that VDACs are involved in apoptosis and undergo extensive conformational changes in response to a variety of stimuli, facilitating the release of cytochrome *c* into the cytosol (Zalk et al., 2005). It has been proposed that the interaction of VDACs with the Bcl-2 family, comprised of pro- and anti-apoptotic proteins, regulates cytochrome *c* release via the opening or closing of VDAC channels in the OMM. The release of cytochrome *c* into the cytosol from the mitochondria in turn activates death-inducing proteolytic enzymes called caspases, which are responsible for destroying the cell (Na et al., 2007; Rostovtseva et al., 2004; 2005; Shimizu et al., 2000; Vander Heiden et al., 2001).

The characteristics and functions of VDACs have been extensively studied in fungi and animals (Blachly-Dyson and Forte, 2001; Kleene et al., 1987; Martinou, 2001; Zamzami and Kroemer, 2001); however, little is known about plant VDACs. Sequence analysis and electrophysiological data of plant VDACs have been reported for only a few species. Most *VDAC* genes are expressed in all plant tissues at various levels (Al Bitar et al., 2003; Elkeles et al., 1995; Geiger et al., 1999; Wandrey et al., 2004). Recently, it was shown that the gene expression of a *PgVDAC* in pearl millet (*Pennisetum glaucum*) was induced by various abiotic stresses (Desai et al., 2006) and a potato VDAC was reported to be involved in the tRNA translocation step through the plant mitochondrial outer membrane (Salinas et al., 2006). However, the biological functions of plant VDACs have been poorly defined. Until now, neither the biochemical nor physiological functions of *Arabidopsis* VDAC isoforms have been characterized. For this reason, the identification and characterization of the VDAC family in a model plant, such as *Arabidopsis*, will help to improve our understanding of the biological functions of plant VDACs.

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MATERIALS AND METHODS

Plant material and yeast strains

Arabidopsis thaliana (ecotype Columbia) was used as plant material. After germination, plants were grown in a growth chamber with a 16/8 h light/dark cycle at 22°C, light intensity of 100 μmol $m^{-2}s^{-1}$ and relative humidity of 57-80%. The following *Saccharomyces cerevisiae* strains were used for complementation and localization: the wild type strain, BY4742 (MATα, *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*) and the *ScVDAC1* (-) mutant strain, YNL055c (*por*) (MATα, *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*; YNL055c::kanMX4, EUROSCAF company).

Plasmid constructs for yeast and plant

The *YX-VDAC-GFP* constructs used in the yeast complementation studies were subcloned from the X*hoI*/*Spe*I fragment of *VDACs* into the X*hoI*/*Spe*I site of the yeast vector, *YX-GFP* (Hong et al., 1999). The *35S-VDAC-GFP* constructs encoding the VDAC-GFP fusion proteins under control of the 35S promoter were constructed by subcloning the X*hoI*/*SpeI* fragment of *VDACs* into the plant binary vector, *35S-GFP-JFH1* (Hong et al., 1999).

Plant transformation to generate transgenic plants

Transformation of *AtVDACs-GFP* into *Arabidopsis thaliana* plants was performed by *Agrobacterium tumerfaciens* (GV3101) using the floral dip method (Clough and Bent, 1998). Dry seeds were harvested, and hygromycin-resistant plants (T_0) were identified. We conducted the experiments with T_1 plants of three independent transgenic plant lines.

Treatment with environmental stresses

Arabidopsis thaliana was germinated and grown on Murashige and Skoog medium (Life Technologies; http://www.invitrogen.com) containing 3% sucrose and 0.25% phyta-gel (pH 5.8). The plants were grown for 10 d in a growth chamber at 22°C under a 16 h light/8 h dark regimen. Cold-, high-salinity- and drought-stress treatments were applied, essentially as reported previously (Seki et al., 2002). For the cold-stress treatment, the plants were grown under dim light (0.7-0.8 μmol $s^{-1}m^{-2}$) at 4°C. For the high-salinity-stress treatment, the plants were transferred and grown in water containing 250 mM NaCl. To impose drought stress, the plants were removed from the plates and desiccated in plastic dishes at 22°C. Pathogen treatment was applied as previously described (Kim et al., 2006; Park et al., 2004). For the pathogen treatment, 2-week-old plants grown on an MS plate were infiltrated with *Pseudomonas syringae* pv *tomato* (*Pst*DC3000). Control plants were infiltrated with $MgCl_2$.

Confocal microscopy

Confocal images were generated using a laser confocal microscope, with an argon ion laser system, as previously described (Lee et al., 2007). An ×100, 1.35 Plan Apo objective lens was used to image the yeast cells and guard cells. Images of yeast cells were taken at a threefold zoom setting. The GFP signal was excited at the 488 nm wavelength and the AF (autofluorescence of chlorophyll) signal was excited at the 543 nm wavelength. The fluorescence images were acquired in the green channel for GFP and the red channel for AF.

Transient expression of RFP fusion protein

For expression in protoplast, the plasmid DNAs were purified using Qiagen (QIANGEN Inc., USA) columns according to the manufacturer's protocol. The fusion construct was introduced into transgenic protoplasts of AtVDACs prepared from leaf tissues by polyethylene glycol-mediated transformation (Lee et al., 2001). Expression of the fusion proteins was monitored at various times after transformation with an Olympus AX-70 fluorescence microscope. The fluorescence images were captured with a cooled CCD camera (Olympus DP-70). The filter sets used were XF116-2 (exciter, 475AF20; dichroic, 500DRLP; emitter, 510AF23) and XF33 (exciter, 535DF35; dichroci, 570DRLP; emitter, 604DF50) for GFP and RFP, respectively.

Reverse transcriptase (RT)-PCR

RT-PCR analysis was carried out as previously described (Lee et al., 2007). Total RNA was prepared from roots, rosette leaves, stems, cauline leaves and flowers. First strand cDNA was synthesized for amplification. Primers for the PCR were: F, 5′-ATG GTG AAA GGT CCC GGT CTC-3′ and R, 5′-AGG CTT GAG TGC GAG AGC CAA-3′ for AtVDAC1; F, 5′-ATG AGC AAA GGT CCA GGA CTC-3′ and R, 5′-AGG TTT GAG AGC AAG AGA GAG-3′ for AtVDAC2; F, 5′-ATG GTT AAA GGT CCA GGA CTC-3′ and R, 5′- GGG CTT GAG AGC GAG AGC AAT-3′ for AtVDAC3; F, 5′-ATG GGA AGC AGT CCA GCT CCG-3′ and R, 5′-TGG TTT GAG GGC GAG GGC GAG-3′ for AtVDAC4; F, 5′-ATG GAT CAA ACA GAG GAA-3′ and R, 5′-CCA GCT CAG CTC CTG ATT-3′ for RD29A (GenBank Accession Number, D13044); F, 5′-ATG GCT GTA TAT GAA CAA ACC GG-3′ and R, 5′-TCA AAT ATC CAC AGA ACT CAA AC-3′ for DREB2B (GenBank Accession Number, NC 003074); F, 5′-GGA GCT ACG CAG AAC AAC TAA GA -3′ and R, 5′-CCC ACG AGG ATC ATA GTT GCA ACT GA-3′ for PR1 (GenBank Accession Number, AY064023); F, 5′-CCA ACA ACG TGA AAT CGA CAG-3′ and R, 5′-TCT TGG TAT TGC TGG TAC TCT-3′ for the internal standard *Tubulin2* (GenBank Accession Number, M84700). The amplification results from the *Tubulin2* mRNA were used to normalize the results obtained from the *VDAC* mRNA. Reactions for the control and tested genes were performed in parallel, but in separate tubes. The products were visualized on 1.2% agarose gels.

RESULTS

Sequence and phylogenetic analysis of Arabidopsis VDACs

In *Arabidopsis*, voltage-dependent anion channels (VDACs) were reported to exist in five different AtVDAC isoforms, based on database searches and a previous report (Clausen et al., 2004). These five genes were named *AtVDAC1* (AGI No. At3g01280), *AtVDAC2* (AGI No. At5g67500), *AtVDAC3* (AGI No. At5g15090), *AtVDAC4* (AGI No. At5g57490) and *AtVDAC5* (AGI No. At3g49920). *AtVDAC* genes encode proteins composed of 227 to 277 amino acids, with calculated molecular masses ranging from 25 to 30 kDa. The five AtVDAC isoforms show amino acid sequence identities ranging from 14% to 50%. To identify the putative secondary structure of AtVDAC isoforms, we compared their secondary structure with that of a previously reported human VDAC1 (HsVDAC1) (Bayrhuber et al., 2008) using the ClustalW2 database and the PRED-TMBB database (Fig. 1A). These predictions revealed remarkably similar structural patterns among AtVDAC isoforms and HsVDAC1. The positions of α-helices and β-strands were highly conserved in the AtVDAC isoforms, although high sequence conservation was not observed. Among the five *Arabidopsis* VDACs, AtVDAC1 and AtVDAC2 contained the eukaryotic mitochondrial porin signature motifs (Young et al., 2007), while AtVDAC3, AtVDAC4 and AtVDAC5 did not (Fig. 1A). The phylogenetic relationships between the five AtVDACs and VDACs from other plants, yeast and mammals is depicted

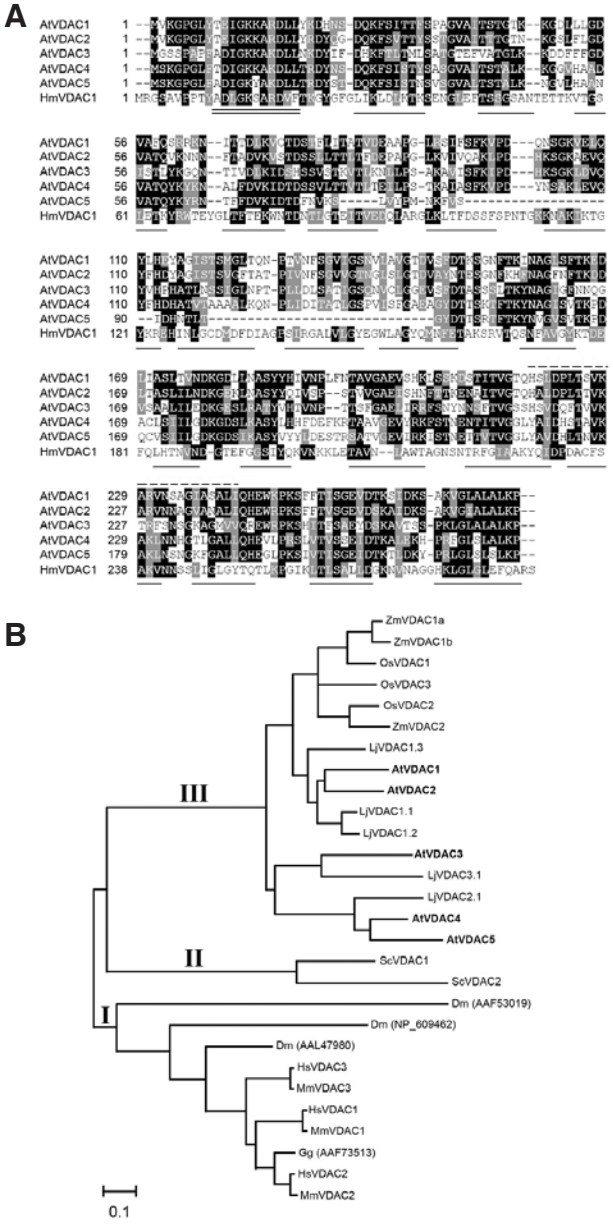

Fig. 1. Comparison of primary and secondary structure of *Arabidopsis* VDAC isoforms. (A) The amino acid sequence alignment of AtVDAC isoforms and comparison of secondary structure of AtVDAC isoforms and HsVDAC1. Identical and similar amino acids are highlighted with black and gray shadings, respectively. Secondary structure prediction from AtVDACs and HsVDAC1 were performed on the ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/) and PRED-TMBB server (http://bioinform atics. biol.uoa.gr/). A double underline indicates a predicted β-helical region and a single underline indicates predicted β-stands of AtVDACs. The sequence marked with a dotted line indicates eukaryotic mitochondrial porin signatures (PROSITE Pattern-ID: PS00558) of AtVDAC1 and AtVDAC2 proteins. (B) Phylogenetic tree of VDAC proteins. A phylogenetic tree was generated by ClustaW2 and visualized by MEGA 4.1 software (http://www.megasoftware.net/). The scale bar represents the evolutionary distance expressed in the number of substitutions per amino acid. *At, Arabidopsis thaliana; Hs, Homo sapiens; Lj, Lotus japonicus; Mm, Mus musculus; Os, Oryza sativa; Sc, Saccharomyces cerevisiae; Zm, Zea mays; Dm, Drosophila melanogaster; Gg, Gallus gallus.*

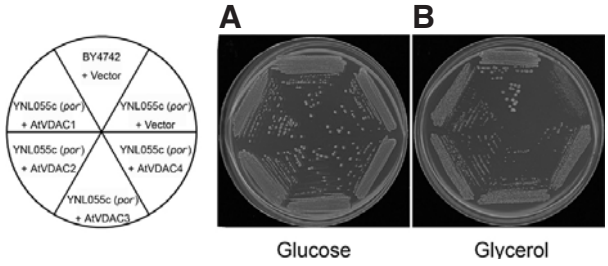

Fig. 2. Functional complementation of a yeast mutant by four AtVDAC isoforms. Wild-type yeast (BY4742) and the yeast VDAC mutant, YNL055c (*por*), were transformed with vector only as positive and negative controls, respectively. The yeast cells were streaked on the synthetic complete (SC) medium, minus uracil, containing 2% (w/v) glucose (A) or 2.5% (w/v) glycerol (B) as sole carbon source. Plates containing glucose were incubated for 3 d at 30°C, whereas glycerol plates were incubated for 5 d at 30°C. The diagram indicates the genotypes of the yeast strains and the transformed constructs.

in Fig. 1B. They are classified into three subfamilies by homology. Branches I and II are composed of mammal and fungal VDACs. Branch III is composed of plant VDACs. The VDACs from dicot and monocot plants are further classified into subbranches.

Arabidopsis AtVDACs complement the defect of a yeast VDAC1 mutant

To determine whether *Arabidopsis* VDACs can functionally restore yeast mutants, we performed yeast complementation studies using the yeast VDAC mutant strain, YNL055c (*por*) (Lee et al., 1998). YNL055c showed delayed growth in the presence of glycerol medium as the sole carbon source but was able to grow well in glucose medium (Figs. 2A and 2B). Four of the *AtVDAC* cDNAs were subcloned into the yeast expression vector *YX-GFP* (Hong et al., 1999) to express proteins constitutively under the control of the triose phosphate isomerase promoter. Unfortunately, we could not perform the yeast complementation experiment with *AtVDAC5* because we failed to amplify *AtVDAC5* cDNA from the *Arabidopsis* cDNA library. The wild type yeast strain, BY4742, transformed with the vector alone, grew well on the synthetic complete (SC) medium containing glucose or glycerol (Figs. 2A and 2B), whereas the yeast mutant strain, YNL055c, transformed with the vector alone did not grow in glycerol medium (Fig. 2B). The transformed yeast strains expressing each of the four AtVDAC proteins were able to grow well on the SC medium plates containing glycerol as the sole carbon source (Fig. 2B). These results suggest that four AtVDACs can functionally replace the yeast VDAC1 (ScVDAC1).

Localization of AtVDACs tagged with GFP in yeast cells

The yeast voltage-dependent anion channel, ScVDAC1 is known to be an outer mitochondrial membrane protein (Lee et al., 1998). Thus, the ability of AtVDACs to complement the yeast VDAC1 mutant may result from the localization of heterologous proteins in the mitochondria of yeast cells. To investigate the localization of plant proteins in yeast cells, the four AtVDAC proteins tagged with GFP were expressed in yeast cells. Free GFP protein was used as a cytosolic marker protein (Fig. 3). GFP signals were visualized by confocal laser scanning microscopy. The GFP fluorescences were observed on small organelles with similar patterns for each of the four iso-

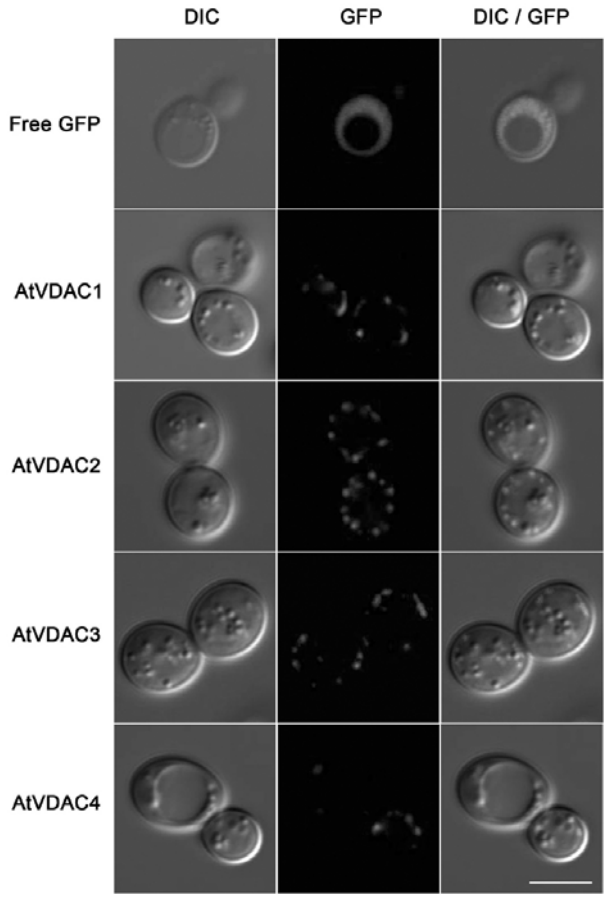

Fig. 3. Subcellular localization of AtVDACs tagged with GFP in yeast cells. Fluorescence of free GFP and of AtVDAC-GFP encoding four different AtVDACs in yeast cells was observed by confocal microscopy. DIC, differential interference contrast; GFP, green fluorescence protein; DIC/GFP, overlapping images of DIC and GFP. Bar = 5 μm.

forms. The localization of fluorescence signals strongly indicated that the AtVDAC proteins might be targeted to the mitochondria of yeast cells.

Localization of AtVDACs tagged with GFP in plant cells

In order to identify the subcellular localization of AtVDAC isoforms in plant cells, we independently constructed stable transgenic plants expressing four GFP-tagged AtVDACs. *GFP* was tagged to the C-terminal end of the *AtVDAC* fragment, together with three glycine residues used to provide a flexible linker between AtVDAC and GFP. The GFP signals in the living cells of the transgenic plants were visualized using confocal microcopy. Three independent transgenic plant lines were analyzed. Figure 4A shows representative green (GFP) and red (AF) fluorescence signals in guard cells of the transgenic plants expressing AtVDAC-GFP fusion proteins. When the GFP images of AtVDAC1, AtVDAC2 and AtVDAC3 fusion proteins were compared with the red AF signals, the fluorescent images could not be overlapped (Fig. 4A). This result indicated that AtVDACs were not targeted into the chloroplast.

To further confirm that AtVDACs were targeted to mitochondria, the *F1-ATPase-RFP* construct encoding a mitochondrial marker protein (Jin et al., 2003) was introduced into protoplasts isolated from AtVDACs-GFP transgenic plants. We then analyzed the fluorescence images of F_1-ATPase-RFP and AtVDACs-GFP fusion proteins in transgenic protoplasts. The green fluorescence distribution of AtVDACs-GFP overlapped with those of the F_1-ATPase-RFP red fluorescence signal, indicating co-localization on the mitochondria (Fig. 4B). We could not detect the GFP fluorescence in AtVDAC4-GFP in the transgenic plants.

Gene expression of four *AtVDACs*

To determine whether the transcript levels of these *AtVDAC* isoforms are affected by biotic and abiotic stresses, we analyzed their expression levels in response to pathogen, cold, salt and drought stresses (Fig. 5). To examine the response of the *AtVDAC* transcripts to a bacterial pathogen, plant leaves were treated with *Pseudomonas syringae* pv *tomato* (*Pst*DC3000) and analyzed by RT-PCR. All four *AtVDAC* transcript levels were significantly increased by bacterial pathogen treatment. Transcript levels were rapidly increased at 6 h and maximized at 12 h after treatment. These increased transcript levels were maintained for up to 24 h (Fig. 5) after treatment. We also examined the four *AtVDAC* transcript levels in response to abiotic stresses such as cold, drought and salt; however, transcript levels were not significantly affected by these stress treatments (Fig. 5). These results indicate that the gene expression of each of the four *AtVDAC* isoforms can be induced by biotic stress in *Arabidopsis*.

DISCUSSION

Despite extensive studies in animal cells, there has been little characterization and investigation of the biological function of VDACs in plant cells. In this study, we isolated and characterized VDAC isoforms at the molecular level in a model plant, *Arabidopsis thaliana*. Four *VDACs* were isolated in *Arabidopsis* and their functions were examined by expressing them in a *VDAC*-deficient yeast mutant. Complementation assays and localization analysis of the plant AtVDACs in yeast cells demonstrated that these proteins can function as VDACs in the yeast OMM (Figs. 2 and 3). Therefore, although the primary amino acid sequence similarities of VDACs between yeast and plant are relatively low, these results indicate that the functions of VDACs are highly conserved (Elkeles et al., 1995; Young et al., 2007).

AtVDACs tagged with GFP were localized to mitochondria, not only in guard cells, but also in the protoplasts of transgenic plants (Fig. 4). GFP fluorescence was observed as a punctate pattern, indicative of mitochondrial expression in guard cells and protoplasts. However, we saw no expression of the AtVDAC fusion protein in chloroplasts. It has been previously reported that the pea VDAC-GFP fusion protein, expressed in tobacco leaves, is also exclusively localized to mitochondria, but not to chloroplasts (Clausen et al., 2004). In contrast, the distribution of VDACs in organelles other than mitochondria has been reported in several plant species. LjVDACs of *Lotus japonicus*, for example, were found to be present in small vesicles at the cell periphery, as well as in mitochondria (Wandrey et al., 2004), and the immuno-positive signal of PgVDAC in *Pennisetum glaucum* was detected in both chloroplasts and mitochondria (Desai et al., 2006). VDACs have also been localized to the glyoxysomal membrane of the castor bean (Reumann et al., 1997). Considering these examples, VDACs in plants seem to play roles in metabolite transport, not only in mitochondria, but also in other organelles.

Unfortunately, we were unable to detect the GFP signals of

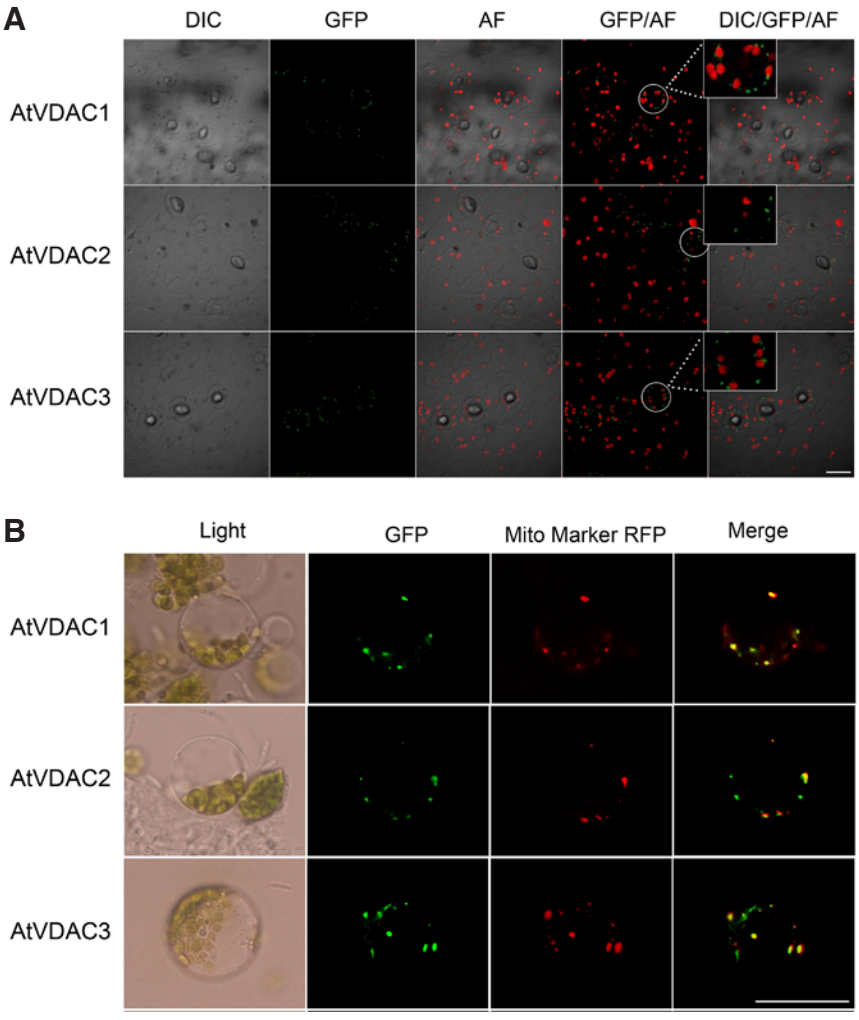

Fig. 4. Subcellular localization of AtVDAC-GFP fusion proteins in transgenic plants. (A) Localization of AtVDAC-GFP fusion proteins in guard cells. The leaves of transgenic plants (AtVDAC1-GFP, AtVADC2-GFP and AtVDAC3-GFP) were harvested, and the epidermis was peeled off. These epidermal fragments were investigated for GFP fluorescence. DIC images of guard cells were taken. The GFP and AF signals were detected by confocal microscopy. GFP/AF and DIC/GFP/AF overlapping images were produced to compare the localization of fluorescence using FV10-ASW software. The magnified image of the white circle in GFP/AF was made using Adobe PhotoShop. Bar = 20 μm. (B) Co-localization of AtVDAC-GFP and F_1-ATPase-RFP fusion proteins in transgenic protoplasts. Protoplasts prepared from AtVDACs-GFP transgenic plants were transformed with the *F_1-ATPase-RFP* construct. The transformed protoplasts were observed with a fluorescent microscope 2 days after transformation. Green and red fluorescences are GFP and RFP signals, respectively. Bar = 30 μm.

AtVDAC4 in transgenic plants. Although heterologous expression of AtVDAC4 was detected in yeast cells, the protein was not expressed in transgenic plants. It is possible that the expression level of AtVDAC4 is simply very low or that the recombinant form of the AtVDAC4 protein is not stable in plant cells.

As shown in Fig. 5, expression levels of the *AtVDAC* transcripts were monitored in response to pathogen, cold, drought and high-salinity stresses. None of the *AtVDAC* isoforms showed significant changes in their expression levels in response to abiotic stresses. However, all four *AtVDAC* genes were up-regulated in response to a bacterial pathogen in *Arabidopsis* (Fig. 5). It has been suggested that the mechanism of cell death during a hypersensitive response (HR) in plants is connected with the mechanism of programmed cell death in animals (Gilchrist, 1998). Despite the fact that Bcl-2 family homologues are not present in plants, an HR-like cell death pathway could be induced in transgenic tobacco plants expressing the animal Bax gene, while cell death induced by fungal necrotrophs could be suppressed by expressing animal anti-apoptotic genes, such as Bcl-2 or Bcl-XL (Dickman et al., 2001; Hofius et al., 2007; Lacomme and Santa Cruz, 1999). Additionally, a rice mitochondrial VDAC has been reported to induce apoptosis in the Jurkat T-cell line (Godbole et al., 2003), suggesting that VDACs can functions as a conserved element in cell death pathways in plants and animals (Lacomme and Roby, 1999; Swidzinski et al., 2004). These results suggest that VDACs can be involved in pathogen-triggered hypersensitive cell death in plants. Further investigation into the mechanism of AtVDACs in pathogen defense will expand our understanding of the functional roles of VDACs in plants.

In this study we have characterized *Arabidopsis* VDACs at the molecular level and shown that AtVDACs can function as voltage-dependent anion channels in yeast. Using the stable expression of GFP-fusion proteins in transgenic plants, we have also shown that AtVDACs were targeted to mitochondria. Interestingly, the *AtVDAC* gene expressions were rapidly induced by a bacterial pathogen. These results suggest that AtVDACs may be involved in the plant defense against pathogens.

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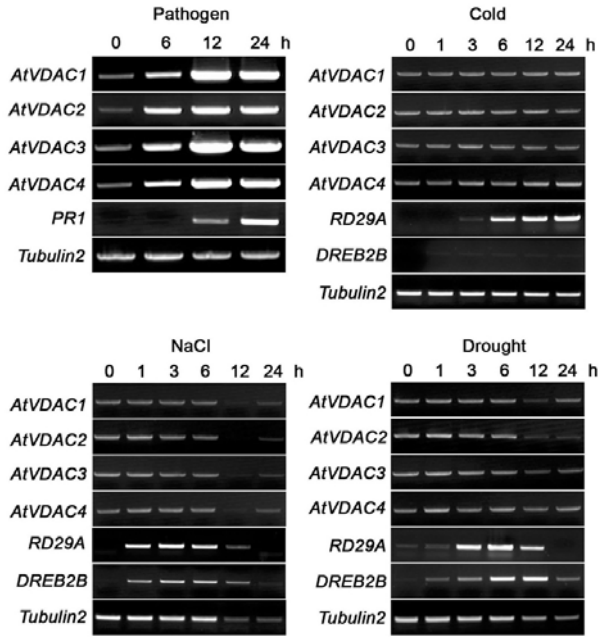

Fig. 5. Gene expression levels of *AtVDAC* isoforms in response to various stresses. Total RNA was isolated from wild-type plants treated with a bacterial pathogen (pathogen), 4°C (Cold), 250 mM NaCl (salt), and no drain (drought). RT-PCRs were performed with *AtVDAC*-specific primers using first strand cDNA as a template. RT-PCRs of *RD29A* and *DREB2B* transcripts were performed to verify the appropriate treatments of cold, salt and drought stresses. RT-PCR of the *PR1* transcript was performed to verify the appropriate treatment of the bacterial pathogen. RT-PCR of *Tubulin2* transcript was employed as a quantitative control.

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