African Journal of Biotechnology Vol. 10(55), pp. 11588-11593, 21 September, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.648
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Arabidopsis thaliana VDAC2 involvement in salt stress response pathway

Guo-qin Wen[1,2], Liang Cai[1], Zhen Liu[1], De-kuan Li[1], Qin Luo[1], Xu-feng Li[1], Jian-mei Wan[1] and Yi Yang[1*]

[1]Key Laboratory of Bio-Resources and Eco-Environment, Ministry of Education, College of Life Science, State Key Laboratory of Hydraulics and Mountain River Engineering, Sichuan University, Chengdu 610064 China.

[2]College of Life Science , China West Normal University , Nanchong 637002 China.

Accepted 6 June, 2011

Soil salinity seriously affects plants distribution and yield, while salt stress induces SOS genes, and voltage-dependent anion channels (VDAC) and a mitochondrial porin, are induced too. In this paper, phenotypes of *AtVDAC2* transgenic lines and wild type (RLD) were analyzed. It was found that *AtVDAC2* over-expressing transgenic plants were more sensitive to NaCl, and produced more H_2O_2 in the NaCl treatment. Also, to find the inner reason, the salt overly sensitive gene 3 (*SOS3*) expression level was changed with the expression of *AtVDAC2*. So, it was conjectured that the signal of salt stress response was first sent to *AtVDAC2*, then *AtVDAC2* expression improved, leading to the down-stream signals changes, such as accumulation of H_2O_2 and improved expression of *SOS3*. So, it was found that in the over-expression of transgenic lines with *AtVDAC2* up-regulation, *SOS3* expression increased significantly, and in the inhibited-expressing lines, it was vice versa. In summary, *AtVDAC2* was involved in salt stress signaling pathway, and it regulated *SOS3* gene expression.

Key words: *Arabidopsis thaliana,* voltage-dependent anion channels (VDAC), salt stress, signaling pathway.

INTRODUCTION

Soil salinity seriously affects plants distribution and yield. High levels of Na^+ are toxic to plants because of their adverse effects on cellular metabolism and ion homeostasis (Niu et al., 1995). Therefore, to maintain low level of Na^+ in the cell, specifically in the cytoplasm, is essential to plants (Hasegawa et al., 2000). *SOS1*, *SOS2* and *SOS3* regulated cellular ion homeostasis under salt stress (Liu and Zhu, 1997; Ishitani et al., 2000; Halfter et al., 2000; Shi et al., 2000). *SOS3* is a myristoylated calcium-binding protein that responds to salt-induced cytosolic Ca^{2+} elevations (Liu and Zhu, 1997; Ishitani et al., 2000). SOS3 interacts directly with *SOS2*, a serine /threonine protein kinase (Halfter et al., 2000). In this signaling pathway, *SOS1* accepts the message from SOS2. SOS1 is a Na^+/H^+ antiporter localized plasma membrane (Shi et al., 2000). On one hand, in salt stress, adjustment of intracellular Na^+ balance depends on the SOS1. On the other hand, NHX1 of vacuole membrane transfers intracellular Na^+ into vacuole. These reduce the cellular damage of Na^+.

Also, some anion channels are salt induced protein that protects the cell. Voltage-dependent anion channel, a mitochondrial porin, mediating the release of mitochondrial proteins, such as cyto c (Shimizu et al., 1999; Sugiyama et al., 2002), is confirmed to response to NaCl in maize (Geiger et al., 1999), rice (Bitar et al., 2003) and so on. However, function of salt induced VDAC protein in salt stress signaling pathway needs further research.

There are so many reports on the cloning and characterization of mammalian, fungal and Drosophila VDACs (Blachly-Dyson et al., 1994; Blachly-Dyson et al., 1993; Kleene et al.; 1987) Ryerse et al., 1987; Troll et al., 1992). Most of the plant VDACs had been studied for).

*Corresponding author. E-mail: yangyi518@126.com. Tel: +86-28-85410957. Fax: +86-28-85410957.

Table 1. Primers of real-time quantitative PCR.

Gene		Primer Sequence (5'→3')	Product (bp)
*β-Actin (*At1g49240)	F	GATGAAGCTCAATCCAAACGA	228
	R	AGCAGGGGCATTGAAAGTCT	
AtVDAC2 (At5g67500)	F	GCTGATGTTGCCACCCAATACAA	105
	R	TGGGAGGATCTCGGTAAGTGTGACT	
SOS3 (At5g24270)	F	GCCATTCACGGTAGAAGAAGTGGAG	213
	R	AAGGACCGGACAAATTCACCAAAC	

their electrophysiological properties after purifying these proteins from plant extracts (Aljamal et al., 1993). However, the cloning and sequence analysis of genes encoding for plant VDACs had been reported only in few plant species, such as potato (Heins et al., 1994), wheat (Elkeles et al., 1995), maize (Geiger et al., 1999) and rice (Bitar et al., 2003) and recently, a family of VDACs was characterized from a legume, *Lotus japonicus* (Wandrey et al., 2004). The expressing levels of these VDACs were detected in all tissues and it was found that there was a different expression of different isoforms in different tissues.

The analysis of expression revealed that VDAC affected plant response to different stresses, including drought, heat shock, salinity (Wang et al., 2006; Desai et al., 2006), as well as defense against pathogen (Tateda et al., 2009).

Arabidopsis thaliana VDAC family have five members and they are highly homology. *AtVDAC2* was chosen as the object to focus on due to its display in salt stress signaling pathway.

In this paper, it was reported that *AtVDAC2* is involved in the salt signaling pathway. *AtVDAC2* over-expressing transgenic *Arabidopsis* was sensitive to NaCl, while the inhibited lines were less sensitive. And the expression level of the salt stress related genes *SOS3* was detected by real-time quantitative PCR. The results show that it was up-regulated in the *AtVDAC2* over-expression lines, as it was down-regulated in the inhibited lines.

Studies to understand the role of *AtVDAC2* in *Arabidopsis* development in salt stress adaptation are underway.

MATERIALS AND METHODS

Plant material and growth conditions

All *Arabidopsis* plants in this study were of the ecotype background accessions Reschiev (RLD). *AtVDAC2* transgenic *Arabidopsis* plants were grown on perlite/soil mixture in growth chambers at 23℃ with 16 h light (250 μmol/m^2·s^1) and 60% humidity (Yan et al., 2009). Two over-expressing lines (V1+2, V1+4), two inhibited lines (V1-2, V1-8) and RLD as the control were analyzed in this experiment.

Evaluation of salt sensitive

Seeds of transgenic and wild type plants after vernalization in 4℃ for 3 days were surface sterilized with 0.1% mercuric chloride and sown on filter paper soaked in 200 mmol/L NaCl solution. The germination characteristics were observed every 24 h. The experiments were repeated for three times.

Seeds after vernalization and sterilization were sown on Murashige and Skoog (MS) agar plates, then the plates were vertically placed in incubator. After 3 days, almost the same seedlings were transplanted to the plates of MS + 200 mmol/L NaCl. Also, the plates were vertically placed in incubator with full light. 14 days later, the length of their root was measured.

Gene expression analysis

Total RNA was isolated from 4-weeks-old *Arabidopsis* seedlings with RNAplant Kit (TIANGEN). 2 μg sample of total RNA from each sample was reverse transcribed to cDNA with ReverTra Ace qPCR RT Kit (TOYOBO). The primer pair sequences for target genes are listed in Table 1. The quantitative real-time PCR was set up as follows: cDNA reverse transcribed from 100 ng of RNA was used as template for each real-time PCR reaction; primer pairs were at 0.3 umol/L; total reaction volume was 20 μl with 10 μL 2×SYBR Green Realtime PCR Master Mix (TOYOBO). The DNA polymerase was activated by heat at 95℃ for 2 min followed by 40 cycles, denaturation at 95℃ for 10 s, annealing at 55℃ for 15 s and elongating at 72℃ for 15 s. Data were collected with Icycler (Bio-Rad, USA). All the reactions were set up in triplicates so that comparable Ct (threshold) values could be obtained and also the standard deviations could be calculated (S.D.s). The authenticity of the amplified product in each case was verified by performing a melt curve analysis immediately after the quantitative PCR. The quantitative analysis of the raw data was done by Bio-Rad iQ5 software.

Detection of H_2O_2 in plants with NaCl treatment

After the seedlings were grown for 2 weeks on MS plates (NaCl 100 mmol/L), H_2O_2 in the seedlings were detected. H_2O_2 was visually detected in the leaves of plants by using 3,3-diaminobenzidine (DAB) as substrate (Thordal-Christensen et al., 1997). Briefly, seedlings root were soaked in a 1 mg/ml solution of DAB, at pH 3.8, for 8 h under light at 25℃. The seedlings were continually supplied with DAB solutions 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 H_2O_2. After cooling,

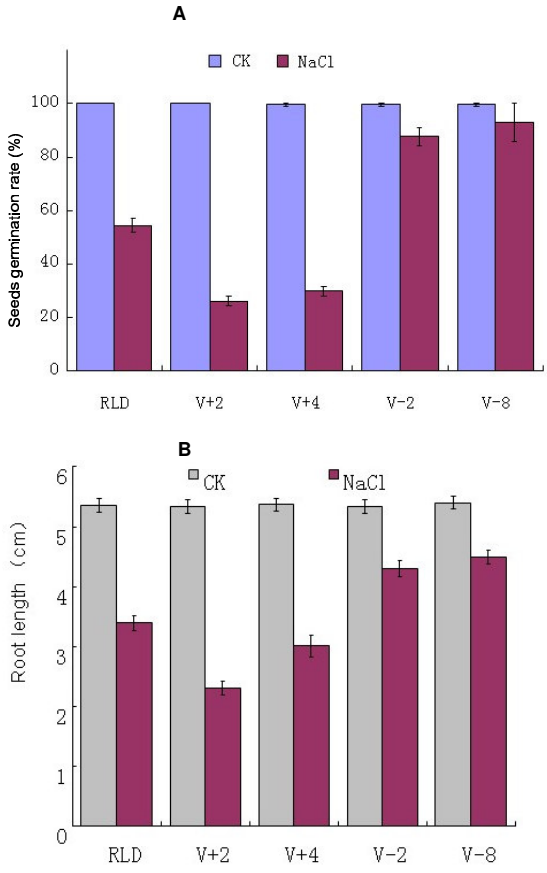

Figure 1. Salt sensitive in *AtVDAC2* transgenic *Arabidopsis* and wild type. RLD was the wild type which is the control, V-2, V-8 were the inhibited-expressing lines, and V+2, V+4 were the over-expressing lines. A, *AtVDAC2* transgenic *Arabidopsis* seeds germination rate with NaCl treatment. CK was the control group treated with H_2O; NaCl was the treatment group with 200 mmol/L NaCl. Statistics of the experiment were done on the third and tenth day of germination. Each test was repeated three times (n >80). B, Root length of *AtVDAC2* transgenic *Arabidopsis* and wide type in NaCl treatment. CK was the control group treated with H_2O; NaCl was the treatment group with 100 mmol/L NaCl. Statistics of the experiment were done after the seedling grew on the MS + NaCl plates for 2 weeks. Each test was repeated three times (n = 15).

seedlings were extracted at room temperature with fresh ethanol for 4 h and were preserved at room temperature in ethanol and photographed.

RESULTS AND DISCUSSION

AtVDAC2 over-expressing *Arabidopsis* seeds sensitivity to NaCl

Seeds on the filter paper of water germinated fast and neatly. Seeds germinated on the 3rd day. The over and inhibited expressing lines had no significant difference. But the seeds with NaCl treatment germinated slowly and differently. First, the over-expressing seeds germinated slowly, and they were sensitive to NaCl as their germination rate was about 40%. The fastest to germinate were the inhibited-expressing seeds, with less sensitive to NaCl, and the germination rate was about 90%. The speed of germination and germination rate of wild type was between the over and inhibited expressing lines. These differences were associated with *AtVDAC2* expressing levels. The results showed that *AtVDAC2* was involved in the NaCl stress response pathway (Figure 1A).

To be sure of the differences of the *AtVDAC2* over and inhibited expression transgenic lines in NaCl stress, the

Figure 2. *AtVDAC2* transgenic *Arabidopsis* stained with DAB under NaCl treatment. Left, over-expressing line; middle, wild type (RLD); right, inhibited-expressing line.

root length was measured. Robust seedlings were picked to the MS + NaCl plates after grown on MS plates for 3 days. Two weeks later, their root length was not same. Over-expressing lines were more sensitive to NaCl than the inhibited lines and wild type, as the root length of the over-expressing lines were shorter. The inhibited lines were less sensitive, as their root length was longer than the others (Figure 1B). This result showed that *AtVDAC2* was involved in the NaCl stress response pathway.

AtVDAC2 over-expressing lines production of more H_2O_2 in NaCl stress

Salt stress leads to oxidative stress in plant by inducing superoxide free radicals which cause cell membrane dysfunction (Hasegawa et al., 2000). H_2O_2 is an important superoxide free radical which cumulates in many stress response, including NaCl stress. To understand the function of *AtVDAC2* in NaCl stress, the H_2O_2 was detected in *AtVDAC2* transgenic lines by DAB staining.

In this experiment, it was found that the stains of the over-expressing lines were dark and widely distributed and those of the inhibited were light (Figure 2). So, it showed that there were more H_2O_2 in the over-expressing lines and less in the inhibited-expressing ones. This means that the signals of NaCl stress response in over-expressing lines passed more quickly and smoothly. However, the signals were blocked in the inhibited ones. This result was similar to the research of Tateda et al. (2009) in *Nicotiana benthamiana* VDAC. In their research, H_2O_2 accumulation responded to *Pseudomonas cichorii* of inhibited-expressing lines which decreased much more than the control. Yet, that of over-expressing lines was increased. Thus, it was shown that in NaCl stress, up-regulated *AtVDAC2* leads to strengthening of H_2O_2 accumulation, while down-regulated *AtVDAC2* leads to reduction of H_2O_2 accumulation.

Change in expressing level of *AtVDAC2* leads to change in expressing level of *SOS3* in *AtVDAC2* transgenic lines

The expressing levels of *AtVDAC2* and *SOS3* (Figure 3) of AtVDAC2 transgenic *Arabidopsis* and RLD were analyzed by real-time quantitative PCR. The results showed that the expressing level of *AtVDAC2* in over-expressing lines was more than 100-fold of the wild type, while that of the inhibition lines was about 70% of the wild type. The expressing level of *SOS3* in over-expressing lines was more than 50-fold of the wild type, while that of the inhibited-expressing lines was less than 20% of the wild type. The up-regulated *AtVDAC2* expression caused the increase of *SOS3* expression. On the contrary, the down-regulated *AtVDAC2* caused the decrease of *SOS3* expression. SOS3, responding to salt-induced cytosolic Ca^{2+} elevations (Liu and Zhu, 1997; Ishitani et al., 2000), interacts directly with SOS2, a serine/threonine protein kinase (Halfter et al., 2000). SOS1, an Na^+/H^+ antiporter localized plasma membrane (Shi et al., 2000), adjusts intracellular Na^+ balance. In the over-expressing transgenic lines with *AtVDAC2* up-regulation, *SOS3* expression increased significantly, and the inhibited-expressing lines were vice versa. These results explained the phenomenon of *AtVDAC2* transgenic plants in salt stress.

Qiu et al. (2002, 2004) proposed that, in salt stress, Ca^{2+} as intracellular second messenger, send out the stress signals to SOS pathway, causing SOS3 activation, and then the *SOS3* can activate *SOS1* and NHX1 to ease the damage of Na^+. In our experiment, it was thought that in the over-expressing lines, *SOS3* gene was up-regulated, resulting in the rapid transduction of stress signals, and that of the inhibited-expressing lines was down-regulated, resulting to signals loss, or blockage of the passage. So, sensitivity of *AtVDAC2* transgenic *Arabidopsis* to NaCl changed. As a result, it was thought that *AtVDAC2* is located upstream of NaCl stress response pathway, sending the salt stress signals down to *SOS3*.

Conclusion

As a result of sessile mode of life, plant is exposed to extreme environments including biotic and abiotic stresses, such as water shortages, salinity, temperature stress and so on. It was reported that expression of VDACs was changed in response to stresses. Four

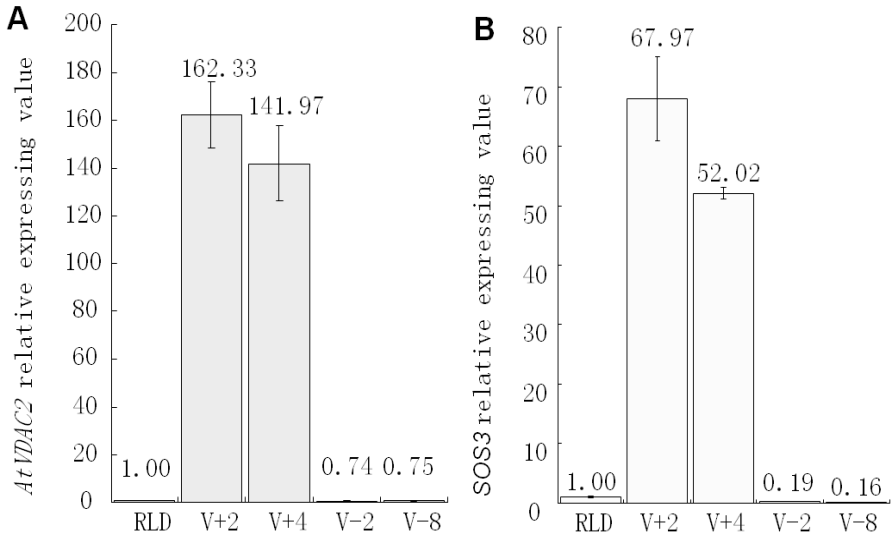

Figure 3. Relative expressing quantity of *AtVDAC2* and *SOS3* in transgenic lines of *Arabidopsis* by real-time quantitative PCR. A, *AtVDAC2* relative expressing value; B, *SOS3* relative expressing value. The expressing quantity of *AtVDAC2* and *SOS3* was analyzed by ΔCT method, ΔCT was gotten by the CT of *AtVDAC2* and *SOS3* to reduce the CT of *β-actin*, then the relative expressing values were accounted for by ΔCT.

isoforms (At3g012080, At5g15090, At5g57490 and At5g67500) of *Arabidopsis* VDACs were down-regulated in high salt stress but not affected by cold and drought stresses (Lee et al., 2009). Some other researchers found that in heat or aging-related cell death, *Arabidopsis* VDAC (At5g15090) protein was up-regulated (Swidzinski et al., 2002, 2004). Rice VDACs were up-regulated in osmotic, salt and drought stresses. Rice VDACs were up-regulated too in the recovery phase of osmotic stress in the root (Bitar et al., 2003). Pearl millet *VDAC*, as a salinity-inducible gene, is also up-regulated by drought, cold and salicylic acid but not by abscisic acid (Desai et al., 2006). VDAC is also an early HR marker gene in *A. thaliana* (Lacomme and Roby, 1999). Three isoforms of *N. tabacum* VDACs are up-regulated in disease resistance (Tateda et al., 2009).

As mentioned earlier, stresses caused VDAC expression change. These results suggest that increased expression of some VDACs can improve the resistance of plants to the environment but there was no clear experimental evidence to explain why VDAC adjust plant stress resistant.

Seed germination and root growth, H_2O_2, *AtVDAC2* and *SOS3* expressing levels in *AtVDAC2* transgenic lines were observed. The results showed that over-expressing transgenic plants were more sensitive to NaCl, as *AtVDAC2* was in the signal transduction. Over-expressing transgenic plants can produce more H_2O_2 in NaCl stress. Also, it was found that the *SOS3* expressing level was changed with *AtVDAC2*. So it was conjectured that in salt stress signaling, the stress signal is first sent to VDAC, and then VDAC expression changes, leading to the down-stream signals change, such as H_2O_2 and *SOS3*. It is known that *SOS3* is an Ca^{2+}-depended kinase; it can send signals to *SOS2*, *SOS1* and *NHX1*. *SOS1* and *NHX1* can help cell adjust balance of intracellular Na^+/K^+. But when *AtVDAC2* is down-regulated, the signals cannot pass down and the stress resistance is weakened.

So, *AtVDAC2* is involved in salt stress response pathway and it regulates *SOS3* expression. Identification of the components and the underlying mechanisms await further experimentation.

REFERENCES

Aljamal JA, Genchi G, PintoVD, Stefanizzi L, Santis AD, Benz R, Palmieri F(1993). Purification and characterization of Porin from corn (*Zea mays* L.) mitochondria. Plant Physiol. 102:15-621.

Bitar FA, Roosens N, Smeyers M, Vauterin M, Boxtel JV, Jacobs M, Homble F (2003). Sequence analysis, transcriptional and posttranscriptional regulation of the rice VDAC family. Biochim. Biophys. Acta. 1625: 43-51.

Blachly-Dyson E, Baldini A, Litt M, McCabe ERB, Forte M(1994). Human genes encoding the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane: mapping and identification of two new isoforms. Genomics. 20: 62-67.

Blachly-Dyson E, Zambronicz EB, Yu WH, Adams V, McCabe ER, Adelman J, Colombini M, Forte M (1993). Cloning and functional expressing in yeast of two human isoforms of the outer mitochondrial membrane channel, the voltage-dependent anion channel. J. Biol. Chem. 268: 1835-1841.

Desai MK, Mishra RN, Verma D, Nair S, Sopory SK, Reddy MK(2006). Structural and functional analysis of a salt stress inducible gene encoding voltage dependent anion channel (VDAC) from pearl millet (*Pennisetum glaucum*). Plant Physiol. Biochem. 44: 483-493.

Elkeles A, Devos KM, Graur D, Ziz i M, Breiman A(1995). Multiple

cDNAs of wheat voltage-dependent anion channels (VDAC): isolation, differential expressing, mapping and evolution. Plant Mol. Biol. 29: 109-124.

Geiger TR, Keith CS, Muszynski MG, Newton KJ (1999). Sequences of three maize cDNAs encoding mitochondrial voltage-dependent anion channel (VDAC) proteins (accession nos. AF178950, AF178951, and AF178952) (PGR 99-156). Plant Physiol. 121: 686.

Halfter U, Ishitani M, Zhu JK(2000). The *Arabidopsis* SOS2 protein kinase physically interacts with and is activated by the calcium- binding protein SOS3. PNAS. 97: 3735-3740.

Hasegawa PM, Bressan RA, Zhu JK (2000). Plant cellular and molecular responses to high salinity. Ann. Rev. Plant. Physiol. Mol. Bio. 51: 463-499.

Heins L, Mentzel H, Schmid A, Benz R, Schmitz UK(1994). Biochemical, molecular, and functional characterization of porin isoforms from potato mitochondria. J. Biol. Chem. 269: 26402–26410.

Ishitani M, Liu JP, Halfter U, Kim CS, Shi WM, Zhu JK (2000). SOS3 function in plant salt tolerance requires N- myristoylation and calcium binding. Plant Cell1. 2: 1667-1677.

Kleene R, Pfanner N, Pfaller R, Link TA, Sebald W, Neupert W, Tropschug M (1987). Mitochondrial porin of Neurospora crassa: cDNA cloning, *in vitro* expressing and import into mitochondria. EMBO J. 6: 2627-2633.

Lacomme C, Roby D (1999). Identification of new early markers of the hypersensitive response in *Arabidopsis thaliana*. FEBS Lett. 459: 149-153.

Lee SM, Hoang MHT, Han HJ, Kim HS, Lee K, Kim KE, Kim DH, Lee SY, Chung WS (2009). Pathogen inducible voltage-dependent anion channel (AtVDAC) isoforms are localized to mitochondria membrane in *Arabidopsis*. Mol. Cell. 27: 321-327.

Liu J, Zhu JK (1997). An *Arabidopsis* mutant with increased calcium requirement for potassium nutrition and salt tolerance. PNAS. 94: 14960-14964.

Niu X, Bressan RA, Hasegawa PM, Pardo JM (1995). Ion homeostasis in NaCl stress environments. Plant Physiol.109: 735-742.

Qiu QS, Guo Y, Dietrich MA, Schumaker KS, Zhu JK (2002). Regulation of SOS1, a plasma membrane Na^+/H^+ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. PNAS. 99: 8436-8441.

Qiu QS, Guo Y, Quintero FJ, Pardo JM, Schumaker KS(2004). Regulation of vacuolar Na^+/H^+ exchange in *Arabidopsis thaliana* by the salt-overly-sensitive (SOS) pathway. J. Biol. Chem. 279: 207-215.

Ryerse J, Blachly-Dyson E, Forte M, Nagel B (1997). Cloning and molecular characterization of a voltage-dependent anion-selective channel (VDAC) from *Drosophila melanogaster*. Biochim. Biophys. Acta. 1327: 204-212.

Shi H, Ishitani M, Kim C, Zhu JK (2000). The *Arabidopsis thaliana* salt tolerance gene SOS1 encodes a putative Na^+/H^+ antiporter. PNAS. 97: 6896-6901.

Shimizu S, Narita M, Tsujimoto Y (1999). Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature. 399: 483-487.

Sugiyama T, Shimizu S, Matsuok Y, Yoneda Y, Tsujimoto Y(2002). Activation of mitochondrial voltage-dependent anion channel by apro-apoptotic BH3-only protein Bim. Oncogene. 21: 4944-4956.

Swidzinski JA, Leaver CJ, Sweetlove LJ (2004). A proteomic analysis of plant programmed cell death. Phytochemistry. 65: 1829-1838.

Swidzinski JA, Sweetlove LJ, Leaver CJ (2002). A custom microarray analysis of gene expressing during programmed cell death in *Arabidopsis thaliana*. Plant J. 30:431-446.

Tateda C, Yamashita K, Takahashi F, Kusano T, Takahashi Y (2009). Plant voltage-dependent anion channels are involved in host defense against *Pseudomonas cichorii* and in Bax-induced cell death. Plant Cell Rep. 28: 41-51.

Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB (1997). Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley—powdery mildew interaction. Plant J. 11: 1187-1194.

Troll H, Malchow D, Muller-Taubenberger A, Humbel B, Lottspeich F, Ecke M, Gerisch G, Schmid A, Benz R (1992). Purification, functional characterization, and cDNA sequencing of mitochondrial porin from *Dictyostelium discoideum*. J. Biol. Chem. 267: 21072-21079.

Wandrey M, Trevaskis B, Brewin N, Udvardi MK (2004). Molecular and cell biology of a family of voltage-dependent anion channel porins in *Lotus japonicus*. Plant Physiol. 134: 1-12.

Wang J, Zhang LD, Zuo KJ, Oian HM, Cao YF, Tang KX (2006). Cloning and expressingal studies of the voltage-dependent anion channel gene from *Brassica ra*pa L. J. Integr. Plant Biol. 48: 197-203.

Yan J, He H, Tong S, Zhang W, Wang J, Li X, Yang Y (2009), Voltage-Dependent Anion Channel 2 of *Arabidopsis thaliana* (*AtVDAC2*) Is Involved in ABA-Mediated Early Seedling Development. Int. J. Mol. Sci. 10: 2476-2486.