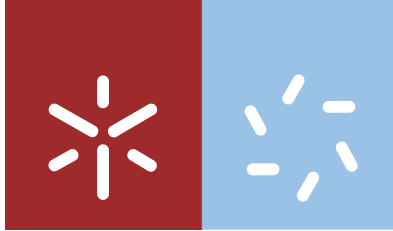


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Escola de Ciências

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**Functional characterization of the
grapevine aquaporin VvXIP1**



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Dissertação de Mestrado
Mestrado em Biofísica e Bionanossistemas

Trabalho efetuado sob a orientação do
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e do
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Functional characterization of the grapevine aquaporin VvXIP1

Abstract

The diffusion of water and small solutes through biological membranes is facilitated by aquaporins, members of the Major Intrinsic Proteins (MIPs) superfamily. As sessile organisms, plant species have been found to have more aquaporin genes than organisms from the other kingdoms of life. Among all the variety of MIPs, a subfamily called Uncharacterized Intrinsic Proteins (XIP) has been recently described in several fungi and eudicot plants. In the present study, the localization, expression and functional characterization of the grapevine VvXIP1 were performed. Phylogenetic analysis revealed that VvXIP1 is evolutionarily close to XIPs from cotton and peach. Co-localization studies with ZmPIP2;5-GFP and VvXIP1-RFP fusion proteins in transiently transformed *Nicotiana bethamiana* leaves after infection with *Agrobacterium tumefaciens* revealed that VvXIP1 is intracellular, and location prediction with the tool PredictProtein suggests that it locates in the tonoplast. Stopped flow spectrometry was used to evaluate water transport capacity of VvXIP1 in microsomal vesicles purified from the yeast strain YSK1172 transformed with the construct *pVW214-VvXIP1*. Results showed that VvXIP1 is unable to transport water but mediates glycerol transport. Growth experiments of the transformed yeast on solid media revealed that VvXIP1 is able to transport (besides glycerol) copper and H₂O₂. Furthermore, when CM-H₂DCFDA was used to measure H₂O₂ uptake by spectrofluorimetry, yeast cells expressing VvXIP1 showed a much higher rate of fluorescence increase after H₂O₂ addition than control cells transformed with the empty vector. Similarly, *VvXIP1*-transformed yeasts showed a 3.5 - fold higher rate of O₂ production than the control, as measured with a Clark electrode, when H₂O₂ was added to the incubation media. Both results strongly corroborated that VvXIP1 is able to transport H₂O₂. Additional growth experiments in solid media with 2.1 M sorbitol showed that the yeast transformed with the construct *pVW214-VvXIP1* exhibited higher water stress sensitivity than the yeast transformed with the empty vector. To provide further insights into gene function, the expression of *VvXIP1* was studied by qPCR in different organs and in response to different treatments/stressors. Results showed *VvXIP1* steady-state transcript levels were much higher in leaves than in berries, canes or flowers from field grown cv. Vinhão grapevines. Moreover, *VvXIP1* expression was downregulated in leaves from potted grapevines cv. Aragonez submitted to severe water deficit. In agreement, *VvXIP1* was downregulated by ABA and salt stress in *in vitro* cultured cells. Based on the observation that VvXIP1 is able to transport

copper, the steady-state levels of the corresponding mRNA were measured in cv. Vinhão leaves from field-grown grapevines treated with the copper based fungicide Bordeaux Mixture. The observed downregulation of the expression of *VvXIP1* should have major importance in plant response to copper toxicity, contributing do the regulation of the intracellular levels of the heavy metal in coordination with the copper transporters VvCTrs recently studied in our group.

Caraterização funcional da aquaporina de Videira VvXIP1

Resumo

A difusão da água e de pequenos solutos através da membrana biológica é facilitada por aquaporinas, membros da superfamília *Major Intrinsic Proteins* (MIPs). Como organismos imóveis, as plantas possuem um número maior de genes de aquaporinas que os organismos de outros reinos. De entre todos os membros da superfamília MIP, uma subfamília, denominada *Uncharacterized Intrinsic Proteins* (XIPs), foi recentemente descrita em fungos e plantas dicotiledóneas. No presente trabalho de tese foi estudada a localização e a função da proteína VvXIP1 de videira, bem como os níveis de expressão do mRNA correspondente em diferentes tecidos. A análise filogenética revelou que a proteína VvXIP1 é evolutivamente próxima de XIPs de algodão e pessegueiro. Os estudos de localização subcelular envolveram uma abordagem de transformação transiente de folhas de *Nicotiana bethamiana* por infiltração com *Agrobacterium tumefaciens*. Os resultados mostraram que a fluorescência verde da fusão ZmPIP2;5-GFP se localizava na membrana plasmática, ao contrário da fluorescência vermelha da fusão VvXIP1-RFP localizada provavelmente ao nível do tonoplasto, tal como previsto pela ferramenta bioinformática *PredictProtein*. A técnica “stopped flow” foi utilizada para avaliar a capacidade da aquaporina VvXIP1 transportar água em vesículas microssomais purificadas de células da estirpe de levedura YSK1172 transformada com o plasmídeo *pVV214 - VvXIP1*. Os resultados mostraram que a aquaporina é incapaz de transportar a água, mas é permeável ao glicerol. Estudos de crescimento em placa da levedura transformada mostraram que VvXIP1 é capaz de transportar cobre e H₂O₂ para além do glicerol. Quando se utilizou a sonda fluorescente CM-H₂DCFDA para medir por espectrofluorimetria a incorporação de H₂O₂, os resultados mostraram que as células da levedura a expressar a proteína VvXIP1 proporcionaram um aumento maior de fluorescência do que o controlo quando se adicionou H₂O₂ ao meio de reação. Em paralelo, estudos com o eléctrodo de Clark mostraram que a levedura a expressar a proteína VvXIP1 produzia mais O₂ que a levedura transformada com o vector vazio após a adição de H₂O₂ ao meio de reação. Ambas a evidências experimentais suportaram que a aquaporina VvXIP1 é capaz de transportar H₂O₂. A estirpe de levedura transformada com o plasmídeo *pVV214 - VvXIP1* mostrou ainda uma sensibilidade aumentada ao stresse hídrico que a levedura transformada com o plasmídeo vazio. Para obter uma melhor compreensão da função do gene *VvXIP1*, os níveis de expressão foram estudados por qPCR em diferentes órgãos e em resposta a diferentes tratamentos / stresses. Em videiras da casta Vinhão de uma vinha comercial o gene *VvXIP1* foi

mais expresso nas folhas do que em bagos, canas ou flores. Foi ainda observado que a expressão do gene *VvXIP1* foi regulada negativamente em folhas de videiras envasadas da casta Aragonez submetidas a stresse hídrico severo. Em paralelo, em culturas celulares de videira a produção de mRNA foi regulada negativamente por 150 μ M de ABA e 200 mM de NaCl. Por se ter descoberto que VvXIP1 é capaz de transportar cobre, foram medidos os níveis de mRNA em folhas de videiras da casta Vinhão tratadas com a Calda Bordalesa que é um fungicida à base de cobre. A diminuição observada dos níveis de transcritos de *VvXIP1* pode constituir uma resposta da planta à toxicidade do cobre, contribuindo para a regulação dos níveis intracelulares do metal pesado em coordenação com os transportadores de cobre VvCTrs recentemente estudados no nosso grupo de investigação.

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Abbreviations and Acronyms

CM-H₂DCFDA -5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester

ABA - Abscisic acid

E_a - Activation energy

AQP - Aquaporin

BLAST - Basic Alignment Search Tool

CSB - Cabernet Sauvignon Berry

CTAB - Cetyltrimethyl ammonium bromide

cv. - Cultivar

DTT - Dithiothreitol

EST - Expressed Sequence Tag

P_{gly} - Glycerol permeability coefficient

GIP - GlypF-like Intrinsic Proteins

GFP - Green Fluorescent Protein

HIP - Hybrid Intrinsic Proteins

MIP - Major Intrinsic Protein

MS - Murashige and Skoog

NIP - NOD26-like Intrinsic Protein

OD - Optic density

P_r - Permeability coefficient

PMSF - Phenylmethylsulfonyl fluoride

PIP - Plasma membrane Intrinsic Protein

PEG - Polyethylene glycol

PCR - Polymerase chain reaction

PVP - Polyvinylpyrrolidone

ROS - Reactive oxygen species

RFP - Red Fluorescent Protein

RT-PCR - Reverse transcriptase-PCR

SA - Salicylic acid

SIP - Small Basic Intrinsic Protein

SC - Synthetic Complement

TIP - Tonoplast Intrinsic Protein

TRIS - Tris(hydroxymethyl)aminomethane

WCTR1 - *Vitis vinifera* Copper Transporter 1

XIP - X Intrinsic protein / Uncharacterized intrinsic protein

YNB - Yeast Nitrogen Base

INTRODUCTION

Aquaporins are multifunctional water and solute channels widespread in living organisms

Transport of nutrients, ions, metabolic waste products and even water across cell membranes is of the utmost importance in all-living systems. Most transport processes across membranes are mediated by integral membrane proteins, sometimes functioning in association with extracytoplasmic receptor proteins and/or cytoplasmic energy-coupling and regulatory proteins. Transport proteins are often referred to as transporters, carriers, permeases, porters or channels. Important solutes like sugars, organic acids, minerals, protons and water cross the plasma membrane and cytosolic membranes through specific transport systems that play key roles in the cell metabolism. This is reflected in the genome of all organisms where up to 14% of transcribed genes code for transport proteins (reviewed by Conde et al., 2010).

The membrane that surrounds the cell and its compartments is known to be impermeable to charged and polar molecules. Classical studies have provided a solid basis for the lipid permeability of water and all physiologically relevant solutes, but they were unable to explain the high velocity of water and solute exchange or the low activation energy observed for such phenomena. More recently, it was shown that in addition to the intrinsic passive permeability of the bilayer to water, aquaporins can mediate the transport of water and small solutes (Gomes et al., 2009). In plants, it is known that the loss of aquaporin function is associated with growth impairment and reduced viability, but the role of these channels for survival in native environments is not fully understood (Ludwig and Dynowski, 2009).

Aquaporins and aquaglyceroporins are the two clades of proteins dividing the Major Intrinsic Proteins (MIP) superfamily. MIPs are channels forming a hydrophilic pathway for small-uncharged molecules across the lipid bilayer of biological membranes that are virtually present in all living organisms. The division between aquaporins and aquaglyceroporins is based not only on sequence similarities but also on transport activities (Bienert et al., 2011). Despite the sometimes large sequence difference between MIPs from different clades, the overall structure is highly conserved. Structures from x-ray and electron crystallography of MIPs revealed a tetrameric quaternary structure in which each monomer consists of six membrane spanning helices (H1 to H6) connected by five loops (A-E). Loop B (cytoplasmic) and loop E (extracellular) form two half-membrane spanning helices (HB and HE) and interact with each other from opposing sides through two highly conserved asparagine-proline-alanine (NPA) boxes, forming a narrow region of the pore (Figure 1). A constriction region about 8 Å from the NPA boxes toward the periplasmic side, termed the aromatic/arginine (ar/R) region, is formed by two residues from

H2 and H5 and two residues from loop E. This region is a primary selection filter and is a major checkpoint for solute permeability (Danielson and Johanson, 2008).

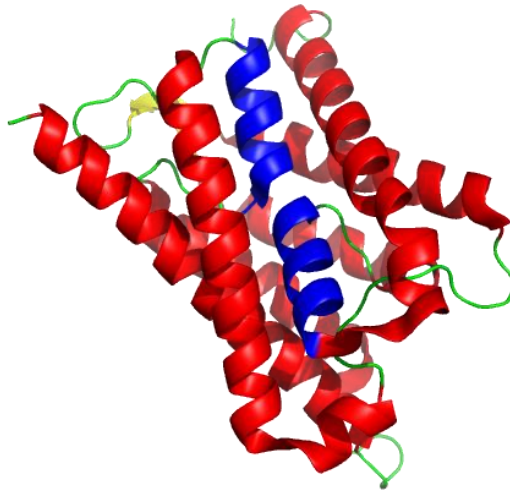


Figure 1 – 3D structure of the mutant aquaporin from spinach SoPIP2;1. Adapted with pyMol (PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC) from Nyblom et al. (2009).

Unlike aquaglyceroporins, AQPs are not widespread in bacteria and archaea, in which water transport occurs through passive diffusion, unspecific pores, or through some aquaglyceroporins like the *Lactococcus lactis* GluLac, which is oddly able to transport water and glycerol (Gomes et al., 2009).

AQy1 and AQy2 were initially identified in *S. cerevisiae*, although, further research revealed that yeasts can have between one and four AQP genes (Soveral et al., 2012). In filamentous fungi a wide variety of AQPs have been described (Gomes et al., 2009).

Although invertebrates represent 97% of the animal species, their AQPs are still poorly described, with only few species having one or more aquaporins functionally characterized. Some are insects, including the two *Drosophila* species, having six homologues each; the mosquitoes *Aedes aegypti*, *Anopholes gambiae* and *Culex pipiens* with five homologues and the leafhopper *Cicadella viridis*. In the nematode *Caenorhabditis elegans* eleven MIP genes were identified, from which eight are aquaporins. In amphibians, over 17 MIPs have been identified and classified into clusters. Furthermore, the heterologous expression of aquaporins in *Xenopus laevis* oocytes is recurrently used as a powerful resource to study transport activities.

In mammals, 13 AQP isoform have been found which play major roles in the body homeostasis like re-absorbing water in the kidney and maintaining the cerebrospinal fluid balance in the brain (Bienert et al., 2011).

Plant aquaporins

Plants display a much larger diversity in MIPs than other organisms. While, as previously referred, 13 isoforms have been identified in mammals, 35 have been found in *Arabidopsis*, 55 in poplar, 71 in cotton and at least 36 in maize (Chaumont et al., 2001). Also, even the genome of the evolutionarily early land plant *Physcomitrella patens* encodes 23 different isoforms (Bienert et al., 2011). Plant MIPs perform key functions in transmembrane water conductance in a broad variety of physiological processes, such as uptake from the soil, transcellular and root-to-shoot transport, cellular water homeostasis and osmotic driven growth control. However, studies have revealed that some isoforms play important roles in many other processes, such as gas and nutrient uptake and translocation, metalloid homeostasis, nitrogen remobilization, signal transduction (Bienert et al., 2011).

The MIP superfamily in plants was initially divided into four subfamilies: the Plasma Membrane Intrinsic Proteins (PIPs), Tonoplast Intrinsic Proteins (TIPs), Nodulin-like Intrinsic Proteins (NIPs) and the Small Basic Intrinsic Proteins (SIPs). More recently, two new subfamilies were identified in the moss *P. patens*: the GlypF-like Intrinsic Proteins (GIPs) and the Hybrid Intrinsic Proteins (HIPs). Another subfamily called the Uncharacterized Intrinsic Proteins (XIPs) was identified, with members in a number of dicotyledonous plants, including tomato and grapevine (Danielson and Johanson, 2008; Hachez and Chaumont, 2010).

Interestingly, XIPs are present in a wide variety of eudicot plant species, but not in Brassicaceae (e.g. *Arabidopsis*). Although XIP genes have also been identified in the genomes of many fungi and a protozoan species, they remain undetected in monocots (Gupta et al., 2009). The most conserved feature of XIP protein sequences, which could be used as a signature for this subfamily, is the NPARC motif, with a cysteine residue located after the second NPA motif (Danielson and Johanson, 2008; Gupta et al., 2009). In addition, XIPs show considerable amino acid variation at both the first NPA motif and the ar/R filter. Based on the four amino acids defining the ar/R filters, XIPs from eudicots can be divided into four subclasses, two of which have an ar/R signature similar to that found in some plant NIPs, while the other two are even more hydrophobic (Bienert et al., 2011).

Aquaporins in grapevine

Grapes (*Vitis* spp.) are economically the most important fruit species in the world (Conde et al., 2007). Both development and maturation of grape berries have received considerable scientific interest 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. In the wine producers' perspective, the most important attribute of *Vitis vinifera* is its capacity to store huge quantities of sugar, phenolics and other key compounds in the berries. From the plant point of view, the ripe phenotype is the summation of biochemical and physiological changes that occur during fruit development and make the organ edible and desirable to allow the dispersion of the seeds by animal activity (Conde et al., 2011). Therefore, understanding how and when various components, including water, accumulate in the berry, such as sugars, organic acids, phenolics, ions, is of critical importance to adjust grape growing practices and thus modify wine typology.

In grapevine, 23 MIPs were identified with genome sequence analysis in a single cultivar, but there are bound to be even more (Shelden et al., 2009; Tyerman et al., 2012). Several studies have linked aquaporin activity to the vine water status. Also, AQP expression varies according to environmental conditions and affects berry development. *V. vinifera* cultivars have different tolerance to water stress. Thus, different patterns of expression of VvPIP1;1 were observed in Chardonnay and Grenache. While Chardonnay exhibited a significant increase in VvPIP1;1 expression under water stress, Grenache did not show any alteration and this was shown to be related with differences in AQP regulation (Vandeleur et al., 2009). MIP expression also changes during maturation of grapes and this phenomenon is correlated with the increase in hydraulic resistance observed in the post-veraison stages (Tyerman et al., 2012).

In a recent study by our group (Noronha et al., 2014) the localization, expression, and functional characterization of a small basic intrinsic protein (SIP) from the grapevine were assessed. Results showed that *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 *S. cerevisiae*, fluorescent-tagged VvSIP1 was localized at the endoplasmic reticulum (ER). 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.

In the present study we characterized two different *XIP* genes from grapevine - *VvXIP1* and *VvXIP2* -, and performed the localization, expression and functional characterization of *VvXIP1*. Co-localization studies with ZmPIP2;5-GFP and *VvXIP1*-RFP fusion proteins in transiently transformed *Nicotiana bethamiana* leaves revealed that *VvXIP1* is intracellular. Heterologous expression in yeast revealed that *VvXIP1* is able to transport copper, glycerol and H₂O₂. Importantly, stopped flow spectrometry with microsomal vesicles purified from a transformed yeast strain showed that *VvXIP1* is unable to transport water. *VvXIP1* steady-state transcript levels were much higher in leaves than in berries, canes or flowers from field grown cv. Vinhão grapevines. *VvXIP1* expression was low in leaves from potted grapevines cv. Aragonez submitted to severe water deficit. Accordingly, ABA and salt stress downregulated *VvXIP1*.

A manuscript based on this work is being prepared for publication in a peer reviewed journal.

Materials and Methods

***In silico* studies**

Phylogenetic analysis was performed *in silico* using amino acid sequences from *Vitis vinifera*, *Arabidopsis thaliana*, *Gossypium hirsutum*, *Populus trichocarpa*, *Prunus persica*, *Nicotiana tabacum*, *Lotus japonicas*, *Ricinus communis*, *Physcomitrella patens*, *Aspergillus terreus*, *Fusarium oxysporum*, *Penicillium marneffeii*, *Hypocrea virens*, *Hypocrea jecorina* and *Selaginella moellendorffii* obtained from the National Center of Biotechnology (NCBI), Uniprot and PlantGDB using the BLAST tool. The alignment of sequences was performed with PRANKSTER (Hasegawa et al., 1985; Tamura et al., 1993; Whelan et al., 2001; Loytynoja et al., 2005) and Genedoc (Nicholas et al., 1997). The trees were created using these alignments with PROTDIST, NEIGHBOR and RETREE from the PHYLIP software package (Felsenstein J. 1989). An alignment with VvXIP1 and several plant XIPs was performed and TOPCONS (Bernsel et al., 2009) was used to identify the transmembrane helix domains. The accession numbers of all the sequences used are listed in Appendix 1.

Plant material

Cv. Vinhão berries, canes, flowers and leaves were collected from a commercial vineyard near Guimarães. Potted cv. Aragonez plants were grown in a greenhouse at ITQB, subjected to different watering regimes during 4 weeks: full irrigation (FI – control), with plant watering every two days; and non-irrigation (NI), no watering. Leaves were collected when NI plants water potential was $-1.3 < \Psi_{pd} < -0.9$ MPa (Conde et al., 2014).

Cell suspensions of *V. vinifera* L. (Cabernet Sauvignon Berry - CSB) were freshly established from somatic callus that had been previously initiated from Cabernet Sauvignon berry pulp. They were maintained on modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962; Decendit et al., 1996), supplemented with 2% (w/v) sucrose in 250 ml flasks on a rotary shaker at 100 rpm in the dark, at 23 °C according to Conde et al. (2014).

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 *VvXIP1* expression, 5 ml aliquots were incubated overnight with 200 mM NaCl, polyethylene glycol (PEG) 2% (w/v), 150 μ M abscisic acid (ABA), or 150 μ M salicylic acid (SA) at 23 °C. After each treatment, cells were immediately frozen in liquid nitrogen and stored at -80 °C as performed by Noronha et al. (2014).

Growth assays

The following yeast strains were used: YSK1172, MPY2 and MPY3. YSK1172 is a deletion mutant for AQy aquaporins and MPY2 and MPY3 are double and triple mutants (MPY17 strain with *ctr1Δctr3Δ*, *ctr1Δctr3Δctr2Δ*), respectively, in copper transporters unable to grow with respiratory substrates such as glycerol and ethanol which requires high amounts of copper. AQy-null strain was transformed with *pVW214-VvXIP* and *pVW214-Empty Vector*. MPY2 and MPY3 strains were transformed with *pVW214-VvXIP*, *pVW214-Empty Vector* and with *pVW214-VvCTR1*. *VvCTR1* is a copper transporter recently identified and functionally characterized (Martins et al., 2014a). The *pVW214-VvXIP1* construct was prepared by Gateway (QIAGEN) and the primers can be found in appendix 1.

YSK yeast cells were grown in synthetic medium with 7 g/l YNB, 1.3 g/l SC, 2% Agar powder, 2% glucose, 300 mg/100 ml leucine and 250 mg/100 ml Tryptophan, washed and resuspended in sterile water to a final $OD_{600\text{ nm}} = 1$. Different dilutions (0.1, 0.01 and 0.001) were made and 10 μ l from each suspension were spotted in solid medium with the following conditions/stressors: i) 2.1M sorbitol, ii) 1% and 2% of ethanol and the same percentages of glycerol, iii) 30 and 60mM Boric acid (adjusted to pH 5.5 with Tris), iv) 5mM CuSO_4 , v) 0.35 and 0.7 mM H_2O_2 . Appropriate control plates inoculated with non-complemented mutants were prepared.

MPY yeast cells were grown in synthetic medium containing 7g/L YNB, 1.3g/L SC, 2% Agar powder, 2% glucose, 25 mg/100mL histidine, 30 mg/100ml Lysine and 25 mg/100mL Tryptophan, washed and resuspended in sterile water to a final $OD_{600\text{ nm}} = 1$; Different dilutions (0.1, 0.01 and 0.001) dilutions were made and 10 μ l from each suspension were spotted in solid medium containing 10 and 100 μ M CuSO_4 . Yeast growth was observed during several days and images were collected with Chemidox XRS (Bio-Rad).

Fluorescence assays

Yeast cells from YSK1172 AQy-null strain were transformed with *pVW214-VvXIP1* and *pVW214-Empty vector* constructs and pre-cultured in YNB+SC solid medium. Liquid cultures were then grown in the dark overnight in presence of 1 μ M 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA, Molecular Probes), a fluorophore previously used to monitor the level of reactive oxygen species (ROS) (Halliwell and Gutteridge,

1999; Bienert et al., 2007). In its acetylated form, the dye can freely diffuse into yeast cells but, once inside, the fluorochrome is deacetylated and unable to cross the membrane making the cells susceptible to oxidation by ROS. After incubation, cells were washed three times with MOPS buffer (pH 7) and resuspended in this buffer to a final $OD_{600\text{ nm}}=1.4$. Fluorescence of 2 ml yeast suspension was followed over time at 20°C in a spectrofluorometer at an excitation/emission of 492/527 nm (Perkin Elmer Luminescence Spectrometer LS 50).

Clark electrode assays

Yeast cells from YSK1172 AQy-null strain were transformed with *pV214-VvXIP1* and *pV214-Empty vector* constructs and pre-cultured in YNB+SC solid medium. Liquid cultures were then grown overnight. Cells were then washed three times and resuspended in water to a final $OD_{600\text{ nm}}=1$. Hydrogen peroxide was then added to the cell suspension to a final concentration of 50 μM and the oxygen formation followed using a Clark electrode coupled to an YSI 5300 Biological Oxygen Monitor.

RNA isolation from grapevine berries and leaves

RNA from grape berries and leaves was isolated using QIAGEN RNeasy Plant Mini kit following the manufacturer's instructions. 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, 2.0M NaCl, and 2% (v/v) β -mercaptoethanol. After an in-column DNase treatment, the RNA integrity was checked in a 1% agarose gel, and the first-strand cDNA synthesis was performed with the LongRange 2Step RT-PCR (Quiagen), following manufacturer's instructions.

Co-localization studies

The *pH7RWG2-VvXIP1-RFP* construct was prepared by Gateway (QIAGEN) and the primers can be found in appendix 1. Recombination sequences were introduced by PCR in the *VvXIP1* cDNA without a stop codon and the fragment recombined into the entry vector pDONR221 using the BP clonase enzyme. *VvXIP1* cDNA was then recombined into the pH7RWG2 vector by the LR clonase enzyme. *pDE1001-ZmPIP2;5-GFP* was kindly supplied by François Chaumont and was obtained as described by Chaumont et al. (2000). The two constructs were separately introduced in

Agrobacterium tumefaciens (GV3101) and transient transformation of *Nicotiana benthamiana* leaf epidermal cells was 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 $OD_{600nm}=0.1$ with infiltration buffer (50 mM MES pH 5.6, 2 mM Na_3PO_4 , 0.5% glucose, and 100 μ M acetosyringone). Diluted cells were cultivated again until the culture reached an $OD_{600nm}=0.2$. Four-week-old *N. benthamiana* plants were infiltrated with the bacterial cultures and leaf discs were examined under the confocal microscope 2 days later.

Real-time PCR studies

Quantitative Real-time PCRs were performed with a QuantiTect SYBR Green PCR Kit (Qiagen) and in a CFX96 Real-Time detection System (Bio-Rad), at an annealing temperature of 55°C. RNA and cDNA samples were obtained as described above. Experiments were carried out in biological triplicates with the software Bio-Rad CFX Manager (Bio-Rad), using *VvGAPDH* as an internal control. To verify the absence of unspecific and primer-dimer amplification melting curves were performed after each run. The primers used to study the expression of *VvXIP1* and *VvGAPDH* can be found in appendix 2.

Yeast membrane Isolation

YSK1172 yeast cells transformed with *pW214-VvXIP1* were cultivated overnight in YNB medium with leucine and tryptophan. Cells were washed, resuspended in digestion buffer (1.35 M sorbitol, 10mM citric acid, 30 mM Na_2HPO_4 , 1 mM EGTA, pH 7.4) with 30 mM dithiothreitol (DTT), centrifuged and spheroplasts were obtained by digestion with zymolyase 20T (in digestion buffer). After complete digestion, monitored in phase contrast microscope, spheroplasts were pelleted and homogenized in a potter homogenizer after resuspended in HEPES-lysis buffer [20 mM HEPES, 50 mM potassium acetate, 100 mM sorbitol, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM DTT, pH 6.8]. The homogenate was centrifuged at 1000 *g* for 10 min and the supernatant saved. The membranes were then pelleted at 100 000 *g* for 30 min, resuspended in Resuspension buffer (100 mM mannitol, 10 mM Tris-HEPES, pH 7.5), centrifuged again at 100 000 *g* for 30 min, flash frozen and stored at -80 °C These procedures were performed according Rodrigues et al. (2013) and Noronha et al. (2014).

Stopped flow spectroscopy

Membrane permeability of the microsomal fraction from transformed yeasts was studied with the stopped flow technique (HI-TECH Scientific PQ/SF-53) in the Laboratory headed by Prof. Graça Soveral at Faculdade de farmácia, Universidade de Lisboa (Lisbon). Five runs were usually stored and analysed in each experimental condition, as described before (Soveral et al., 1997; Noronha et al., 2014). To study water transport, vesicles resuspended in 100 mM mannitol, 10 mM TRIS-HEPES (pH 7.5) (0.1 ml, 0.4 mg protein ml⁻¹) were mixed with a 340 mM mannitol, 10 mM TRIS-HEPES (pH 7.5) solution at 23 °C to produce an inwardly directed gradient of impermeant solute (osmotic gradient 240 mOsmol). 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 reached. Experiments were performed at 14-33 °C to study activation energies.

To study glycerol transport, vesicles resuspended in 100 mM mannitol, 10 mM TRIS-HEPES (pH 7.5) (0.1 ml, 0.4 mg protein ml⁻¹) were mixed with a solution containing 100 mM mannitol, 240 mM glycerol and 10 mM TRIS-HEPES (pH 7.5) at 23 °C to produce an inwardly directed gradient (osmotic gradient 240 mOsmol). The kinetics of vesicle shrinkage and swelling were measured from the time course of 90 ° scattered light intensity at 400 nm until a stable light scatter signal was reached. The water permeability coefficient (P) and the glycerol permeability coefficient (P_{gly}) were estimated by fitting the light scatter signal to a single exponential curve with the equation $P_t = k(V_o/A) [1/V_w/(osm_{out})_{\infty}]$, where V_w is the molar volume of water, V_o/A is the initial volume to area ratio of the vesicle population and $(osm_{out})_{\infty}$ is the final medium osmolarity 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 energies (E_a) were obtained from the slope of an Arrhenius plot ($\ln P$ or $\ln P_{gly}$ 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 Noronha et al. (2014).

Statistical analysis

The results obtained were statistically verified by analysis of variances tests (one-way and two-way ANOVA using Prism v. 6 (GraphPad Software, Inc.) Post-hoc multiple comparisons were performed using the HSD Tukey test.

RESULTS

VvXIP1 sequence analysis

A phylogenetic tree constructed with amino acid sequences from SIPs, PIPs, TIPS, NIPs and XIPs from *Vitis vinifera*, *Gossypium hirsutum*, *Populus trichocarpa*, *Arabidopsis thaliana* and *Nicotiana tabacum* shows a clear clustering of the analyzed MIP sub-families in 5 separated groups (Figure 2). The tree depicted in Figure 3 was constructed only with XIP members from different plant and fungi species to make a close-up analysis of the phylogenetic relationships within XIP family. As can be seen, VvXIP1 is phylogenetically close to GhXIP1 from cotton and to PpXIP1 from peach.

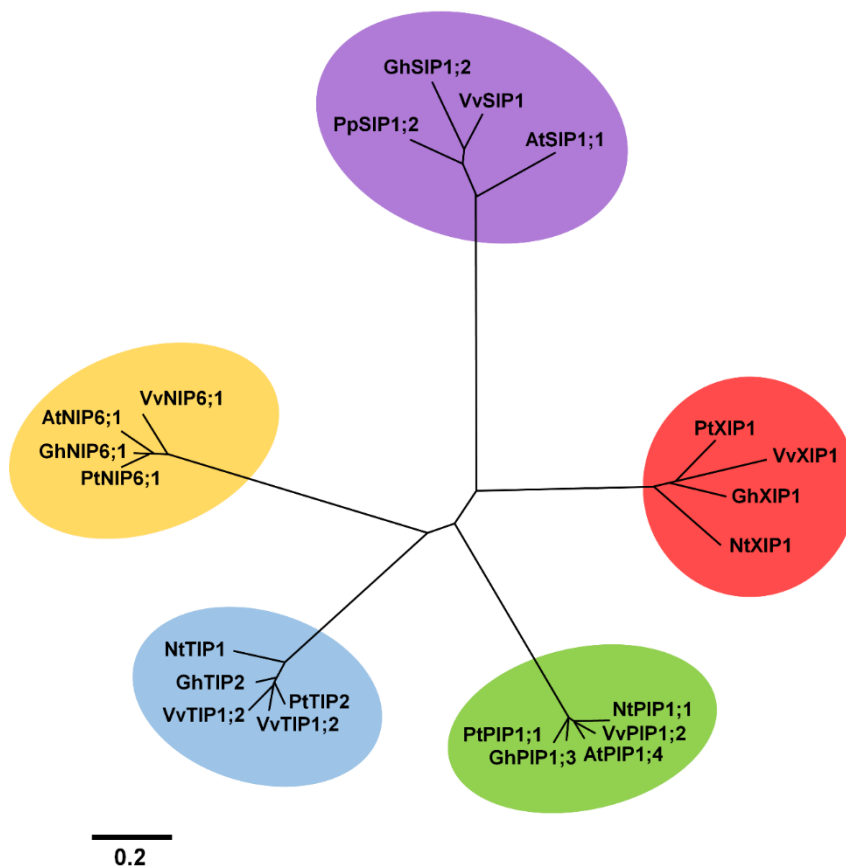


Figure 2 – Phylogenetic analysis of several Plant MIPs. Sequences from PIPs, NIPs, SIPs, TIPs and XIPs from *Vitis vinifera* (Vv), *Arabidopsis thaliana* (At), *Gossypium hirsutum* (Gh), *Populus trichocarpa* (Pt) and *Nicotiana tabacum* (Nt) (NCBI and Uniprot access numbers in Appendix) were aligned using PRANKSTER (Hasegawa et al., 1985; Tamura et al., 1993; Whelan et al., 2001; Loytynoja et al., 2005). The phylogenetic tree was built using genedoc (Nicholas et al., 1997), Phylip (Felsenstein J, 1989) and MEGA4 (Tamura et al., 2007).

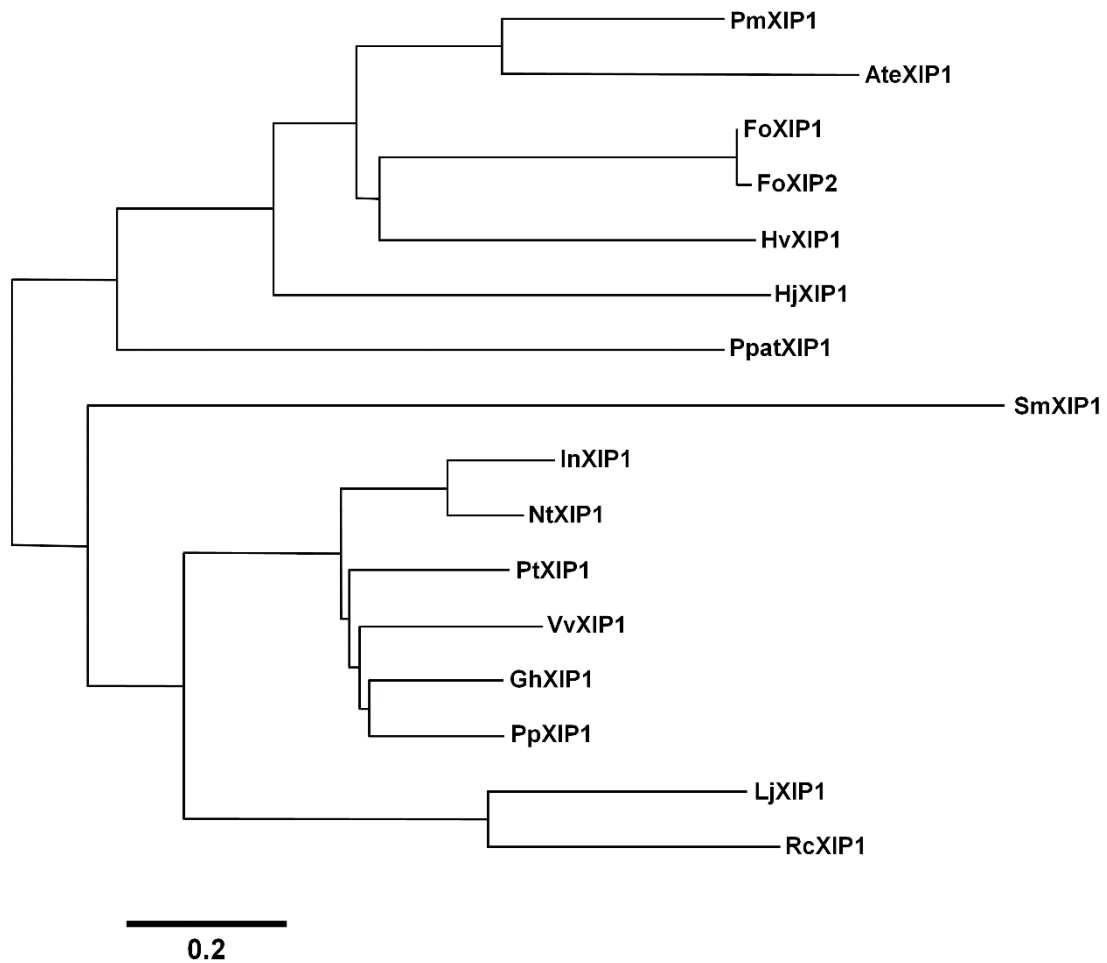


Figure 3 – Phylogenetic tree comparing XIP proteins from plants and fungi. Amino acid sequences from *V. vinifera* (Vv), *P. trichocarpa* (Pt), *Prunus. persica* (Pp), *G. hirsutum* (Gh), *Ipomoea nil* (In), *N. tabacum* (Nt), *Lotus japonicas* (Lj), *Ricinus communis* (Rc), *Physcomitrella patens* (Ppat), *Aspergillus terreus* (Ate), *Fusarium oxysporum* (Fo), *Penicillium marneffeii* (Pm), *Hypocrea virens* (Hv), *Hypocrea jecorina* (Hj) and *Selaginella moellendorffii* (Sm) were aligned using PRANKSTER (Hasegawa et al., 1985; Tamura et al., 1993; Whelan et al., 2001; Loytynoja et al., 2005). The phylogenetic tree was built using genedoc (Nicholas et al., 1997), Phylip (Felsenstein J) and MEGA4 (Tamura et al., 2007).

Figure 4 shows the alignments of amino acid sequences from XIPs from *V. vinifera* (VvXIP1), *P. trichocarpa* (PtXIP1), *P. persica* (PpXIP), *G. hirsutum* (GhXIP1;1), *I. nil* (InXIP1;1), *N. tabacum* (NtXIP1;1), *L. japonicas* (LjXIP1), *R. communis* (RcXIP). As can be seen, all the proteins share the highly conserved NPARC motif, despite the slight variations in the first NPA (NPV in most of the aligned species). Using TOPCONS it was also possible to identify the regions corresponding to the six transmembrane helices.

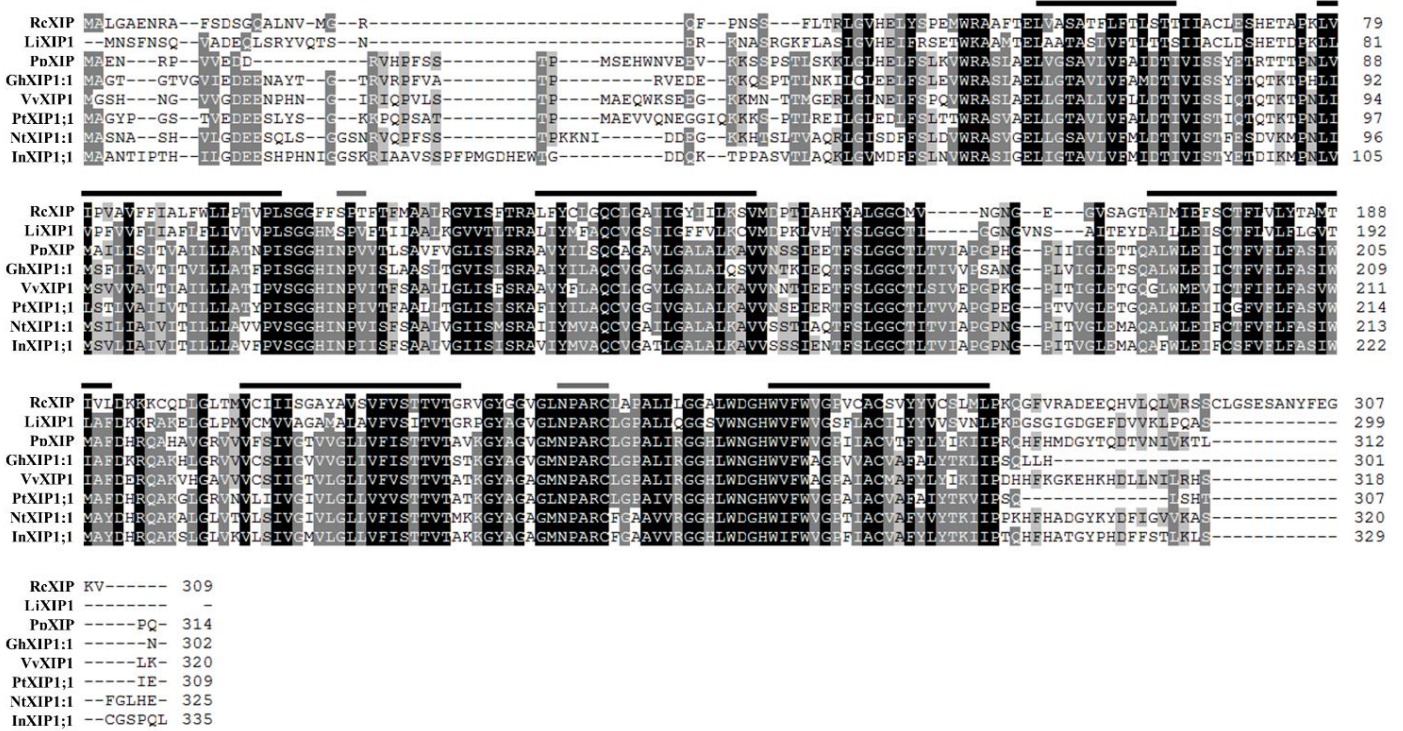


Figure 4 – Alignment of eight plant XIPs (VvXIP1, PtXIP1, PpXIP, GhXIP1;1, InXIP1;1, NtXIP1;1, LjXIP1 and RcXIP) showing six transmembrane helix domains (Black lines) and the conserved ‘NPV’ and ‘NPARC’ motifs (gray lines).

Subcellular localization of VvXIP1

As referred to in “Material and Methods”, the subcellular localization of VvXIP1-RFP was studied after *Agrobacterium tumefaciens* mediated transient expression in *Nicotiana benthamiana* leaf cells. Results are shown in Figure 5 when leaves were observed under the confocal microscope. As can be seen, the green fluorescence from ZmPIP2;5-GFP is clearly located at the cell periphery, at the plasma membrane, as shown before (Chaumont et al., 2000). However, the red fluorescence from RFP does not co-localize with the green fluorescence signal, showing a more diffuse pattern, probably between the vacuole - that occupies most of the intracellular space forcing the cytosol with its surrounding plasma membrane against the cell wall - and the plasma membrane (Figure 5). Thus VvXIP1 does not localize to the plasma membrane, in agreement with the prediction performed by the program “PredictProtein”.

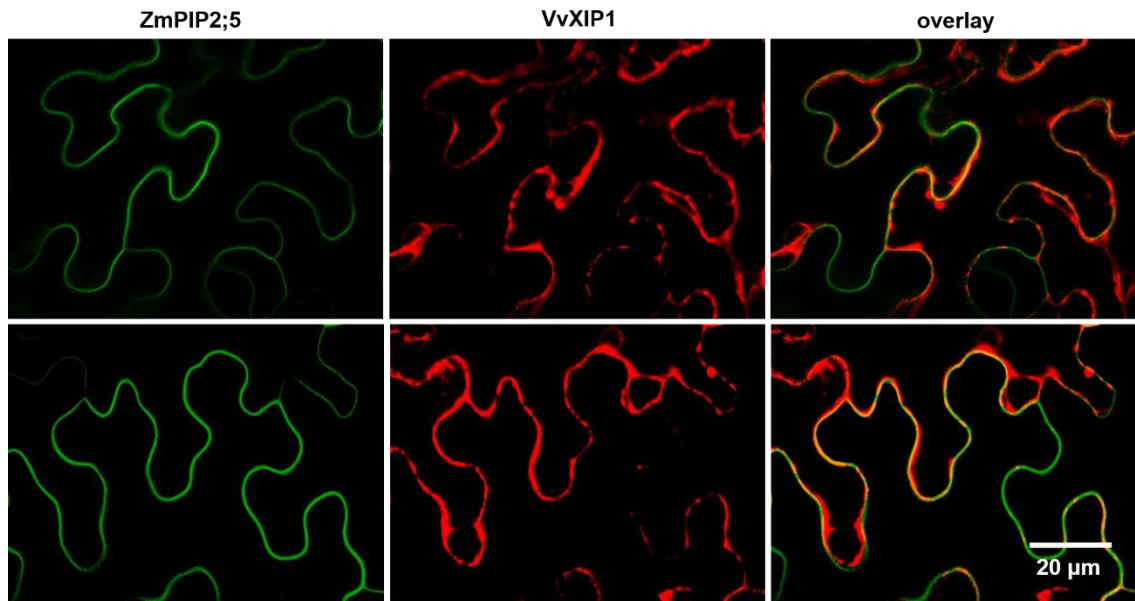


Figure 5 – Subcellular localization of VvXIP1 in *Nicotiana benthamiana*. Laboratory grown plants were infiltrated with *Agrobacterium tumefaciens* strains transformed with *pH7RWG2-VvXIP1* and *pDE1001-ZmPIP-GFP* plasmids. Images were acquired 2 days after infiltration in a confocal microscope.

Transport Studies

H₂O₂ transport

To test whether VvXIP1 can mediate the transport of H₂O₂, the YSK1172 AQy-null yeast mutant strain was transformed with the construct *pVV214-VvXIP1* and H₂O₂ incorporation was studied with three distinct approaches: i) rate of fluorescence variation of the fluorescent probe CM-H₂DCFDA, ii) rate of O₂ production resulting from intracellular breakdown of H₂O₂, and iii) yeast growth inhibition in solid medium supplemented with H₂O₂.

When CM-H₂DCFDA was used to measure H₂O₂ uptake by spectrofluorimetry, yeast cells expressing VvXIP1 showed a much higher rate of fluorescence increase after H₂O₂ addition than control cells transformed with the empty vector (Figure 6A). Similarly, *VvXIP1*-transformed yeast showed a 3.5 - fold higher rate of O₂ production than the control, as measured with a Clark electrode, when H₂O₂ was added to the incubation media (Figure 6B).

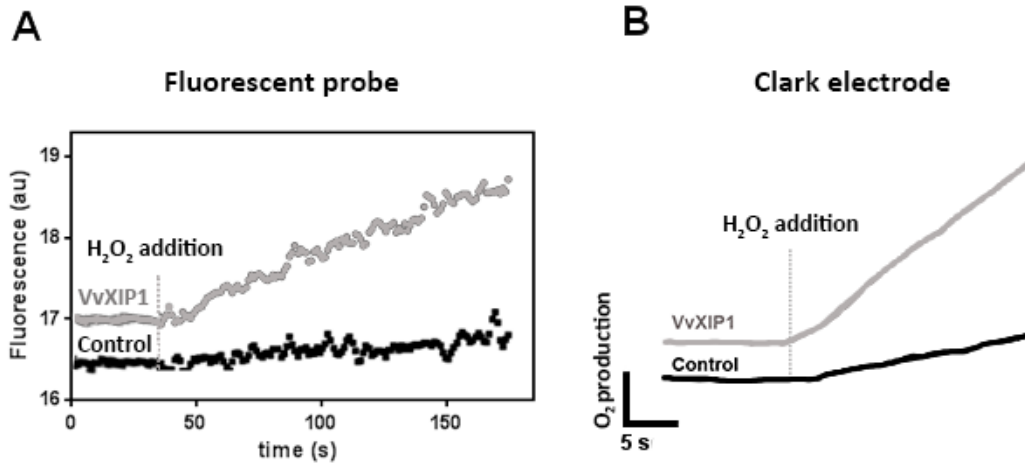


Figure 6 – H₂O₂ transport by yeast cells expressing *VvXIP1*. Cultures of the yeast strain YSK 1172 ΔQynull transformed with the empty vector *pV214* or *pV214* carrying *VvXIP1* were incubated with CM-H₂DCFDA probe overnight and the fluorescence response after 600 μM H₂O₂ addition was monitored with a spectrofluorimeter (A). O₂ release monitored with a Clark electrode in response to 50 μM H₂O₂ (B).

Regarding the growth experiments in solid media, results showed that the growth of the yeast transformed with *pV214-VvXIP1* was severely inhibited when YNB media were supplemented with toxic levels of H₂O₂, 0.35 and 0.7 mM, when compared to controls (Figure 7).

Altogether, results clearly show that VvXIP1 is able to mediate the transport of H₂O₂.

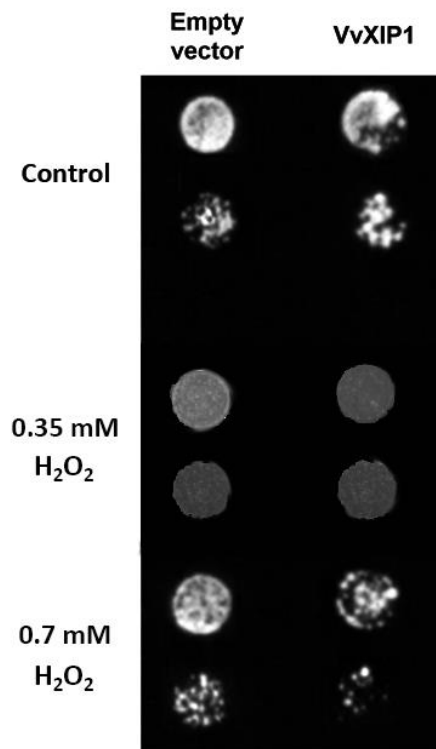


Figure 7 – H₂O₂ sensitivity of yeast cells expressing *VvXIP1*. Cultures of YSK 1172 ΔQynull yeast cells transformed with the *pVW214-Empty vector* and *pVW214-VvXIP1* were spotted at an OD₆₀₀ nm of 0.1 and 0.01 on medium containing the indicated concentrations of H₂O₂ and growth was recorded after 3 days at 30 °C.

Stopped flow experiments to study water transport

Stopped flow light-scattering spectrophotometry was used to check if VvXIP1 is a water channel. As reported in “Material and Methods”, a microsomal fraction from YSK1172 yeast cells expressing *VvXIP1* was used to study membrane permeability. Control microsomal vesicles were obtained from yeasts transformed with the empty vector. The vesicle size, measured by QELS analysis, was homogeneous in all batches with a mean hydrodynamic diameter of 375 ± 62 nm (n=8) (data not shown). Vesicles were challenged with a hypertonic mannitol solution to study water efflux. Results showed that the rate of water efflux (Figure 8A), and thus the osmotic permeability coefficient (P_f ; Figure 8C), in yeast membrane vesicles with VvXIP1 were similar to control vesicles. Also, the activation energies (E_a) were similar in both vesicle batches (Figure 9A and C). Altogether, these clear-cut results strongly suggested that VvXIP1 is unable to transport water.

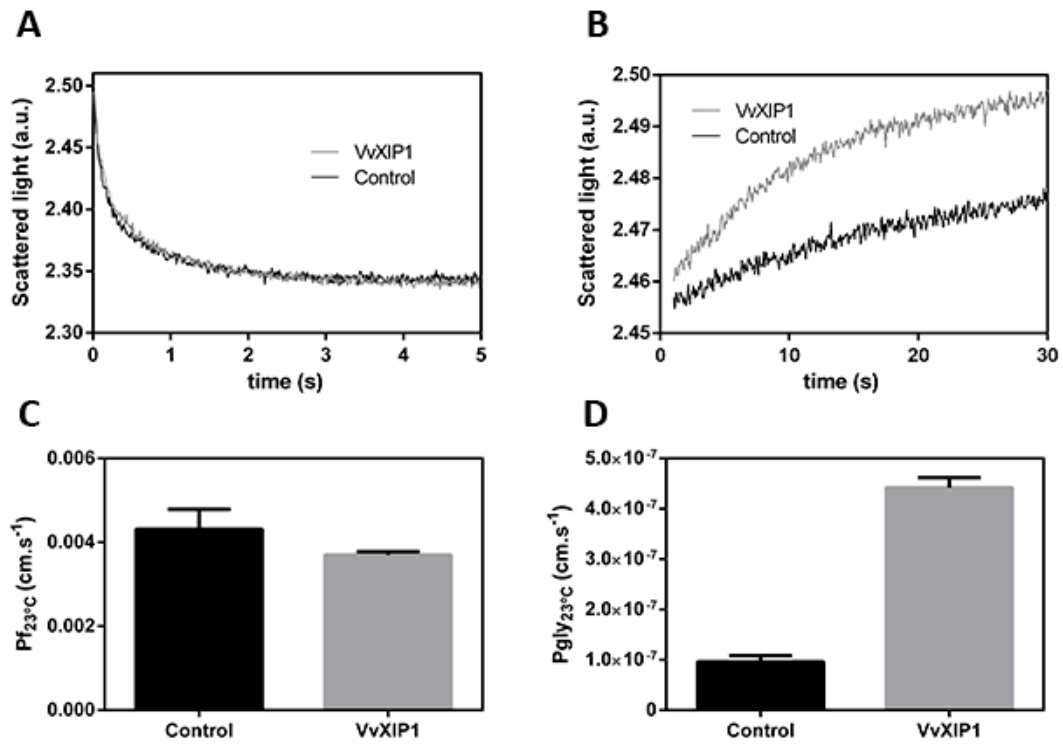


Figure 8 – Stopped flow experiment for evaluation of the water and glycerol permeability in yeast membrane vesicles. Normalized scattered light intensity obtained from stopped flow experiments with membrane vesicles collected from yeast cells transformed with *pVV214-VvXIP1* (grey) or the empty vector (black), suddenly exposed to an osmotic gradient of 240 mOsm with a mannitol solution (A) and with a glycerol solution (B). The water and glycerol permeability coefficients are represented in C and D respectively.

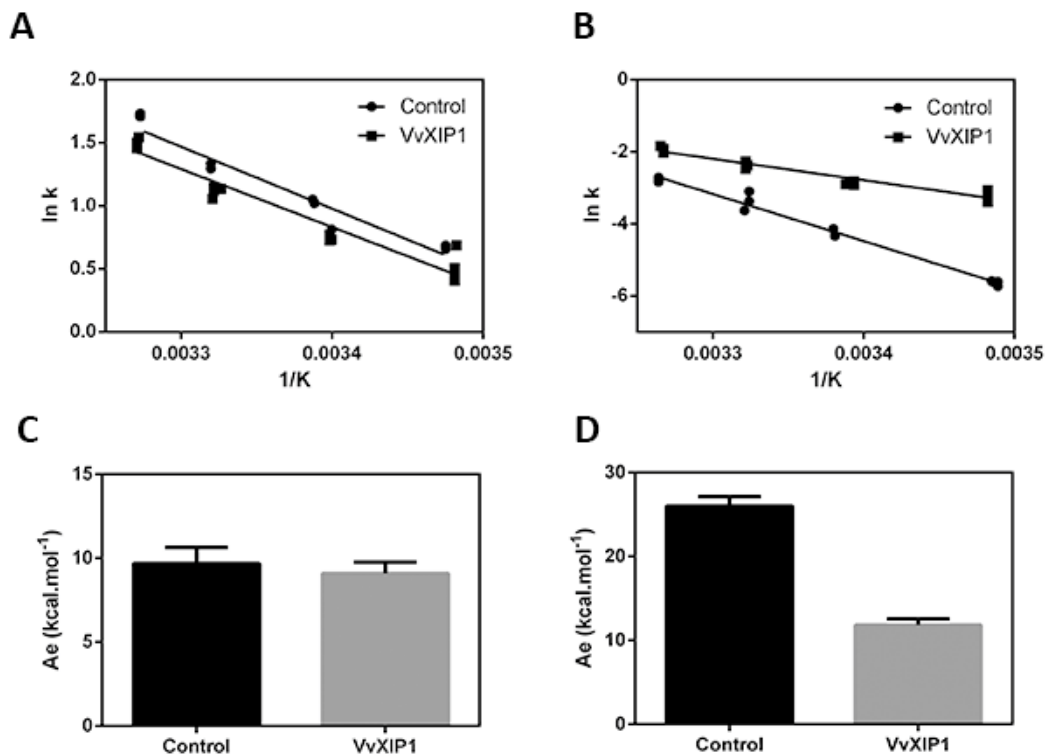


Figure 9 – Stopped flow experiment to evaluate the energetics of water and glycerol permeability in yeast vesicles. Normalized scattered light intensity was obtained from stopped flow experiments performed according to a temperature gradient. Membrane vesicles collected from yeast cells transformed with *pV214-VvXIP1* (grey) or the empty vector (black) were suddenly exposed to an osmotic gradient of 240 mOsm. The gradient was built with mannitol (A) to evaluate water transport and with glycerol (B) to evaluate glycerol transport. The activation energy for water (C) and glycerol (D) transport were calculated from the Arrhenius plots.

Glycerol transport

Taking advantage of the stopped flow approach, the capacity of VvXIP1 to transport glycerol was studied. As shown in Figure 8B the swelling rate of the membrane vesicles from VvXIP1-expressing yeast cells when challenged with a hypertonic glycerol solution was much higher than in control vesicles. The corresponding permeability coefficients are shown in Figure 8D. Channel mediated glycerol transport was confirmed when the E_s were measured. As can be seen in Figures 9B and D, E_s value associated with glycerol transport in vesicles from VvXIP1-expressing yeast cells was much lower than in vesicles from the yeast transformed with the empty vector, strongly suggesting protein-mediated diffusion.

To further demonstrate that VvXIP1 can transport glycerol, transformed yeast cells were grown in glycerol rich medium. Results showed that cells expressing *VvXIP1* grew better than control cells (empty vector) in media supplemented with 1 and 2% ethanol and glycerol, confirming that glycerol is a substrate of VvXIP1 (Figure 10), and, therefore, validating the results from the stopped flow experiments

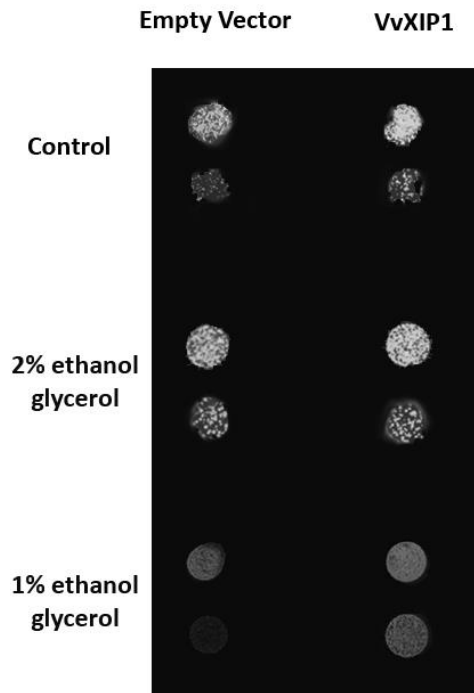


Figure 10 –Yeast growth assays in glycerol rich media by cells expressing *VvXIP1*. Cultures of YSK 1172 AQynull yeast cells transformed with *pVW214-Empty vector* and *pVW214-VvXIP1* were spotted at OD₆₀₀ nm of 0.1 and 0.01 on medium containing the indicated concentrations of ethanol and glycerol and growth was recorded after 3 days at 30 °C.

Copper transport

As referred to in “Material Methods”, the yeast strains MPY2, MPY3 and YSK1172 were used to study if VvXIP1 can transport Cu. MPY2 and MPY3 are double and triple mutants in copper transporters, respectively, and cannot survive when cultivated with respiratory substrates due to the lack of means to uptake high amounts of Cu required for respiration. These yeast strains were transformed with *pVW214-VvXIP1* construct to evaluate if respiration capacity was recovered. Furthermore, MPY strains were also transformed with *pVW214-VvCTR1*, a copper transporter recently identified and characterized in our group (Martins et al., 2014a). Results showed that VvXIP1 and VvCTR1 were able to restore yeast growth when both mutant strains were plated onto 100 μM CuSO₄ (Figure 11A). Also, the transformation of YSK yeast strain with

VvCTR1 increased yeast sensitivity to the toxic effects of Cu, and thus reduced the growth in YNB media supplemented with 5 mM CuSO₄ (Figure 11B), suggesting that in addition to H₂O₂ and glycerol VvXIP1 also transports Cu.

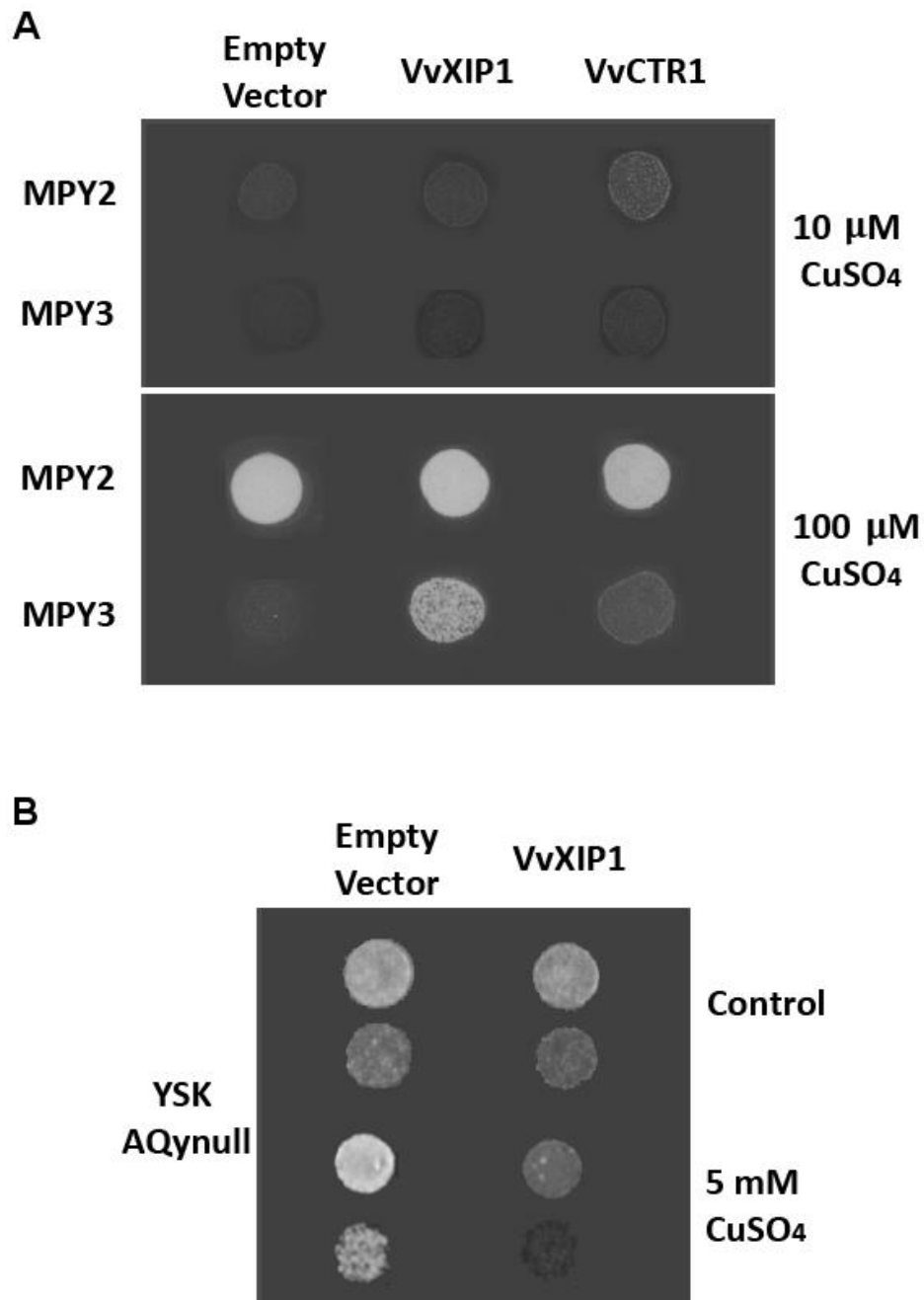


Figure 11 – Cu effect on the growth of yeast cells expressing VvXIP1. Cultures of MPY2 (A), MPY3 (A) and YSK 1172 (B) yeast cells transformed with pV214-Empty vector, pV214-VvCTR1 (only used in MPY strains) and pV214-VvXIP1 were spotted at OD_{600 nm} of 0.1 and 0.01 on medium containing the indicated concentrations of CuSO₄ and growth was recorded after 3 days at 30 °C.

Role of VvXIP1 in osmotic stress response

It has been reported that aquaporin expression in yeast may lead to an osmotic stress oversensitivity (Leitão et al., 2012). In the present study YSK 1172 Aqy-null yeast cells were transformed with *pV214-VvXIP1* and incubated in YNB media supplemented with 2.1 M sorbitol. As shown in Figure 12, transformed cells with VvXIP1 grew less than control cells transformed with the empty vector suggesting that they are more sensitive to osmotic stress.

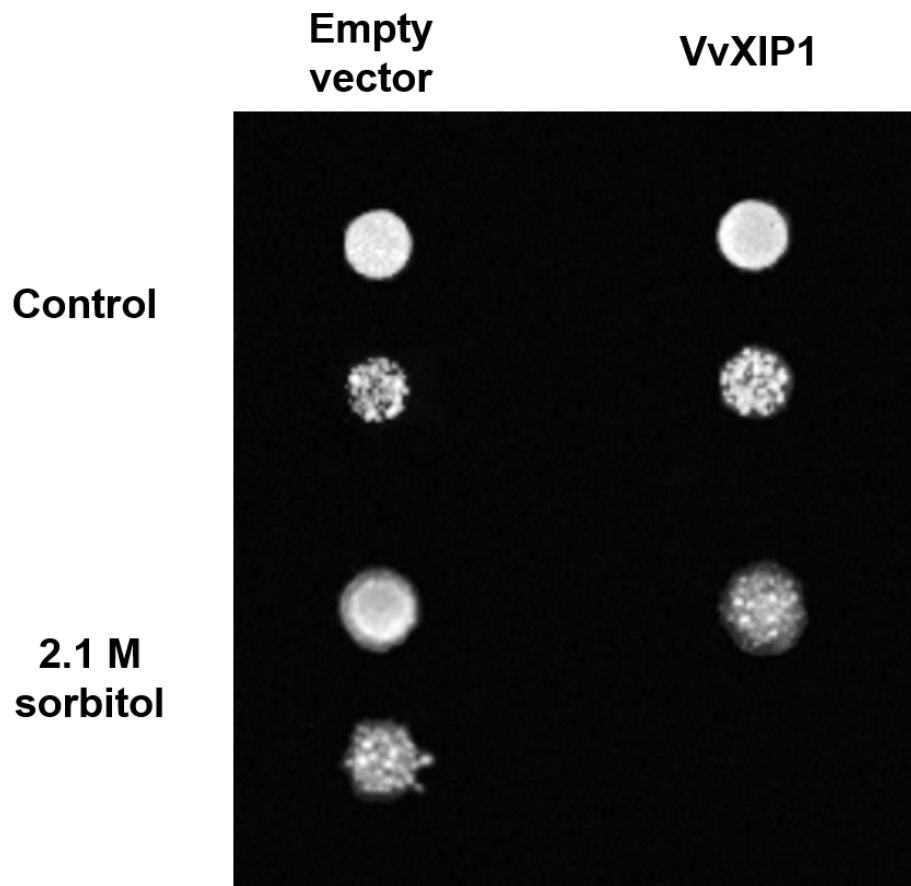


Figure 12 – Sorbitol induced osmotic stress sensitivity of yeast cells expressing *VvXIP1*. Cultures of YSK 1172 Aqynull yeast cells transformed with the *pV214-Empty vector* and *pV214-VvXIP1* were spotted at OD₆₀₀ nm of 0.1 0.01 on medium containing the indicated concentration of sorbitol and growth was recorded after 3 days at 30 °C.

VvXIP1 expression studies

The expression of *VvXIP1* was studied in different organs in field grown cv. Vinhão grapevines. Total RNA was isolated from leaves, berries, canes and flowers. As shown in Figure 13, steady-state transcript levels of *VvXIP1* were much higher in leaves than in the remaining organs.

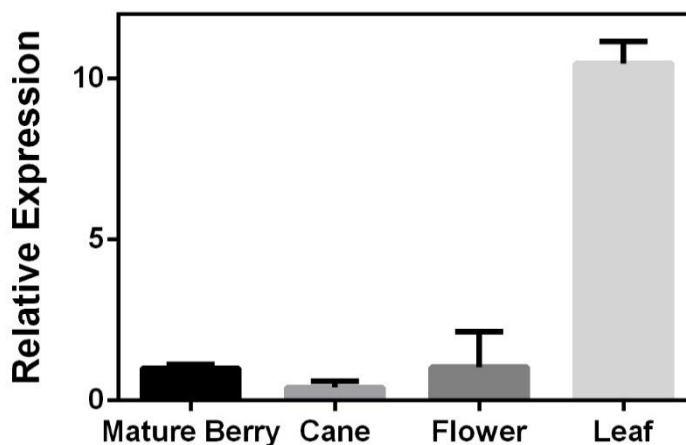


Figure 13 – *VvXIP1* expression in several organs from grapevine determined by quantitative real-time PCR. Transcript levels in berries, canes, flowers and leaves from cv. Vinhão.

To study the effect of water stress on the expression of *VvXIP1*, transcript levels were measured in leaves from potted cv. Aragonez grapevines cultivated in a greenhouse at ITQB (Oeiras – Portugal) in the scope of a collaboration in progress with the group headed by Prof. Manuela Chaves. As can be seen in Figure 14A, *VvXIP1* steady-state transcript levels were lower in leaves from non-irrigated plants than in leaves from plants watered every two days, suggesting that water deficit downregulates *VvXIP1*.

Following the results from heterologous expression experiments which suggested that *VvXIP1* is able to mediate Cu transport, we decided to study whether the application of Bordeaux mixture in field conditions could affect the expression of *VvXIP1*. This task took advantage of the work developed in the scope of the PhD project of Viviana Martins (Martins et al., 2014a; Martins et al., 2014b). As shown in Figure 14B, in leaves from cv. Vinhão vines treated with Bordeaux mixture (+ Cu) a downregulation of *VvXIP1* expression was observed when compared to plants treated with a conventional triazole-based fungicide (- Cu).

Despite the necessary cautions needed to extrapolate the results to a multicellular level, suspension-cultured cells have provided a convenient experimental system to study several important aspects of plant cell physiology, including gene expression and functional studies

(Conde et al., 2006; Noronha et al., 2014; Conde et al., 2014). Additionally, in cell suspensions, the plasma membrane is readily amenable for challenging with exogenous stressors/elicitors. In the present study cultured CSB cells were used to study *VvXIP1* expression after an overnight incubation with 100 mM NaCl, 150 μ M ABA, 150 μ M SA and 2% (w/v) PEG. *VvXIP1* transcript levels were downregulated by ABA and NaCl but not affected by SA and PEG (Figure 14C).

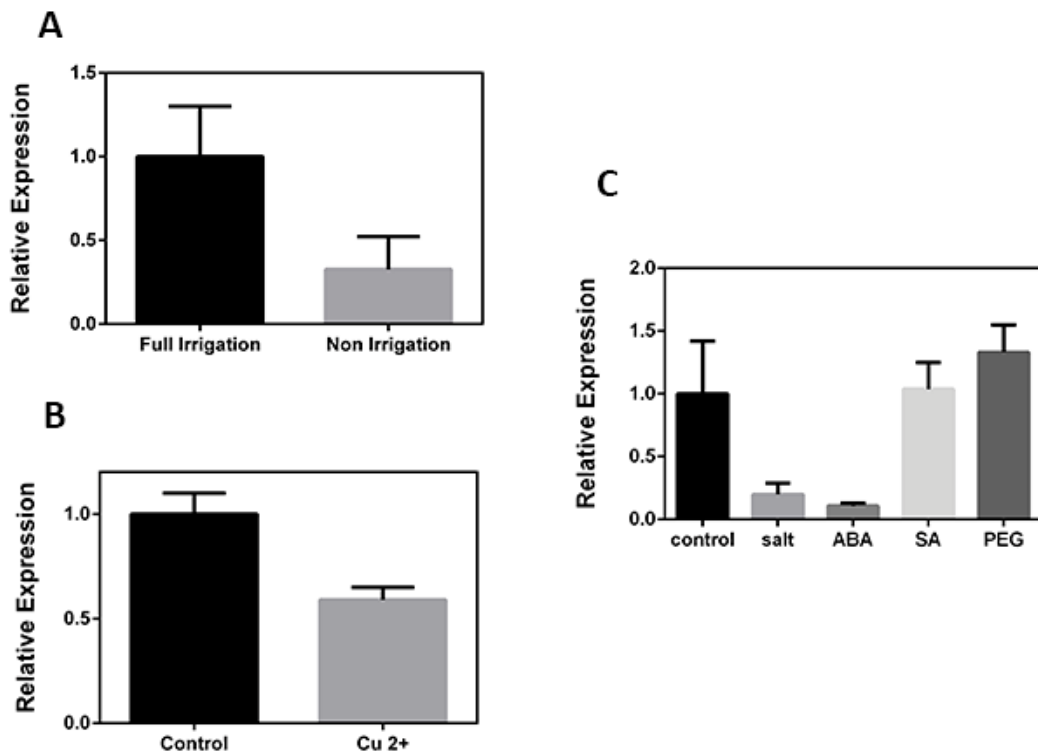


Figure 14 - *VvXIP1* expression in response to potential stressors/elicitors determined by q PCR. *VvXIP1* steady-state transcript levels in leaves from field grown cv. Vinhão grapevines in response to copper treatments (Bordeaux mixture) (A), in potted grapevines (cv. Aragonéz) under drought stress (B) and in CSB cell suspensions treated with salt, ABA, SA and PEG (C).

DISCUSSION

VvXIP1 is a grapevine intracellular solute channel

Despite the overall structure similarities of aquaporins, they are highly divergent among different subfamilies (Gomes et al., 2009; Lopez et al., 2012). The differences in amino acid sequences are even more relevant in plants because as sessile organisms they have much more MIP genes than animals, bacteria or fungi (Bienert et al., 2007; Gomes et al., 2009). As reported to before, XIPs - Uncharacterized Intrinsic Proteins - are present in some plant species and fungi, but their nature and true physiological role are still no clear. As shown in Figure 2, when aligned with several plant TIPs, PIPs, SIPs and NIPs, plant XIPs cluster in a clearly distinct monophyletic group, completely separated from the other MIP subfamilies, suggesting specific transport characteristics. Moreover, VvXIP1 identified in the present study is highly similar to other XIPs from cotton and peach and show strong evolutionary divergence from fungi XIPs.

The divergence in the composition of the NPA motives has been proposed to reflect differences in the substrate specificity, as previously observed in NIPs (Bienert et al., 2011). In the present study, results showed that the composition of the first NPA motive might change considerably within XIP sequences from different species. As shown in “Results”, the alanine of VvXIP1 is replaced by valine in XIPs from *P. persica*, *G. hirsutum* and *N. tabacum*, by isoleucine in *Ipomoea nil* and *P. trichocarpa*, and by threonine in *R. communis*. The specificity of the aquaporin VvXIP1 revealed rather striking, as discussed below.

After their discovery at the plasma membrane of erythrocytes, researchers found that, similarly to other transporters and channels, aquaporins could also localize at intracellular membranes and transport other substrates than water (Wudick et al., 2009). Actually, it is known that TIPs have a key role in water transport at the tonoplast level and are tightly regulated by water deficit conditions (Tyerman et al., 2012). In addition, several aquaporins can be located to the membrane of other organelles, including the endoplasmatic reticulum. This is the case of VvSIP1 that specifically transports water across the ER of grapevine cells (Noronha et al., 2014). In a previous work it was shown that the XIP aquaporin from tobacco NtXIP1;1 is located at the plasma membrane (Bienert et al., 2011), but in the present study clear-cut results showed that VvXIP1 is indeed not targeted to the plasma membrane. After a transient transformation of tobacco leaves, the red fluorescent protein-tagged VvXIP1 did not co-localize with the green fluorescent protein-tagged ZmPIP2;5, which is a known plasma membrane protein. To ascertain the precise localization of VvXIP1, future work will involve the utilization of intracellular membrane

markers, including the endoplasmatic reticulum marker *HDEL-YFP*, the Golgi apparatus marker *ST-YFP*, and the tonoplast marker *ZmTIP2;1-YFP*.

VvXIP1 specificity is puzzling

What should the physiological role be of an intracellular aquaporin which mediates the incorporation H_2O_2 , Cu, glycerol, but not water? Why substrates structurally and functionally so different could share the same channel? Is VvXIP1 involved in detoxifying damaging molecules by trapping them in the vacuole? Is there any physiological redundancy between VvXIP1 and VvNIPs? The addressing of these key questions in future work will expand our knowledge on the role of intracellular aquaporins and will shed light into their structure-function relationships.

H_2O_2 is an important molecule with dual functions, because it is an oxidative stress inducer but also a signaling molecule in plants (Bolwell, 1999; Foyer et al., 1999; Neill et al., 2001). In the present study we demonstrated with distinct approaches that VvXIP1 indeed transports H_2O_2 after its heterologous expression in yeast. But, intriguingly, this aquaporin is unable to transport water, which is indeed a very peculiar finding since H_2O and H_2O_2 display high structural and electrostatic similarity, and MIPs from other subfamilies that are able to transport H_2O_2 are also water channels (Bienert et al., 2011).

H_2O transport capacity of VvXIP1 was studied by stopped flow scattered-light spectrophotometry in yeast membrane vesicles, a technique recently used in our group to confirm water transport capacity of the intracellular aquaporin VvSIP1 (Noronha et al., 2014). Clear-cut results showed that the permeability to water of membrane vesicles isolated from the yeast strain YSK1172 overexpressing VvXIP1 was similar to control. Also, no changes were observed in E_a of water transport across the membrane of the vesicles. This behavior is similar to XIPs from Solanaceae (Bienert et al., 2011), but PtXIP2;1 and PtXIP3;3 from *P. trichocarpa* are water channels, although their water permeability is low when compared to members of the PIP subfamily (Lopez et al., 2012).

Stopped flow experiments also showed that glycerol permeates VvXIP1. Glycerol is a common substrate for non-water conducting MIPs, particularly from the NIP and XIP subfamilies (Bienert et al., 2011).

The role of VvXIP1 in Cu transport was unequivocally demonstrated in transformed yeasts. Cu is an important plant micronutrient, but highly toxic at high concentrations, leading to a reduction in photosynthetic activity, damage to lipids, proteins and DNA and, eventually, cell

death. Recent results obtained in our group showed that the treatment of the grapevine with the Cu-based fungicide Bordeaux mixture caused a transcriptional reprogramming of the expression of the *VvCTrs* (*V. vinifera* copper transporters) and a significant shift in grape berry composition and wine quality (Martins et al., 2012; Martins et al, 2014a; Martins et al., 2014b). Following the observation that VvXIP1 may transport Cu, we found that *VvXIP1* transcript levels in leaves are downregulated after Bordeaux mixture application in the vineyard. These results open a new avenue of research on how VvXIP1 cooperates with the Cu transporters VvCTrs in the regulation of Cu compartmentation/detoxification in grape cells.

***VvXIP1* is expressed in leaves and downregulated by water-deficit stress**

Results suggested that VvXIP1 should play an important role in grapevine leaves because *VvXIP1* steady-state transcript levels were very high in leaf tissues. Also, *VvXIP1* is likely regulated by drought stress, because the levels of mRNAs were reduced by water deficit in leaves from cv. Aragonez. In agreement, in CSB cultured cells, *VvXIP1* transcripts were downregulated by ABA and salt. The role of ABA on plant response to abiotic stresses, including water deficit and salt stress is well-known (Tyerman et al., 2012). Thus, the treatment of maize roots with ABA results over 1–2 h in a transient increase in hydraulic conductivity of the whole organ and of cortical cells and also rapidly enhances the expression of some PIP isoforms (Maurel et al., 2008). In the present study we also showed that VvXIP1 greatly increased yeast cell sensitivity to water-deficit stress imposed by sorbitol, clearly confirming that VvXIP1 has an important role in osmotic regulation. Considering that this protein was proven to be unable to transport water, this effect could be due to its role on the homeostasis of osmotically active solutes. The involvement of aquaporins in osmotic stress has been extensively studied (Tyerman et al., 2012), but the specific role of VvXIP1 deserves further investigation.

In summary, the functional analysis of VvXIP1 revealed an aquaporin with rather atypical features, being unable to transport water but facilitating the transport of heavy metals like Cu. The complete understanding of the puzzling role of this protein may still need intense investigation in the coming years, but the way to get there will be exiting.

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APPENDICES

Appendix 1 - Sequence Accession Numbers

Table I – Accession numbers used to perform phylogenetic analysis between different plant MIPs (Figure 2).

Aquaporin subfamily	Description	Accession Number
PIPs	<i>Gossypium hirsutum</i> PIP1;6	DAA33860
	<i>Populus trichocarpa</i> PIP1;1	EEE92649
	<i>Nicotiana tabacum</i> PIP1	AAL33585
	<i>Arabidopsis thaliana</i> PIP1;4	NP_567178
	<i>Vitis vinifera</i> PIP1;2	ABN14348
NIPs	<i>Gossypium hirsutum</i> NIP6;1	DAA33875
	<i>Populus trichocarpa</i> NIP6;1	XP_002297797
	<i>Arabidopsis thaliana</i> NIP6;1	NP_178191
	<i>Vitis vinifera</i> NIP6;1	XP_002272988
SIPs	<i>Populus trichocarpa</i> SIP1;2	XP_002325395
	<i>Gossypium hirsutum</i> SIP1;2	DAA33876
	<i>Arabidopsis thaliana</i> SIP1;2	NP_187059
	<i>Vitis vinifera</i> SIP1	NP_001267877
TIPs	<i>Gossypium hirsutum</i> TIP2	ACP28878
	<i>Populus trichocarpa</i> TIP2	XP_006381378
	<i>Arabidopsis thaliana</i> TIP2	AAM65100
	<i>Nicotiana tabacum</i> TIP1	BAF95576
	<i>Vitis vinifera</i> TIP1;2	NP_001267926
XIPs	<i>Gossypium hirsutum</i> XIP1;1	D9DBX6
	<i>Populus trichocarpa</i> XIP1	EEE86940
	<i>Nicotiana tabacum</i> XIP1;1	ADO66667
	<i>Vitis vinifera</i> XIP1	F6I152

Table II – Accession numbers used to build a phylogenetic tree comparing XIP proteins from plants and fungi (Figure 3). The sequences from VvXIP1, PtXIP1, PpXIP, GhXIP1;1, InXIP1;1 NtXIP1;1, LjXIP1 and RcXIP were also used in the alignment displayed in Figure 4 and Appendix 3

Description	Accession number
<i>Vitis vinifera</i> XIP1	F6I152
<i>Gossypium hirsutum</i> XIP1;1	D9DBX6
<i>Populus trichocarpa</i> XIP1	EEE86940
<i>Nicotiana tabacum</i> XIP1;1	ADO66667
<i>Prunus persica</i> XIP1	M5VM16
<i>Ipomoea nil</i> XIP1;1	E3UMZ5
<i>Ricinus communis</i> XIP1	B9T717
<i>Penicillium marneffei</i> XIP1	B6QIR3
<i>Fusarium oxysporum</i> XIP1	N4U8H1
<i>Selaginella moellendorffii</i> XIP1	XP_002971714
<i>Physcomitrella patens</i> XIP1	XP_001758094
<i>Aspergillus terreus</i> XIP1	Q0CWK8
<i>Hypocrea virens</i> XIP1	G9N6L5
<i>Hypocrea jecorina</i> XIP1	G0RFI6

Appendix 2 - Primer Sequences

Table III – Primer sequences used to perform quantitative real-time PCR and to insert VvXIP1 in expression vectors

	Primer forward	Primer reverse
qPCR GAPDH	CACGGTCAGTGG AAGCATCATGA	CCTTGTCAGTGAACACACCAGTTGACTC
qPCR VvXIP1	ATCATGTCCGTTGTTGTTGC	CAGCGCGTGAGAAAGAGATA
GATEWAY VvXIP1	GGGGACAAGTTTGTACAAAAAAG CAGGCTTCCAAATGGGTTACACAC AATGGGGTTG	GGGGACCACTTTGTACAAGAAAGCTGGG TCTATCAATG AATGCCTCAATATATTC