

Molecular and Cell Biology of a Family of Voltage-Dependent Anion Channel Porins in *Lotus japonicus*¹

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Voltage-dependent anion channels (VDACs) are generally considered as the main pathway for metabolite transport across the mitochondrial outer membrane. Recent proteomic studies on isolated symbiosome membranes from legume nodules indicated that VDACs might also be involved in transport of nutrients between plants and rhizobia. In an attempt to substantiate this, we carried out a detailed molecular and cellular characterization of VDACs in *Lotus japonicus* and soybean (*Glycine max*). Database searches revealed at least five genes encoding putative VDACs in each of the legumes *L. japonicus*, *Medicago truncatula*, and soybean. We obtained and sequenced cDNA clones from *L. japonicus* encoding five full-length VDAC proteins (LjVDAC1.1–1.3, LjVDAC2.1, and LjVDAC3.1). Complementation of a yeast (*Saccharomyces cerevisiae*) mutant impaired in VDAC1, a porin of the mitochondrial outer membrane, showed that LjVDAC1.1, LjVDAC1.2, LjVDAC2.1, and LjVDAC3.1, but not LjVDAC1.3, are functional and targeted to the mitochondrial outer membrane in yeast. Studies of the expression pattern of the five *L. japonicus* VDAC genes revealed largely constitutive expression of each throughout the plant, including nodules. Antibodies to LjVDAC1.1 of *L. japonicus* and the related POM36 protein of potato (*Solanum tuberosum*) recognized several proteins between 30 and 36 kD on western blots, including LjVDAC1.1, LjVDAC1.2, LjVDAC1.3, and LjVDAC2.1. Immunolocalization of VDACs in *L. japonicus* and soybean root nodules demonstrated their presence on not only mitochondria but also on numerous, small vesicles at the cell periphery. No evidence was found for the presence of VDACs on the symbiosome membrane. Nonetheless, the data indicate that VDACs may play more diverse roles in plants than suspected previously.

Porins are a diverse group of β -barrel proteins that fulfill a variety of functions in prokaryotes and eukaryotes. They are located in the outer membranes of Gram-negative (Delcour, 2002) and -positive bacteria (Riess et al., 1999, 2001; Lichtinger et al., 1998), mitochondria of eukaryotes (Benz, 1994), and plastids of plants (Fischer et al., 1994; for review, see Bolter and Soll, 2001). In addition to the common β -barrel fold and perhaps related to this pore-forming structure, porins share the electrophysiological property of symmetric voltage gating (Bainbridge et al., 1998). Porins can adopt two conformational states: an open state, which is selective for small anions, and a closed state, in which conductivity decreases and selectivity for cations increases. Eukaryotic porins show voltage gating at potentials of ± 20 to 40 mV, whereas an electrical potential of ± 100 mV is needed to close certain bacterial porins (Benz, 1994). The physiological significance of voltage gating remains unclear, however, given that porins are generally located in "nonenergized" membranes (Blachly-Dyson and Forte, 2001).

Despite uncertainty about the physiological significance of voltage gating in porins, this feature has been used to name one important family of these proteins, the voltage-dependent anion channel (VDAC) family in eukaryotes. VDAC protein sequence and function have been conserved during evolution. Thus, homologs have been found in yeast (*Saccharomyces cerevisiae*), animals, and plants, and VDACs from animals (Blachly-Dyson et al., 1993; Xu et al., 1999) can suppress mutations in yeast VDAC1. Genetic studies have shown that yeast VDAC1, which is located in the outer mitochondrial membrane (OMM), is essential for mitochondrial respiration (Dihanich et al., 1987; Lee et al., 1998). Similar data is lacking for plants. Evolution of multicellularity was accompanied by an expansion of the number of VDAC genes in eukaryotic genomes. This makes genetic studies of the physiological role(s) of VDACs in higher eukaryotes intrinsically more difficult than in yeast, which has only two VDAC genes, both encoding proteins of the OMM. Adding to the complexity in higher eukaryotes is the observation that VDAC porins may not be confined to the OMM. In animals, VDACs also appear to be located in the caveolae domains of the plasma membrane (Bathori et al., 1999; Buettner et al., 2000). Caveolae are microdomains that form a unique endocytotic and exocytotic compartment at the cell surface, which is ca-

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pable of importing molecules and delivering them to specific locations within the cell and exporting molecules to the extracellular space (Anderson, 1998). In plants, VDAC proteins may be located in the boundary membrane of glyoxisomes, in addition to the OMM (Corpas et al., 2000).

We were drawn to the study of VDACs in legumes after learning that they might be located in the peribacteroid or symbiosome membrane (SM) of pea (*Pisum sativum*) and *Lotus japonicus* nodules (Saalbach et al., 2002; Wienkoop and Saalbach, 2003). Given the transport properties of VDAC porins and the strategic role of the SM in controlling nutrient exchange between the plant and nitrogen-fixing bacteroids in legume root nodules (Udvardi and Day, 1997), we sought to verify these results, using a method that leaves cell structures intact. Thus, in addition to presenting the molecular and functional characterization of a family of five new legume VDACs, we present data here on the immunolocalization of VDAC proteins in root nodule cells.

RESULTS

Isolation and Sequence Analysis of Full-Length cDNAs Encoding Five Different VDACs in *L. japonicus*

Amino acid sequence data obtained from proteins of isolated SM from soybean (*Glycine max*; M. Wandrey, B. Trevaskis, and M.K. Udvardi, unpublished data), pea (Saalbach et al., 2002), and *L. japonicus* (Wienkoop and Saalbach, 2003) indicated the possible presence of VDACs in this membrane of legume root nodules. Given the broad substrate range of porins that includes dicarboxylic acids (Reumann et al., 1998), which are a primary source of carbon for nitrogen-fixing bacteroids (Udvardi et al., 1988; Udvardi and Day, 1997), we sought to confirm the SM location of these proteins. To facilitate the production of antibodies to VDACs for immunolocalization, we began by isolating partial and then full-length VDAC cDNA from soybean and *L. japonicus*, respectively. A 400-bp VDAC gene fragment was PCR amplified from soybean nodule cDNA using degenerate primers (forward, 5'-CARTAYYTICAYGARTAYGCNNGG-3'; and reverse, 5'-TTIGGICKCCAYTCRTGYTG-3'), designed from conserved regions of plant porins (*Zm-Por1*, GenBank accession no. X73429; *PsPor1*, GenBank accession no. Z25540; *POM34*, GenBank accession no. X80386; *POM36I*, GenBank accession no. X80388; *POM36II*, GenBank accession no. X80387; and *SV-DAC1*, GenBank accession no. U50900). This gene fragment, which showed 83% identity to *PsPor1*, was used to screen a nodule cDNA library from *L. japonicus* at low stringency. Six positive clones were selected and sequenced. All six clones were derived from the same gene, which we named *LjVDAC1.1*. Subsequent in silico screening of the growing *L. japonicus* expressed sequence tag (EST) databases (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov>; Kazusa DNA Research Institute, Chiba, Japan, <http://www.kazusa.or.jp>; and The Institute for Genomic Research, <http://www.tigr.org>) identified potential full-length cDNA clones encoding a further four porins. These were obtained, fully sequenced, and named *LjVDAC1.2*, *LjVDAC1.3*, *LjVDAC2.1*, and *LjVDAC3.1*. These VDACs correspond to EST clones MWM207c01 (GenBank accession no. AV411556), MWM211g08 (GenBank accession no. AV411841), MWM066g10 (GenBank accession no. AV426384), and MWM239d07 (GenBank accession no. AV414031), respectively. The GenBank accession numbers for the full-length sequences are AY316737 to AY31641, respectively.

The five *L. japonicus* VDACs encode proteins that are either 276 or 277 amino acids in length with calculated molecular masses in the range from 29 to 30 kD. They show between 44% to 88% sequence identity and form three main evolutionary branches (Fig. 1).

To identify conserved or variable domains that may be involved in secondary structure formation, targeting, or regulation of channel activity, deduced protein sequences of all five clones were aligned. In addition, secondary structure prediction using PROFsec (Rost, 1996), and a pattern search using the PROSITE database (Hofmann et al., 1999) were performed (Fig. 2). Alignment of *L. japonicus* VDAC proteins revealed highly conserved regions at the N terminus, including a loop (light-gray box under the aligned sequences) and an α -helical region (boxed H). The positions of β -sheets (dark gray) among the different VDACs are also highly conserved, although sequence conservation is not greater than in the rest of the protein. The PROSITE database pattern search revealed a 23-amino acid-long eukaryotic mitochondrial porin pattern (Pattern-ID: PS00558), beginning at position 219, for *LjVDAC1.1* to *LjVDAC1.3* (Fig. 2, underlined). This pattern was not identified in *LjVDAC2.1* or *LjVDAC3.1*.

Phylogenetic Analysis of Plant VDACs

BLAST searches (Altschul et al., 1990) of public databases identified numerous homologs of the *L. japonicus* VDACs in other higher plants, with sequence identities ranging from 58% to 81%. Searches

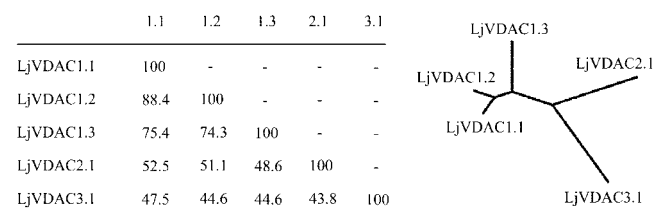


Figure 1. Homology of *L. japonicus* VDACs. Amino acid identities are given in percentages. The multiple sequence alignment and unrooted phylogenetic tree were carried out using ClustalW (Jeanmougin et al., 1998; <http://clustalw.genome.ad.jp>).

Table 1. Threshold cycle (C_T) values and relative abundance of *LjVDAC* transcripts in different organs

Transcript levels for each gene were measured by real-time RT-PCR, normalized to the level of ubiquitin transcript in the same sample, and then expressed relative to the level of *LjVDAC1.1* transcript in leaves (set at 1). C_T values (mean \pm SD, $n = 3$) are presented above the relative gene expression level in each case.

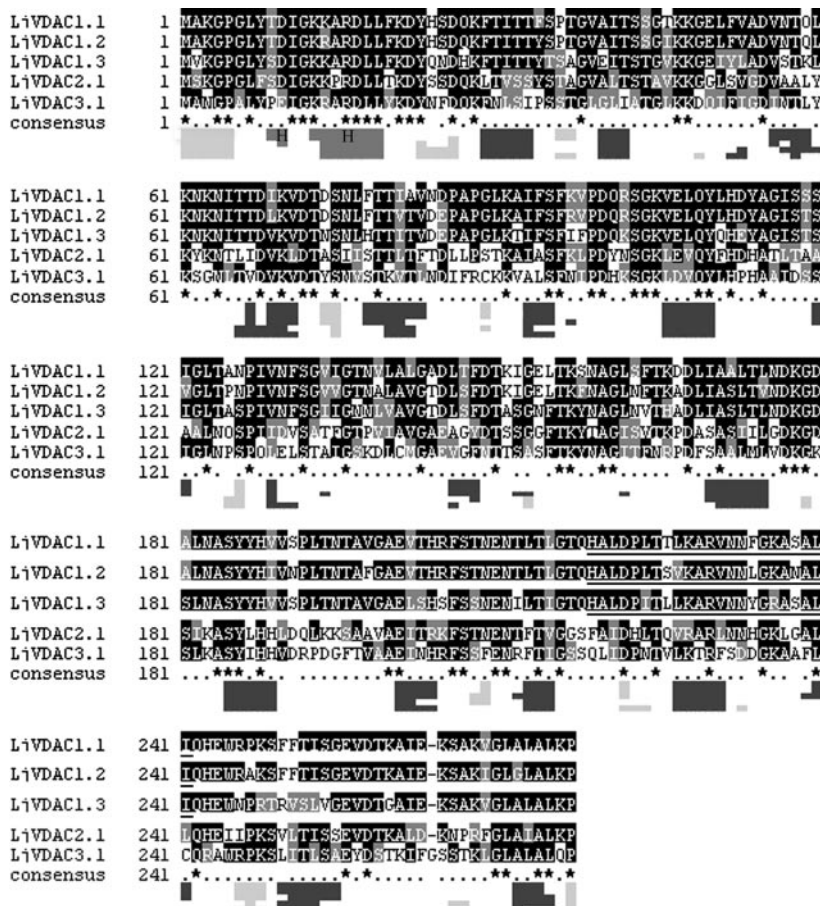
Transcript	Leaf	Root	Nodule
Ubiquitin	22.40 \pm 0.19	23.73 \pm 0.73	22.99 \pm 0.07
<i>LjVDAC1.1</i>	19.75 \pm 0.18	20.09 \pm 0.25	20.92 \pm 0.39
Ratio	1	1.99	0.67
<i>LjVDAC1.2</i>	19.04 \pm 0.08	20.49 \pm 0.12	20.66 \pm 0.25
Ratio	1.64	1.51	0.80
<i>LjVDAC1.3</i>	19.28 \pm 0.06	20.05 \pm 0.17	19.93 \pm 0.19
Ratio	1.39	2.04	1.33
<i>LjVDAC2.1</i>	19.12 \pm 0.11	19.20 \pm 0.09	19.09 \pm 0.08
Ratio	1.55	3.69	2.37
<i>LjVDAC3.1</i>	21.77 \pm 0.07	22.36 \pm 0.12	21.43 \pm 0.08
Ratio	0.25	0.41	0.47

of the Plant Gene Index at TIGR (<http://www.tigr.org>) revealed at least five different Tentative Consensus sequences corresponding to VDACs in each of soybean, *Medicago truncatula*, and *L. japonicus*. The Arabidopsis genome contains five VDAC genes. Significant homology was also found between *L. japonicus* VDACs and proteins of other eukaryotes,

including mammals, insects, nematodes, amoebae, and fungi. For instance, the VDAC from yeast (*VDAC1*, GenBank accession no. NP 014343) shares at least 18% amino acid sequence identity with *L. japonicus* porins. No sequence homology was found with prokaryotic proteins, which is typical for VDAC porins.

The phylogenetic relationships between VDACs from legumes and other plants are depicted in Figure 3. The dendrogram splits into five main branches, some of which contain monocot or dicot VDACs exclusively. Branch I contains only two plant VDACs, one from the single-celled organism *Chlamydomonas reinhardtii* and one from the legume *M. truncatula*. BLAST searches of these proteins revealed that they are more closely related to eukaryotic VDACs of fungi, mammals, and insects. Branch II contains VDACs from both dicotyledonous and monocotyledonous plants. Branches III and IV contain VDACs from dicots and monocots, respectively. Branch V is the largest branch with multiple VDAC isoforms of the dicots *L. japonicus*, soybean, *M. truncatula*, and potato (*Solanum tuberosum*). Some of these may have evolved by gene duplication after the monocot/dicot split, such as the *L. japonicus* VDACs *LjVDAC1.1* and *LjVDAC1.2*, which exhibit high sequence identity (88%).

Figure 2. Comparison of primary and secondary structure of *L. japonicus* VDACs. The multiple sequence alignment was carried out using ClustalW (Jeanmougin et al., 1998; <http://clustalw.genome.ad.jp>). Black shading indicates similar amino acids in at least three VDAC sequences. The graphic below the protein sequence alignment displays a comparison of the secondary structure of *L. japonicus* VDACs in the same order as above. Secondary structure prediction and protein pattern search were performed at the "PredictProtein server" (<http://cubic.bioc.columbia.edu/predictprotein>) with PROFsec (Rost, 1996) and PROSITE (Hofmann et al., 1999), respectively. Dark-gray boxes, β -Sheets; boxed "H," α -helical protein structures; light-gray boxes, likely formation of a loop, predicted with an average accuracy of 82%. The underlined sequences show eukaryotic mitochondrial porin signatures (PROSITE Pattern-ID: PS00558) of the *LjVDAC1.1*, *LjVDAC1.2* and *LjVDAC1.3* proteins following the pattern [YH]-x(2)-D-[SPCAD]-x-[STA]-x(3)-[TAG]-[KR]-[LIVMF]-[DNSTA]-[DNS]-x(4)-[GSTAN]-[LIVMA]-x-[LIVMY].



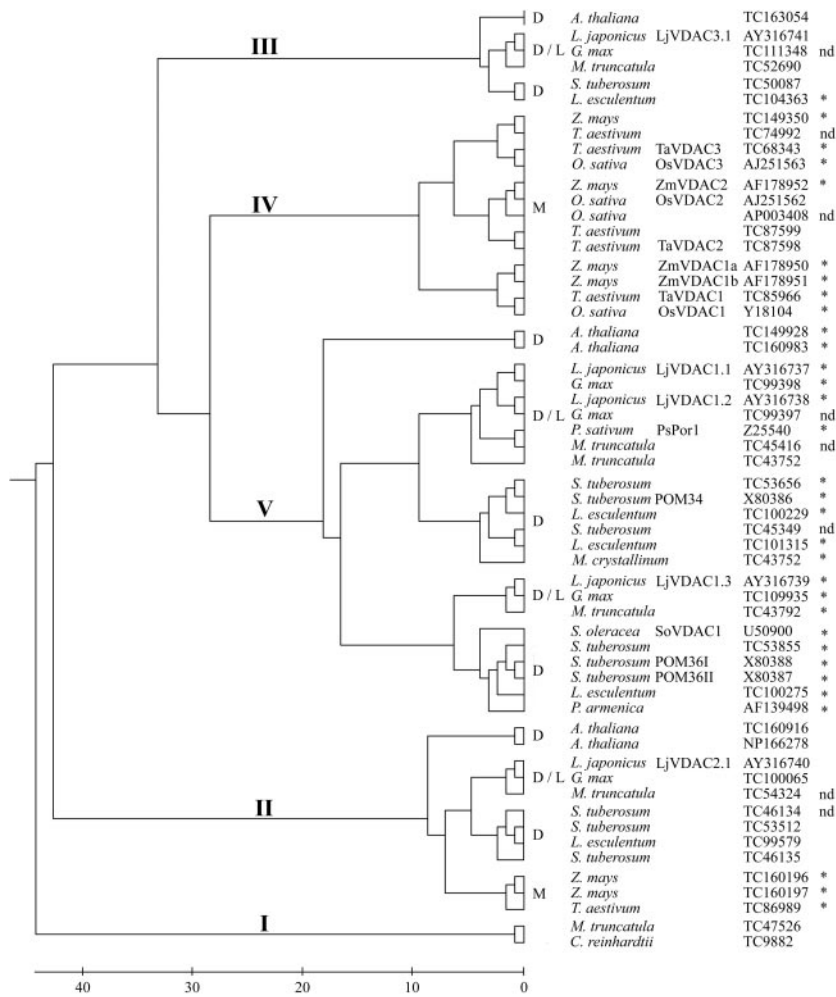


Figure 3. Phylogenetic tree of plant VDACs. Multiple alignment of plant VDAC protein sequences was performed as described in the legend of Figure 1. The phylogenetic tree was calculated applying the neighbor-joining method (Saitou and Nei, 1987; <http://clustalw.genome.ad.jp>). The scale below indicates the number of amino acid differences calculated with the open source software MEGA2 (<http://www.megasoftware.net>). VDACs of legumes (L), other dicots (D), and monocots (M) are shown. GenBank accession numbers or tentative consensus sequence (TC) numbers from the TIGR Gene Index (<http://www.tigr.org/tdb/tgi/plant.shtml>) are given. Proteins marked with an asterisk contain the eukaryotic mitochondrial porin signature (PROSITE ID: PS00558). Cases where incomplete N-terminal sequence precluded identification of the porin signature are also indicated (nd).

VDAC Expression Analysis

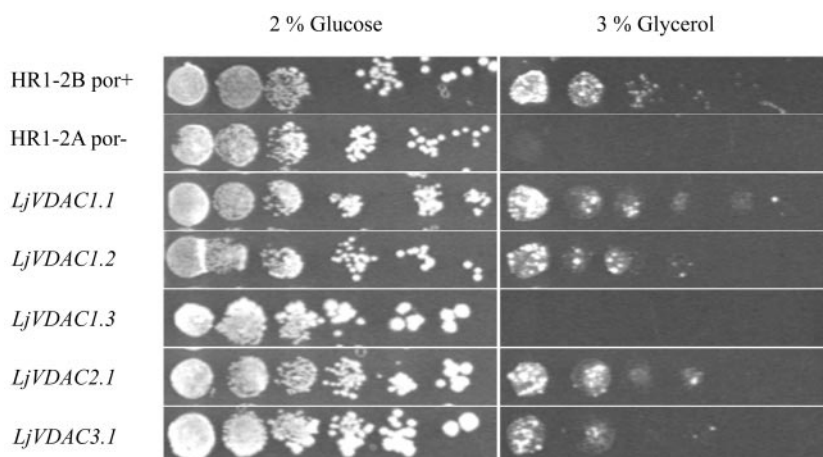
Division of labor between different proteins of the same family is often indicated by distinct patterns of gene expression in time and/or space within an organism. To determine whether specialization of *L. japonicus* VDAC function could be programmed at the level of transcription, we used RNA gel-blot analysis and real-time reverse transcriptase (RT)-PCR to monitor expression in different organs. Northern-blot analysis was performed with total RNA from leaves, roots, flowers, and nodules of 8-week-old *L. japonicus* plants and showed constitutive expression of VDAC genes throughout the plant (data not shown). However, dot-blot analysis with in vitro-transcribed RNA revealed cross-hybridization among cloned *LjVDAC* genes. Therefore, we employed real-time RT-PCR to increase specificity and sensitivity of transcript detection. Leaf, root, and nodule poly(A⁺) RNA from 8-week-old plants was reverse transcribed and the resulting cDNA subjected to PCR analysis. Primers were designed to bind to the 3'-untranslated regions of *LjVDAC1.1* to *1.3*, *LjVDAC2.1*, and *LjVDAC3.1* and tested for their

specificity before use. Transcript levels of a polyubiquitin gene, which is expressed constitutively in *L. japonicus* (Colebatch et al., 2002), were measured to facilitate normalization of data. Real-time RT-PCR revealed only slight differences in transcript levels for the various VDAC genes in different organs (Table 1). *LjVDAC2.1* was the most highly expressed and *LjVDAC3.1* the least highly expressed of the VDAC genes in different organs. However, transcript levels of *LjVDAC2.1* were never more than 10 times higher than those of *LjVDAC3.1*, and transcripts of each VDAC gene were as abundant as those of the highly expressed ubiquitin gene.

Functional Complementation of a Yeast VDAC1 Mutant

The functionality of *LjVDAC1.1* to *1.3*, *LjVDAC2.1*, and *LjVDAC3.1* was assessed by complementation of yeast mutant strain HR1-2A (Dihanich et al., 1987), which lacks VDAC1, the VDAC of the OMM. This mutant is impaired in respiration and relies mainly on fermentation for energy production. The metabolic defect results in an inability to grow on non-

Figure 4. Functional complementation of a yeast VDAC1 mutant with *L. japonicus* VDACs. The VDAC1-deficient yeast mutant strain HR1-2A was transformed with cDNAs encoding *LjVDAC1.1*, *LjVDAC1.2*, *LjVDAC1.3*, *LjVDAC2.1*, and *LjVDAC3.1*, cloned in yeast expression vector p112A1NE. As controls, wild-type yeast strain (HR1-2B) and the VDAC1-deficient mutant (HR1-2A) were transformed with the cloning vector. A series of 10-fold dilutions of overnight cultured cells, starting with an OD₆₀₀ of 1.0, was made, and 3 μ L of cells was dropped onto plates containing minimal medium and 2% (w/v) Glc or 3% (v/v) glycerol as sole carbon sources. Plates containing Glc were incubated for 3 d at 30°C, whereas glycerol plates were incubated for 6 d at 30°C.



fermentable carbon sources like glycerol. Transformation of the yeast mutant with *L. japonicus* porins *LjVDAC1.1* and *1.2*, *LjVDAC2.1*, and *LjVDAC3.1*, cloned in the expression vector p112A1NE (Riesmeier et al., 1992), suppressed the growth defect (Fig. 4). In contrast, *LjVDAC1.3* was unable to complement the mutation, although the *LjVDAC1.3* protein was expressed in the yeast mutant, as shown by western analysis (Fig. 5, A and B). The results show that all of the *L. japonicus* VDACs except *LjVDAC1.3* are functional in yeast and indicate that four of the five proteins are targeted to the OMM.

Expression of VDAC Proteins in *L. japonicus*

The analysis of VDAC expression in *L. japonicus* was extended to the protein level. Western blots were performed with affinity-purified anti-*LjVDAC1.1* and anti-POM36 antibodies (Heins et al., 1994). In preliminary experiments with proteins expressed in yeast, it was shown that both antibodies cross-react with *LjVDAC1.1*, *LjVDAC1.2*, *LjVDAC1.3*, and *LjVDAC2.1*. However, cross-reaction of the anti-*LjVDAC1.1* antibody with *LjVDAC2.1* was weak. Neither of the two antibodies recognized *LjVDAC3.1* (Fig. 5, A and B). Despite the similarities in predicted M_r of the *L. japonicus* VDACs (29–30 kD), they were separated and distinguishable on western blots. Interestingly, both antibodies recognized yeast VDAC1. Blocking of both antibodies with purified *LjVDAC1.1* protein resulted in disappearance of bands corresponding to *LjVDAC1.1* to *1.3* and *LjVDAC2.1* (data not shown).

The two antibodies were used to detect VDAC proteins in crude extracts and microsomal fractions from different organs of *L. japonicus* and soybean. Western-blot analysis using anti-*LjVDAC1.1* antibody revealed two or three distinct VDAC bands around 32 to 36 kD in extracts of leaves, roots, and nodules (Fig. 5C). In *L. japonicus* extracts, these bands may correspond to *LjVDAC1.1*, *LjVDAC1.2*, and *LjVDAC1.3*. The anti-POM36 antibody recognized these and two

additional bands at 30 and 38 kD (Fig. 5E). Both antibodies also cross-reacted with soybean VDACs. Specificity of the antibodies was tested using antibodies that were blocked with purified *LjVDAC1.1* protein. Blocked anti-*LjVDAC1.1* antibody detected no proteins on western blots of *L. japonicus* or soybean extracts (Fig. 5D), whereas blocked anti-POM36 antibody still cross-reacted with the 30- and 38-kD bands, which, therefore, might not be VDACs (Fig. 5F).

The presence of VDACs in microsomal fractions of *L. japonicus* roots and nodules was also investigated. Anti-*LjVDAC1.1* antibody detected only two protein bands in microsomal fractions from *L. japonicus* roots and nodules, instead of the three bands detected in total protein extracts (Fig. 5G). This indicated that one of the *LjVDAC* isoforms, possibly *LjVDAC1.3*, was located on a membrane that was removed by centrifugation during preparation of microsomal fractions.

Subcellular Localization of VDAC Proteins

The subcellular location of VDACs in *L. japonicus* and soybean nodules was investigated further using western blot and immunolocalization methods. SM was purified from soybean nodules via Percoll gradients (Panter et al., 2000) and aqueous two-phase partitioning (Christiansen et al., 1995). A protein was detected using anti-*LjVDAC1.1* and anti-POM36 antibodies on western blots of SM prepared using both Percoll gradients (Fig. 5G) and aqueous two-phase partitioning (Fig. 5, C and E).

To establish more clearly the subcellular location of VDACs in *L. japonicus* and soybean nodules, we carried out immunofluorescence and immunogold labeling of tissue sections, using affinity-purified anti-*LjVDAC1.1* antibody. Anti-*LjVDAC1.1* antibody bound to punctate structures in the cell periphery close to, or associated with, the plasma membrane in *L. japonicus* and soybean nodules (Fig. 6, A and B). Control labeling with antibodies against the mitochondrial protein, Cyt c (Fig. 6C); the peroxisomal

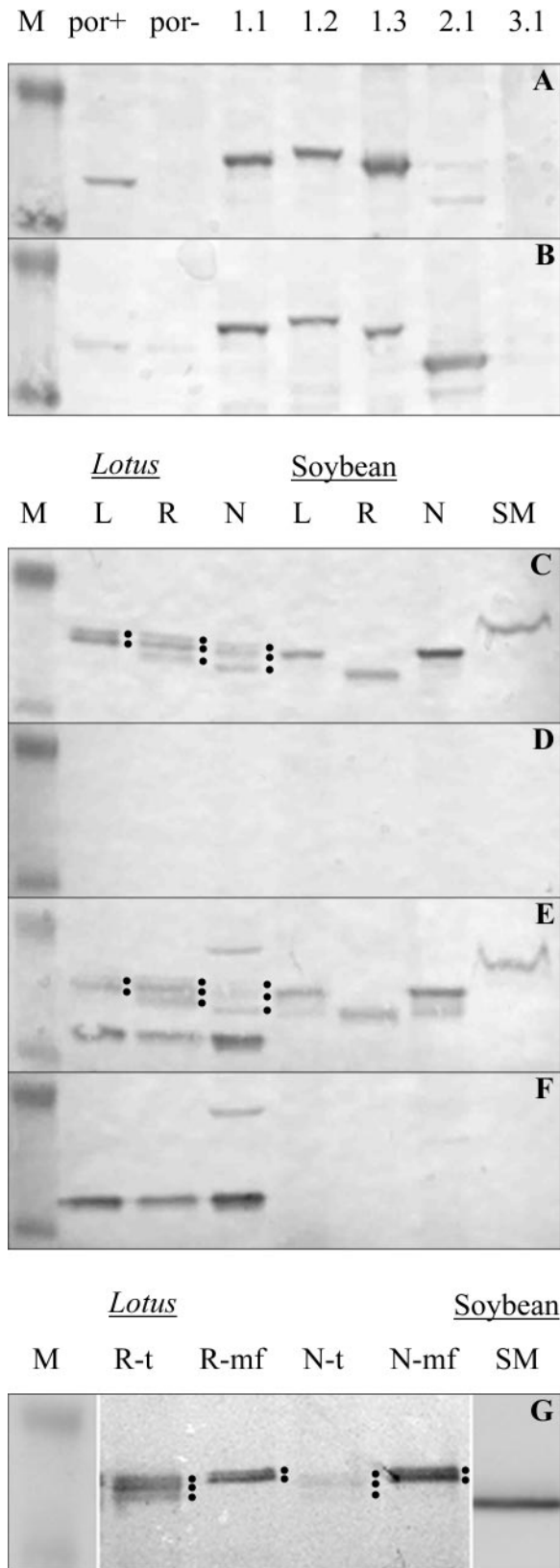


Figure 5. Western-blot analysis of *L. japonicus* VDAC expressing yeast and VDACs of *L. japonicus* and soybean. A and B, *L. japonicus* VDACs were expressed in a VDAC1-deficient yeast mutant (*por⁻*).

protein, uricase (Fig. 6D); and the plastidial protein, ferritin (Fig. 6E) were carried out on soybean nodule sections because of lack of cross-reactivity of some of the antibodies with the corresponding *L. japonicus* proteins. Peroxisomes and plastids were clearly larger (2–4 μm) than the structures labeled with anti-LjVDAC1.1. Thus, peroxisomes and plastids were not labeled by anti-LjVDAC1.1 antibody. An overlay of Cyt c labeling (Fig. 6F) and LjVDAC labeling (Fig. 6G) showed that a subset of LjVDAC-labeled structures were mitochondria (Fig. 6H, yellow). However, the anti-LjVDAC1.1 antibody also labeled vesicular, non-mitochondrial structures close to, or associated with, the plasma membrane that were not labeled with anti-Cyt c antibody (Fig. 6H, green).

Further characterization of VDAC-containing structures was done using immunogold labeling of *L. japonicus* nodule sections with the anti-LjVDAC1.1 antibody. Once again, two types of structures were labeled: abundant 200- to 500-nm vesicular structures near the plasma membrane (Fig. 7A) and less abundant but larger (typically $>1 \mu\text{m}$) mitochondria (Fig. 7B). Mitochondria exhibited internal cristae typical of this organelle, whereas the small vesicles did not.

Symbiosomes in nodule sections were not labeled with anti-LjVDAC1.1 antibody (Figs. 6 and 7), even though a VDAC antigen band was detected in SM preparations on western blots (Fig. 5). This apparent contradiction suggests that the SM preparations illustrated in Figure 5 were actually contaminated with another membrane type.

DISCUSSION

VDAC genes are present in fungi, animals, and plants. However, the numbers of homologs in different plant species exceed those in other eukaryotes. For example, Arabidopsis has five VDAC genes, whereas humans have three and yeast only two. At least five VDAC genes have been uncovered in each

Approximately 20 μg of total protein was loaded per lane. *por⁺*, Strain HR1-2B; *por⁻*, strain HR1-2A; 1.1, LjVDAC1.1; 1.2, LjVDAC1.2; 1.3, LjVDAC1.3; 2.1, LjVDAC2.1; 3.1, LjVDAC 3.1. Western blots were incubated with anti-LjVDAC1.1 (A) and anti-POM36 (B) antibody. C to F, Total protein extracts from leaves, roots, and nodules from *L. japonicus* and soybean and SMs purified via aqueous two-phase partitioning were prepared from 8-week-old plants. Around 20 μg of protein was loaded per lane. L, Leaf; R, root; N, nodule. Western blots were incubated with anti-LjVDAC1.1 (C) and anti-POM36 (E) antibody or with the same antibodies blocked with purified LjVDAC1.1 proteins (D and F, respectively). G, Microsomal fractions of roots and nodules from *L. japonicus* and SMs from soybean prepared via Percoll density centrifugation were obtained from 8-week-old plants. Around 20 μg of microsomal protein extract and 5 μg of symbiotic membranes were loaded. For comparison, total protein extract of *L. japonicus* roots and nodules was loaded. VDAC proteins were detected with anti-LjVDAC1.1 antibody. R, Root; N, nodule; t, total protein extract; mf, microsomal fraction. Protein bands representing *L. japonicus* VDACs are marked with dots. Molecular masses of protein standard are 28 and 39 kD.

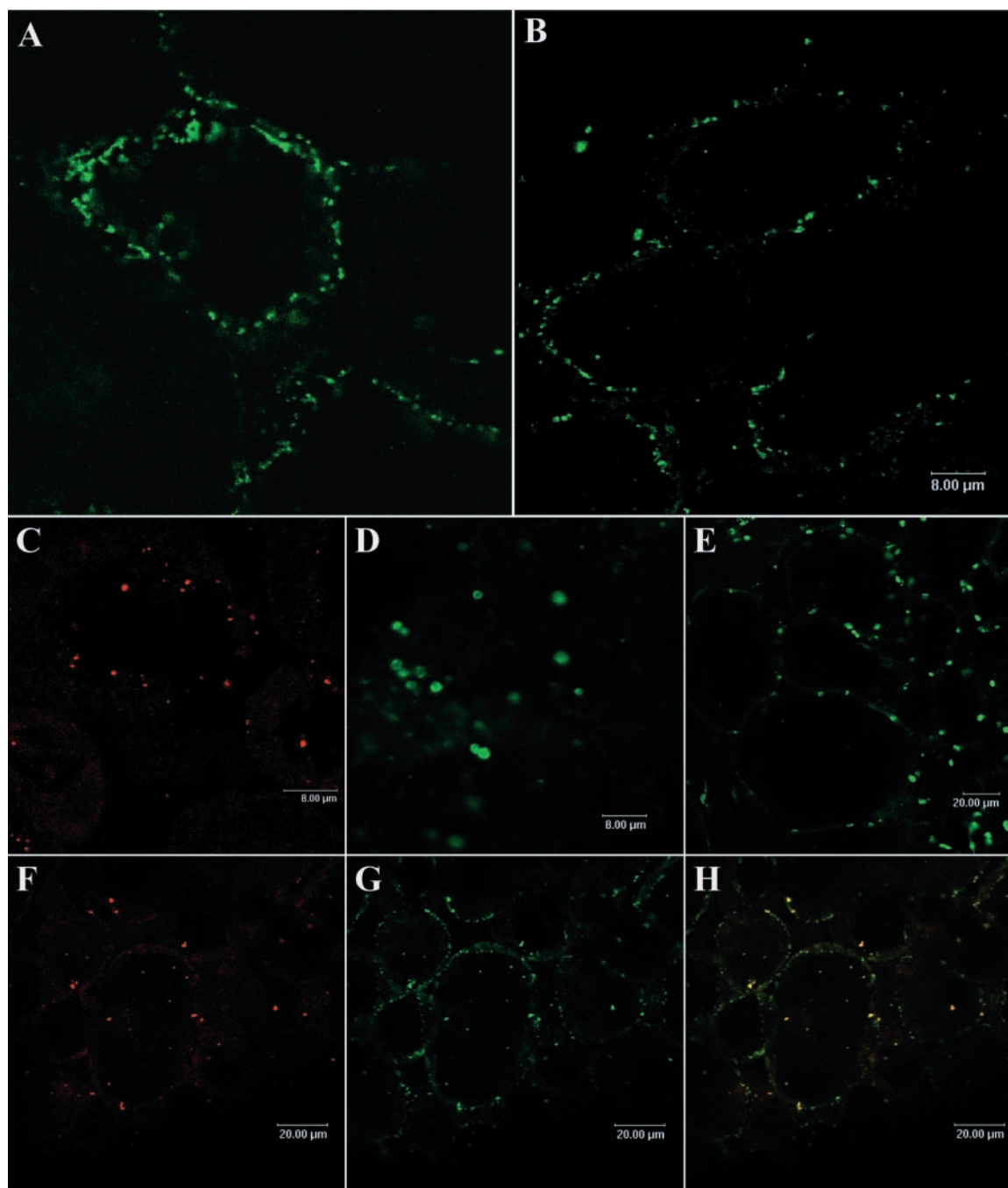


Figure 6. Immunolocalization of VDAC proteins in root nodules of soybean and *L. japonicus*. A and B, Single labeling studies with affinity-purified anti-LjVDAC1.1 antibody of *L. japonicus* and soybean nodule sections, respectively. C to E, Labeling of soybean mitochondria, peroxisomes, and plastids with anti-cytochrome c (Cyt c), anti-uricase, and anti-ferritin antibody, respectively. Colocalization of mitochondrial VDACS and Cyt c in soybean sections, using anti-Cyt c antibody (F) and LjVDAC1.1 antibody (G), is shown by image overlay (H, yellow). Note the non-mitochondrial signal in green in the overlay.

of *L. japonicus*, *M. truncatula*, and soybean by EST sequencing projects. Plant VDAC genes fall into five distinct subfamilies; the largest of these appear to have arisen since the divergence of monocots and dicots (Fig. 3). Three of the five *L. japonicus* VDAC genes characterized here fall into this category. Two

of these, *LjVDAC1.1* and *LjVDAC1.2*, are very similar in sequence and have a matching pair of genes in soybean, which may reflect a gene duplication event before the divergence of the *Glycine* and *Lotus* genera. Relatively recent gene duplication events in several other lineages are revealed by phylogenetic analysis

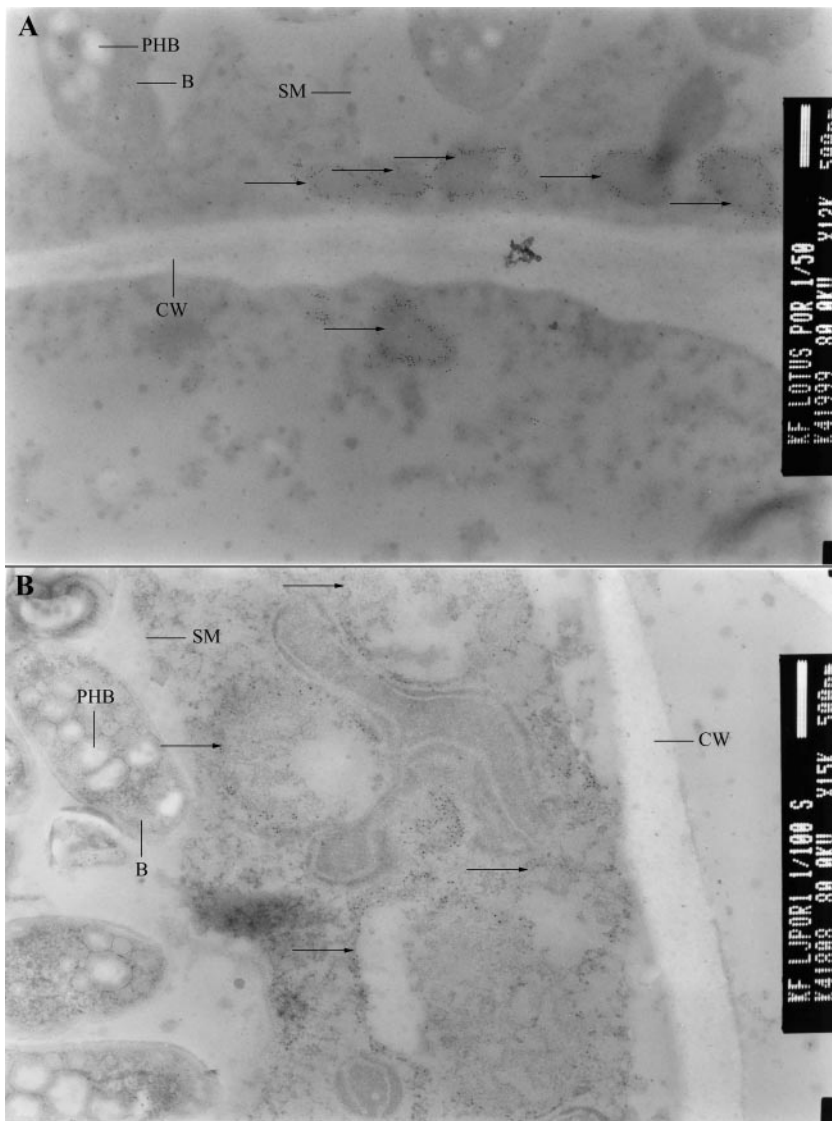


Figure 7. Immunogold labeling of *L. japonicus* VDACs on root nodule sections. Nodules from 8-week-old *L. japonicus* nodules were used for immunogold labeling. Ultrathin sections (90 nm) were incubated with affinity-purified LjVDAC1.1 antibody. Secondary antibody was conjugated with 15-nm gold particles. Immunogold-labeled structures are marked with arrows. A, Small peripheral vesicles; B, mitochondria. SMs, bacteroids (B), polyhydroxybutyrate (PHB), and cell walls (CW) are also indicated.

(Fig. 3). Presumably, such events have provided the plant kingdom with an opportunity to diversify the physiological roles of VDAC proteins.

One way in which specialization of gene/protein function may come about during evolution is through a change in the pattern of gene expression. To determine whether any of the *L. japonicus* VDAC genes are expressed in an organ-specific manner, we carried out real-time RT-PCR analysis of leaves, roots, and nodules from mature *L. japonicus* plants. All five *L. japonicus* VDAC genes were expressed at high levels, although there were slight differences in the level of expression between genes and organs. Differential expression of VDAC genes during development has been reported for wheat (*Triticum aestivum*) and rice (*Oryza sativa*; Elkeles et al., 1995; Al Bitar et al., 2003).

Protein specialization can arise from changes in protein sequence and structure, which can alter the biochemical properties of a protein and/or its sub-

cellular location. Clear differences in primary structure were revealed by comparisons of the deduced amino acid sequences of the five *L. japonicus* VDACs (Fig. 2). However, the predicted secondary structural elements, including an α -helical domain and numerous β -sheets, are conserved among all five proteins (Fig. 2). Thus, these structural elements may be essential for channel formation and functionality. A 23-amino acid-long eukaryotic mitochondrial porin pattern (PROSITE Pattern-ID PS00558), which is conserved among plant, animal, and fungal VDACs, is present in LjVDAC1.1 to 1.3 but not in LjVDAC2.1 or LjVDAC3.1. This difference, together with sequence divergence, contributed to our naming of three separate VDAC subfamilies in *L. japonicus*. With the identification of an increasing number of VDAC sequences, the 23-amino acid porin pattern is losing its generality. Thus, most plant VDACs of branches I to III of the phylogenetic tree (Fig. 3) do not possess this porin signature. Surprisingly, the plant VDACs that

posses the eukaryotic mitochondrial porin signature (branches IV and V of the tree; Fig. 3) are most distantly related to yeast and animal VDACs, which also posses the signature. Thus, although VDACs of yeast, animals, and plants have diverged during evolution, the porin signature has been maintained in some plant porin families, suggesting a common function of the motif in the different kingdoms. The functional significance of this pattern remains unknown, however.

The N-terminal α -helix is conserved among eukaryotic VDAC porins. Although positively charged N-terminal α -helices often function as mitochondrial signal sequences, and VDACs are imported via proteins of the Translocator of outer membrane (TOM) complex (Krimmer et al., 2001), it has been found that this domain is not involved in mitochondrial targeting (Court et al., 1996). On the other hand, circular dichroism, crystal structure analysis of yeast VDAC1 (Mannella, 1998), and point mutation analysis (Thomas et al., 1993) have shown that the N-terminal helix forms a mobile domain, which is involved in voltage sensing and opening and closing of the channel. Thus, the conserved N-terminal region appears to be involved in basic mechanisms of channel function, like voltage sensing and channel gating, rather than in specific functions like targeting or selectivity. This is supported to some extent by our observation that LjVDAC1.3, which like all other *L. japonicus* VDACs contains the conserved N-terminal α -helix, is apparently not targeted to the OMM (see below).

Functionality and intracellular location of *L. japonicus* VDAC proteins was examined by expressing them in a VDAC1-deficient yeast mutant. Complementation or suppression of the *por*⁻ mutation in yeast strain HR1-2A by LjVDAC1.1, LjVDAC1.2, LjVDAC2.1, and LjVDAC3.1 demonstrated not only that these proteins function in yeast but also that they are targeted to the OMM (Fig. 4). This is the first time, to our knowledge, that plant VDACs have been shown to function in yeast, which opens up the possibility to carry out more detailed biochemical characterization of these proteins in the future. LjVDAC1.3 was unable to complement the yeast mutant, although the protein was expressed in transformed cells (Fig. 5, A and B). Immunolocalization showed that LjVDAC1.3 was not targeted to the OMM in yeast (data not shown), which provides an explanation for its inability to complement the mutant. Interestingly, LjVDAC1.3 is the least related of all the *L. japonicus* VDACs to animal and yeast VDACs. Sequence differences must account for the altered intracellular targeting of this protein in yeast, and this result raises the possibility that the protein is not located on the OMM in *L. japonicus*.

The anti-LjVDAC1.1 antibody that we produced is highly specific for VDACs. It binds strongly to LjVDAC1.1, LjVDAC1.2, and LjVDAC1.3, weakly to LjVDAC2.1, and not at all to LjVDAC3.1 (Fig. 5A) or

other *L. japonicus* proteins (Fig. 5, C and D). The anti-LjVDAC1.1 antibody also recognizes VDAC proteins in soybean (Fig. 5, C and D). In contrast, the anti-POM36 antibody cross-reacted strongly with two proteins in *L. japonicus* that could not be confirmed as VDACs (Fig. 5, E and F). Localization studies, using affinity-purified LjVDAC1.1 antibody together with Cyt c antibody, confirmed the mitochondrial location of some VDACs in root nodules (Fig. 6, F–H). However, anti-LjVDAC1.1 antibody also labeled distinct vesicular structures close to the plasma membrane, which were not labeled with anti-Cyt c antibody (Fig. 6, F–H). Because the anti-LjVDAC1.1 antibody cross-reacts with LjVDAC1.1 to 1.3, and LjVDAC1.1 and LjVDAC1.2 are targeted to the OMM in yeast, these proteins are likely to be located in the OMM in root nodules as well. In contrast, the LjVDAC1.3 protein, which was not targeted to the OMM of yeast and was probably absent from microsomal fractions (Fig. 5G), may be located in the non-mitochondrial, vesicular structures observed in root nodules. These structures were apparently not peroxisomes or plastids (Fig. 6). We can only speculate on the identity of these vesicles at this stage. VDAC proteins have been found in extra-mitochondrial membranes in animals, in the caveolea, or Triton-insoluble fraction domains of plasma membranes (Bathori et al., 1999; Bathori et al., 2000). Such domains have not been described in plants. However, because the ultrastructure of caveolea varies and can take on a vesicular form also (Anderson, 1998), the VDAC-decorated vesicles in nodules (Fig. 7A) could represent caveolae-like domains. Alternatively, they could represent vesicles involved in the secretory pathway. Salient in this regard is the observation that VDAC fusion proteins are targeted to secretory pathway vesicles in animals (Buettner et al., 2000). Previous work on plant VDACs localized an anonymous protein in the boundary membrane of glyoxisomes in cucumber (*Cucumis sativus*) plants, using the anti-POM36 antibody (Corpas et al., 2000). However, the same antibody cross-reacted strongly with two *L. japonicus* proteins that may not be VDACs (Fig. 5E). Therefore, results obtained from the use of the anti-POM36 antibody in immunolocalization studies should be interpreted with caution.

Results from proteomics studies have indicated the presence of VDACs in SM preparations from pea and *L. japonicus* nodules (Saalbach et al., 2002; Wienkoop and Saalbach, 2003). The VDAC sequenced from *L. japonicus* SM preparations is identical to LjVDAC1.1 described here. However, using cytological methods and the affinity-purified LjVDAC1.1 antibody, we were unable to detect the presence of VDAC proteins on the SM in sections of soybean or *L. japonicus* nodules. Therefore, we conclude that VDAC proteins are probably not located on the SM in intact legume root nodules. Although we cannot exclude the possibility that the VDAC antigens recognized by the

LjVDAC1.1 antibody are somehow masked and not detectable in the SM in situ, our results urge caution in interpreting the results of proteomics experiments with isolated, potentially contaminated organelles, such as symbiosomes. Ideally, the results of subcellular proteomics should be confirmed by in situ methods, such as immunolocalization.

To summarize, we have characterized five VDAC porins from the model legume, *L. japonicus*, four of which are functional at the OMM when expressed in yeast. Transcripts of all five genes accumulate to similar, high levels throughout the plant. Immunolocalization of VDACs in *L. japonicus* and soybean root nodules revealed their presence not only on mitochondria but also on distinct, small vesicles, which remain to be identified. Our results indicate that the biological roles of VDAC proteins have diversified during the evolution of plants.

MATERIALS AND METHODS

Plant Culture

Lotus japonicus GIFU B-129-S9 and soybean (*Glycine max* cv Stevens) plants were grown in quartz sand-filled pots in a greenhouse under artificial light in a 16-h-light/8-h-dark rhythm. Inoculation of *L. japonicus* with *Mesorhizobium loti* strain R7A and of soybean with *Bradyrhizobium japonicum* USDA110 was done at the time of sowing and repeated 4 to 7 d later.

DNA Manipulation

DNA manipulations such as plasmid purification, restriction digests, agarose gel electrophoresis, ligations, and screening of cDNA libraries were performed using standard protocols (Sambrook et al., 1989). Synthesis of cDNA was carried out with the Superscript Lambda cDNA synthesis kit (Gibco BRL, Eggenstein, Germany).

RNA Gel-Blot Analysis

Leaves, stems, roots, flowers, and nodules from 6-week-old *L. japonicus* plants were harvested and ground to a fine powder in liquid nitrogen, using mortar and pestle, and RNA was extracted using the detergent-based method of Jacobsen-Lyon et al. (1995). After separation of RNA by gel electrophoresis under denaturing conditions (Lehrach et al., 1977), RNA was transferred to nylon membranes (Porablot NY amp plus, Macherey & Nagel, Düren, Germany) and fixed using a UV crosslinker (Stratagene, Heidelberg). Blots were prehybridized for 4 h at 65°C and hybridized overnight at 65°C in 250 mM sodium phosphate buffer (pH 7.2) containing 7% (w/v) SDS, 1% (w/v) bovine serum albumin (BSA), and 1 mM EDTA. DNA probes were ³²P-labeled using the RediPrime random primer labeling kit (Amersham, Braunschweig, Germany) and purified with NAP5 columns (Amersham), which removed unincorporated nucleotides. Membranes were washed twice at 65°C in 2× SSC (1× SSC contains 0.15 M NaCl and 0.015 M sodium citrate) plus 0.1% (w/v) SDS, and once in 0.2× SSC and 0.1% (w/v) SDS. Blots were subjected to autoradiography overnight at -80°C, using Kodak X-Omat AR Films (Sigma, Deisenhofen, Germany).

Real-Time RT-PCR

Poly(A⁺) RNA was purified from 10 μg of DNase-treated total RNA, using the Qiagen Oligotex mRNA kit (Qiagen, Hilden, Germany). Approximately 10 ng of mRNA was then reverse transcribed with Superscript II RT (Invitrogen, Karlsruhe, Germany) using an oligo(dT) primer, to generate 50 μL of first strand cDNA. Real-time RT-PCR was performed using 1 μL of a 1/10 (v/v) dilution of the first strand cDNA reaction and the SYBRGreen reagent (Eurogentec, Seraing, Belgium) in a reaction volume of 25 μL on a

GeneAmp 5700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA). Primers were designed to the 3'-untranslated region of *LjVDAC1.1* (forward, 5'-GATAATT TGATCCTTGCCAAGAC-3'; and reverse, 5'-GAACACTTCTTAGCCAAGAAGAG-3'), *LjVDAC1.2* (forward, 5'-ATGA TTTTGAATTGATAGGCTGCG-3'; and reverse, 5'-ACCCAGC AAGAAA-TTAACAGCTC-3'), *LjVDAC1.3* (forward, 5'-TTTTGAGCCATTATGGC-AAGA GC-3'; and reverse, 5'-AACACTT ATGTCCAATAGAACCAG-3'), *LjPor2.1* (forward, 5'-TTG AAAGGAGGGCAGAATAA TTAG-3'; and reverse, 5'-GTCAAACAGAAGCCATTGGTGAT-3'), and *LjPor3.1* (forward, 5'-GCCTTGTCAAAGGTAACCA-3'; and reverse, 5'-CTCTAGCCATTATGTAAGTATT TC-3'). Each primer pair amplified a single product, as shown by the melting temperature of the amplicons and agarose gel electrophoresis. Likewise, ubiquitin primers (forward, 5'-TTCACCTGTGCTC-CGTCTC-3'; and reverse, 5'-AACAACAGCACACAGACAATCC-3') were designed against the *L. japonicus* polyubiquitin gene (GenBank accession no. AW720576). Samples without template were used as negative controls. Expression data were normalized to ubiquitin and then compared using the formulae:

$$nC_T = C_{T_{LjVDAC}} - C_{T_{LjUbi}}$$

$$\Delta C_T = nC_{T_x} - nC_{T_y}$$

$$nRatio = 2^{-\Delta C_T}$$

where C_T is the PCR cycle number at which a set threshold value (usually 0.1) of SYBR Green fluorescence is reached, nC_T is the normalized C_T value, ΔC_T is the difference in normalized C_T values in tissues x and y, and $Ratio_n$ is the ratio of gene transcript level in tissue x compared with y.

Yeast (*Saccharomyces cerevisiae*) Complementation

Yeast strains HR1-2B (por^+) and HR1-2A (por^-) were a kind gift of Dr. Carla Koehler (University of California, Los Angeles; Dihanich et al., 1987). Rich-medium yeast peptone dextrose for yeast growth contained 1% (w/v) yeast extract, 1% (w/v) peptone, and 2% (w/v) Glc. Full-length VDAC cDNAs were cloned into yeast expression vector p112A1NE (Riesmeier et al., 1992) and transformed into the por^- strain, using the lithium acetate method (Gietz et al., 1995). As controls, por^+ and por^- strains were transformed with the non-recombinant vector. Transformants were selected for Trp prototrophy on solid minimal medium containing 0.67% (w/v) yeast nitrogen base (Difco, Augsburg, Germany), 2% (w/v) Glc, 0.14% (w/v) yeast synthetic drop-out mix (Sigma), and 2% (w/v) Bacto-Agar (Gibco BRL) supplemented with 20 mg L⁻¹ uracil, 30 mg L⁻¹ Leu, and 20 mg L⁻¹ His.

Protein Extraction and Western-Blot Analysis

All protein extraction methods were carried out at 4°C, unless otherwise stated. Total protein extracts from *L. japonicus* and soybean organs were prepared from 8-week-old plants by harvesting fresh tissue into liquid nitrogen and grinding the frozen tissue with mortar and pestle to a fine powder. The powder was resuspended in an equal volume of extraction buffer (10 mM Tris [pH 7.5], 140 mM NaCl, 5 mM EDTA, 1% [v/v] Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). Cell debris and insoluble components were pelleted twice by centrifugation at 14,000 rpm for 10 min. Protein concentration of supernatants was determined according to Bradford using the Bio-Rad Protein Assay Kit I (Bio-Rad, Munich). Approximately 20 μg of protein was loaded per lane.

SMs from soybean root nodules of 8-week-old plants were prepared as described by Panter et al. (2000) and by aqueous polymer two-phase partitioning after Christiansen et al. (1995). Membrane pellets were resuspended in 20 mM Tris (pH 7.2) and 1% (w/v) SDS and subjected to SDS-PAGE. Approximately 20 μg of protein was loaded per lane.

Crude yeast protein extracts were prepared by first pelleting 5 mL of cells of an overnight culture and resuspending them in 150 μL of LDS-Sample buffer (Invitrogen). After heating for 10 min at 95°C, a half volume of glass beads was added, and the cells were vortexed for 10 min before being subjected to SDS-PAGE. Approximately 20 μg of protein, in 10 μL of extract, was loaded per lane.

To obtain polyclonal anti-LjVDAC1.1 antiserum, the open reading frame of the *LjVDAC1.1* cDNA was amplified with primers containing either

*Bam*HI or *Hind*III restriction sites (forward, 5'-GGATCCATGGCTAAGGGTCTGGTCTC-3'; and reverse, 5'-AAGCTTCC CAATGTCTTGC-AAGGATC-3'). After subcloning the 871-bp PCR fragment into pGEMTEasy (Promega, Mannheim, Germany), the *Bam*HI-*Hind*III fragment was isolated and ligated to a poly-His-encoding sequence in vector pQE30 (Qiagen). Protein expression in *Escherichia coli* and protein purification using nickel affinity columns was performed as per the manufacturer's instructions (QIAexpressionist, Qiagen). Purified protein was then injected into rabbits (Pineda Antikörper Service, Berlin), and polyclonal antisera were collected 61 and 90 d after immunization. Antiserum was then affinity purified using Sepharose columns with immobilized LjVDAC1.1 protein (Pineda Antikörper Service).

After separation on NuPage precast gels (Invitrogen), proteins were electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Einbeck, Germany) overnight at 70 mA or for 150 min at 400 mA with an XCell II Blot Module (Invitrogen) in 25 mM Tris-Base, 190 mM Gly, and 20% (v/v) methanol. Protein detection on western blots using 1:1,000 (v/v) diluted primary antibody, alkaline phosphatase-conjugated anti-rabbit secondary antibody (1:1,500 [v/v] dilution, Promega), and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate tablets (Roche Diagnostics, Mannheim, Germany) was performed as described by Blake et al. (1984).

Immunolocalization by Laser-Scanning Confocal Microscopy

Root nodules of around 8-week-old *L. japonicus* and soybean plants were fixed overnight in PEM (50 mM 1,4-Piperazinediethanesulfonic acid (PIPES) [pH 6.8], 5 mM EDTA, and 5 mM MgSO₄), containing 3.7% (w/v) paraformaldehyde, under a slight vacuum. Using a VT1000S Vibratome (Leica, Bensheim, Germany), 100- μ m sections were taken and mounted onto poly-L-Lys-coated slides (Poly-Prep-Slides, Sigma). Sections were treated for 10 min with 0.5% (w/v) Cellulase R10 in PEM (Yakult, Tokyo) before blocking with PEM, containing 3% (w/v) BSA, 0.1% (v/v) Tween20, and 20 mM Lys for 2 h at room temperature. Affinity-purified anti-LjVDAC1.1 and anti-Cyt c antibodies (BD Biosciences, Heidelberg) were applied in a 1:10 (v/v) dilution, anti-uricase antibody (VandenBosch and Newcomb, 1986) was applied in a 1:250 (v/v) dilution, and anti-ferritin antibody (Lucas et al., 1998) in a 1:50 (v/v) dilution in blocking solution overnight at 4°C. Sections were then washed in 200 mL of blocking solution for 1 h at room temperature with gently stirring. As fluorescent conjugates, anti-Rabbit antibody labeled with Alexa Fluor 488 and anti-mouse antibody labeled with Alexa Fluor 568 (Molecular Probes Europe, Leiden, Netherlands) were used and applied at 1:50 (v/v) dilution in blocking solution for 4 h at RT. After washing in blocking solution for 2 to 4 h, the sections were viewed on a laser-scanning confocal microscope (Leica DM IRBE microscope, TCS SPII confocal scanner). Images were generated using processing software from Leica.

Protein Localization by Immunogold Labeling and Electron Microscopy

Root nodule pieces from 8-week-old plants were fixed overnight at 4°C in 0.1 M sodium phosphate buffer (pH 7.3), containing 4% (w/v) paraformaldehyde. Samples were then dehydrated for 6 h in 30% (v/v) ethanol, 3 h in 50% (v/v) ethanol at room temperature, and overnight in 70% (v/v) ethanol at 4°C. Afterward samples were embedded in LR-White (LRW) Resin Medium Grade (Plano, Wetzlar, Germany) by infiltration for 6 h in a 1:1 (v/v) LRW:ethanol mixture and for 24 h in pure LRW at 4°C. Polymerization was carried out for 2 d at 50°C. Ultrathin sections (90 nm) were taken with an Ultracut E ultramicrotome (Leica) and mounted onto pyroxylin- and carbon-coated 200-mesh gold grids (Plano). For immunogold staining, the sections were blocked for 1 h at RT in phosphate-buffered saline (PBS; 10 mM sodium phosphate buffer [pH 7.5] and 130 mM NaCl), containing 1% (w/v) Aurion-BSA (Science-Services, Munich) and 0.1% (v/v) Tween 20. Affinity-purified LjVDAC1.1 antibody was diluted 1:10 (v/v) in incubation solution (PBS, 0.1% [w/v] Aurion-BSA, and 0.01% [v/v] Tween20) and applied overnight at 4°C. After three washes for 15 min in wash buffer (PBS and 0.1% [v/v] Tween20), the gold-conjugated secondary antibody (10 nm of gold, British BioCell International, Cardiff, UK) diluted 1:30 (v/v) in incubation solution was applied for 3 to 4 h at room temperature. After washing three times for 15 min in (successively) wash buffer, PBS, and distilled water, the grids

were contrast stained with uranyl acetate and lead citrate. The labeled sections were monitored with a Joel Transmission electron microscope (Tokyo, Japan) at a voltage of 80 mV and photographed.

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