Copper-based fungicide Bordeaux mixture regulates the expression of *Vitis vinifera* copper transporters

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Abstract

Background and Aims: The Bordeaux mixture has been widely used in viticulture, but the response of grape cells (*Vitis vinifera* L.) to copper is still largely unknown. In the present study, we investigated if copper application in the vineyard could interfere with copper homeostasis through the regulation of the expression of copper transporters (*VvCTrs*).

Methods and Results: The *VvCTrs* characterised in silico are phylogenetically related to COPTs from Arabidopsis and rice. In a field trial, we studied the expression of each *VvCTr* in grape berries and leaves throughout the season by quantitative real-time polymerase chain reaction. Transcripts of *VvCTr1* and *VvCTr8* were the most abundant in leaves and berries, respectively, while *VvCTrs 4*, *5* and *7* were the least expressed, but the application of copper resulted in a significant change in the transcript levels. In plants treated with Bordeaux mixture, the copper concentration increased twofold in the pulp and ninefold on the skin of surface-washed mature berries.

Conclusions: The expression pattern of *VvCTr1*-8 depends on the tissue and developmental stage. Four applications of copper following a methodology used in organic farming are sufficient to induce a significant increase in copper content in berry tissues which is either a cause or a consequence of the observed transcriptional reprogramming of the expression of the *VvCTrs*.

Significance of the Study: The application of Bordeaux mixture should have major consequences on grapevine copper homeostasis, regulating copper transport at the transcriptional level. A new research avenue emerged towards the characterisation of VvCTr-mediated transport at the molecular level in response to copper.

Keywords: *Bordeaux mixture*, *copper*, *gene expression*, Vitis vinifera, VvCTr

Introduction

At the plant level, a major consequence of copper application in viticulture is altered metabolism of grape cells (Romeu-Moreno and Mas 1999, Llorens et al. 2000) and changes in leaf physiology (Moutinho-Pereira et al. 2001). At the cellular level, copper tightly binds to target sites in polypeptides, contributing to its cytotoxicity; and copper ions can lead to the generation of harmful reactive oxygen species via Fenton and Haber–Weiss reactions that cause severe oxidative damage under conditions of excessive copper supply (reviewed by Peñarrubia et al. 2010). The accumulation of copper in the vacuole may constitute a mechanism of toxic avoidance in grape cells (Martins et al. 2012).

Despite its negative effects, copper is a key micronutrient having a central role in respiration, photosynthesis, antioxidant activity, hormone perception and cell metabolism in general, as the cofactor of several enzymes, including plastocyanin, copper/zinc superoxide dismutase and cytochrome-c oxidase (Himelblau and Amasino 2000, Pilon et al. 2006, Cohu and Pilon 2010).

A complex network of transport pathways tightly regulates copper uptake by cells to sustain its essential functions, while also ensuring it is not detrimental at high concentration. Found ubiquitously in eukaryotes, copper transporter (Ctr) proteins are small integral membrane proteins that catalyse copper uptake into cells (Puig and Thiele 2002). They are present in many organisms, including fungi (Dancis et al. 1994, Peña et al. 2000, Korripally et al. 2010), animals (Lee et al. 2002, Zhou et al. 2003, Mackenzie et al. 2004, Eisses and Kaplan 2005) and plants (Sancenón et al. 2003, Yuan et al. 2011, Martins et al. 2012). These transporters are responsible for transport of copper to the cytosol, while P-type ATPases mediate copper efflux to the extracellular space or its compartmentation into the vacuole (Prohaska 2008). The first Ctr was identified in baker's yeast by a genetic selection for mutants defective in iron uptake and revealed a connection between the uptake of copper and the ability of cells to accumulate ferrous iron (Dancis et al. 1994). The presence of Ctr1 was apparently essential for copper supply to the multi-copper ferroxidase Fet3p that in turn is essential for ferrous iron uptake. Two more Ctrs were later identified in

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Saccharomyces cerevisiae (Peña et al. 2000, Rees et al. 2004) and extensive studies in the human Ctr1 allowed the detailed characterisation of the structure-function of Ctr proteins (Lee et al. 2002, Eisses and Kaplan 2005, Aller and Unger 2006, De Feo et al. 2010). Plant Ctrs, also known as COPTs, have only recently been identified and are best characterised in *Arabidopsis thaliana* (Sancenón et al. 2003, Andrés-Colás et al. 2010, Garcia-Molina et al. 2011, 2013, Jung et al. 2012) and in *Oryza sativa* (Yuan et al. 2011). Although the localisation and function of most Ctr-like proteins remain unclear, COPT proteins localised at the plasma membrane appear to mediate copper uptake to the cytosol while COPTs localised at the tonoplast appear to mediate its efflux from the vacuole, making it available for use in the cell (Sancenón et al. 2004, Klaumann et al. 2011).

In the present study, eight putative *Vitis vinifera* copper transporters (VvCTrs) were initially characterised in silico. To gain insights about the contribution of each VvCTr during grape berry development and ripening, the expression of each gene was studied by quantitative real-time polymerase chain reaction (qPCR) in grape berries and leaves throughout the fruiting season. The regulation of each *VvCTr* by copper was investigated in a commercial vineyard treated with Bordeaux mixture. The expression pattern of *VvCTr1-8* was tissue and development dependent. Copper application following a methodology used in organic farming induced a significant increase in copper content in grape berries which was associated to a transcriptional reprogramming of the expression of *VvCTr*s.

Material and methods

Promoter analysis of VvCTr genes

The five-adjacent genomic sequences to *VvCTr* genes were identified by performing BLAST of *VvCTr* sequences on the grapevine genome (*V. vinifera* cv. Pinot Noir clone PN40024 genome sequence; Jaillon et al. 2007). The promoter motifs and *cis*-regulatory elements of these putative promoter sequences were analysed with the Plant Promoter Analysis Navigator – PlantPAN software (http://plantpan.mbc.nctu.edu.tw; Chang et al. 2008).

Sequence analysis of COPT/Ctr proteins

The eight putative *VvCTr* genes characterised in this study are annotated in GenBank: *VvCTr1* (accession no. HQ108185), *VvCTr2* (HQ108186), *VvCTr3* (HQ108187), *VvCTr4* (HQ108188), *VvCTr5* (HQ108189), *VvCTr6* (HQ108190), *VvCTr7* (HQ108191) and *VvCTr8* (HQ108192). Alignment of VvCTr amino acid sequences was performed with Prankster software (http://www.ebi.ac.uk/goldman-srv/prank/prankster) and analysed on GeneDoc (http://www.nrbsc.org/gfx/genedoc). Amino acid sequence similarity and phylogenetic trees of COPT/Ctr proteins were obtained with Phylip-3.69 software (http://evolution.genetics.washington.edu/phylip) and analysed using 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).

Vineyard treatments and sample collection

Field experiments during 2012 were conducted with 8-year-old grapevines cv. Vinhão from a commercial vineyard established in the north of Portugal (coordinates: 41°25′14.06″ latitude and 8°14′38.80″ longitude), in the region of Vinhos Verdes. Fungicide was applied as follows: 'control' grapevines were treated throughout the season with a conventional triazole-based fungicide, and 'copper-treated' grapevines were sprayed with Bordeaux mixture [20 g/L $CuSO_4$ + 20 g/L $Ca(OH)_2$], following the regular vineyard management practices applied in commercial farms. Both control and copper-treated grapevines were cultivated under the same microclimate. Four copper treatments were applied during the season, every 15 days, the first at the pea size and the last one 3 weeks before harvest. Three bunches and nine leaves were collected from each grapevine at each of the following grape berry developmental stages: green stage [pea size, E-L 31; Coombe (1995)], veraison stage (berry begins to colour and enlarge, E-L 35) and mature stage (berries harvest-ripe, E-L 38). Samples were frozen immediately in liquid nitrogen and stored separately at −80C.

Copper quantification in grape berries

For copper quantification studies, grape berries from copper-treated and control plants were rinsed thoroughly in Milli-Q water (EMD Millipore, Billerica, MA, USA) to remove any trace of residual external copper and dried briefly with filter paper. The skin and pulp of grape berries were carefully separated, and both whole berries and isolated tissues were ground in liquid nitrogen and lyophilised for 7 days. The lyophilised berry homogenates were digested by high-pressure microwave digestion in a Milestone ETHOS Plus Microwave Labstation (Milestone, Sorisole, Italy), and copper was measured by electrothermal atomisation atomic spectrometry in a PerkinElmer Model 4110 ZL graphite furnace atomic absorption spectrometer (PerkinElmer Life and Analytical Sciences, Shelton, CT, USA), using Zeeman-effect background correction, equipped with a model AS-72 autosampler and the PerkinElmer AAWinLab software, version 2.5 (PerkinElmer Life and Analytical Sciences, Shelton, CT, USA) (Catarino et al. 2005, 2010).

Gene expression analysis by quantitative real-time polymerase chain reaction

The relative accumulation of *VvCTrs* messenger ribonucleic acid (mRNA) was analysed in grape berries and leaves sampled throughout the season as reported above. Frozen samples were ground in liquid nitrogen, and total RNA was extracted in a buffer containing 100 mmol/L Tris-HCl (pH = 8.0), 2 mol/L NaCl, 25 mmol/L ethylenediaminetetraacetic acid, 2% cetyltrimethylammonium bromide, 2% polyvinylpyrrolidone and 2% β-mercaptoethanol. Ribonucleic acid was purified with the RNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands), and samples were treated with DNAse to remove any contaminating DNA. Messenger RNA was converted to complementary DNA (cDNA) by reverse transcription with an Omniscript RT Kit and oligo (dT) primers (Qiagen). Quantitative real-time PCR was performed in 96-well plates with QuantiTect SYBR Green Master Mix (Qiagen). For each sample (biological replicate), qPCR reactions were undertaken in triplicate (technical replicates) using 10 µL Master Mix, 300 nmol/L of each primer, 1 µL of diluted cDNA and nuclease-free water to a final volume of 20 µL. Aliquots from the same cDNA sample were used with all primer sets in each experiment. 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 each *VvCTr* gene: *VvCTr1* (Fwd 5′-AGGTGGT GGAGGTGGAGAACT-3′; Rev 5′-ACAGAGCCAATACAAAGC CA-3′), *VvCTr2* (Fwd 5′-GGTGTGACGGCTTCAGATGA-3′; Rev 5′-GAAACCCAATTGAAAGGCCGA-3′), *VvCTr3* (Fwd 5′- GTCTT GTCCATCCCTCCCA-3′; Rev 5′-TGCCACAGAGAGTGCACGAT AT-3′), *VvCTr4* (Fwd 5′-GTGGCTTTCCCATTTCCAGCT-3′; Rev 5′-ACGGCCGCAACGTTTCACT-3′), *VvCTr5* (Fwd 5′-AATGGT

GTCGTTGGAGAAGA-3′; Rev 5′-GTCCTTACACAGACGCAGTA-3′), *VvCTr6* (Fwd 5′-CTCTACAAACAGCCTTCTTGGT-3′; Rev 5′-TGAATTTCGCTGAGGAGCCGGA-3′), *VvCTr7* (Fwd 5′-CAAT AAGCTTGTGGCTGGCCTT-3′; Rev 5′-CTCATAAGGCAACATC TCTGA-3′), *VvCTr8* (Fwd 5′-GTG GAGTGCGACGAAGTTC-3′; Rev 5′-GCAAGCATTATCAACGACAGCA-3′). Gene expression was normalised 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′ (Gainza-Cortés et al. 2012, Pérez-Castro et al. 2012); ACT1_Fwd, 5′-CTTGCATCCCTCA GCACCTT-3′; ACT1_Rev, 5′-TCCTGTGGACAATGGATGGA-3′ (Reid et al. 2006, Le Henanff et al. 2009). 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 analysed using the CFX Manager Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and comparison of gene expression was performed following the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Statistical analysis

The data presented consist of the results obtained from three independent experiments (samples from each grapevine being one biological replicate) for each treatment at each stage, and three technical replicates for each sample, and are represented as the mean ± SEM. *VvCTr* transcript levels of samples collected from copper-treated plants were compared with those of samples collected from control plants by the Student's *t*-test using Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). In figures, the values are marked with asterisks to denote the significance level as compared to those of the control: *, $P \leq 0.05$; **, $P \leq 0.01$; *** and $P \leq 0.001$.

Results

In silico analysis of Vitis vinifera *copper transporters*

The eight putative *VvCTr* genes identified are spread along the grapevine genome, localising to chromosomes 4, 6, 8 and 11 (Table 1); chromosome 6 harbours *VvCTrs 4* to *7*, which are arranged consecutively. No introns are present in the gene sequences. BLAST analysis led to the identification of ~1 kb long regulatory regions located upstream of the transcription start site of each *VvCTr* gene, containing putative regulatory elements within the two strands (Table 2). The localisation of the putative *cis*-acting regulatory elements showed that expression of the *VvCTr* genes may be regulated at the distal region, evenly across the 1 kb 5′ regulatory region (Supporting Information Figure S1). Several functional significant *cis*-acting regulatory elements that are associated with tissue-specific expression, hormonal and light regulation, disease response and cellular growth elements were identified (Table 2). The latter were highly represented in all regulatory regions, together with abscisic acid-responsive elements, seed-specific tissue expression and light-regulated motifs. Elements associated with disease resistance response were found in only a few regulatory regions, namely that of *VvCTr1*, *VvCTr3*, *VvCTr4* and *VvCTr5*. Root and pollen specific expression elements were found in all regulatory regions, except in that of *VvCTr3* and that of *VvCTr7* and *VvCTr8*, respectively. The regulatory region of *VvCTr7* also lacked ARR1 binding elements, while that of *VvCTr4* lacked Myb/Myc boxes.

Table 1. Organisation of *VvCTr* genes in the grapevine (*Vitis vinifera* L.) genome.

	VvCTr1	*VvCTr2*	*VvCTr3*	*VvCTr4*	*VvCTr5*	*VvCTr6*	*VvCTr7*	*VvCTr8*
Accession no.	HQ108185	HQ108186	HQ108187	HQ108188	HQ108189	HQ108190	HQ108191	HQ108192
Chromosome	11	8	8	6	6	6	6	4
Gene size (bp)	900	1001	420	534	681	495	546	733
Position (Mb)	*802906–803805*	*15576024–15577024*	*15561847–15562266*	*6511022–6511555*	*6515604–6514924*	*6517054–6517548*	*6521117–6521662*	*1702677–1703409*
Strand	+	–	–	–	–	–	–	–
Number of exons	1	1	1	1	1	1	1	1
mRNA/ORF (bp)	900/447	1001/459	420/420	534/534	681/456	495/495	546/522	733/435

Table 2. *Cis*-acting elements identified by PlantPAN analysis in *VvCTr* promoter sequences.

Cis-acting element	Number of copies							
	VvCTr1	*VvCTr2*	*VvCTr3*	*VvCTr4*	*VvCTr5*	*VvCTr6*	*VvCTr7*	*VvCTr8*
Disease resistance response	2	0	6	6	1	0	0	0
Light regulated motif	10	12	4	12	8	8	12	7
Abscisic acid responsive element	10	13	8	11	14	15	8	15
Seed-specific tissue expression	9	9	8	10	14	14	11	10
Pollen expression element	11	9	12	11	10	7	0	0
Root motif	1	2	0	4	4	7	13	3
Cellular growth motif	17	18	19	16	13	16	13	13
ARR1 binding element	8	9	6	6	7	5	0	7
WRKY binding site	8	0	7	4	16	0	0	5
Myb/Myc box	9	6	6	0	5	6	6	5

Table 3. Amino acid sequence similarity (%) among members of the *Vitis vinifera* CTr family.

	VvCTr1	VvCTr2	VvCTr3	VvCTr4	VvCTr5	VvCTr6	VvCTr7	VvCTr8
VvCTr1	100	32	32	34	37	30	32	59
VvCTr2	—	100	43	58	58	52	49	37
VvCTr3	—	—	100	49	50	45	47	32
VvCTr4	—	—	—	100	84	55	50	32
VvCTr5	—	—	—	—	100	54	53	35
VvCTr6	—	—	—	—	—	100	49	27
VvCTr7	—	—	—	—	—	—	100	35
VvCTr8	—	—	—	—	—	—	—	100

WRKY binding sites were identified in all promoter regions, with the exception of that of *VvCTr2*, *VvCTr6* and *VvCTr7*.

Analyses of the putative VvCTr proteins showed that the amino acid sequences share 27–84% similarity (Table 3). VvCTr4 and 5 share the highest similarity, while VvCTr6 shares the lowest similarity with VvCTr8. As shown in Figure 1a,b, all VvCTrs contain three highly conserved transmembrane domains. Most proteins contain Met motifs in the N-terminus and Cys residues in the C-terminus, which faces the cytosol. For instance, VvCTr2 and VvCTr6 are lacking the Met motifs, while the Cys residues are absent in VvCTr2, VvCTr3 and VvCTr5. All proteins contain a MLAxMSF (x representing any amino acid) motif in the second transmembrane domain (TMD) which is separated by an NxG motif from the third TMD that contains a GxxxG motif. The evolutionary relationship between VvCTrs and other COPT/Ctr proteins was examined through a phylogenetic analysis (Figure 1c). VvCTrs cluster together with different COPTs from Arabidopsis and rice, comprising a major group composed of COPTs/Ctrs of higher plants. For instance, VvCTr1 and VvCTr8 cluster together with AtCOPT5 and OsCOPT7, while VvCTr2, 4, 5 and 6 are closely related with AtCOPT4. Moreover, VvCTr7 appears isolated from other COPT/Ctr transporters in the phylogenetic tree, which may be connected to the higher number of Met motifs present in the N-terminus of this protein.

Expression of VvCTrs *throughout grape berry development*

The expression of *VvCTr1–VvCTr8* was studied throughout the fruiting season in grape berries and leaves of cv. Vinhão, with qPCR. As shown in Figure 2 most genes were more strongly expressed in leaves than in berries, except for *VvCTr7* which was not expressed in leaves. Transcripts of *VvCTr1*, *2* and *8* were the most abundant in leaves and in berries, whereas *VvCTr4*, *5* and *7* were the least expressed. The expression of each gene over the course of the fruiting season was not uniform. Particularly in leaves, the transcript level of some genes peaked at veraison (*VvCTr1*, *3*, *6* and *8*), while others displayed an abrupt decrease at this stage (*VvCTr2*, *4*, *5*).

Effect of Bordeaux mixture application on VvCTrs *expression*

To investigate if *VvCTrs* expression was regulated by copper status in plant tissues, a set of grapevines was treated with Bordeaux mixture following the regular vineyard management practices, and samples were also collected at green, veraison and mature stages of fruit development. As confirmed by atomic spectrometry, the treatments with Bordeaux mixture caused an increase in the copper concentration in whole grape berries at all stages of development (Figure 3). In mature berries, this increase was substantial in the skin (ninefold), but copper concentration was also higher in the pulp (twofold) in comparison to that detected in control fruit (Figure 3). In each stage, the gene expression in whole grapes and leaves was compared between control plants (Figure 2) and copper-treated plants (Figure 4). The expression of some genes was repressed by copper at all stages of development, such as *VvCTr7* and *8* in berries and *VvCTr3* and *6* in leaves, highlighting that expression in each organ responded differently to copper application. Accordingly, *VvCTr3* was upregulated by copper in the berry, while *VvCTr8* was strongly upregulated in leaves at all stages. Nonetheless, some genes did not show a homogeneous response

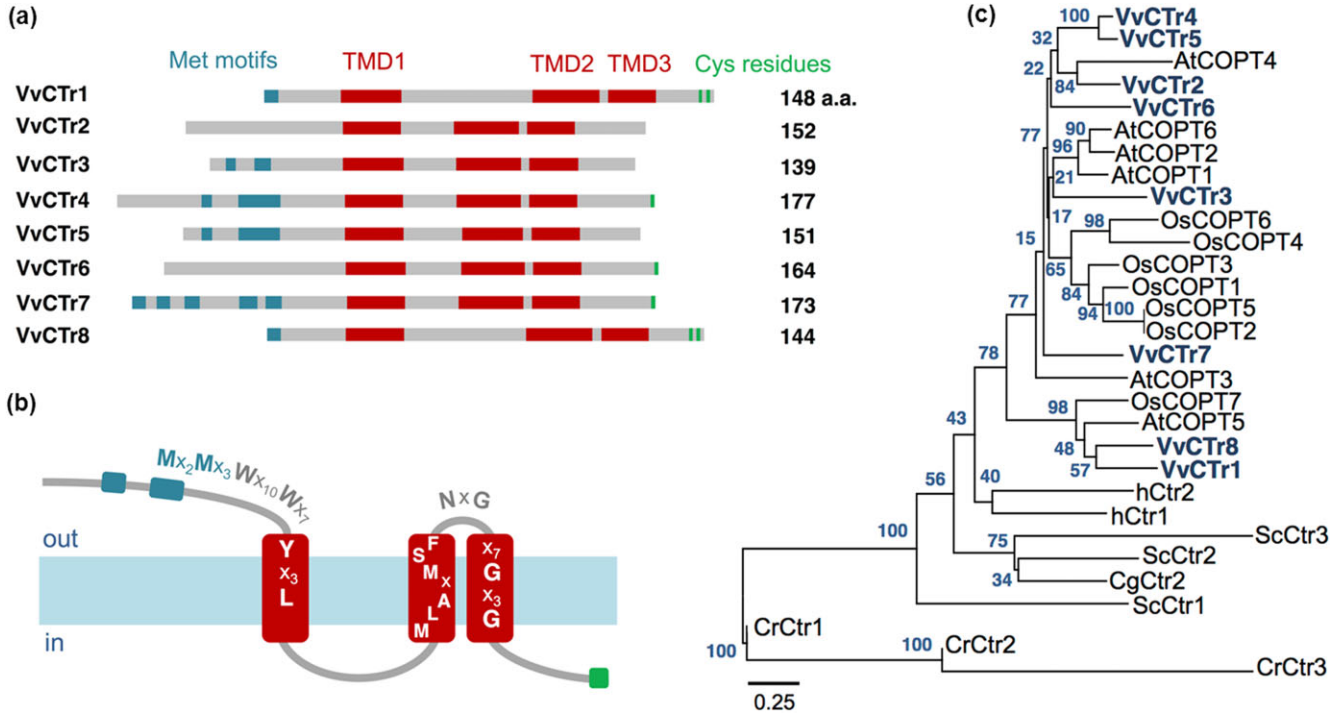

Figure 1. Sequence analysis of grapevine CTrs and other members of the COPT/Ctr family. (a) Alignment of the VvCTrs. Conserved features comprise three transmembrane domains (TMD1–3, shown in red), methionine-rich motifs in the N-terminal consisting of two to seven Met residues in a row or separated by one or two amino acids (in blue) and Cys residues in the C-terminal (in green). The length of each protein in amino acids is shown on the right. (b) Proposed topological model of VvCTrs. Several conserved amino acids among the eight proteins are indicated. (c) Phylogenetic relationship of COPT/Ctr proteins from different species: grapevine (VvCTr1, accession number of GenBank or Protein database of National Center for Biotechnology Information: HQ108185; VvCTr2, HQ108186; VvCTr3, HQ108187; VvCTr4, HQ108188; VvCTr5, HQ108189; VvCTr6, HQ108190; VvCTr7, HQ108191; VvCTr8, HQ108192), Arabidopsis (AtCOPT1, NP_200711; AtCOPT2, NP_190274; AtCOPT3, NP_200712; AtCOPT4, NP_850289; AtCOPT5, NP_197565; AtCOPT6, NP_850091), rice (OsCOPT1, NP_001044379; OsCOPT2, NP_001055594; OsCOPT3, NP_001044380; OsCOPT4, NP_001173438; OsCOPT5, NP_GQ387495; OsCOPT6, NP_001173929; OsCOPT7, HQ833657), baker's yeast (ScCtr1, NP_015449; ScCtr2, NP_012045; ScCtr3, NP_013515), *Colletotrichum gloeosporioides* (CgCtr2, ABR23641), human (hCtr1, NP_001850; hCtr2, NP_001851) 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.

to copper in all sampling stages, such as *VvCTr2*, *4* and *5* in both organs. *VvCTr7* displayed a unique expression pattern, since it was exclusively expressed in berries from control plants, being completely repressed by copper application, while in leaves it was not expressed and remained unchanged in copper-treated plants.

Discussion

The characterisation of plant Ctrs, also known as COPTs, has been restricted to studies in Arabidopsis (Sancenón et al. 2004, Andrés-Colás et al. 2010, Garcia-Molina et al. 2011, 2013, Klaumann et al. 2011, Jung et al. 2012) and in rice (Yuan et al. 2011), where six and seven COPTs were identified, respectively. After the characterisation of putative CTr orthologues in the grapevine we investigated if copper application in the vineyard could interfere with copper homeostasis through the regulation of the expression of *VvCTrs*.

All identified VvCTrs contain three transmembrane domains, typical of Ctr-like proteins (Puig and Thiele 2002, Peñarrubia et al. 2010), each apparently giving a particular contribution for copper transport. A conserved MLAxMSF sequence is present in TMD2, and this transmembrane domain is separated from TMD3 by a short extracellular loop comprising the motif NxG, suggesting that these amino acids may have a particular role in the assembly of VvCTr transporters. A conserved GxxxG motif is present in TMD3 and has been reported to be responsible for the close packing of the three TMDs in other Ctrs, being critical for forming a functional and structurally intact transporter that enables the movement of copper ions across the lipid bilayer (Aller et al. 2004, De Feo et al. 2009, Peñarrubia et al. 2010). Extensive studies on the yeast Ctrs and the human Ctr1 revealed that Ctrs appear to exist as integral membrane oligomers forming a pore that allows the transport of copper (Aller et al. 2004, Eisses and Kaplan 2005, Aller and Unger 2006, De Feo et al. 2010), TMD2 representing the principal pore-lining helix due to the presence of the MxxxM motif that appears to regulate the size of the pore (De Feo et al. 2010, Peñarrubia et al. 2010). The N-terminus of most VvCTrs contains Met motifs that have been reported to be important in copper binding for facilitated import (Puig and Thiele 2002, Peñarrubia et al. 2010). In contrast, the Cys present in the C terminal cytosolic domain appears to contribute to oligomerisation and stability of Ctr oligomers (Eisses and Kaplan 2002). These features are absent in some VvCTrs, namely VvCTr2, which lacks both Met and Cys residues at the N- and C-terminus, respectively, and closely clusters with AtCOPT4, which also lacks these motifs and has been shown to be toxic when expressed in yeast (Sancenón et al. 2003). These proteins are closely related to VvCTr6 which also lacks the Met motifs, and to VvCTr4 and VvCTr5, which share a highly similar amino acid composition (84%).

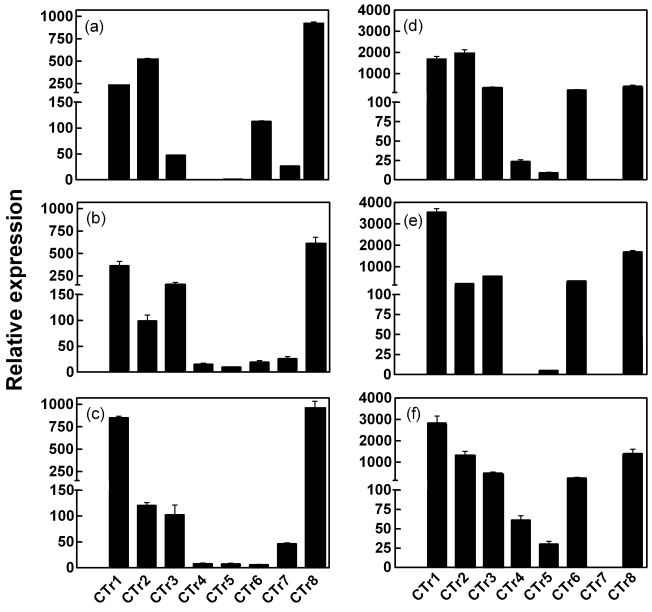

Figure 2. Expression of *VvCTr* genes in (a–c) grape berries and (d–f) leaves cv. Vinhão as measured by quantitative real-time polymerase chain reaction. Transcript levels in control plants at the (a,d) green, (b,e) veraison and (c,f) mature stages of grape berry development and ripening are shown. *VvCTrs* expression was normalised to the transcript levels of *ACT1* and *GAPDH* (internal standards). Results indicate mean ± SEM of three independent experiments (biological replicates) and three technical replicates for each sample.

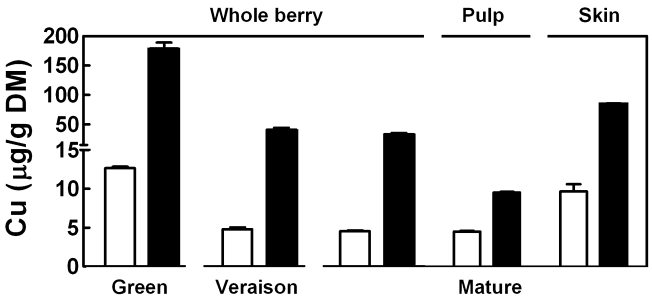

Figure 3. Copper concentration in grape berries of cv. Vinhão, collected at the green, veraison and mature stages and in the pulp and skin of mature berries, from control plants (□) and from plants treated with Bordeaux mixture (■). Results indicate mean ± SEM of three independent experiments (biological replicates).

Most *VvCTrs* were expressed in both grape berries and leaves, throughout all stages of fruit development. The expression of different *VvCTrs* was not constant throughout the fruiting season suggesting a regulation of copper transport at the transcriptional level that is dependent on the developmental stage. Moreover, the overall expression of all genes was greater in leaves than in berries, probably due the role of copper in the photosynthetic apparatus, with the exception of *VvCTr7*, which was present only in the berry. Several putative regulatory elements identified in the promoter regions of most *VvCTrs*, namely ARR1 binding elements, WRKY binding sites, disease resistance response and pollen expression elements, are absent in those of *VvCTr7*. Also, phylogenetic data showed that VvCTr7 is not closely related to other VvCTrs, containing the highest number of Met motifs. *VvCTr1* and *VvCTr8* which share

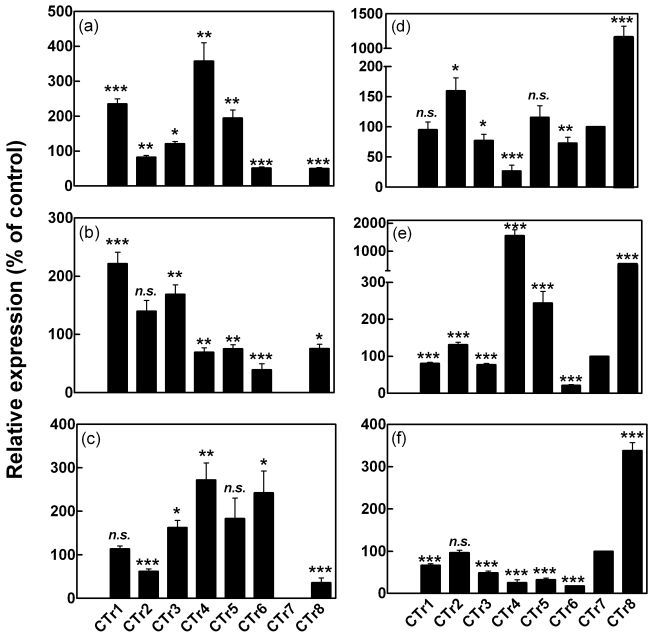

Figure 4. Effect of Bordeaux mixture on the expression of *VvCTr* genes, as measured by quantitative real-time polymerase chain reaction, in (a–c) grape berries and (d–f) leaves of cv. Vinhão collected at the (a,d) green, (b,e) veraison and (c,f) mature stages. Expression of *VvCTrs* was normalised to the transcript levels of *ACT1* and *GAPDH* (internal standards) and compared to the expression detected in control plants for each gene. Results indicate mean ± SEM of three independent experiments (biological replicates) and three technical replicates for each sample. The values are marked with asterisks to denote the significance level as compared to the control: *, $P \leq 0.05$; **, $P \leq 0.01$; *** and $P \leq 0.001$.

a similar secondary structure were the most expressed in both leaves and berries, suggesting their preponderant role in copper homeostasis. The presence of *VvCTr1* and *VvCTr8* transcripts in other grapevine cultivars, namely Alvarinho and Cabernet Sauvignon (Martins et al. 2012) supports their major function in copper transport within grape cells. The diversity in transcript abundance of plant *COPTs* has been reported previously in rice and Arabidopsis, each gene having its specific pattern of expression among different plant tissues (Kampfenkel et al. 1995, Sancenón et al. 2003, 2004, Garcia-Molina et al. 2011, Klaumann et al. 2011, Yuan et al. 2011, Jung et al. 2012). Our data are in line with these observations in rice and Arabidopsis, each *VvCTr* probably playing a specific physiological and biochemical role. Each *VvCTr* responded differently to copper treatment, further supporting the notion that the application of Bordeaux mixture should have major consequences on grapevine copper homeostasis. *VvCTr1* and *VvCTr3* were mostly upregulated in berries in response to copper, with a correspondent downregulation in leaves. Accordingly, the upregulation of *VvCTr1* by excess copper has been observed previously in grape berry cell suspensions (Martins et al. 2012). In contrast, *VvCTr2* and *VvCTr8* were downregulated in berries and upregulated in leaves. In Arabidopsis and rice, a downregulation of gene expression or a lack of regulation by copper have been reported, in some cases in a tissue-dependent manner (Sancenón et al. 2003, Del Pozo et al. 2010, Yuan et al. 2011, Jung et al. 2012). Our results showed that the regulation of the expression of *COPT/Ctr* genes by copper is rather complex. The presence of several *cis*-elements in the regulatory regions of *VvCTr* genes suggests

specific tissue-dependent regulation by environmental factors, including light, and by hormones, namely abscisic acid, which is involved in the coordination of berry ripening (Coombe and Hale 1973) and signalling of stomatal aperture (Galbiati et al. 2011). The proteins MYB and MYC have been shown to be involved in the regulation of abscisic acid signalling (Abe et al. 2003). The presence of ARR1 and WRKY binding elements in the regulatory regions of *VvCTr* genes further suggests hormonal regulation, because they function as transcriptional activators or repressors of cytokinin (Sakai et al. 2000) and gibberellin and abscisic acid signalling (Zhang et al. 2004, Xie et al. 2005), respectively, in addition to their role in pathogen-defense processes, senescence, trichome development and plant growth and development (Eulgem et al. 2000). As defects in COPT-mediated copper transport induced changes in copper distribution that affected root elongation, vegetative growth, chlorophyll content, responses to iron deficiency and low-phosphate signalling and pollen development in Arabidopsis (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), it is likely that VvCTrs are also intimately associated to specific physiological processes. Also, the complex regulation of *VvCTr* genes may be related to the distinct subcellular localisation of VvCTr proteins that could mediate copper transport in particular subcellular compartments. It is likely that plant Ctrs are present not only in the plasma membrane and vacuole (Sancenón et al. 2004, Klaumann et al. 2011) but also in other subcellular locations. For instance, the AtCOPT5 has been reported to be located both in pre-vacuolar vesicles (Garcia-Molina et al. 2011) and in the tonoplast (Klaumann et al. 2011), highlighting the existence of complex transport pathways needed to maintain copper homeostasis. The formation of homo- or hetero-oligomers between Ctr transporters is also necessary to form functionally mature transporters as reported in *S. cerevisiae* (Sinani et al. 2007), *S. pombe* (Zhou and Thiele 2001), humans (Aller and Unger 2006, De Feo et al. 2009), Arabidopsis (Jung et al. 2012) and rice (Yuan et al. 2011), indicating that there is also a potential for the association of different VvCTr subunits, that might represent another level of transport regulation depending on cellular demands. Recent studies have shown that VvCTr1 mediates intracellular copper transport, being mobilised via the trans-Golgi network, through the pre-vacuolar compartment and located to the vacuolar membrane. Moreover, bimolecular fluorescence complementation demonstrated that Ctr monomers are self-interacting (Martins et al. 2014).

Conclusions

The understanding of the mechanisms and regulation of copper homeostasis in grapevine tissues has an important basic and applied relevance. The in silico characterisation and expression studies by qPCR of the eight members of the *V. vinifera* CTr family of copper transporters provided key insights on the complexity of copper transport mechanisms in grape berries and leaves. Four applications of copper following a methodology used in organic farming were enough to induce a significant increase in copper content in berry tissues which was either a cause or a consequence of the observed transcriptional reprogramming of the expression of *VvCTr*s. The puzzle of this highly complex network of copper-delivering systems will gradually be assembled with the cloning of each *VvCTr*, localisation and characterisation of VvCTr-mediated transport (Martins et al. 2014), ultimately aiming at the elucidation of the particular role of each protein in copper homeostasis.

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Supporting information

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Figure S1. Map of the 5′ regulatory regions of the *VvCTr* family. Positions are with respect to the first base of the transcription start site (TSS).