Overexpression of the voltage-dependent anion channel 2 (VDAC2) gene induces drought resistance in *Arabidopsis thaliana*

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Abstract

Abiotic stresses, especially drought, trigger abscisic acid (ABA) signal conduction in plants. However, the molecular mechanisms are not well understood. We investigated the role of the voltage-dependent anion channel 2 gene (*AtVDAC2*) in drought resistance of *Arabidopsis* through ABA signalling. Some characteristics such as seed germination, root elongation, drought resistance, ABA related genes expressing, etc. were studied in plant transgenic lines. The *AtVDAC2* overexpressing plants displayed an ABA-hypersensitive phenotype with higher seed germination and root elongation, while plants with *AtVDAC2* knockdown (*AtVDAC2-Dn*) exhibited an ABA-insensitive phenotype. Additionally, *AtVDAC2*-OE plants showed enhanced resistance (about 40%) to drought than wide-type and *AtVDAC2*-Dn plants. In *AtVDAC2* transgenic plants, some downstream targets in the ABA and/or drought-signalling pathways were altered at various levels, suggesting the involvement of *AtVDAC2* in ABA-dependent drought resistance in *Arabidopsis*. Moreover, *AtVDAC2*-OE plants exhibited increased 9-cis-epoxycarotenoid dioxygenase 3 (*NECD3*) gene expression and generation of the reactive oxygen species hydrogen peroxide, suggesting that functional *AtVDAC2* may be involved in ABA and H$_2$O$_2$ production process. The overexpression of the *AtVDAC2* gene can confer drought resistance in *Arabidopsis*.

Keywords: ABA signalling, voltage-dependent anion channel, drought resistance, *Arabidopsis*.

Abbreviations: ABA_abscisic acid, ABI_abscisic acid-insensitive, DAB_3,3-diaminobenzidine, Dn_knockdown, H$_2$O$_2$_hydrogen peroxide, MS_Murashige and Skoog, NECD_9-cis-epoxycarotenoid dioxygenase, OE_overexpression, PP2Cs_the protein phosphatase 2Cs, RCAR_regulatory component of ABA receptors, RD29B_regulated by drought 29B, ROS_reactive oxygen species, VDAC_voltage-dependent anion channel.

Introduction

Plants encounter various abiotic stresses such as drought, salinity, waterlogging, and low temperature during their life cycles. Therefore, they produce various biochemical and physiological responses to overcome these unfavourable conditions. For example, plants have to initiate adaptive mechanisms to survive under dehydration which leads to inhibition of physiological processes.

Phytohormone abscisic acid (ABA) regulates plant growth and developmental processes in response to changes in water status. In *Arabidopsis*, elevation of ABA levels can stimulate the activity of downstream targets to mediate drought stress adaption. The additional ABA-independent signal transduction pathway may also have a role in counteracting the effects of dehydration (Yamaguchi-Shinozaki and Shinozaki, 2006). Numerous studies have been conducted yet on ABA-regulated adaptation to drought stress such as RCAR (regulatory component of ABA receptors), ABI (abscisic acid-insensitive), RD29B (regulated by drought), NECD (9-cis-epoxycarotenoid dioxygenase) (Schroeder et al., 2001; Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006; Razem et al., 2008; Ma et al., 2009; Park et al., 2009). However, identification of novel genes involved in ABA signalling is required to improve our understanding of mechanism and action of this important phytohormone.

As it is known, the growth of plants is suppressed under drought stress, i.e., plants become smaller and production is reduced and ABA levels change as well (Westgate et al., 1985; Sharp et al., 1988; Bartels et al., 1988). Considering the change in matter accumulation and energy metabolism, voltage-dependent anion channel (VDAC) gene activates filtering of transport across the mitochondrial outer and inner membrane.

Recent research has demonstrated that abnormalities in VDAC gene lead to respiratory impairment in brewer’s yeast (*Saccharomyces cerevisiae*) (Dihanich et al., 1987; Lee et al., 1998; Graham and Craigen, 2004). In mammals, abnormalities of the VDAC gene lead to impairment of the male reproductive system, central nervous system, and glucose homeostasis (Craigen and Graham, 2008). Recently, studies have also demonstrated a role for the VDAC gene in plants. For example, in *Arabidopsis thaliana*, the VDAC3 gene (*AtVDAC3*) improved germination and growth under low temperature conditions (Yang et al., 2011). Under high salinity condition such with osmotic stress and drought, the VDAC expression is up-regulated in rice (Al Bitar et al., 2003). Moreover, salt exposure induces the VDAC gene expression in pearl millet (Desai et al., 2006). The VDAC expression is also upregulated by drought, freezing and salicylic acid treatment, but is not
upregulated after ABA treatment (Desai et al., 2006). Interestingly, VDAC expression has been shown to alter followed bacterial infections in tobacco plants (Tateda et al., 2009). Thus, these previous studies demonstrate that VDAC expression is always changed under biotic and abiotic stimuli, suggesting its importance in adaptive mechanisms to environmental changes.

Although we already know that plant growth is restrained under drought stress, and ABA levels is also changed, it is not clear whether VDAC is associated with growth inhibition under drought stress and plays an important role in ABA signalling. *A. thaliana* contains 5 VDAC genes that exhibit high homology to one another (Clausen et al., 2004). In our lab, we have found some evidences that display AtVDAC2 is involved in ABA-mediated early seedling development, such as germination (Yan et al., 2009). However, the functions of VDACs in *A. thaliana* have not been fully characterized and their role in the ABA-mediated drought stress response is still unclear.

In this study, we analysed ABA sensitivity under drought stress condition in *A. thaliana* using transgenic plant including overexpressing AtVDAC2 (AtVDAC2-OE) or knockdown AtVDAC2 (AtVDAC2-Dn) lines. It provided information on AtVDAC2 and phenotypes associated with ABA-mediated drought stress response. This study will provide a good complement for studies of ABA and drought signal transduction networks.

**Results and Discussion**

**AtVDAC2-OE plants displayed an ABA-hypersensitive phenotype**

As a part of standardization, we first confirmed the up- or down-regulation of VDAC2 in our transgenic lines. The expression of VDAC2 was 140-fold higher in AtVDAC2-OE plants than in related wild-type plants, while the expression of VDAC2 in AtVDAC2-Dn plants decreased to about 0.7-fold than wild-type (Fig. 1). Then, we selected 2 AtVDAC2-OE lines (OE2 and OE4) and 2 AtVDAC2-Dn lines (Dn2 and Dn8) for phenotypic analysis. Wild-type RLD plants were used as the control.

To confirm whether VDAC2 played an essential role in ABA signalling in plants, we first analysed phenotypic differences between AtVDAC2-OE and AtVDAC2-Dn lines after treatment with ABA. The *ArvDac2* transgenic plants were selected for their ability to germinate and root growth in MS medium with or without ABA. The findings from germination trail were similar to that reported by Yan et al. (2009). The AtVDAC2-Dn plants were capable of germinating in MS medium with 0.7 μM ABA. However, the germination rate of AtVDAC2-OE plants was relatively depressed (Fig. 2), while that of wide-type plants was between the 2 transgenic lines.

ABA was also observed to affect root growth in transgenic plants. As shown in Fig. 2, 0.5 μM ABA treatment resulted in reduced root lengths in the AtVDAC2-OE lines compared to wild-type plants. Conversely, ABA treatment in AtVDAC2-Dn lines led to increased root lengths compared to wild-type plants. Taken together, we speculated that the AtVDAC2 gene strengthens the conduction of ABA signals. Excessively expressed AtVDAC2 made plant sensitive to ABA and inhibited the seed germination and growth of root, while a low AtVDAC2 gene expression led to ABA signal inhibition with less affected plant growth.

**Response to drought stress in AtVDAC2 transgenic plants**

Since AtVDAC2 transgenic plants showed a marked change in ABA sensitivity, in terms of seed germination and root elongation, we next examined their responses to drought stress. Four-week-old plants were grown without water for 3 weeks until differences between the plants were visible and plants were then hydrated by watering for 1 week. After rehydration for 1 week, most of the AtVDAC2-OE plants, about 36% of the wild-type plants, and a few of the AtVDAC2-Dn plants were recovered (Fig. 3). These data suggested that AtVDAC2-OE plants had enhanced resistance to drought compared to wide-type and AtVDAC2-Dn plants.

**AtVDAC2-OE plants produced more hydrogen peroxide as a result of ABA treatment**

Drought stress triggers ABA-induced accumulation of reactive oxygen species (ROS). Thus, plants require ROS detoxification mechanisms to survive following drought stress (Smirnoff, 1995). The H$_2$O$_2$, an important superoxide free radical, is accumulated in plants after exposure to many types of stressors, such as drought, salinity, etc. Therefore, to characterize whether ROS production occurred in response to ABA treatment in AtVDAC2 transgenic plants, we analysed H$_2$O$_2$ accumulation by DAB staining. The AtVDAC2-OE lines stained much darker and into a greater extent than wide-type plants. In turn, AtVDAC2-Dn lines were stained more lightly than wild-type plants (Fig. 4). These data showed that H$_2$O$_2$ accumulation was increased in AtVDAC2-OE lines, but decreased in AtVDAC2-Dn lines, suggesting the involvement of VDAC in mediating H$_2$O$_2$ accumulation similar to previous studies of VDAC in Nicotiana benthamiana (Tateda et al., 2009). Thus, it is possible that H$_2$O$_2$ production was activated in response to ABA in AtVDAC2-OE lines but was blocked in AtVDAC2-Dn lines. The stain of DAB in the root of AtVDAC2-Dn lines was much deeper than that of AtVDAC2-OE lines (Fig. 4). We suggest that it might be due to migration ability of H$_2$O$_2$. The AtVDAC2-Dn lines produced H$_2$O$_2$ in root, and it could not be transferred to the upper parts of plant. This situation was opposite in AtVDAC2-OE lines. However, this needs more investigation to be sufficiently explained.

**Transcriptional alterations in downstream targets in AtVDAC2 transgenic plants**

To better understand the mechanisms mediating drought resistance in AtVDAC2 transgenic plants, we analysed the expression of downstream targets of ABA signalling (Fig. 5). The expression of Rdc2, a foregane ABA receptor, was increased by 37- or 105-fold in AtVDAC2-OE plants compared to wild-type plants. However, it was decreased by about 0.2-fold in AtVDAC2-Dn plants. The Rdc2 has been shown to act as a positive regulator of ABA signalling and ABA receptor and to interact with the protein phosphatase 2Cs (PP2Cs) ABI1 and ABI2 (Park et al., 2009; Ma et al., 2009; Szostkiewicz et al., 2010). The Rdc2 acts as a negative regulators of ABA response which is inactivated by the ABA receptor. Therefore, we analysed the expression of ABI1. Interestingly, this gene showed 4- or 58-fold up-regulation in AtVDAC2-OE plants when compared to wild-type plants. In contrast, ABI1 expression in AtVDAC2-Dn plants was reduced to about 0.1-fold, compared to wild-type plants. Other ABA-inducible genes, such as RD29B and NACD7 were also up-regulated in AtVDAC2-OE plants and down-regulated in AtVDAC2-Dn plants. Indeed, the expression of RD29B, which contains an
Table 1. Primers for quantitative real-time PCR.

<table>
<thead>
<tr>
<th>Gene (access number)</th>
<th>Sequence (5′→3′)</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTIN (AT1G01360)</td>
<td>F GATGAGGCTCAATCCAAACGA</td>
<td>228</td>
</tr>
<tr>
<td>VDAC2 (AT5G67500)</td>
<td>R AGCAGGGGCAATTGAAGTCT</td>
<td>105</td>
</tr>
<tr>
<td>RD29B (AT5G52300)</td>
<td>F GCTGATGTTCGCCCACAATACAA</td>
<td>244</td>
</tr>
<tr>
<td>ABI1 (AT4G26080)</td>
<td>R TCCTCTGGATGTGACTAG</td>
<td>165</td>
</tr>
<tr>
<td>RCAR1 (AT1G49240)</td>
<td>F TGATCGGACCAGGAGTGTGATT</td>
<td>257</td>
</tr>
<tr>
<td>VDAC2 (AT5G67500)</td>
<td>R TGGAGATCTCGGAAGTGTGACT</td>
<td>105</td>
</tr>
<tr>
<td>ABI1 (AT4G26080)</td>
<td>R TGATCGGACCAGGAGTGTGATT</td>
<td>257</td>
</tr>
<tr>
<td>RCAR1 (AT1G49240)</td>
<td>F TGGAGATCTCGGAAGTGTGACT</td>
<td>105</td>
</tr>
<tr>
<td>VDAC2 (AT5G67500)</td>
<td>R TGATCGGACCAGGAGTGTGATT</td>
<td>257</td>
</tr>
</tbody>
</table>

ABA-responsive element in its promoter, was up-regulated by 60- or 35-fold in AtVDAC2-OE plants, compared to wild-type plants. However, it was down-regulated to about 0.4-fold in AtVDAC2-Dn plants. The expression of NECD3, which is involved in ABA biosynthesis, was increased by 14- or 90-fold in AtVDAC2-OE plants, but was decreased to about 0.2-fold in AtVDAC2-Dn plants, compared with wild-type plants. These findings suggested that overexpression of VDAC2 enhanced the ABA biosynthesis and activated ABA signalling and ABA-related gene (RCAR1, RCAR3, ABI1, and RD29B) expression. So, the adaptation to drought stress in AtVDAC2-OE plants may be regulated by overexpression of VDAC2 related to ABA signal transduction.

Discussion

VDAC is the most abundant protein of the outer mitochondrial membrane and is involved in transportation across the mitochondrial outer and inner membranes. Although studies have revealed the functions of VDACs in various physiological processes during the respiratory process (Graham and Craigen, 2004), glucose homeostasis and reproduction (Craigen and Graham, 2008), and seed germination under low temperature conditions (Yang et al., 2011), little is known about the involvement of VDAC in abiotic stress responses. The findings in the current study demonstrated that the overexpression of AtVDAC2 enhanced ABA sensitivity during seed germination and root elongation and increased the accumulation of H$_2$O$_2$, promoting adaptive drought resistance in Arabidopsis.

Drought is one of the major abiotic stresses that can trigger severe damage to plants. Most plants generate ABA in response to drought. The ABA is a vital hormone that regulates plant growth, leaf abscission, seed and bud dormancy, and stress responses, and many drought- or ABA-induced genes, such as RCAR1, ABI1, RD29B, etc. (Park et al., 2009; Ma et al., 2009; Szostkiewicz et al., 2010). However, the interactions between ABA signalling and the drought stress response are still not clear, and it is imperative to identify novel genes that may be involved in plant adaptation to drought stress and the ABA response. In this study, we identified VDAC as an ABA-sensitive target of the drought stress response in Arabidopsis. In Arabidopsis, drought stress may initiate responses to either ABA-dependent or ABA-independent signal transduction (Yamaguchi-Shinozaki and Shinozaki, 2006). We found that overexpression of AtVDAC2 enhanced ABA sensitivity, in terms of seed germination and root elongation and improved drought resistance. The opposite was the AtVDAC2 knockdown. These data support that VDAC is involved in mediating the ABA signalling pathways in response to drought stress. Additionally, the expression levels of upstream components of the ABA-signalling pathway, such as RCAR1 and ABI1 and downstream components such as RD29B were all increased in AtVDAC2-OE plants and decreased in AtVDAC2-Dn plants. These expression patterns suggested that AtVDAC2 most likely functions upstream of these components. Consistent with these results, RCAR proteins, of which RCAR1 was up-regulated in response to VDAC2 overexpression, are essential for ABA signal transduction and are able to bind to the PP2Cs, upstream drought- and/or ABA-responsive targets that initiate downstream responses to drought stress (Park et al., 2009; Ma et al., 2009; Szostkiewicz et al., 2010).

Fig 1. Relative expression of VDAC2 in transgenic lines of Arabidopsis by qPCR. Relative expression of VDAC2 was analysed by the ΔCT method after normalization of the CT of VDAC and related genes to CT of β-actin. The WT is Arabidopsis thaliana of the Reschiev (RLD) ecotype. The OE2 and OE4 are plants with AtVDAC2 overexpression, while Dn2 and Dn8 are plants with AtVDAC2 knockdown.
Moreover, the elevated expression of *RCAR1* in *ArVDAC2*-OE plants may stimulate expression of downstream targets of ABA signalling. This was confirmed by the significantly increased expression of *RD29B* in *ArVDAC2*-OE plants (conversely, decreased expression in *ArVDAC2*-Dn plants). Thus, these results strongly suggested that *ArVDAC2* may confer drought resistance to plants via ABA signalling.

Drought stress causes endogenous ABA biosynthesis, and in turn, ABA triggers ROS production to mediate downstream responses (Pei et al., 2000; Zhang et al., 2001; Kwak et al., 2003). In this study, we observed that NEC3 expression increased, which may induce more endogenous ABA biosynthesis. The ROS production (i.e., H$_2$O$_2$) was promoted in plants after ABA treatment. These data indicated the involvement of *ArVDAC2* in ROS-mediated ABA signal transduction.

In summary, the findings of this study demonstrated the functions of *ArVDAC2* in the ABA-dependent drought stress response. Upregulation of the *ArVDAC2* gene induced NEC3 gene expression, triggering ABA biosynthesis and activating the ABA signal transduction pathway. This promoted the expression of *RCAR1*, *ABI1*, and *RD29B*. Furthermore, *ArVDAC2* also activated ROS production (i.e., H$_2$O$_2$) by increasing ABA biosynthesis. Therefore, in *ArVDAC2*-OE plants, the ABA signals were conducted fast and smoothly, leading to the plants sensitive to ABA and tolerant to drought stress. However, in *ArVDAC2*-Dn plants, the ABA signalling path were blocked by the decreased expression of *ArVDAC2* and the plants remained sensitive to drought stress. Through these mechanisms, plants were able to adapt to drought stress more effectively, revealing an important role for *ArVDAC2* in the drought stress response. We show that *ArVDAC2* improved the ABA synthesis related gene (NECD3) expression. Further research required to understand the mechanism and identify whether there are other signalling molecules involved.

**Materials and Methods**

**Plant materials**

*Arabidopsis thaliana* wild-type and transgenic plants used throughout this study were Reschiev (RLD) ecotype. *ArVDAC2* transgenic *Arabidopsis thaliana* plants were stored in our lab (Yan et al., 2009). We selected 2 *ArVDAC2*-OE lines (OE2 and OE4) and 2 *ArVDAC2*-Dn lines (Dn2 and Dn8) for phenotypic analysis. Wild-type RLD plants were used as the control. Plants were routinely grown in a growth chamber under 60% humidity at 23°C with a 16-h light/8-h dark photoperiod (250 μmol/m$^2$/sec$^{-1}$) in pots containing 1:1 vermiculite: soil mixture. For in vitro culture, seeds were surface sterilized in 75% alcohol solution for 15 s and 0.1% HgCl$_2$ for 5 min and were then washed 3 times in sterile distilled water. Stratification of the seeds was conducted during day 3 at 4°C, unless otherwise indicated. Afterwards, seeds were sown on Murashige and Skoog (MS) plates containing solid medium composed of MS basal salts and 3% Glc, solidified with 0.7% agar, and adjusted to pH 5.7 with KOH before autoclaving. Plates were sealed and incubated in a controlled-environment growth chamber.

**Quantitative real-time PCR**

Seeds from wild-type and transgenic plants were grown in pots. Plant material was collected and frozen in liquid nitrogen. Total RNA for quantitative reverse transcription (RT)-PCR was
The root continuously supplied with water, primer pairs, Tiangen Biotech, −8°C ed by cooling, seedlings were extracted at room. Ace qPCR RT Kit (TOYOBO, Osaka, Japan). The primer pair sequences for target genes are listed in Table 1.

Quantitative real-time PCR was conducted as follows: The cDNA reverse transcribed from RNA was used as a template for each real-time PCR reaction. Primer pairs were used at a final concentration of 0.3 μmol/L in a total reaction volume of 20 μL containing 10 μL 2x SYBR Green Realtime PCR Master Mix (TOYOBO). The DNA polymerase was activated by heat at 95°C for 2 min followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 15 s, and elongation at 72°C for 15 s. Data were collected on an iCycler instrument (Bio-Rad, Hercules, CA USA). All reactions were set up in triplicates so that comparable Ct (threshold) values could be obtained and also to calculate the standard deviations (S.Ds). The authenticity of the amplified product in each case was verified by performing melt curve analysis immediately after the quantitative PCR protocol. Quantitative analysis of the raw data was conducted in iQ5 software (Bio-Rad).

Germination and root growth assays

To measure ABA sensitivity, seeds were plated on MS medium with or without 0.7 μM ABA. Germination characteristics were observed every 24 h for 7 days. The experiments were repeated at least 3 times. The root growth assay for scoring ABA sensitivity was conducted by measuring root growth at 14 days after transferring of 3-day-old seedlings onto vertical MS plates containing 0.5 μM ABA.

3,3-diaminobenzidine (DAB) staining of tissues

H2O2 was visually detected in leaves using 3,3-diaminobenzidine (DAB) as a substrate (Thordal-Christensen et al., 1997). Briefly, seedlings were grown for 2 weeks on MS plates containing 0.5 μM ABA. Roots were then soaked in a 1 mg/mL solution of DAB, pH 3.8, for 8 h under light at 25°C. The seedlings were continually supplied with DAB solution until the experiments were terminated by immersion in boiling ethanol (96%) for 10 min. This treatment decolorized the leaves except for the deep brown polymerization product produced by the reaction of DAB with H2O2. After cooling, seedlings were extracted at room temperature with fresh ethanol for 4 h, preserved at room temperature in ethanol, and photographed.

Analysis of the effects of drought on Arabidopsis thaliana

Four-week-old Arabidopsis plants (wild-type and transgenic) were not watered for 3 weeks. After this time, images were taken to document morphological differences between plants. Plants were then rescued with irrigation for 1 week, and differences between plants were observed and recorded photographically. At the same time, counted the survival plants and analyzed the survival ration. The experiments were repeated 3 times.

Conclusion

Study of plant response to drought is becoming increasingly important, as drought is the most important environmental constraints to plant survival and to crop productivity. The understanding of drought stress signal transduction in plants is necessary to improve management practices in agriculture. Fortunately, many studies have been performed to build the net of drought stress signal transduction. But some details are still not clear. To supplement fundamental studies on drought stress

Fig 4. DAB Staining of VDAC transgenic Arabidopsis plants after ABA treatment. The WT is Arabidopsis thaliana of the Reschiev (RLD) ecotype. The OE are plants with AtVDAC2 overexpression, while Dn are plants with AtVDAC2 knockdown.

Fig 5. Relative expression of ABA signalling related genes in transgenic lines of Arabidopsis by qPCR. Relative expression of RCAR1 (A), ABI1 (B), RD29B (C), NECD3 (D) was analysed by the ΔCT method after normalization of the CT of VDAC and related genes to that CT of β-actin. The WT is Arabidopsis thaliana of the Reschiev (RLD) ecotype, OE2 and OE4 are plants with AtVDAC2 overexpression, while Dn2 and Dn8 are plants with AtVDAC2 knockdown.

extracted from Arabidopsis 4-week-old seedlings using Trireagent (TIANGEN RNA Plant Kit, Tiangen Biotech, Beijing, China), according to the manufacturer’s protocol. A 2-μg sample of total RNA from each sample was reverse transcribed to cDNA with ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). The primer pair sequences for target genes are listed in Table 1.
signal transduction in plants, we analysed ABA sensitivity of *A. thaliana* in drought stress condition using transgenic plant lines, including overexpressing AtVDAC2 (AtVDAC2-OE) or with knockdown of AtVDAC2 (AtVDAC2-Dn). We found that by changing the RNA expression of AtVDAC2 in transgenic plant lines, the sensitivity of ABA and drought stress is changed. The overexpressing AtVDAC2 plant lines showed inhibited seed germination and relative root length, while the AtVDAC2-Dn plant lines were quite the reverse. We also found that hydrogen peroxide and ABA related genes expression were changed as well. These findings indicate a novel functional link between changes in AtVDAC2 and modulation of ABA and drought stress signal transduction. The excessive expression of AtVDAC2 gene reinforces ABA and drought stress signalling, which inhibit the expression of AtVDAC2 gene that suffocates these signals.

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