The grape aquaporin VvSIP1 transports water across the ER membrane

Henrique Noronha1,2, Alice Agasse2, Ana Paula Martins3, Marie C. Berny1, Dulceneia Gomes2, Olfa Zarrouk3, Pierre Thiebaud6, Serge Delrot7, Graça Soveral3, François Chaumont4 and Hernâni Gerós1,2,*

1 Centro de Investigação e de Tecnologias Agro-ambientais e Biológicas CITAB, Portugal
2 Grupo de Investigação em Biologia Vegetal Aplicada e Inovação Agroalimentar (AgroBioPlant), Departamento de Biologia, Escola de Ciências, Universidade do Minho, Braga, Portugal
3 Research Institute for Medicines and Pharmaceutical Sciences (iMed.UL) and Department of Biochemistry and Human Biology, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal
4 Institut des Science de la Vie, Université catholique de Louvain, Croix du Sud 4-L7.07.14, B-1348 Louvain-la-Neuve, Belgium
5 Instituto de Tecnologia Química e Biológica, Apartado 127, 2781-901 Oeiras, Portugal
6 CNRS UMR 5164, Bordeaux, France
7 Université de Bordeaux, INRA, ISVV, Ecophysiologie et Génomique Fonctionnelle de la Vigne, UMR 1287, F-33140 Villenave d’Ornon, France

* To whom correspondence should be addressed. E-mail: geros@bio.uminho.pt

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Abstract

Water diffusion through biological membranes is facilitated by aquaporins, members of the widespread major intrinsic proteins (MIPs). In the present study, the localization, expression, and functional characterization of a small basic intrinsic protein (SIP) from the grapevine were assessed. VvSIP1 was expressed in leaves and berries from field-grown vines, and in leaves and stems from in vitro plantlets, but not in roots. When expressed in tobacco mesophyll cells and in Saccharomyces cerevisiae, fluorescent-tagged VvSIP1 was localized at the endoplasmic reticulum (ER). Stopped-flow spectroscopy showed that VvSIP1-enriched ER membrane vesicles from yeast exhibited higher water permeability and lower activation energy for water transport than control vesicles, indicating the involvement of protein-mediated water diffusion. This aquaporin was able to transport water but not glycerol, urea, sorbitol, glucose, or inositol. VvSIP1 expression in Xenopus oocytes failed to increase the water permeability of the plasma membrane. VvSIP1-His-tag was solubilized and purified to homogeneity from yeast ER membranes and the reconstitution of the purified protein in phosphatidylethanolamine liposomes confirmed its water channel activity. To provide further insights into gene function, the expression of VvSIP1 in mature grapes was studied when vines were cultivated in different field conditions, but its transcript levels did not increase significantly in water-stressed plants and western-exposed berries. However, the expression of the aquaporin genes VvSIP1, VvPIP2;2, and VvTIP1;1 was up-regulated by heat in cultured cells.

Key words: Aquaporin, protein purification, proteoliposomes, Vitis vinifera, VvSIP1, water transport.

Introduction

The physiological role of intracellular aquaporins (AQP)s in plants is not yet clear and remains a stimulating matter of debate (for reviews, see Ishibashi, 2006; Nozaki et al., 2008; Maeshima and Ishikawa, 2008; Gomes et al., 2009; Wudick et al., 2009; Conde et al., 2010). Intracellular AQP)s may play important roles in organelle water transport and intracellular water homeostasis, but they may also transport small solutes important for cell signalling (Gomes et al., 2009).
In comparison with other organisms, plants appear to have a remarkably large number of ubiquitously expressed AQPs (Javot and Maurel, 2002; Javot et al., 2003). Following the identification of Arabidopsis thaliana AtTIP1;1 as the first plant water channel (Höhle et al., 1992; Maurel et al., 1993), several intracellular AQPs have been described, particularly at the tonoplast (reviewed by Wudick et al., 2009). However, three SIPs (small basic intrinsic protein) and one NIP (nodulin-like intrinsic protein) from Arabidopsis have shown to localize at the endoplasmic reticulum (ER) (Ishikawa et al., 2005; Mituzani et al., 2006). Also, an AQP able to transport CO₂, NaAQP1, was shown to be localized at the chloroplast membranes (Uchelín et al., 2003, 2008). AQP8 and AQP9 from mammals were localized in the membrane system of the mitochondria (Calamita et al., 2005; Amiry-Moghaddam et al., 2005) and, more recently, Arabidopsis AtTIP5;1 was specifically found in the mitochondria of pollen tubes (Soto et al., 2010).

SIPs constitute a subfamily of major intrinsic proteins (MIPs) that was identified for the first time by database mining and phylogenetic analyses (Johansson and Gustavsson, 2002), and are related to mammalian AQP11 and AQP12 in their intracellular localization and function (reviewed by Ishibashi, 2006). Each SIP member may exhibit a particular pattern of expression in the plant and play a role specific to each cell. Arabidopsis thaliana AtSIP1;1 is expressed in the roots and flowers, especially in stamens, and pollen, and in trichomes of rosette leaves. AtSIP1;2 is expressed in the cotyledon and hydathode tissue of rosette leaves. AtSIP2;1 is expressed in the vascular tissue of roots and the leaf veins, and in flowers, pollen, and siliques (Ishikawa et al., 2005).

Regarding key distinctive structural characteristics of SIPs, the first NPA motif of loop B, which participates in the formation of an essential constriction region, is changed to NPT, NPC, or NPL in AtSIP1;1, AtSIP1;2, and AtSIP2;1, respectively, and to NPC or NPT in the human AQPs AQP11 and AQP12, respectively. The two NPA motifs are involved in the selection of substrate through hydrogen bond formation between a water molecule and the asparagine residue. Thus, any variation of the NPA motif might directly reflect the substrate specificity and/or velocity of water transport (reviewed by Ishibashi, 2006; Maehama and Ishikawa, 2008; Gomes et al., 2009). The SIP members are also relatively rich in basic residues such as lysine, and their isoelectric points are higher than in AQPs from other subfamilies.

Grape berries are sophisticated biochemical factories of major economic importance. They import and accumulate water, minerals, sugars, and amino acids, and synthesize organic acids, tannins, and anthocyanins, as well as flavour and aroma compounds. The development and maturation of grape berries have received considerable scientific scrutiny because of both the uniqueness of such processes to plant biology and the importance of these fruits as a significant component of the human diet and wine industry (Conde et al., 2007). The grape genome has only two SIP genes, VvSIP1 and VvSIP2, encoding a SIP1 and SIP2 subtype, respectively. The present study investigates the expression of VvSIP1 in leaves and grape berries throughout the season and the subcellular localization of the protein fused to a fluorescent tag both in tobacco leaves and in yeast. Expression of VvSIP1 in Xenopus oocytes did not increase the water permeability of the plasma membrane. In contrast, when expressed in yeast, VvSIP1-enriched ER membrane vesicles exhibited higher water permeability than control vesicles, as determined by stopped-flow spectroscopy, and the protein was unable to accept other substrates, including glycerol, urea, sorbitol, glucose, and inositol. VvSIP1 protein was also purified to homogeneity and its water transport activity was reconstituted in phosphatidylethanolamine artificial vesicles. The potential role of VvSIP1 in stress response was studied in field-grown grapevines and grape cell cultures.

**Materials and methods**

**Plant material**

Field-grown grapevines (Vitis vinifera L.) of cv. Aragonez and Vinhão were used in the present study. Cv. Aragonez vines were collected from commercial vineyards in Reguengos de Monsaraz and Estremoz (south of Portugal) and cv. Vinhão from a commercial vineyard in Guimarães (north of Portugal). Rows were oriented north–south. Expression studies were also performed in 3-month-old grapevine plantlets of the cv. Trincadeira growing in vitro.

The cv. Aragonez vines cultivated in Reguengos de Monsaraz were subjected to RDI (regulated deficit irrigation) and SDI (sustained deficit irrigation), within the scope of the European Project Innovine. RDI vines were supplied with 50% less water than SDI vines, and berries from SDI vines were collected from the green to mature phase to study VvSIP1 expression during maturation. At the mature stage, the following values for leaf water potential were measured: –0.7 MPa (RDI) and –0.5 MPa (SDI). Also, the oscillations in berry temperature were continuously monitored. Average daily maximum temperatures in grapes from clusters exposed to the west (RDI-W and SDI-W) were 4–5 °C higher than in east-exposed clusters (RDI-E and SDI-E) because ambient temperatures are higher after mid-day. The expression of VvSIP1 was also studied in berries from SDI-E, RDI-E, SDI-W, and RDI-W vines.

The cv. Aragonez vines in Estremoz were subjected to full irrigation (100% evapotranspiration) and non-irrigation (rain-fed only). Watering was applied according to crop evapotranspiration and soil water content.

Grape berry clusters from 4–6 plants, located in three different rows, were collected, and grapes from three different berry clusters per plant were harvested and immediately frozen in liquid nitrogen. Berries and leaves were sampled at green [4 weeks after flowering (WAF)], veraison (9 WAF), and mature (15 WAF) stages of berry development and ripening.

Cells of V. vinifera L. (CSB, Cabernet Sauvignon Berry) were cultivated in liquid medium according to Descendit et al. (1996), and maintained in 250 ml flasks on a rotatory shaker at 100 rpm in the dark, at 25 °C. The mineral medium was supplemented with 2% (w/v) sucrose. Cells were subcultured weekly by transferring 10 ml aliquots into 40 ml of fresh medium. In order to study the effect of different treatments on VvSIP1 expression, 5 ml aliquots were incubated overnight with 100 mM NaCl, polyethylene glycol (PEG) 2% (w/v), 150 μM abscisic acid (ABA) (Gagné et al., 2011), and 150 μM salicylic acid (SA) (Laura et al., 2007) at 23 °C. The effect of heat was evaluated after an overnight incubation at 38 °C. Cells were immediately frozen in liquid nitrogen and stored at −80 °C.

**In silico studies**

SIP sequences were obtained from the database of the National Center of Biotechnology (NCBI). Protein alignment was performed...
RNA isolation from grape berries and leaves

Total RNA was isolated from grape berries and leaves with a QIAGEN RNeasy Plant Mini Kit following the manufacturer's instructions, except that the extraction buffer was changed to 2% cetyltrimethyl ammonium bromide (CTAB), 2% soluble polyvinylpyrrolidone (PVP) K-30, 300 mM TRIS-HCl (pH 8.0), 25 mM EDTA, 2.0 M NaCl, and 2% (v/v) β-mercaptoethanol. After an incubation DNase treatment, the RNA integrity was checked in a 1% agarose gel, and first-strand cDNA synthesis was performed with the LongRange 2Step RT-PCR (Qiagen), following the manufacturer's instructions.

Subcellular localization of VvSIP1

The pH7RGW2-VvSIP1-RFP construct was obtained using Gateway (Qagen) recombination technology. Briefly, the recombination sequences (forward, GGG GAC AAG TTT GTA CAA AAA AGC AGG CT; and reverse, GGG GAC CAC TTT GTA CAA GAA AGC TGG GTI) were introduced by PCR (primers are shown in Supplementary Table S1 available at JXB online) in the VvSIP1 cDNA without a stop codon and the fragment was recombined into the entry vector pDONR221 using the BP clonase enzyme. The VvSIP1 cDNA was recombined into the pH7RGW2 vector by the LR clonase enzyme, introduced in Agrobacterium tumefaciens (GV3101), and transient transformation of tobacco (Nicotiana tabacum) leaf epidermal cells constitutively expressing green fluorescent protein (GFP)-HDEL (Batoko et al., 2006) were performed according to Sparkes et al. (2006). Bacterial cells were cultivated overnight in liquid LB medium up to the exponential—stationary phase and then diluted to OD600nm=0.1 with infiltration buffer (50 mM MES pH 5.6, 2 mM NaPO4, 0.5% glucose, and 100 μM acetoxyrindone). Diluted cells were cultivated again until the culture reached an OD600nm=0.2. Four-week-old tobacco plants were infiltrated with the bacterial cultures and leaf discs were examined under the confocal microscope 3 d later.

VvSIP1 was cloned into pUG35-GFP behind the cDNA encoding GFP. Restriction sites for BamHI were introduced by PCR (primers are shown in Supplementary Table S1 at JXB online), and the fragment was cloned into pUG35-GFP vector after digestion with BamHI. VvSIP1 expression was regulated by the inducible MET25 promoter. Saccharomyces cerevisiae strain CEN.PK 135-5D (ura–) was transformed with the pUG35-SIP1-GFP vector with the LiAc/SS-DNA/PEG method (Gietz and Woods, 2002). To study fluorescence localization, transformed yeast cells were cultivated overnight in YNB minimal medium without methionine and uracil, washed with deionized water, and observed with a Zeiss 710 confocal microscope (Carl Zeiss, Jena, Germany), with excitation at 488 nm and detection between 506 nm and 538 nm.

Real-time PCR studies

Quantitative real-time PCRs were prepared with a QuantiTect SYBR Green PCR Kit (Qiagen) and were performed in a CFX96 Real-Time Detection System (Bio-Rad), at an annealing temperature of 50 ºC. RNA and cDNA were obtained as detailed above. Experiments were done in triplicate (biological replicates) with the software Bio-Rad CFX Manager (Bio-Rad), using VvGAPDH as internal control. After each run, melting curves were performed to check for unspecific and primer dimer amplification. The primers used to study the expression of VvSIP1, VvGAPDH, and VvGPT are shown in Supplementary Table S1 at JXB online.

Semi-quantitative PCR studies

Semi-quantitative PCR was performed with HotStarTaq DNA Polymerase (Qiagen) to study the effect of heat on AQP expression. Briefly, all the primers used were previously tested to determine the exponential phase of the amplification curve for each condition. Three reactions for each condition were run in the same gel and quantified with the software Quantity One (Biorad). The primers used for VvHT1, VvPIP2, VvIP1;1, and VvACT1 are shown in Supplementary Table S1 at JXB online.

Isolation of yeast endoplasmic reticulum

The recombination sequences of Gateway technology were introduced in the VvSIP1 cDNA with the stop codon and the primers shown in Supplementary Table S1 at JXB online. The fragment was introduced into the vector pDONR221, recombined with pYES-DEST52, and the resulting vector pYES-DEST52-VvSIP1 introduced into yeast cells by the method described above. ER-enriched vesicles were obtained following the method of Wuestehube and Schekman (1992), which has been routinely used to purify ER membranes from yeasts. Briefly, yeast cells were cultivated overnight in YNB medium without uracil supplemented with 2% galactose, and spheroplasts were obtained by digestion with zymolyase 20T (Rodrigues et al., 2013) dissolved in digestion buffer (1.35 M sorbitol, 10 M citric acid, 30 M NaHPO4, 1 M EGTA, pH 7.4). After 45 min digestion, the spheroplasts were lysed in a Dounce tissue homogenizer in HEPES-lys buffer [20 mM HEPES, 50 mM potassium acetate, 100 mM sorbitol, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), pH 6.8], and a crude membrane fraction was obtained by centrifugation at 18 000 g for 15 min at 4 ºC. This membrane fraction was resuspended in HEPES-lys buffer, layered on top of a 1.2/1.5 M sucrose interface, centrifuged at 18 000 g for 15 min at 4 ºC, and the pellet was resuspended in 100 mM mannitol, 10 mM TRIS-HEPES (pH 7.5), and stored at −80 ºC. The protein amount was estimated by the Lowry method (Lowry et al., 1951). The purity of the ER fraction was checked by immunoblot with an anti-calreticulin antibody.

Functional characterization by stopped-flow spectroscopy

Water permeability of membrane vesicles was assessed with the stopped-flow technique (HI-TECH Scientific PQ/SF-53). Experiments were performed at 10–37 ºC to study activation energies. Five runs were usually stored and analysed in each experimental condition, as described by Sorvel et al. (1997). Briefly, vesicles resuspended in 100 mM mannitol, 10 mM TRIS-HEPES (pH 7.5) (0.1 mM, 0.4 mg protein ml−1) were mixed with an equal amount of hyperosmotic mannitol solutions at 23 ºC to produce an inwardly directed gradient of impermeable solute (osmotic gradient 120 M-osmol). The kinetics of vesicle shrinkage were measured from the time course of 90 ° scattered light intensity at 400 nm until a stable light scatter signal was attained. The osmotic water permeability coefficient (P0) was estimated by fitting the light scatter signal to a single exponential curve with the equation P(t)=k(Vf/VO) [1/(Vo-osmol∞)], where V∞ is the molar volume of water, Vf/VO is the initial volume to area ratio of the vesicle population, and (osmol∞) is the final medium osmolality after the application of the osmotic gradient. The osmolarity of each solution was determined from freezing point depression by a semi-micro-osmometer (Knauer GmbH, Germany). The activation energy (Ea) of water transport was obtained from the slope of an Arrhenius plot (ln Pf as a function of 1/T) multiplied by the gas constant R. Vesicle size (initial volume) was determined by quasi-elastic light scattering (QELS) by a particle sizer (BI-90 Brookhaven Instruments) as described by Sorvel et al. (1997). To determine VvSIP1 specificity for water, mannitol was replaced by several solutes (glycerol, urea, glucose, sorbitol, and inositol) with

by Prankster and the result was visualized in Genedoc (Nicholas et al., 1997). The 3D representation was performed by I-TASSER (Zhang, 2008) using SoPIP2;1 as a template (Tornroth-Horsefield et al., 2006). The resulting 3D model was visualized using the PyMol software (DeLano, 2002).
the same osmotic potential, and solute uptake was measured as stated above.

**VvSIP1 purification and reconstitution into phosphatidylethanolamine liposomes**

The construct pYES-DEST52-VvSIP1-His-tag was obtained by Gateway recombination. Briefly, the pDONR221-VvSIP1 without a stop codon (see above) was recombined with pYES-DEST52-His-tag in front of the GAL1 promoter, and behind six histidines. This vector was used to transform S. cerevisiae, and purified ER membranes were obtained as described above. The sample was diluted to 0.2 mg protein ml⁻¹ in Na₂CO₃, incubated for 30 min at 4 °C, and centrifuged for 40 min at 50 000 g. The pellet was washed with ice-cold water and resuspended in purification buffer [20 mM imidazole, 100 mM KCl, 10% (w/v) glycerol, pH 7.5] before protein solubilization. Three detergents were tested at different concentrations [1 and 2% (w/v)] and protein:lipid ratios (1/10 and 1/20): octyl-glucoside (OTG), n-dodecyl β-d-maltoside (DDM), and lysophosphatidylcholine (LPC). The best results were obtained with 2% LPC at a 1/10 protein:lipid ratio after incubation for 2 h at 42 °C under shaking. Non-digested proteins were pelleted at 100 000 g for 30 min at 4 °C. The purification step was started by mixing the supernatant with Ni-NTA agarose (Quigen), and, after incubation for 2 h at 37 °C under shaking, the mixture was loaded into an empty Bio-Spin Chromatography Column (Bio-Rad). The column was sequentially eluted with the following buffers: purification buffer supplemented with 0.05% (w/v) LPC, 40 mM imidazole buffer [40 mM imidazole, 10% glycerol (v/v), 100 mM KCl, 0.05% (w/v) LPC, pH 7.5], and 300 mM imidazole buffer [300 mM imidazole, 10% glycerol (v/v), 100 mM KCl, 0.05% (w/v) LPC, pH 7.5]. In each eluate, the purity of VvSIP1 was checked by western blot with an anti-ZmSIP1 antibody raised in maize against the C-terminal peptide of ZmSIP1;1 (FLPPPAPKPTTKKA). To reconstitute VvSIP1 in artificial vesicles, phosphatidylethanolamine lipids were mixed by sonication in a buffer with 100 mM mannitol, 10 mM TRIS-HEPES (pH 7.5), and 2% (w/v) OTG before addition of the purified protein (protein:lipid ratio of 1/50). After 30 min incubation on ice, the mixture was dialysed against 100 mM mannitol, 10 mM TRIS-HEPES (pH 7.5) to remove LPC, and the proteoliposomes were frozen in liquid nitrogen and stored at −80 °C until used (Gerós et al., 1996).

**Western blot analysis**

Protein samples obtained as described above were separated on 10% acrylamide gels as described by Laemmli (1970). Proteins were transferred to a nitrocellulose membrane during 1 h 30 min at 100 V, and were blocked during 1 h in TRIS-buffered saline containing 0.1% (v/v) Tween-20 (TBS-T) with 5% (w/v) skimmed milk powder, 1% (w/v) bovine serum albumin (BSA), 0.1% goat serum, and 0.05% (v/v) Tween-20. The membranes were probed against ZmSIP1 (1:1000 dilution) and calreticulin (1:10 000) (Carduejio et al., 2013) during 1 h at room temperature in blocking solution, followed by an incubation with an anti-rabbit peroxidase-conjugated antibody (Sigma) at 1:160 000 dilution in TBS-T, for 45 min. The immunodetection was accomplished with the chemiluminescent ECL detection substrate (Biorad).

**Oocyte injection and permeability assays**

*VvSIP1:2* and *VvSIP1* cDNAs were cloned into the oocyte expression vector pX77 with the primers shown in Supplementary Table S1 at JXB online, and *in vitro* complementary RNAs (cRNAs) were obtained with the Transcript Aid T7 High Yield Transcription Kit (Fisher, Thermo Scientific). *Xenopus laevis* oocytes were isolated and defolliculated by digestion at room temperature for 1.5 h with 4 mg ml⁻¹ collagenase A in Barth’s solution [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 10 mM HEPES-NaOH, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, pH 7.4, and 200 mOsm kg⁻¹].

*In vitro* transcripts or distilled water (50 nl) were injected and the oocytes were incubated at 18 °C in Barth’s solution for 2 d. Oocyte swelling was measured by transferring the oocytes to 5-fold diluted Barth’s solution, and the changes in the cell volume were calculated as described before (Fetter et al., 2004).

**Flow cytometry**

Flow cytometry analysis of proteoliposomes was performed in an Epics XL Beckman Coulter flow cytometer equipped with an argon-ion laser with a beam emitting at 488 nm at 15 mW. Green fluorescence was collected through a 525 nm band-pass filter. Data were analysed with Flowing Software 2.0 (Rodrigues et al., 2013).

**Statistical analysis**

The results were statistically analysed by Student’s *t*-test and by analysis of variances tests (one-way and two-way ANOVA) using Prism v. 5 (GraphPad Software, Inc.). Post-hoc multiple comparisons were performed using the Hsu Tukey test. For each condition, differences between mean values are identified with different letters or asterisks.

**Sequence accession numbers**

VvSIP1 (DQ086835), VvGPT (GSV1VT00006900001), VvPIP2:2 (EF364436), VvTIP1:1 (DQ834701), MtSIP1:1 (G7JKD7), MiSIP1:2 (G7KYE4), AtSIP1:1 (Q9M8W5), AtSIP1:2 (Q9FK43), ZmSIP1:1 (Q9ATM3), ZmSIP1:2 (Q9ATM2), OsSIP1:1 (B0L1W3), OsSIP1:2 (B5KGP0), OsSIP1:1 (Q5VR89), PsSIP1:1 (A9RDU1), PsSIP1:2 (A9U3Q2).

**Results**

**Analysis of SIP1 protein sequences**

The amino acid sequence of *V. vinifera* VvSIP1 was compared with that of SIP1 AQPs from *A. thaliana*, *Zea mays* (maize), *Olea europaea* (olive), *Oryza sativa* (rice), *Medicago truncata*, and the moss *Physcomitrella patens* (Fig. 1). All proteins share six transmembrane domains and two intracellular and extracellular loops that fold into the membrane and interact with each other through two ‘NPA’ motifs. A 3D computer simulation of VvSIP1 using SoPIP2;1 as a template (Törnroth-Horensfiel et al., 2006) confirmed that these ‘NPA’ motifs form a central constriction (Supplementary Fig. S1 at JXB online). Except for MtSIP1:1 and AtSIP1:2, the first ‘NPA’ motif is changed to NPT (Fig. 1). The R1 (I, L, V, or N), R2 (I, L, or V), R3 (P), and R4 (I or N) residues, which form the Ar/R filter, meet proximal to the central constriction formed by the two ‘NPA’ motifs (Supplementary Fig. S1). In contrast to most PIPs, TIPs, and NIPs, which contain an arginine (R) in R4 residues, the SIP1 proteins analysed show a conserved asparagine (N), except in the case of AtSIPs. In SIP1 sequences, the R3 residue is a conserved proline (P), which seems to be a characteristic of SIP1 members. Furthermore, a highly conserved AFGWAY1 motif is present in loop E of all SIP1s.

**Expression studies of VvSIP1**

To study the expression of *VvSIP1*, total RNA was isolated from cv. Vinhão leaves and berries and from grape berries of cv. Aragonez at three different developmental stages. RNAs
from grapevine plantlets growing in vitro and from liquid-cultured cells were also isolated to study the transcript levels of VvSIP1. In cv. Vinhão, VvSIP1 transcripts were detected in all samples, but were more abundant at the end of the season, both in leaves and in mature grapes (Fig. 2A, B). Conversely, in berries from cv. Aragonez, VvSIP1 expression decreased during maturation (Fig. 2C). In 3-month-old grapevine plantlets growing in vitro VvSIP1 transcripts were very abundant in leaves and stem but were not detected in roots (data not shown).

Aragonez vines cultivated under field conditions were also used to study VvSIP1 expression in berries in response to water deficit and different sun exposures (Fig. 2D, E). As described in the Materials and methods, grapevines subjected to RDI were irrigated with 50% less water than those subjected to SDI. In addition, average daily maximum temperatures were 4–5 ºC higher in grapes exposed to the west (RDI-W and SDI-W) than in those exposed to the east (RDI-E and SDI-E). The results showed that transcript levels of VvSIP1 did not increase significantly in berries from water-stressed plants.
and in berries from western-exposed clusters. The expression of \textit{VvSIP1} was also studied in mature berries from vines cultivated under more pronounced water deficit conditions (non-irrigation), but again the expression of \textit{VvSIP1} did not increase significantly (Fig. 2E).

In suspension-cultured cells (CSB, Cabernet Sauvignon Berry), \textit{VvSIP1} expression (Fig. 2F) did not change after treatments with salt (150 mM NaCl) and osmotic stresses [2\% (w/v) PEG], nor did it change after elicitation with the stress-related hormones ABA (150 \textmu M) and SA (150 \textmu M). These stress conditions have been reported to induce physiological changes in plant cells. In contrast, \textit{VvSIP1} transcript levels were significantly increased after an overnight incubation at 37 °C, while the expression of \textit{VvGPT} (\textit{Vitis vinifera} Glucose-Pi Transporter) decreased in the same experimental conditions (Supplementary Fig. S2 at \textit{JXB} online; negative control). Heat seems to be a positive signal for the expression of AQPs, because plasma membrane \textit{VvPIP2;2} and tonoplast \textit{VvTIP1;1} were also up-regulated (Supplementary Fig. S3).

Subcellular localization of \textit{VvSIP1}

The subcellular localization of \textit{VvSIP1}–red fluorescent protein (RFP) was studied after transient expression in tobacco epidermal cells. Figure 3 shows that \textit{VvSIP1}–RFP co-localized with the ER marker GFP–HDEL (Batoko \textit{et al.}, 2000). This reticular nature of the ER of tobacco cells has been clearly shown in other reports (Más and Beachy, 1999). \textit{VvSIP1} also localized in yeast internal membranes resembling the ER after transformation with \textit{VvSIP1}–GFP (Fig. 4A). Western

\textbf{Fig. 2.} \textit{VvSIP1} expression determined by quantitative real-time PCR. Transcript levels in leaves (A) and berries (B) from cv. Vinhão and cv. Aragonez (C) during development. \textit{VvSIP1} expression in mature berries from cv. Aragonez under different irrigation regimes and sun exposures (D, E). Expression of \textit{VvSIP1} in cultured cells in response to ABA and SA, and salt, osmotic, and heat stresses. Results indicate the mean ±SD of three independent experiments. Letters denote significant differences.
VvSIP1 transports water across the ER

Blot analysis with anti-ZmSIP and anti-calreticulin antibodies confirmed that VvSIP1 co-purified with the marker calreticulin, further corroborating that VvSIP1 is localized at the ER of transformed yeast.

Water transport by VvSIP1

The osmotic permeability coefficient ($P_f$) of *Xenopus* oocytes injected with *VvSIP1* cRNA did not increase, in contrast to the $P_f$ of the positive control cells injected with tobacco *NtPIP2;1* cRNA (Fig. 5). These results suggest that VvSIP1 is not correctly targeted to the plasma membrane of oocytes or that it is inactive.

To study VvSIP1 function, ER vesicles were isolated from yeast cells expressing *VvSIP1*, and its water permeability monitored by stopped-flow light-scattering spectrophotometry. QELS analysis showed that the vesicle size in all batches was homogeneous. Unimodal distributions were observed, with a mean hydrodynamic diameter of $379 \pm 65$ nm ($n=8$) (data not shown). To analyse VvSIP1...
activity, vesicles were challenged with a hypertonic mannitol solution. The change in the light scatter signal due to water efflux was used to calculate the $P_f$ and the $E_a$ of water transport. As shown in Fig. 6A, the shrinking rate of ER vesicles from VvSIP1-expressing yeast cells was twice as high as that of the control (Table 1). The increase in water permeability was consistent with the decrease in $E_a$ (Fig. 6B; Table 1), clearly indicating the involvement of protein-mediated water diffusion.

To purify VvSIP1, yeast cells were transformed with the construct pYesDes52-VvSIP1-6-His-tag followed by the purification of the ER fraction. Approximately 7 mg of total ER protein was obtained from 1.5 litres of yeast culture at OD$_{640}$=1.5. After solubilization with 2% LPC at a 1/10 protein to detergent ratio, VvSIP1 was eluted from the Ni-NTA column with 300 mM imidazole. Western blot analysis with the anti-ZmSIP1 antibody confirmed that VvSIP1 was purified to homogeneity (Fig. 7). In addition to a main band with a molecular mass of ~26 kDa, corresponding to the AQP monomer, a second band of ~52 kDa was detected, which probably corresponds to a VvSIP1 dimeric assembly (Bienert et al., 2012). Finally, a smaller band (~20 kDa) was also detected in fractions #3 and #4 that could correspond to partially degraded VvSIP1. The preparation of phosphatidylethanolamine proteoliposomes was optimized for low protein to lipid ratios (1/50 lipid to protein ratio) to avoid the use of high amounts of purified protein.

Flow cytometry analysis showed that proteoliposomes formed a homogenous population and stained positively when loaded with the fluorescent sugar 2-NBDG, suggesting that sealed vesicles were formed, which is a prerequisite for transport experiments (Supplementary Fig. S4 at JXB online). To confirm the water transport activity mediated by VvSIP1, the shrinkage rate of proteoliposomes resuspended in a hypertonic medium was assayed by stopped-flow. These proteoliposomes displayed higher water transport activity and lower $E_a$ than empty liposomes (Table 1), confirming that VvSIP1 is able to facilitate water diffusion.

### Table 1. Permeability ($P_f$) and activation energy ($E_a$) for water transport in ER membranes and liposomes obtained by stopped flow (for details see the Materials and methods)

<table>
<thead>
<tr>
<th></th>
<th>$P_f$ ($10^{-3}$ cm s$^{-1}$)</th>
<th>$E_a$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER vesicles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Empty vector</td>
<td>1.99 ± 0.23</td>
<td>13.7 ± 2.6</td>
</tr>
<tr>
<td>VvSIP1</td>
<td>5.09 ± 0.77*</td>
<td>6.4 ± 1.5*</td>
</tr>
<tr>
<td>Liposomes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Empty</td>
<td>3.8 ± 0.1 x 10$^{-1}$</td>
<td>16.6 ± 0.5</td>
</tr>
<tr>
<td>Proteoliposomes (1/50)</td>
<td>5.0 ± 0.4 x 10$^{-1}$*</td>
<td>12.2 ± 0.7*</td>
</tr>
</tbody>
</table>

Asterisks denote significant differences compared with the control: $^*P < 0.01.$
Fig. 7. Purification of VvSIP1 from S. cerevisiae. ER membranes from yeast transformed with pYESDES52-VvSIP1-6-His were isolated, solubilized with 2% LPC (w/v) at a protein:detergent ratio of 1/10, and purified in an Ni-NTA agarose column (for details see the Materials and methods). VvSIP1 monomeric (~26 kDa) and dimeric (~52 kDa) assembly can be observed. FT, flow-through of the column; W, eluates after washing the column. Eluates after addition of 300 mM imidazole (1–10).

Discussion

Comparison of VvSIP1 with other SIP aquaporins

Three members of the SIP subfamily were found in Arabidopsis (SIP1;1, SIP1;2, and SIP2;1) (Johanson et al., 2001) and maize (Chaumont et al., 2001), and two in rice (OsSIP1;1 and OsSIP2;1) (Sakurai et al., 2005) and grape (VvSIP1;1 and VvSIP2;1) (Fouquet et al., 2008). SIPs have also been described in Populus trichocarpa (Gupta and Sankararamakrishnan, 2009), the moss P. patens (Danielson and Johanson, 2008), and a putative ER AQP was described for the first time in the arbuscular mycorrhizal fungi Glomus intraradices (Aroca et al., 2009). As shown in Fig. 1, the VvSIP1 amino acid sequence has a high degree of similarity with SIP1 AQPs from monocots, dicots, and moss. In particular, all the sequences analysed share R3 and R4 residues of the Ar/R filter, except AtSIP1;1 and AtSIP1;2 where asparagine is replaced by iso-leucine in R4, and all the sequences contain the motif AFGWAY in loop E. The importance of loop E for the oligomerization of AQPs has already been shown (Duchesne et al., 2002; Fetter et al., 2003), and a single amino acid substitution in loop E of AQP11 is responsible for a mice lethal phenotype (Tkchekneva et al., 2008). Nonetheless, there is still little information on the oligomerization of intracellular AQPs.

Three types of ER retention signals were identified at the C-termini of transmembrane proteins: dihydrophobic, diacidic, and dibasic (reviewed by Barlowe, 2003; Giraud and Maccioni, 2003). Interestingly, a dihydrophobic motif (LF) and a dibasic signal (KQKK) are present in the C-terminus of VvSIP1, but their role in ER retention remains to be determined. The presence of a sequence rich in positively charged amino acids is a common feature of SIP AQPs (reviewed by Gomes et al., 2009).

VvSIP1 co-localizes at the ER

So far, only one experimental study was dedicated to SIP localization and function (Ishikawa et al., 2005). AtSIP1;1, AtSIP1;2, and AtSIP2;1 are localized at the ER of Arabidopsis protoplasts. Likewise, VvSIP1 clearly co-localized at the ER of tobacco epidermal cells (Fig. 3). This ER localization was further demonstrated in yeast cells expressing VvSIP1–GFP and by ER purification followed by western blotting with an anti-ZmSIP1 (Fig. 4). A similar approach was used to localize AtSIPs (Ishikawa et al., 2005) and mouse AQP11 and AQP12 (Itoh et al., 2005; Morishita et al., 2005) at the ER.

VvSIP1 facilitates membrane water diffusion in ER vesicles and in proteoliposomes

Oocytes have often been used as a tool to study the water channel activity of AQPs, especially PIPs and TIPs (Gomes et al., 2009), but, in the present study, oocytes injected with VvSIP1 cRNA did not show increased membrane water permeability (Fig. 5). This could be due to a failure in VvSIP1 protein expression or trafficking to the plasma membrane, which might indicate that this cell model is not suitable to study SIP AQPs. However, it cannot be excluded that VvSIP1 was targeted to the plasma membrane but the protein was inactive or was able to facilitate the diffusion of other substrates rather than water. In agreement, oocytes injected with the intracellular AQP11 did not show water transport although the protein was apparently targeted to the plasma membrane (Gorelick et al., 2006), in contrast to what was observed previously (Morishita et al., 2005).

To clarify the function of VvSIP1 further, water transport activity was monitored by stopped-flow light-scattering spectrophotometry in ER membrane vesicles isolated from yeast cells expressing VvSIP1 (Fig. 6). The observed increase in water permeability, together with the decrease in activation energy for water permeation, strongly supports that VvSIP1 mediates water fluxes. Furthermore, it was shown that VvSIP1 does not transport other solutes, including urea, glycerol, glucose, inositol, and sorbitol, suggesting that it is specific for water. In crude membranes from AtSIP-expressing yeast cells, AtSIP1;1 and AtSIP1;2, but not AtSIP2;1, also displayed water transport activity (Ishikawa et al., 2005).
However, it was not excluded that AtSIP2;1 could facilitate water diffusion after heterodimerization with other SIPS.

Some AQPs, but no SIP members, were purified to homogeneity and reconstituted in liposomes (Zeidel et al., 1992; Liu et al., 2006; Yakata et al., 2007; Tanimura et al., 2009). Recently, the intracellular AQP11 from mouse was shown to exhibit water channel activity after its reconstitution in proteoliposomes followed by stopped-flow measurements (Yakata et al., 2006). Here, the solubilization of VvSIP1-His6-tag from yeast ER membranes using LPC, its purification to homogeneity through an Ni-NTA column, and its incorporation into phosphatidylethanolamine proteoliposomes by detergent dialysis were reported (Fig. 7). As observed in ER vesicles, stopped-flow studies with proteoliposomes (Table 1) suggested that VvSIP1 plays a role as an intracellular water channel.

VvSIP1 is not responsive to different vine water regimes and berry sun exposures

As already mentioned, AtSIP1;1 and AtSIP1;2 are expressed in a variety of Arabidopsis tissues, but low expression levels were detected in leaves and fruits (Ishikawa et al., 2005). In grapevine, expression of VvSIP1 in leaves and berries of cv. Vinhão increased during development, peaking at the mature phase (Fig. 2A, B). In contrast, VvSIP1 expression in cv. Aragonez decreased during berry development (Fig. 2C), suggesting that the VvSIP1 expression pattern may depend on the variety or be affected by the terroir.

It has been shown that water deficit is one of many environmental conditions that regulate plant aquaporins (Tyerman et al., 2002; Hachez et al., 2008). This is particularly relevant since grape berries are highly susceptible to excessive sun exposure and their quality is affected by temperature and water availability (Kliewer and Torres, 1972; Spayd et al., 2002; Pillet et al., 2012). Generally, plants modulate the expression of plasma membrane and tonoplast AQPs in response to drought (Tyerman et al., 2002). In the present study, the transcript levels of VvSIP1 did not increase significantly in berries from vines subjected to water stress and in berries from western-oriented clusters (Fig. 2D, E), suggesting that VvSIP1 does not play an important role in stress response under the field conditions tested. In contrast, in in vitro cultures the expression of VvSIP1 (Fig. 2D), VvPIP2:2, and VvTIP1;1 (Supplementary Fig. S3 at JXB online) was up-regulated after an overnight incubation at 37 °C. This treatment did not affect the viability of the cells and induced heat-shock proteins. However, the cells kept their morphology substantially unchanged, with a prominent central vacuole (not shown). Thus, the observed transcription profile of AQPs could be involved in the regulation of the intracellular water status under heat stress. In agreement with this, it cannot be excluded that more extreme temperature conditions in the field can also promote the increase of VvSIP1 expression and that of other AQP members in grapevine tissues.

Besides regulation at the transcriptional level, several factors may affect the gating of AQPs, including phosphorylation, heteromerization, pH, Ca2+, pressure, solute gradients, and temperature (Chaumont et al., 2005). Post-transcriptional regulation of AQPs by protonation was shown in AtPIP2;2 in response to anoxia (Tournaire-Roux et al., 2003). In this regard, the possible involvement of post-transcriptional regulation of VvSIP1 deserves further investigation.

Much work is also needed to clarify the specific physiological role of SIPS in the ER membrane. In mammals, it was observed that AQP11-knockout mice died before weaning due to advanced renal failure, and two important abnormalities were detected: vacuolization and cyst formation in the kidney proximal tubule. Because the observed vacuoles apparently originated from the ER, it was suggested that AQP11 might have some role in vesicle homeostasis (Morishita et al., 2005). In plants, the analysis of Arabidopsis mutants followed by complementation experiments is a major tool for the assignment of a physiological role to a gene from a non-model plant. However, in preliminary experiments with T-DNA insertion mutants of AtSIP1;1 and AtSIP2;1, visible modifications in growth, morphology, and stress responses were not observed (Maeshima and Ishikawa, 2008). In this regard, a thorough exploitation of phenotypes at the cellular level, including the study of ER ultrastructure, could provide important information on the role of SIPS.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Lateral (A) and top (B) views of the VvSIP1 3D structure predicted by I-Tasser software using SoPIP2;1 as template.

Figure S2. VvGPT expression determined by quantitative real-time PCR in CSB (Cabernet Sauvignon Berry) suspension cells incubated overnight at 37 °C and in control cells incubated at 23 °C.

Figure S3. VvPIP2;2 and VvTIP1;1 expression determined by real-time PCR in Cabernet Sauvignon Berry suspension cells incubated overnight at 37 °C and in control cells incubated at 23 °C.

Figure S4. Flow cytometry analysis of phosphatidylethanolamine liposomes showing a homogeneous population (A) able to accumulate the fluorescent sugar analogue 2-NBDG (B).

Table S1. Primers used for PCR.

Acknowledgements

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