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ORIGINAL ARTICLE

Copper homeostasis in grapevine: functional characterization of the *Vitis vinifera* copper transporter 1

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Abstract

Main conclusion **The *Vitis vinifera* copper transporter 1 is capable of self-interaction and mediates intracellular copper transport.**

Abstract An understanding of copper homeostasis in grapevine (*Vitis vinifera* L.) is particularly relevant to viticulture in which copper-based fungicides are intensively used. In the present study, the *Vitis vinifera* copper transporter 1 (VvCTr1), belonging to the Ctr family of copper transporters, was cloned and functionally characterized. Amino acid sequence analysis showed that VvCTr1 monomers are small peptides composed of 148 amino acids with 3 transmembrane domains and several amino acid residues typical of Ctr transporters. Bimolecular fluorescence complementation (BiFC) demonstrated that Ctr monomers are self-interacting and subcellular localization studies revealed that VvCTr1 is mobilized via the *trans*-Golgi network, through the pre-vacuolar compartment and located to the vacuolar membrane. The heterologous expression of *VvCTr1* in a yeast strain lacking all Ctr transporters fully rescued the phenotype, while a deficient complementation was observed in a strain lacking only plasma membrane-bound Ctrs. Given the common subcellular localization of VvCTr1 and AtCOPT5 and the highest amino acid sequence similarity in comparison to the remaining AtCOPT proteins, *Arabidopsis copt5* plants were stably transformed with *VvCTr1*. The impairment in root growth observed in *copt5* seedlings in copper-deficient conditions was fully rescued by VvCTr1, further supporting its involvement in intracellular copper transport. Expression studies in *V. vinifera* showed that *VvCTr1* is mostly expressed in the root system, but transcripts were also present in leaves and stems. The functional characterization of VvCTr-mediated copper transport provides the first step towards understanding the physiological and molecular responses of grapevines to copper-based fungicides.

Keywords Copper transport · Ctr-like proteins · Functional complementation · Metal homeostasis · *Vitis* · VvCTr1

Abbreviations

ACT1	Actin 1
BCS	Bathocuproine disulfonic acid
BiFC	Bimolecular fluorescence complementation
CCH	Copper chaperone
CFP	Cyan fluorescent protein
CTAB	Cetyltrimethylammonium bromide
Ctr/COPT	Copper transporter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

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GFP	Green fluorescent protein
LB	Luria–Bertani medium
pCOPT5	Promoter region of *Arabidopsis COPT5*
PIP	Plasma membrane intrinsic protein
PVP	Polyvinylpyrrolidone
RAN1	Responsive to antagonist 1
RFP	Red fluorescent protein
RRG	Relative root growth
SC-URA	Synthetic complete medium without uracil
SNARE	Syntaxin family of soluble *N*-ethyl maleimide sensitive factor adaptor protein receptors
TMD	Transmembrane domain
VvCTr	*Vitis vinifera* copper transporter
YFP	Yellow fluorescent protein
YPEG	Yeast extract/peptone/ethanol/glycerol medium

Introduction

The uptake of copper in plant cells is tightly regulated to ensure its accurate distribution to copper-demanding proteins while also avoiding the inherent toxicity of this highly reactive element. Copper ions play important roles in a number of physiological processes associated with cell expansion, fruit ripening and leaf abscission (Fry et al. 2002), namely photosynthesis, respiration, antioxidant activity, cell metabolism and hormone perception (Himelblau and Amasino 2000; Pilon et al. 2006; Cohu and Pilon 2010). Redox cycling between Cu^{2+} and Cu^{+} can catalyze the production of toxic hydroxyl radicals, with deleterious effects on lipids, proteins, DNA and other biomolecules (Dučić and Polle 2005).

The compartmentation, chelation and exclusion of metal ions are performed by a complex network of metal transport pathways that regulate copper homeostasis in response to changes in external and internal supply (Dučić and Polle 2005; Gasic and Korban 2006; Yruela 2009). Within the plant cell, copper is required in several subcellular compartments: the cytosol, endoplasmic reticulum (ER), mitochondrial inner membrane, chloroplast stroma and thylakoid lumen as well as the apoplast. Both the mitochondria and plastids are copper sinks, and the vacuole sequesters significant amounts of copper and contribute to copper delivery within the cell (Pilon et al. 2006; Martins et al. 2012). A copper chaperone (CCH) and a responsive to antagonist 1 (RAN1) were the first copper delivery systems identified in plant cells (Himelblau and Amasino 2000). The latter is a member of The P_{IB}-type family of membrane transport ATPases (Peñarrubia et al. 2010) which couple copper transport from the cytosol into secretory compartments or the extracellular space to the hydrolysis of ATP (Mandal et al. 2004; Pilon et al. 2006).

The initial uptake of copper into plant cells is performed by the family of COPT transporters belonging to a highly conserved Ctr-like copper transporter family (Puig and Thiele 2002; Peñarrubia et al. 2010; Yuan et al. 2011). Six genes encoding COPT transporters were identified in *Arabidopsis*, COPT1-6 (Kampfenkel et al. 1995; Sancenón et al. 2003; Puig et al. 2007; Jung et al. 2012; Garcia-Molina et al. 2013; Perea-García et al. 2013) and a family comprising seven members was identified in rice (*Oryza sativa*; Yuan et al. 2011). Whereas all the OsCOPTs appear to be plasma membrane-bound proteins, the AtCOPTs are localized in different cellular compartments including the plasma membrane (COPT1, COPT2, COPT6; Andrés-Colás et al. 2010; Jung et al. 2012; Perea-García et al. 2013) where they function in copper uptake, and the tonoplast (COPT5; Klaumann et al. 2011) where they are believed to mediate copper export to the cytosol.

Defects in COPT-mediated copper transport induce changes in copper distribution that affect root elongation, vegetative growth, chlorophyll content, responses to iron deficiency and low-phosphate signaling, pollen development, and, ultimately, plant survival (Sancenón et al. 2004; Andrés-Colás et al. 2010; Garcia-Molina et al. 2011; Klaumann et al. 2011; Jung et al. 2012; Perea-García et al. 2013).

Previously, we identified eight putative CTrs in *Vitis vinifera*, but their role in copper transport has not yet been determined. In the present study, we characterized the *Vitis vinifera* Copper Transporter 1 (VvCTr1) and assessed its subcellular localization. The heterologous expression of *VvCTr1* in yeast *ctrΔ* strains and in *Arabidopsis COPT5* mutants suggested that VvCTr1 functions as copper transporter and provided insights about its contribution for copper mobilization within the cell. Following previous studies showing that *VvCTr1* expression is modulated by several stress conditions, including copper excess (Martins et al. 2012), the presence of *VvCTr1* transcripts in the roots, stem and leaves of grapevines cv. "Trincadeira" suggests that it may act as a core regulator of copper homeostasis in grapevine. The characterization of CTr-mediated copper transport is of particular importance in the viticulture context in which copper is widely used as the active component of several fungicides (McCallan 1948; Deacon 2006), raising concerns regarding negative impacts in grapevine physiology and in the metabolism of grape cells (Fleming and Trevors 1989; Martins et al. 2012).

Materials and methods

In silico sequence analysis of COPT/Ctr proteins

Alignment of multiple amino acid sequences was performed with Prankster software (http://www.ebi.ac.uk/goldman-srv/prank/prankster) and analyzed on GeneDoc

(http://www.nrbsc.org/gfx/genedoc). Phylogenetic trees were obtained with Phylip-3.69 software (http://evolution.genetics.washington.edu/phylip) and analyzed on Mega 4.0 (http://www.megasoftware.net/mega4). Bootstrap values from 1000 trials were used. Predictions of transmembrane-spanning domains were performed with PSIPRED Server (http://bioinf.cs.ucl.ac.uk/psipred).

Plasmid constructs

The complete *VvCTr1* coding sequence was amplified via PCR from grape berry cDNA using gene-specific primers (Online Resource Fig. S1) flanked by attB sites, and cloned into the vector pDONR207 through a BP reaction (Invitrogen Gateway® Technology). The resulting entry clone was used for recombination with plant and yeast expression vectors through LR reactions. In fusions of fluorescent proteins to the C terminus of VvCTr1, the stop codon was omitted in the reverse primer.

For subcellular localization studies, the entry clone carrying *VvCTr1* was recombined into pH7FWG2 for green fluorescent protein (GFP) fusion and pH7RWG2 for red fluorescent protein (RFP) fusion (Karimi et al. 2002). For protein interaction studies through bimolecular fluorescence complementation the entry clone carrying *VvCTr1* was recombined into the binary BiFC vectors pDEST-VYNE^{GW} and pDEST-^{GW}VYNE for N- or C-terminal fusions to Venus^{N}, respectively, and into pDEST-SCYCE^{GW} and pDEST- ^{GW}SCYCE for N- or C-terminal fusions to S(CFP)3A^{C}, respectively (Gehl et al. 2009). For expression of *VvCTr1* in yeast cells, the entry clone was recombined with the destination vector pVV214 containing the strong PGK promoter (Van Mullem et al. 2003). For expression of *VvCTr1* in *Arabidopsis copt5* plants, the pH7FWG2-VvCTr1 construct was digested with SacI and SpeI (New England BioLabs®, Inc.) following the manufacturer's instructions, resulting in the excision of the *CaMV35S* promoter. In parallel, the promoter region of AtCOPT5 gene (*pCOPT5*, from nucleotide −357 to the ATG; Garcia-Molina et al. 2011) was amplified using primers flanked by the restriction sites of SacI and SpeI: Fwd 5′-AATGAGCTCACCAGAATCAGG TTAACAC-3′, Rev 5′-TTACTAGTCTTTGCGAGCTTG ATTTGAGC-3′). T4 ligase (New England BioLabs®, Inc.) was used to introduce the amplicon into the digested pH7FWG2-VvCTr1 plasmid, placing *VvCTr1* under the control of *pCOPT5*. All constructs were confirmed by sequencing.

Subcellular localization of VvCTr1

Expression vectors were introduced in *Agrobacterium tumefaciens* (GV3101) and transient transformation of tobacco leaf epidermal cells was performed based on the method of Sparkes et al. (2006). Briefly, bacterial cultures were grown overnight on liquid LB medium with the appropriate antibiotic selection to exponential-stationary phase and then diluted to $OD_{600\ nm} = 0.08$ in infiltration buffer (50 mM MES pH 5.6, 2 mM Na_3PO_4, 0.5 % glucose and 100 μM acetosyringone) and grown to $OD_{600\ nm} = 0.2$. Four-week-old tobacco plants (*Nicotiana benthamiana* L.) were infiltrated with the bacterial cultures and leaf disks were examined under the confocal microscope after 3 days.

To study VvCTr1 subcellular localization, tobacco plants were co-infiltrated with *Agrobacterium* strains carrying one of *VvCTr1* plant expression vectors and constructs of *Arabidopsis* proteins with well-known cell locations. The construct wave138Y consisting of the aquaporin *PIP1;4* fused to *EYFP* in pNIGEL backbone was used to label the plasma membrane. Similarly, wave13Y consisting of the SNARE *VTI12* fused to *EYFP* was used to target the *trans*-Golgi/early endosome compartment (Geldner et al. 2009). A construct consisting of the coding sequence of *AtCOPT5* fused to *RFP* in pH7RWG2 plasmid was used to label the vacuole membrane/pre-vacuolar compartment (Garcia-Molina et al. 2011; Klaumann et al. 2011).

Protein–protein interactions

The association between VvCTr1 monomers was tested through BiFC in which the N- and C-terminal sub-fragments of the fluorescent protein Venus^{N}/S(CFP)3A^{C} were fused separately to VvCTr1. Tobacco plants were co-transformed with the constructs of VvCTr1 fused to Venus^{N} and VvCTr1 fused to S(CFP)3A^{C} by its N or C terminus. The combination of VvCTr1-S(CFP)3A^{C} and Remorin-Venus^{N} was used as negative control and Remorin-Venus^{N} with Remorin-S(CFP)3A^{C} was used as positive control for protein–protein interactions (Tajima and Blumwald, unpublished).

Fluorescence microscopy

Fluorescence microscopy was performed using a Zeiss confocal laser scanning microscope (LSM 710 AxioObserver). The excitation wavelength was 488 nm for GFP and YFP and 594 nm for RFP, and emission was 500–535 nm for GFP, 530–590 nm for YFP and 600–660 nm for RFP. For multicolor imaging of GFP/RFP and YFP/RFP, sequential scanning was used to avoid crosstalk between fluorescence channels. For BiFC studies, the excitation and emission wavelengths were set at 488 and 515 nm, respectively. Images were processed with ZEN Lite 2011 software (Carl Zeiss Microscopy).

Yeast transformation and growth assays

The lithium acetate procedure was used to transform plasmid DNA into the yeast *ctr1Δctr3Δ* double-mutant strain MPY17 (*MATα, ctr1::ura3::kan*^{R}*, ctr3::TRP1, lys2-801, his3*), and the yeast *ctr1Δctr3Δctr2Δ* triple-mutant strain, which display a severe or total loss of Ctr high-affinity copper transport (Peña et al. 1998; Rees et al. 2004). Yeast cells transformed with the empty vector (pVV214) or with *VvCTr1* were grown in SC-ura (glucose-rich medium) to $OD_{600\ nm} = 1.0$, washed, and several tenfold diluted clones were plated as drops on selective media (YPEG: 2 % ethanol, 3 % glycerol-rich medium) with or without supplement of $CuSO_4$ (0–100 μM). Plates were incubated for 9 days at 30 °C.

Transformation of *Arabidopsis copt5* plants and assessment of relative root growth

The *Arabidopsis* T-DNA insertion line used in this study (Online Resource Fig. S2; NASC ID: N593550–*copt5-2*; Garcia-Molina et al. 2011) was kindly provided by Dr Lola Peñarrubia (Departament de Bioquímica y Biología Molecular, Universitat de València, Spain) and the presence of the T-DNA insertion within the *COPT5* locus was confirmed after genotyping. The *copt5* homozygous line was transformed by floral dipping (Clough and Bent 1998) with the modified pH7FWG2–VvCTr1 construct in which *VvCTr1* was placed under the control of *pCOPT5*. The transformed *copt5* plants were self-pollinated, and homozygous lines were obtained. The presence of *VvCTr1* in the genome of transformed *copt5* lines was confirmed by PCR (Online Resource Fig. S3). For assessment of the root phenotype, seeds of homozygous *copt5* lines carrying *VvCTr1* were initially germinated in ½ Murashige and Skoog solid medium (10 mM MES, pH 5.7; Murashige and Skoog 1962), at 20 °C, in a 16-h light/8-h dark photoperiod, together with wild-type Col 0 and untransformed *copt5* lines. Four-day-old seedlings were then transferred to ½ MS medium supplemented with 75 μM BCS (bathocuproine disulfonic acid, Sigma-Aldrich Co. LLC) to create copper deficiency, and incubated in the same conditions. The relative root growth (RRG) was evaluated after 8 days.

Grapevine growing conditions

Grapevines (*V. vinifera* L.) cv. "Trincadeira" were obtained from the "Instituto Nacional de Investigação Agrária" (Oeiras, Portugal). Plants were grown for 5 months, at 24 °C, under a 16-h light/8-h dark photoperiod, after subculture of apical meristems in basal Murashige and Skoog medium (Murashige and Skoog 1962). The grapevine organs, namely root, stem and leaves, were carefully separated, immediately frozen in liquid nitrogen and stored at −80 °C.

Analysis of *VvCTr1* expression in grapevine

Frozen samples were ground in liquid nitrogen and total RNA was extracted in buffer containing 100 mM Tris–HCl (pH 8), 2 M NaCl, 25 mM EDTA, 2 % CTAB, 2 % PVP and 2 % β-mercaptoethanol. RNA purification was performed with the RNeasy® Plant Mini Kit (Qiagen) and samples were treated with DNAse to remove any contaminating DNA. mRNA was converted to cDNA by reverse transcription with an Omniscript® RT Kit and oligo(dT) primers (Qiagen). Quantitative real-time PCR (qPCR) was performed in 96-well plates with QuantiTect SYBR Green® Master Mix (Qiagen). For each sample (biological replicate), qPCR reactions were performed in triplicate (technical replicates) using 10 μl Master Mix, 300 nM of each primer, 1 μl of diluted cDNA and nuclease-free water to a final volume of 20 μl. The following cycler conditions were used: 15 min at 95 °C and 45 cycles of 15 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C. Fluorescence was measured at the end of each amplification cycle. Primers were designed to specifically anneal with *VvCTr1*: Fwd 5′-AGGTGGTGGAGGTGGAGAACT-3′, Rev 5′-ACAGAGCCAATACAAAGCCA-3′ (Online Resource Fig. S1). Gene expression was normalized to the *V. vinifera* glyceraldehyde-3-phosphate dehydrogenase gene (*VvGAPDH*; NCBI/GenBank Database accession no. XM_002263109) and the *V. vinifera* actin 1 gene (*VvACT1,* XM_002282480). The following primers were used: GAPDH_Fwd 5′-CTTTCCGTGTTCCTACTGTTG-3′, GAPDH_Rev 5′-CCTCTGACTCCTCCTTGAT-3′; ACT1_Fwd 5′-CTTGCATCCCTCAGCACCTT-3′, ACT1_Rev 5′-TCCTGTGGACAATGGATGGA-3′. The correctness of all qPCR products obtained was controlled on agarose gels and the specificity of the individual PCR reactions was checked through dissociation curves at the end of each qPCR reaction, by heating the amplicons from 65 to 95 °C. Data were analyzed using the CFX Manager Software (Bio-Rad Laboratories, Inc.).

Statistical analysis

The relative root growth of *Arabidopsis copt5* seedlings was compared to that of *VvCTr1* complemented lines by the Student's *t* test using Prism® 5 (GraphPad Software, Inc.). In graphs, the values are marked with asterisks to denote the significance level: $***P \leq 0.001$. The expression of *VvCTr1* in the different grapevine organs was compared by one-way ANOVA (GraphPad Software, Inc.). In graphs, significant differences are marked by different letters (a, b).

Results

In silico characterization of VvCTr1

The amino acid sequence of VvCTr1 was examined in silico and a phylogenetic analysis was performed to study its evolutionary relationship with other COPT/Ctr proteins (Fig. 1a). This analysis revealed that VvCTr1 belongs to a major group of Ctr-like proteins from higher plants comprised by six COPTs from *Arabidopsis thaliana* and seven COPTs from *O. sativa*, and clusters together with the AtCOPT5 and the OsCOPT7. Similarly to other Ctr-like proteins, VvCTr1 monomers are small peptides composed of 148 amino acids, contain 3 transmembrane domains and MxxxM and GxxxG (x representing any amino acid) motifs present in TMD2 and TMD3, respectively (Fig. 1b).

Subcellular localization of VvCTr1

To assess the subcellular localization of VvCTr1, tobacco plants were transiently co-transformed with constructs where *VvCTr1* was fused to *GFP* or *RFP* and constructs of fusion proteins with known cell locations. As shown in Fig. 2, there was no co-localization between aquaporin PIP1;4 targeted to the plasma membrane (Boursiac et al. 2005; Geldner et al. 2009) and the signal corresponding to VvCTr1-RFP. To investigate the possible localization of VvCTr1 to intracellular membranes, VvCTr1-RFP and VTI12-YFP were co-expressed. VTI12 belongs to the family of SNARE proteins which are involved in fusion of transport vesicles with specific organelles and targeted the *trans*-Golgi/early endosome network (Sanderfoot et al. 2001; Geldner et al. 2009). Similar to VTI12-YFP, VvCTr1-RFP appeared as small vesicles consistent with trafficking endosomal bodies (Fig. 2). A partial co-localization was seen between VvCTr1 and VTI12, suggesting the localization of VvCTr1 to the *trans*-Golgi/early endosome network. In addition, VvCTr1-GFP and COPT5-RFP displayed significant co-localization (Fig. 2). Recent studies showed a clear localization of COPT5 to pre-vacuolar vesicles (Garcia-Molina et al. 2011) and to the vacuole membrane (Klaumann et al. 2011). The partial localization of VvCTr1 to the early endosomal network and its clear co-localization to COPT5-RFP suggested the trafficking of VvCTr1 via the *trans*-Golgi network, through the pre-vacuolar compartment and its destination to the tonoplast. Further confirmation of the localization of VvCTr1 to the vacuole membrane was achieved after subjecting the cells to an osmotic shock (Online Resource Fig. S4).

VvCTr1 monomers form a multimeric functional complex

To assess whether VvCTr1 proteins were able to assemble as multimers, a bimolecular fluorescence complementation (BiFC) technique was performed. We used the N terminus of the yellow fluorescent protein Venus ($Venus^N$) and the C terminus of the cyan fluorescent protein S(CFP)3A ($S(CFP)3A^C$) to assess the formation of a complex between monomeric forms of VvCTr1. Each of the fragments by itself displays no fluorescence; however, following the close interaction between the fusion proteins, $Venus^N$ associates with $S(CFP)3A^C$ restoring the fluorescent protein $Venus^N/S(CFP)3A^C$ and resulting in a chimerical signal for Venus plus CFP which is detected at 515 nm (green fluorescence; Gehl et al. 2009). As shown in Fig. 3, when tobacco plants were transiently co-transformed with the fusion proteins VvCTr1-$Venus^N$ and VvCTr1- $S(CFP)3A^C$ a strong fluorescent signal was observed indicating that VvCTr1 monomers had the ability to form homo-multimers. When the fragments of the fluorescent protein were fused to the N terminus of VvCTr1 (N' fusion) the signal was significantly weaker than that observed in the C-terminal fusion. Moreover, the co-expression of VvCTr1-$S(CFP)3A^C$ and of the membrane-associated protein Remorin tagged to $Venus^N$ resulted in no detectable fluorescence (negative control), confirming that only the close association between fusion proteins would allow reassembly of the fluorescent protein, as in the co-expression of Remorin-$Venus^N$ and Remorin-$S(CFP)3A^C$ (positive control; Tajima and Blumwald, unpublished).

Functional complementation of yeast *Ctr* mutants

The potential role of VvCTr1 in copper transport was initially investigated in *S. cerevisiae ctr1Δctr3Δ* and *ctr1Δctr3Δctr2Δ* strains. While the former lacks only the Ctrs located to the plasma membrane, the latter also lacks its vacuolar Ctr, being deprived of all high-affinity copper transporters. These mutants are characterized by a defective mitochondrial respiratory chain since cytochrome c oxidase cannot obtain its copper cofactor (Peña et al. 1998). Both mutants were transformed with *VvCTr1* and their growth was analyzed in several growth media. As shown in Fig. 4, both mutants containing the vector alone were unable to grow in ethanol/glycerol medium (YPEG) with no supplement of copper (0 μM $CuSO_4$). Unlike the double mutant, the triple mutant carrying the vector alone could not grow even in the presence of 10 μM $CuSO_4$. However, the presence of *VvCTr1*

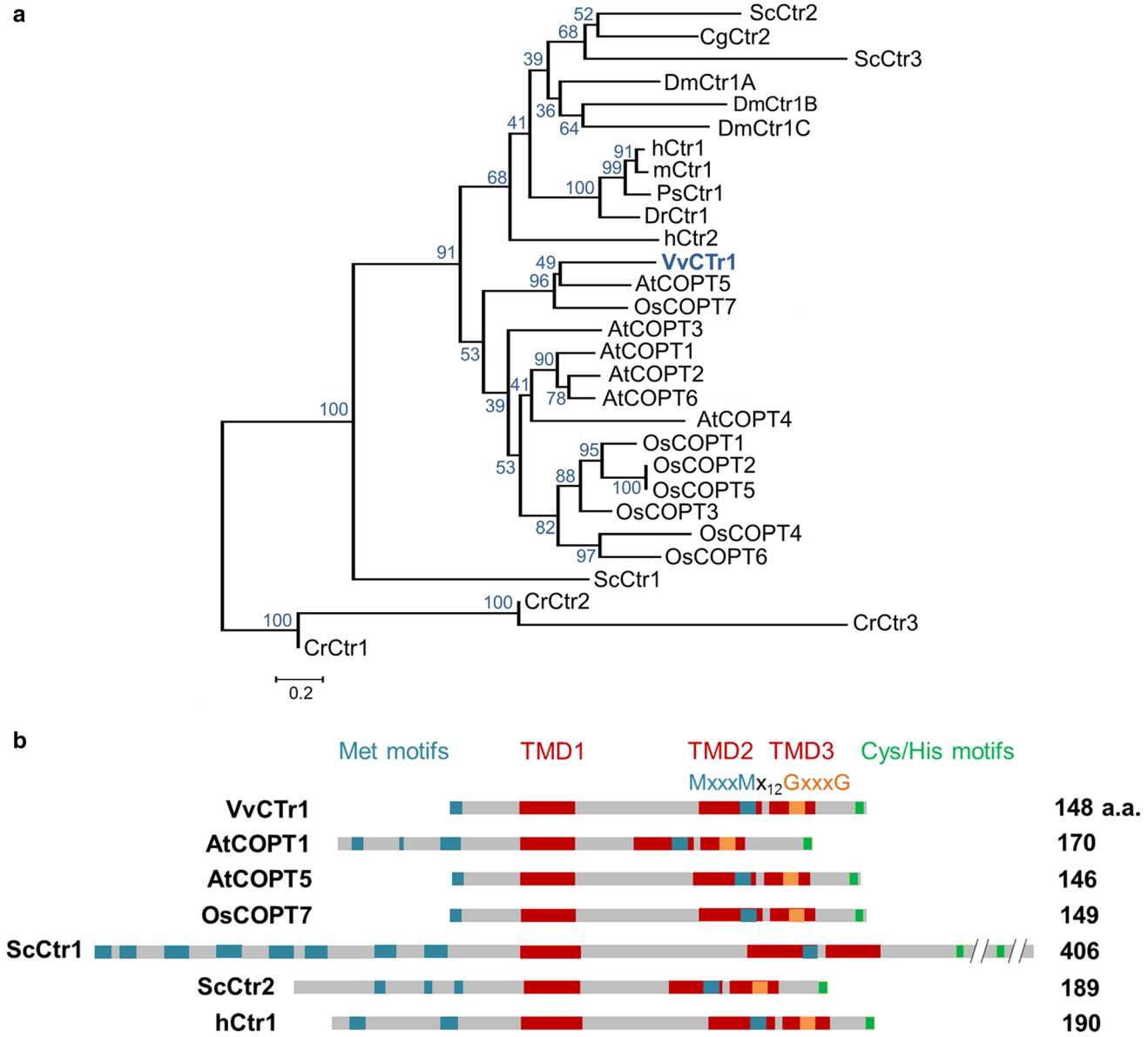

Fig. 1 Primary sequence analysis of VvCTr1 and other members of the COPT/Ctr family. **a** Phylogenetic relationship of VvCTr1 (accession number of GenBank or Protein database of National Center for Biotechnology Information: HQ108185) and other COPT/Ctr proteins from different species: *A. thaliana* (AtCOPT1, NP_200711; AtCOPT2, NP_190274; AtCOPT3, NP_200712; AtCOPT4, NP_850289; AtCOPT5, NP_197565; AtCOPT6, NP_850091), *O. sativa* (OsCOPT1, NP_001044379; OsCOPT2, NP_001055594; OsCOPT3, NP_001044380; OsCOPT4, NP_001173438; OsCOPT5, NP_GQ387495; OsCOPT6, NP_001173929; OsCOPT7, HQ833657), *S. cerevisiae* (ScCtr1, NP_015449; ScCtr2, NP_012045; ScCtr3, NP_013515), human (hCtr1, NP_001850; hCtr2, NP_001851), mouse (mCtr1, NP_780299), *lizard* (PsCtr1, CAD13301), zebrafish (DrCtr1, NP_991280), fruitfly (DmCtr1A, NP_572336; DmCtr1B, NP_649790; DmCtr1C, NP_651837), *C. gloeosporioides* (CgCtr2, ABR23641) and green algae (CrCtr1, XP_001693726; CrCtr2, XP_001702470; CrCtr3, XP_001702650). The numbers for interior branches indicate the bootstrap values (%) for 1000 replications. The scale at the bottom is in units of number of amino acid substitutions per site. **b** Alignment of COPT/Ctr family copper transport proteins. Conserved features comprise three transmembrane domains (TMD1–3, shown in *red*), methionine-rich motifs consisting of 2–5 methionine residues usually separated by three or fewer amino acids (in *blue*), Gly motifs arranged as GxxxG (in *orange*) and Cys/His motifs composed of three amino acids in which two are cysteines or histidines (in *green*). The length of each protein in amino acids is shown on the *right*

fully restored the growth defect of the triple mutant in this condition, suggesting its involvement in copper transport. In contrast, the lack of growth of the double mutant containing the vector alone in a medium with no supplement of copper (0 μM $CuSO_4$) was only partially restored by VvCTr1.

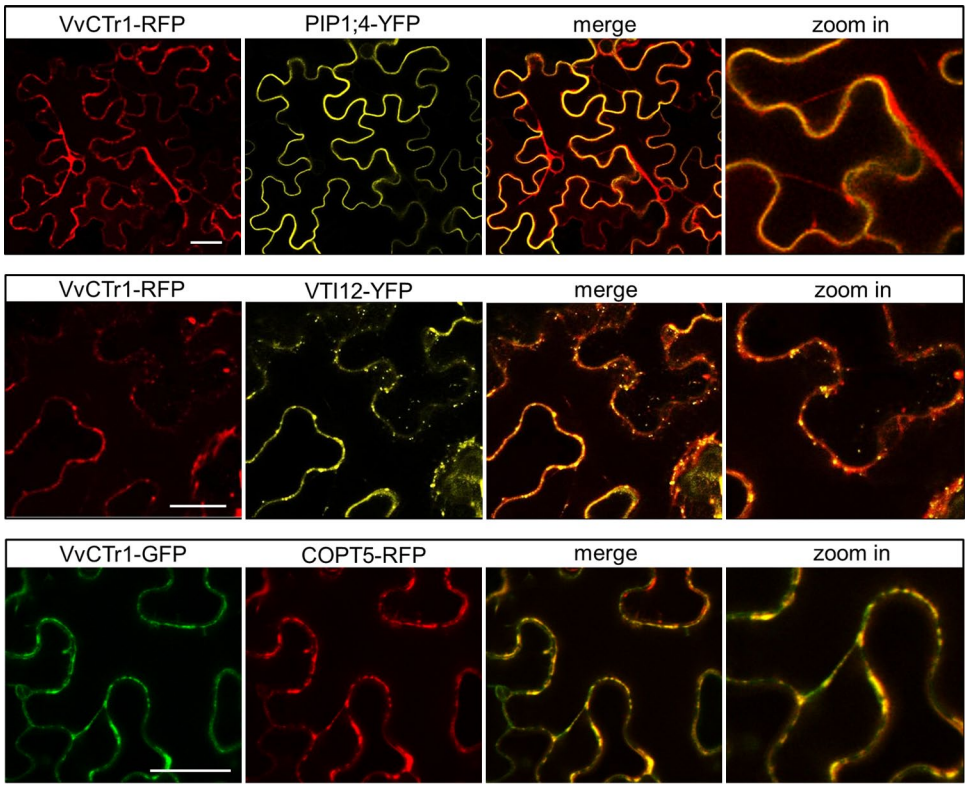

Fig. 2 Subcellular localization of VvCTr1. Tobacco plants were transiently co-transformed with *Agrobacterium* strains carrying the construct of *VvCTr1* fused to *RFP* or *GFP* and constructs of fusion proteins with known cell locations: PIP1;4 (plasma membrane), VTI12 (*trans*-Golgi network/early endosome) and COPT5 (pre-vacuolar compartment/vacuole). Images were acquired in a confocal microscope and show *green fluorescence* (VvCTr1-GFP), *red fluorescence* (VvCTr1-RFP, COPT5-RFP), *yellow fluorescence* (PIP1;4-YFP, VTI12-YFP) and the overlap of fluorescence signals (merge, zoom in). *Bar* 30 μm

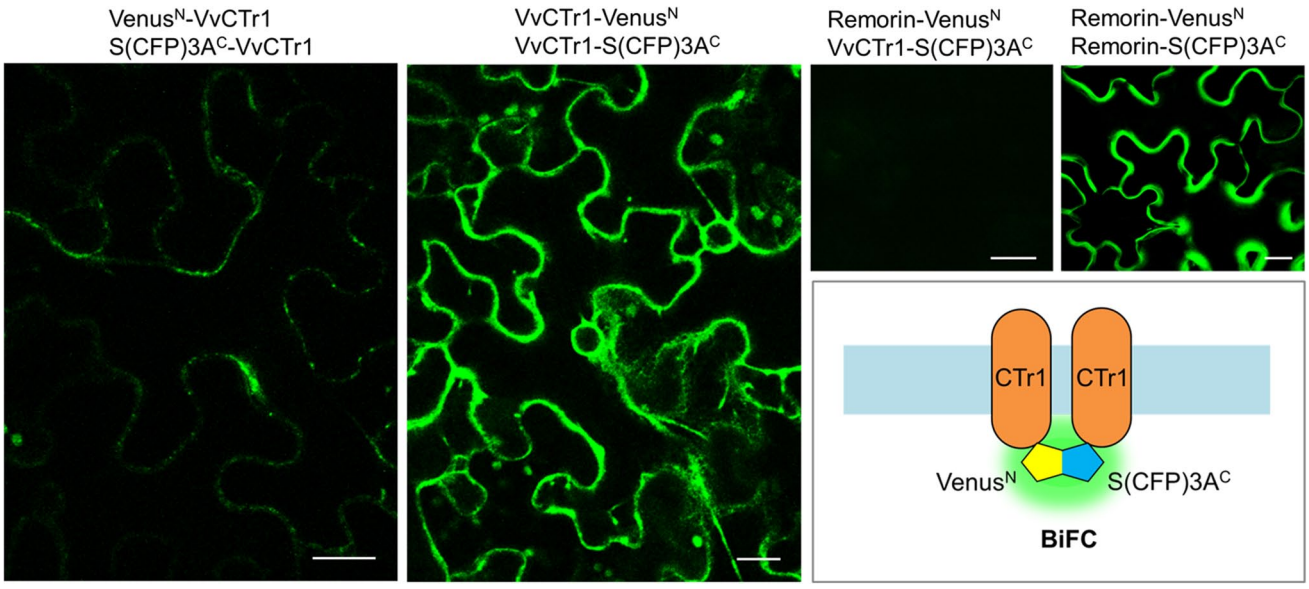

Fig. 3 Formation of oligomeric complexes by VvCTr1 monomers. A bimolecular fluorescence complementation technique was performed in tobacco plants transiently transformed with constructs consisting of N′ or C′ fusions of VvCTr1 with the N- or C-terminal sub-fragments of Venus and S(CFP)3A, respectively. The tight interaction of the fusion proteins mediates refolding and reconstitution of the fluorescent protein, resulting in efficient fluorescence emission that was analyzed under a confocal microscope. A negative control for protein interaction was performed with VvCTr1 fused to S(CFP)3A^C and the membrane-associated protein Remorin fused to Venus^N. The interaction between Remorin-Venus^N and Remorin-S(CFP)3A^C was used as positive control. *Bar* 20 μm. *Inset* model of the multimer assembly and BiFC between VvCTr1 monomers fused with Venus^N or S(CFP)3A^C

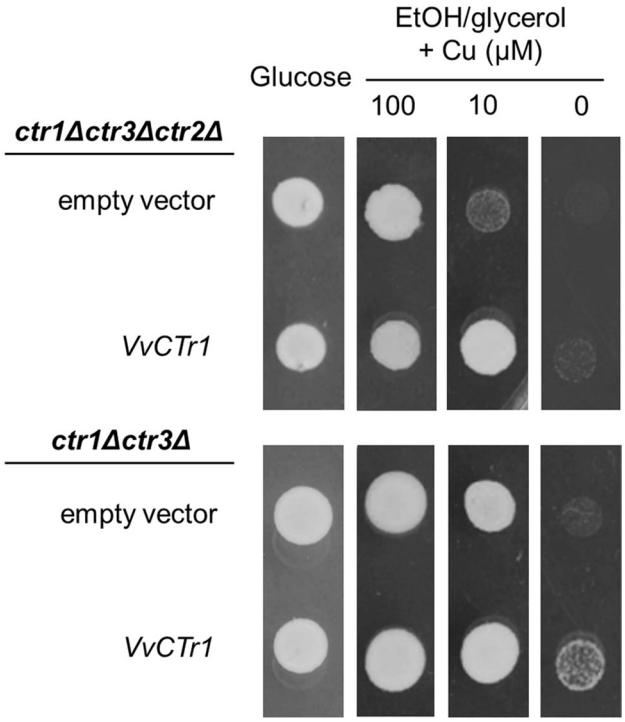

Fig. 4 Functional complementation of *S. cerevisiae* *ctr1Δctr3Δctr2Δ* and *ctr1Δctr3Δ* strains by VvCTr1. Yeast mutants were transformed with the vector pVV214 alone (empty vector) or with the same vector carrying *VvCTr1*. Cells were grown on glucose (SC-ura) or on ethanol/glycerol (YPEG)-selective media supplemented with 10–100 μM $CuSO_4$ or without supplementation of $CuSO_4$ (0), for 9 days

Functional complementation in *Arabidopsis copt5* seedlings

In addition to the good co-localization of VvCTr1 and AtCOPT5, the former shares the highest amino acid sequence similarity with the latter (53 %), in comparison to the sequences of the remaining *Arabidopsis* COPT proteins. To confirm the role of VvCTr1 in mediating copper transport in a plant system, *VvCTr1* was stably expressed in *Arabidopsis COPT5* knockout mutants. The mutant seedlings are characterized by reduced root growth in copper-deficient conditions (Garcia-Molina et al. 2011; Klaumann et al. 2011) and the ability of VvCTr1 to rescue this phenotype was evaluated. As shown in Fig. 5, the relative root growth (RRG) of *copt5* seedlings was similar to that observed in wild-type plants and in homozygous *copt5* lines transformed with *pCOPT5-VvCTr1*, in copper-sufficient conditions (½ MS). However, in copper-deficient conditions (+75 μM BCS), the RRG of *copt5* seedlings was impaired, decreasing by 35 % in comparison to wild-type plants. VvCTr1 successfully rescued the root phenotype of *copt5* seedlings, where a full recovery of RRG was observed.

Expression of *VvCTr1* in planta

To assess the involvement of *VvCTr1* in *V. vinifera* copper homeostasis, its expression was studied by real-time PCR in distinct grapevine organs cv. "Trincadeira". As shown in Fig. 6 *VvCTr1* transcripts were present in the roots, stem and leaves. The highest expression was detected in the roots, followed by the leaves and the stems.

Discussion

The characterization of copper transport pathways in grapevines holds important basic and applied relevance due to the intensive use of copper-based fungicides, mainly in organic viticulture (Deacon 2006; García-Esparza et al. 2006). In silico analysis revealed that, like other COPT/Ctr proteins, VvCTr1 contains three transmembrane domains (TMDs), an N terminus rich in methionine motifs and a C terminus containing two cysteine residues. Extensive studies performed in the human Ctr1 and yeast Ctrs contributed to elucidate the role of these key residues in the assembly and function of Ctr transporters (Aller et al. 2004; Eisses and Kaplan 2005; Aller and Unger 2006; De Feo et al. 2010). TMD1 appears to participate in molecular interactions of Ctrs with ferric reductases that reduce Cu^{2+} to Cu^{+} (Rees and Thiele 2007), while TMD2 contains the highly conserved motif MxxxM that seems to be the key for regulation of the size of the pore and is required for Ctr function (De Feo et al. 2010; Peñarrubia et al. 2010; Jung et al. 2012). TMD3 contains the highly conserved GxxxG motif that appears to be responsible for the close packing of the three TMDs, being critical for forming a functional and structurally mature transporter (Aller et al. 2004; De Feo et al. 2009; Peñarrubia et al. 2010). The methionine motifs present in the N terminus of most Ctrs are important in copper binding for facilitated import, especially in copper-limiting conditions (Jung et al. 2012), while the cysteines present in the C-terminal cytosolic domain seem to contribute to oligomerization and stability of the protein and serve as intracellular copper donors for its mobilization to copper chaperones (Eisses and Kaplan 2002; Nose et al. 2006).

Bimolecular fluorescence complementation (BiFC) performed in this study showed that VvCTr1 monomers interact with each other, forming homodimers or higher-molecular-mass oligomers with the potential to assemble as functional mature copper transporters. These findings are in support of previous observations that showed that mature Ctrs may form multimeric complexes capable of creating a selective pore for copper (De Feo et al. 2009; Peñarrubia et al. 2010). When the fluorescent tags were placed before the N terminus of VvCTr1, this interaction was significantly weaker than in C-terminal fusions, indicating

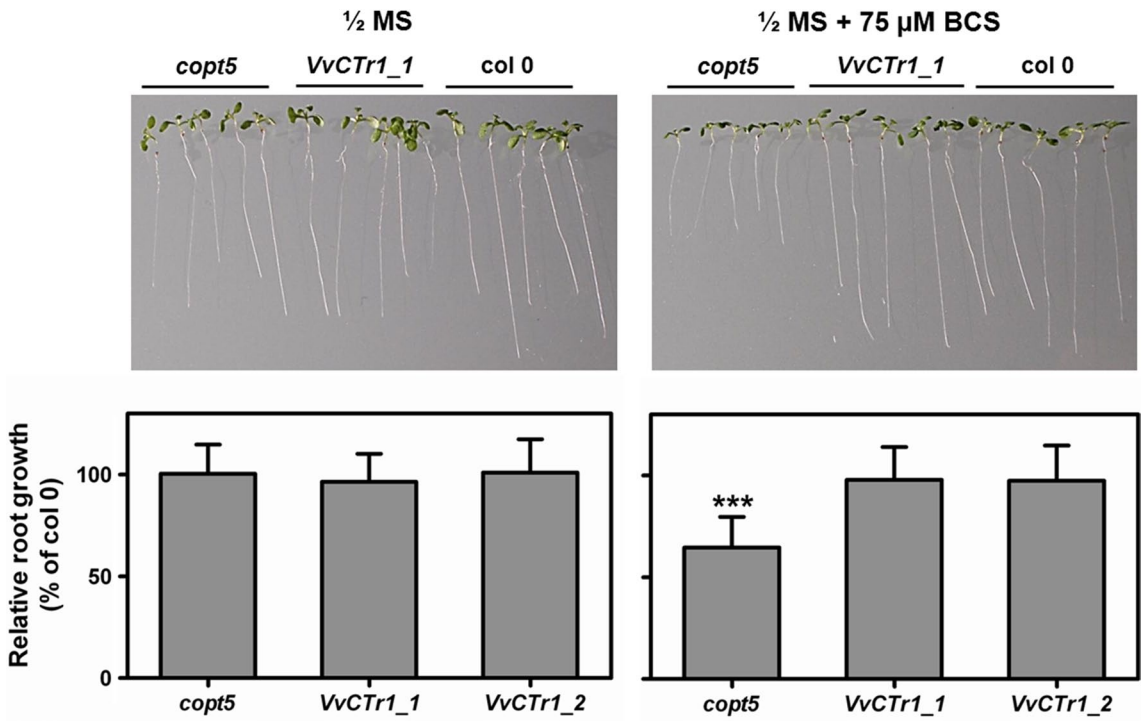

Fig. 5 Functional complementation of *A. thaliana copt5* seedlings by VvCTr1. *copt5* plants was stably transformed with *pCOPT5-VvCTr1* and homozygous lines were obtained (see "Materials and methods"). The relative root growth of wild-type Col 0, *copt5*, and *VvCTr1* complemented independent lines (*VvCTr1_1* and *VvCTr1_2*) was evaluated in copper-sufficient (½ MS) and in copper-deficient conditions (½ MS + 75 µM BCS) after 8 days. Results are expressed as percentage of mean ± SD of RRG compared to Col 0; $n = 24$. *Asterisks* denote the significance level as compared to *VvCTr1* complemented lines: $***P \leq 0.001$

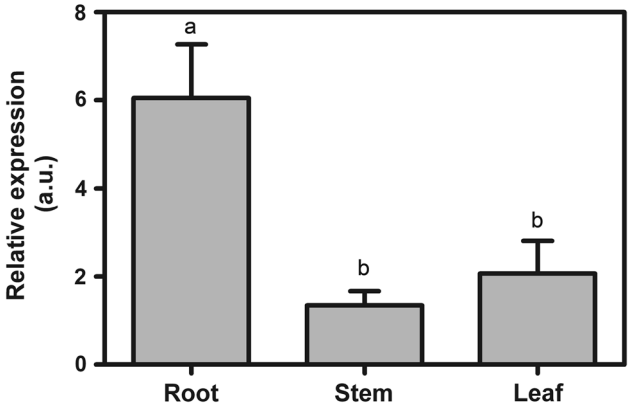

Fig. 6 Expression of *VvCTr1* in grapevines cv. "Trincadeira" by real-time PCR. Gene expression was normalized to the transcript levels of *ACT1* and *GAPDH* (internal standards). Results indicate mean ± SD of values obtained for five biological replicates. In bars, *different letters* indicate significant differences ($P \leq 0.001$)

that the N terminus of VvCTr1 has a major role in VvCTr1-VvCTr1 interactions. Accordingly, yeast two-hybrid studies have shown that the N-terminal portion of human hCtr1 is capable of self-interaction (Klomp et al. 2003). It is well established that the hCtr1 forms symmetrical homotrimers containing nine transmembrane helices with a selective pore between the subunit interfaces (Aller and Unger 2006; De Feo et al. 2009) and this model was initially extrapolated to Ctrs from other eukaryotes, and sometimes validated, as the case of *S. cerevisiae* Ctr3 (Peña et al. 2000). However, it is now known that the *S. cerevisiae* Ctr1 appears to form trimers as well as higher molecular weight complexes (Sinani et al. 2007). The complexity of Ctr transport becomes even higher with the possible formation of hetero-oligomeric complexes, as the case of the *Schizosaccharomyces pombe* Ctr4 and Ctr5 whose interaction is necessary for the proper localization of each subunit to the plasma membrane and transporter activity (Zhou and Thiele 2001). In plants such as rice and *Arabidopsis* where 7 and 6 COPTs were already identified, respectively, the number of possible combinations is even higher and the formation of specific homo- and heteromeric complexes between COPTs has been shown (Yuan et al. 2011; Jung et al. 2012). These studies suggested that the specific assembly of Ctr proteins could determine their subcellular localization and affinity for copper.

Complementation assays using *Saccharomyces cerevisiae* strains defective in copper uptake allowed the characterization of some Ctr-like proteins, including the human

Ctr1 (Zhou and Gitschier 1997) and several COPTs (Kampfenkel et al. 1995; Sancenón et al. 2003; Yuan et al. 2011; Jung et al. 2012). In the present study, VvCTr1 fully complemented the growth defect of the yeast *ctr1Δctr3Δctr2Δ*, lacking all high-affinity copper transport, validating its function as a copper transporter. However, the phenotype complementation by VvCTr1 in yeast *ctr1Δctr3Δ,* lacking only plasma membrane Ctrs, was only partial, suggesting its localization to internal membranes. In fact, it has been previously shown that *Arabidopsis* AtCOPT1 and AtCOPT2 localized to the plasma membrane fully complemented the growth defect of the yeast double mutant, while AtCOPT3 and AtCOPT5 that locate to internal membranes only partially complemented it (Sancenón et al. 2003; Garcia-Molina et al. 2011).

Subcellular localization of VvCTr1 was performed in tobacco plants co-expressing fusion proteins with well-known cell locations. Sharing a high amino acid sequence similarity with AtCOPT5, VvCTr1 co-localized with this protein, being mobilized through the *trans*-Golgi network to the pre-vacuolar compartment/vacuolar membrane where it could mediate copper release to the cytosol (Klaumann et al. 2011), and also be involved in copper movements through the pre-vacuolar compartment, which could act in copper recycling through vesicles that provide the metal cofactor to key copper-dependent processes such as photosynthesis (Garcia-Molina et al. 2011). *Arabidopsis copt5* lines show defective root growth in copper-limiting conditions because the route for copper export is blocked and the metal remains trapped inside the vacuole, preventing long-distance transport (Garcia-Molina et al. 2011; Klaumann et al. 2011). In the present study, VvCTr1 was able to complement the root phenotype of *copt5* seedlings, suggesting its essential role in plantlet development under copper-deficient conditions. The major role of VvCTr1 in the root system was further supported by expression studies in grapevines. Being highly expressed in the roots, but also present in other grapevine organs, including the stem and leaves, VvCTr1 could have a major role in the regulation of copper mobilization. The presence of *VvCTr1* transcripts in other grapevine cultivars, namely, "Alvarinho" and "Cabernet Sauvignon" (Martins et al. 2012), supports its function as a core regulator of copper homeostasis, whose expression is nonetheless finely modulated by environmental factors, including copper stress (Martins et al. 2012). Further studies on the other VvCTr members and their possible interactions will contribute to elucidate the roles of CTrs in regulating copper homeostasis in the grapevine.

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Conflict of interest The authors declare that they have no conflict of interest.

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