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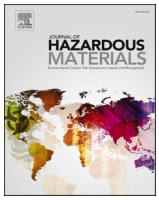

Copper excess transcriptional responses in roots of grapevine (*Vitis* sp.) rootstocks

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Abbreviations: Cu, copper; Zn, zinc; ROS, reactive oxygen species; HMA, Heavy Metal Associated; SOD, superoxide dismutase; COPT, Copper Transporter; IAC, IAC-572 101–14; ISA, Isabel; IBCA, IBCA-125; Paulsen or PLS, Paulsen 1103; LiCl, lithium chloride; PSII, photosystem II; Fo, initial fluorescence; Fm, maximum fluorescence; Fv, variable fluorescence; YII, maximum quantum yield of PSII; Fo', minimal fluorescence from light-adapted leaf; ΦPSII, effective quantum yield of PSII; F', fluorescence recorded before the beginning of a strong light pulse; Fm', maximum fluorescence yield when the reaction centers of PSII are closed by a strong light pulse; *qP*, photochemical quenching; *qN*, non-photochemical quenching of variable fluorescence; ETR, electron transport rate; ICP-OES, inductively coupled plasma optical spectroscopy; N, nitrogen; FDR, false discovery rate; SRA, Sequence Read Archive; NCBI, National Center for Biotechnology Information; P, phosphorus; Ca, calcium; Fe, iron; Mn, manganese; K, potassium; Mg, Magnesium; PCA, Principal Component Analysis; DEGs, differentially expressed genes; GO, Gene Ontology; ZIP, Zinc-regulated/Iron-regulated Transporter Protein; PHT, Phosphate Transporters; CSDs, Cu-containing superoxide dismutases; FSDs, Fe-SODs; NRAMP, Natural Resistance-Associated Macrophage Protein; YSL, yellow stripe like; VIT, vacuolar iron transporter; FRO, ferric-chelate reductase/oxidase; PAP, Purple Acid Phosphatase; PCR2, PLANT CADMIUM RESISTANCE 2; Cd, Cadmium.

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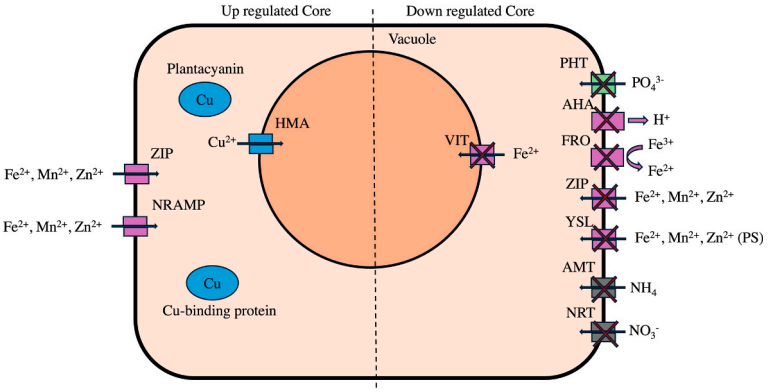
HIGHLIGHTS

- We identified core copper excess responsive genes in grapevine roots.
- Leaf and root ionomes are altered by copper excess.
- Copper is differentially partitioned in grapevine rootstock genotypes.
- We identified candidate genes that might be involved in copper tolerance.
- Some genes might be interesting for engineering copper tolerance in grapevine.

GRAPHICAL ABSTRACT



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ABSTRACT

Copper (Cu) is an essential element for plants, participating in photosynthesis, oxidative metabolism and cell wall synthesis. However, excessive Cu may become toxic, as Cu participates in Fenton chemistry and cause oxidative stress. Grapevine (*Vitis* sp.) is an important perennial crop, used for in natura consumption as well as for wine and juice. Vineyards are susceptible to fungal diseases that are commonly controlled by using Cu-based fungicides, which can lead to Cu accumulation in the soil. Since grape production is based on grafting scions of consumed-friendly varieties onto rootstocks that can withstand soil-borne diseases and stresses, it is important to identify rootstock genotypes that are tolerant to Cu excess. In this work, we compared physiological and molecular responses of four *Vitis* sp. rootstock genotypes to Cu excess, namely IAC, IBCA, Paulsen and Isabel. While IAC, IBCA, Paulsen were similarly tolerant, Isabel was the most sensitive to Cu excess. IAC and IBCA showed higher Cu accumulation in shoots, suggesting distinct partitioning strategy. We identified core Cu excess-responsive genes in grapevine roots of all four genotypes, including a putative HMA vacuolar Cu transporter and Cu-binding proteins. Genes related to the homeostasis of other elements are altered, such as iron (Fe) and phosphorus (P), suggesting that Cu excess alters the ionome balance. IAC and IBCA had extensive changes in their laccase gene repertoire, suggesting that could be related to the distinct Cu partitioning. Moreover, genes associated specifically with Isabel could be related to the genotype Cu excess sensitivity. Our work provides a valuable dataset for understanding variation in Cu tolerance how roots respond transcriptionally to Cu stress, and provide candidate genes for engineering Cu tolerance in grapevines.

1. Introduction

Copper (Cu) is an essential micronutrient for plants. It can be found in two transition states, Cu^{+} and Cu^{2+}. Cu ability to lose and gain electrons makes it useful in electron transfer reactions, and it is involved in photosynthesis, respiration, antioxidant metabolism and cell wall synthesis [1]. Cu is part of the structure of plastocyanin, the thylakoid lumen protein that transfers electrons from cytochrome b6f to photosystem I; cytochrome c oxidases, involved in mitochondrial electron transport; superoxide dismutase, that convert O_2^- into H_2O_2; and polyphenol oxidases, amine oxidases and multicopper oxidases, involved in several aspects of plant growth and development [1–3]. Cu deficiency leads to a reorganization of Cu homeostasis to save Cu for photosynthesis while decreasing or substituting other functions such as Cu/Zn superoxide dismutase, laccases and Cu chaperones [3,4]. However, Cu excess can also be toxic since Cu can produce reactive oxygen species (ROS) through Fenton chemistry, substitute other metals, disrupt membrane integrity and other cellular functions [1,2,5,6]. Therefore, plants must keep Cu concentration optimal for adequate growth.

Grapevine (*Vitis vinifera* sp.) is a major cultivated plant worldwide, and a crop with deep connections with human history and culture, being consumed as table grape, raisins, alcoholic drinks such as wine and non-alcoholic beverages [7,8]. Secondary metabolites derived from grapes are used in cosmetics, food and pharmaceutical applications [9]. Grapevine plants have a diploid, relatively small genome, and good genomic resources, such as reference genome and resequenced genomes for several *Vitis* sp. genotypes, including cultivated and wild species [10–13]. Since its domestication ~6,000 years ago, grapevine diversified into more 12,000 documented cultivars. Although only a fraction of these are important economically [14,15], the genetic diversity of *Vitis* sp. can be explored to identify interesting traits.

In vineyards, control of fungal diseases, such as downy mildew, is of paramount importance. One of the most common fungicides used are Cu-based solution, such as the Bordaux mixture, which also function as insecticide [16–19]. However, continuous use leads to Cu accumulation in vineyard soils, which can reach toxic levels, as reported in Europe and South America [5,20–23]. Especially for young plants with shallow roots, high Cu levels in soils can cause oxidative stress, decrease primary root growth while increasing lateral root length, decreasing photosynthesis, and reducing chances of vine successful establishment [6,24,25]. Since grapes are produced by grafting a scion onto a rootstock, it is important to identify rootstock genotypes with the ideal characteristics, such as genotypes showing higher exudation of organic acids and with higher ability to secrete phenolic compounds which are involved in Cu chelation [25]. However, little is known about the molecular response of grapevine plants to Cu excess, as well as the variation in response between distinct genotypes.

The Cu uptake mechanism is relatively well known in the model

species *Arabidopsis thaliana*. In the rhizosphere, Cu is reduced from Cu^{2+} to Cu^{+} by the plasma-membrane reductases AtFRO4/AtFRO5, and Cu^{+} is transported into root cells by members of the COPT (Copper Transporter) family [1]. Inside the cells, Cu is chelated by chaperones to avoid deleterious effects of free Cu. Chaperone proteins deliver Cu safely to transporters, such as members of Heavy Metal Associated (HMA) family. HMA proteins are involved in xylem loading, vacuolar detoxification and plastid/chloroplast transport [1,3]. Under Cu excess, oxidative stress responses involving superoxide dismutase (SOD), catalase and other ROS scavenging molecules are up-regulated. The COPT family in grapevine has eight members, some of which are transcriptionally regulated by Cu [17,25,26]. VvCtr1 (VvCOPT1) was the only grapevine Cu-related protein characterized to date [17]. Other putative Cu homeostasis-related genes where shown to be regulated by Cu excess in leaves in transcriptomic studies [27,28]. However, there is no transcriptomic analyses in grapevine roots exposed to excess Cu in the literature.

Given (1) the economic importance of grapevines; (2) the prevalence of Cu toxicity in vineyards, especially the negative impact on young ones; (3) the need to understand the molecular responses to Cu excess in grapevine roots; and (4) how this response can vary between diverse *Vitis* sp. genotypes; we aimed to compare the physiological and transcriptional responses of four rootstocks exposed to Cu excess. We identify core genes that are up- and down-regulated in roots of plants exposed to high Cu concentration, which can have functional significance to how grapevine plants detoxify Cu. We also identified differences comparing genotypes, which may be involved in variation in Cu homeostasis, and might be used as candidates for engineering Cu tolerance grapevines. Our study provides valuable molecular datasets for how *Vitis* sp. roots respond to Cu excess, as well as identify promising candidate genes for Cu tolerance.

2. Material and methods

2.1. Plant material

We generate plants by regenerating micropropagated calli from tissue culture. The following genotypes were used: IAC-572 101–14 [(*V. riparia* x *V. rupestris*) x *V. caribaea*]; IBCA-125 (*V. labrusca* x *V. rotundifolia*); Isabel (*V. labrusca*); and Paulsen 1103 (*V. berlandieri* x *V. rupestris*) – hereafter IAC, IBCA, Isabel and Paulsen, respectively. The selection of genotypes is based on their genetic wide variability, use in vineyards in southern Brazil and inclusion as materials in breeding programs for several traits at Embrapa Uva e Vinho. Plantlets with one fully expanded leaf were transplanted from axenic conditions to 500 mL containers filled with soil. The plants were grown in samples of Humic Cambisol soil [29], characterized by the following attributes: clay 225.0 g kg^{-1}; organic matter 30.0 g kg^{-1}; pH in water 5.9; exchangeable Ca 9.7 $cmol_c$ dm^{-3} and exchangeable Mg 1.8 $cmol_c$ dm^{-3}, both using 1 mol L^{-1} KCl; available P 16.5 mg dm^{-3} and available K 140 mg dm^{-3}, measured by the Mehlich 1 method. The samples were collected from the top layer of soil, 0 to 20 cm deep, in an uncultivated area covered by natural pasture in the municipality of Bento Gonçalves, Rio Grande do Sul State, Brazil (29°9'48" S and 51°31'55" O). After collection, the soils were air-dried, sieved through a 2 mm mesh, fertilized, and subjected to liming, following the regional recommendation of CQFS-RS/SC (2004) for grapevine cultivation. In the treatments, we used two copper (Cu) concentrations – 0.1 μM (as control) and 250 μM (as excess) – sourced from copper sulfate ($CuSO_4 \cdot 5H_2O$). To prepare the higher concentration (250 μM), we dissolved the necessary amount of Cu in water, followed by mixing and distribution in a pot containing 1 kg of soil. After complete drying, we combined the soil from this pot with an additional volume of the same soil type, reaching the required amount for all treatments. We carried out this combination using a concrete mixer, ensuring uniform Cu distribution. Similarly, we performed liming and nutrient addition using the concrete mixer, aiming for uniformity in these processes. Plant irrigation occurred by adding water to the pot saucers whenever they were dry.

The experiment was conducted at Embrapa Uva e Vinho in Bento Gonçalves, Rio Grande do Sul, Brazil (29°09′48″S, 51°31′42″O, at 616 m altitude). Pots were organized in a growth chamber (26–27ºC, 60–66 % humidity, 16/8 light / dark cycle). Plants were produced from in vitro regeneration and acclimated in greenhouse conditions before the start of the experiment. We conducted experiments with young grapevine plants because Cu contamination in vineyards usually affects young plants that are used to install new vines after old ones die. Plants were completely randomized in three replicates containing 15 plants per experimental unit (n = 15). After 28 days of treatment, plants were collected and had their roots washed to remove soil, and dried in paper towel to remove excess water. These samples were either used to measured root and shoot dry weight measurements, or were frozen in liquid nitrogen, stored at −80ºC, and used for RNA extraction.

2.2. Root RNA extraction and sequencing

Roots from each replicate were pooled for RNA extraction. In total, we prepared 24 libraries (4 genotypes x 2 treatments x 3 replicates). Root samples were extracted according to the protocol described [30] using RNA selective precipitation with LiCl 7.5 M (Invitrogen®). After RNA extraction, quantification was performed using NanoDrop 2000 (Thermo Scientific™), and RNA integrity was inferred in 1.2 % agarose gel visualization. Afterwards, the 24 samples were treated with DNAse (DNase I, RNase-free – Invitrogen®) to remove genomic DNA. For sequencing, 20 μg of total RNA for each of the 24 libraries were sent to the Roy J. Carver Biotechnology Center at the University of Illinois, USA. Sequencing of root transcriptomes was carried out using the Illumina NovaSeq 6000 platform, using the manufacturer standard protocols. Libraries were sequenced using paired-end reads of 150 bp, and read quality was analyzed using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). All low-quality reads and adaptor sequences were removed from analyses.

2.3. Photosynthesis and chlorophyll parameters

Leaf analyzes were performed at 26 days after planting (days of treatments), considering three plants per replicate in each combination of genotype and soil copper treatments. The gas exchange measures were carried out at the 3rd or 4th expanded leaf blade of each plant, using an infrared gas analyzer (IRGA, model LI-6400 XT, LI-COR, Lincoln, USA) equipped with a light source model LI-6400–2B. The analysis was performed from 9 am to 2 pm, setting the speed of airflow at 500 μmol s^{-1} and the intensity of photosynthetic active radiation (PAR) to obtain the net photosynthesis in the light saturation point (1000 μmol CO_2 m^{-2} s^{-1}) and the dark respiration point (0 μmol CO_2 m^{-2} s^{-1}). The environmental CO_2 was used, which the concentration ranged from 415 to 438 μmol mol^{-1} during the measurements. The leaf temperature ranged from 21.4 to 30.6 °C, while the air relative humidity was between 42 % and 53.9 %. Gas exchange data were recorded only when the system variation coefficient was less than 0.5 %.

After this analysis and in the same leaves, the indices of chlorophyll a (Chl.a) and b (Chl.b) were measured, using a portable measuring device (ClorofiLOG, model CFL 1030, Falker). From these indices, chlorophyll a/b ratio and total chlorophyll were calculated. For the same leaves, the variables of chlorophyll fluorescence were determined using a pulse-amplitude modulated fluorometer (Junior-PAM, Walz). The leaves were previously kept in the dark for 30 min, using a dark adaptation clip, for the opening of the reaction centers of photosystem II (PSII). For the determination of initial fluorescence (Fo), a modulated light pulse (< 0.1 μmol m^{-2} s^{-1}) was used; while a pulse of 0.6 s of saturating white light (10000 μmol m^{-2} s^{-1}) was used for the maximum fluorescence (Fm). From these parameters, the variable fluorescence (Fv = Fm - Fo) and the maximum quantum yield of PSII [(Fv/Fm or Y(II), just after dark period]

were calculated. Immediately after this initial condition, a light curve was performed with pulses of PAR at 125, 190, 285, 420, 625, 820, 1150 and 1500 μmol m^{-2} s^{-1}, interspersed with actinic light of 285 μmol m^{-2} s^{-1} and using the activated mode of far-red radiation for the measurement of Fo' (minimal fluorescence from light-adapted leaf). From this curve, the effective quantum yield of PSII [Φ_{PSII} = (Fm' - F')/Fm; where F' = fluorescence recorded just before the beginning of a strong light pulse and Fm' = maximum fluorescence yield when the reaction centers of PSII are closed by a strong light pulse]; the photochemical quenching [qP = (Fm' - F') / (Fm' - Fo')]; the non-photochemical quenching of variable fluorescence [qN = (Fm - Fm') / (Fm – Fo')]; and the electron transport rate in PSII ($E\ TR$ = 0.84. 0.5. PAR. Φ_{PSII}) were obtained.

2.4. Elemental profiling

The protocol for analyzing the elemental profile in tissues of young grapevine plants was developed to establish a method for the quantitative determination of essential elements for plant growth. The initial sample collection focused on plant selection, using tools to collect root and shoot samples, avoiding contamination, and preserving tissue integrity. During the preparation phase, collected roots and aerial parts were washed with distilled water to eliminate soil residues and debris, reducing external contamination. Then, the tissues were dried in an oven with forced ventilation at 60 °C until they reached constant weight. The samples were then decomposed in an acidic medium, according to [31] and the resulting solutions were analyzed by inductively coupled plasma optical spectroscopy (ICP-OES) using the Perkin-Elmer Optima 7300 DV equipment. The total concentration of nitrogen (N) was determined by the Kjeldahl digestion method (BUCHI, Digest automat K-439, and Distillation Kjelflex K-360, Switzerland).

2.5. Transcriptome analyses

Transcript abundance was quantified according to an *in-house* protocol from *Embrapa Recursos Genético e Biotecnologia*. Reads were quality filtered using filterbytile script from BBMap v. 38.33, with the following parameters: ud= 0.75, qd= 1, ed= 1, ua= 0.5, ea= 0.5, overwrite=true (Bushnell B - sourceforge.net/projects/bbmap/). High-quality reads were mapped to gene regions of the reference genome *Vitis vinifera* v2.1 from Phytozome v13 (Phytozome genome ID: 457). Alignment was performed using the STAR v2.7.2a program [32]. To calculate gene expression based on aligned data, raw counts mapped per gene were calculated using the Python script HTSeq-count with the following parameters: -r pos –mode=union –format=bam –type=gene –idattr=ID –stranded=no (https://htseq.readthedocs.io/en/release_0.11.1/) [33]. Differential expression analyses were conducted using EdgeR's exact test [34]. First, the reproducibility of biological replicates and the differences between genotypes were analyzed using the normalized counts and DeSeq and rlog commands from DESeq2 package [35]. The distant matrix was visualized by heatmap and Principal Component Analysis (PCA) and plots were produced by web tool [36]. Transcripts from different genotypes or from different treatments were considered differentially expressed when the false discovery rate (FDR) corrected p-value $\leq$ 0.05 and a log2foldchange $\geq$ 2 (up-regulation) or ≤ -2 (down-regulation). Raw data can be found at Sequence Read Archive (SRA) database at NCBI (National Center for Biotechnology Information), accession number PRJNA1110246. Heatmap and one-dimensional hierarchical clustering were generated based on the expression levels of differentially expressed genes among the IAC, IBCA, Isabel, and Paulsen genotypes, according to the applied filtering and statistical criteria. Each column represents a genotype, while each row corresponds to a differentially expressed gene. The heatmap was created using Clustvis [36] with no row centering, no row scaling, and no clustering distance applied to the columns. Transcripts from different genotypes or from different treatments were considered differentially expressed when the false discovery rate (FDR) corrected p-value $\leq$ 0.05 and a log2foldchange $\geq$ 1 (up-regulation) or ≤ -1 (down-regulation).

Gene Ontology (GO) enrichment analysis were done using func package [37] with *in-house* scripts. Up- and down-regulated gene IDs were created for each genotype differential expression results. The hypergeometric statistics were used. The statistical significance threshold was FDR < 0.05. A bubble plot presenting the enriched GO terms was created using ggplot2 package [38].

2.6. Statistical analyses

Data analysis was performed using the GraphPad Prism software (version 8.0.2). Difference between treatment groups within genotypes was measured by t test, while the difference among genotypes within group treatments was measured by two-way ANOVA, followed by Tukey's multiple range tests. The statistical significance threshold was established at $p < 0.05$.

3. Results

3.1. Copper excess affects grapevine rootstocks' growth when applied to the soil

We exposed four distinct rootstock genotypes, namely IAC, IBCA, Isabel and Paulsen to Cu excess in the soil. Overall, we found that Cu excess affected all genotypes (Fig. 1). Root dry weight was decreased, with Paulsen (PLS) showing the least pronounced decrease in dry weight compared to control, while IBCA and Isabel (ISA) showed the most pronounced (Fig. 2A). IBCA and Isabel also had more root biomass under control condition compared to Paulsen, which suggests that those with more carbon investment in the root system are more severely affected by Cu excess (Fig. 2A). In shoots, IAC and Paulsen had the clearest decrease in biomass when exposed to Cu excess. IBCA and Isabel showed no decrease in shoot dry weight. Paulsen also showed the lowest biomass compared to other genotypes, whereas IBCA and IAC showed the highest (Fig. 2B). These results suggested that rootstock genotypes responded differently to Cu excess.

3.2. Copper excess impact on photosynthesis in grapevine rootstocks

To gain insight into how Cu excess may affect photosynthesis in each genotype, we measured ETR in leaves of plants from the four genotypes under control and Cu excess after 26 days of treatment. There was wide variation in values comparing the four genotypes, with IBCA showing lower ETR values, and the other three having comparable values (Fig. 3). When plants were exposed to Cu excess, ETR decreased only in Isabel (Fig. 3A-D). This suggests that Isabel was the most affected genotype.

We also measured net photosynthesis (Fig S1). The observations agreed with ETR measurements, with IBCA plants showing the lower value in net photosynthesis. However, we did not find significant changes when comparing control and Cu excess, suggesting that although ETR is affected in plants exposed to excessive Cu, carbon assimilation is not. We also measured chlorophyll concentrations and observed no change comparing control and Cu excess in all four genotypes (Fig S2). Taken together, our data suggest that Cu excess affected ETR but no other photosynthesis-related parameters in grapevine plants.

3.3. Copper compartmentalization and ionomic changes in rootstock genotypes

To gain insight into elemental accumulation in rootstock genotypes, we measured elements concentrations in roots and shoots of plants under control and Cu excess treatments. As expected, all genotypes accumulated Cu in the roots when exposed to metal excess. Comparing Cu concentration in roots from plants cultivated under control and Cu excess, the increase was between 22-fold (Isabel) and 42-fold (IBCA). The concentrations, however, were similar when comparing genotypes,

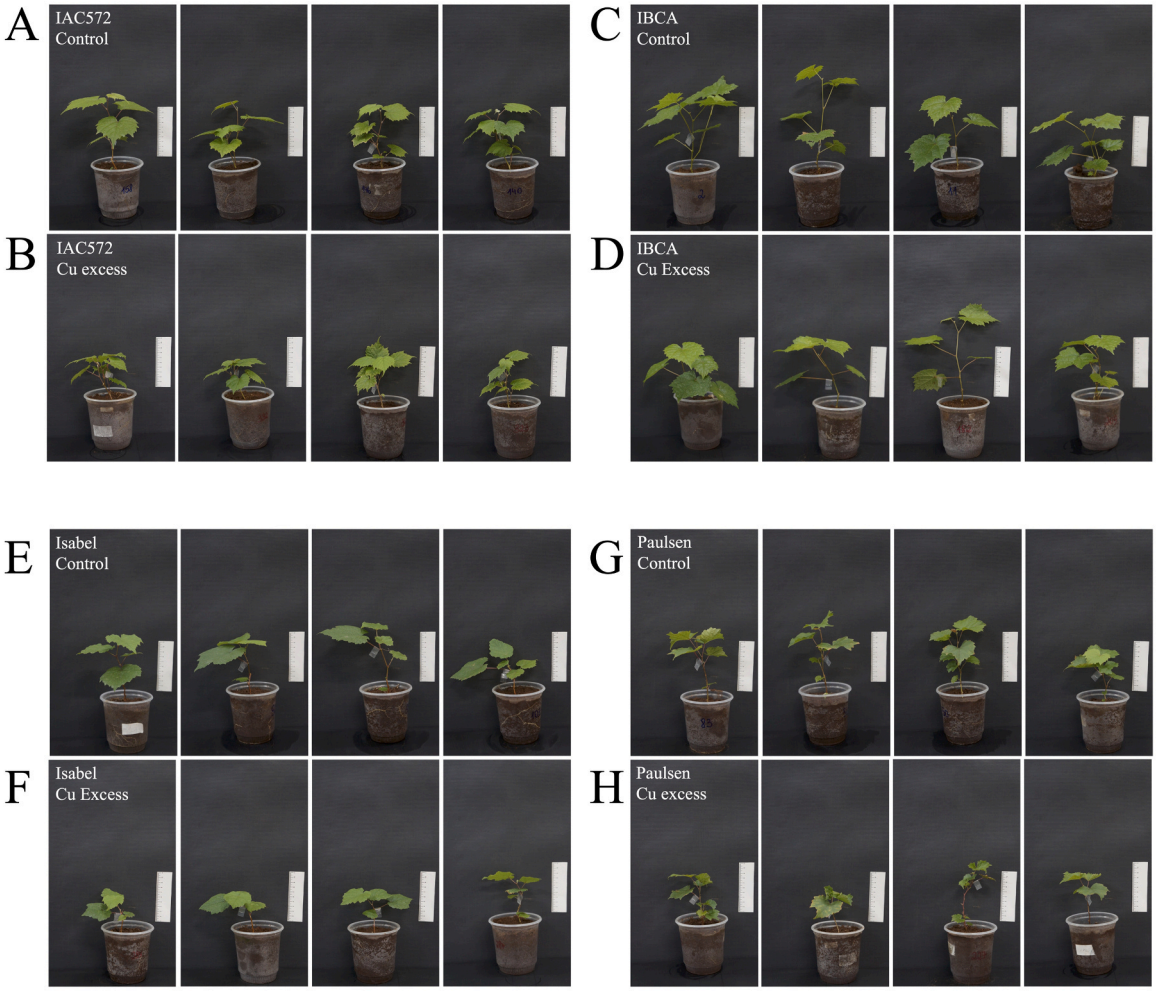

Fig. 1. Copper excess affects grapevine rootstock genotypes. (A) IAC plants under control conditions. (B) IAC plants under copper excess. (C) IBCA plants under control conditions. (D) IBCA plants under copper excess. (E) Isabel plants under control conditions. (F) Isabel plants under copper excess. (G) Paulsen plants under control conditions. (H) Paulsen plants under copper excess. Images represent four independent replicates at the end of the experiment (28 days).

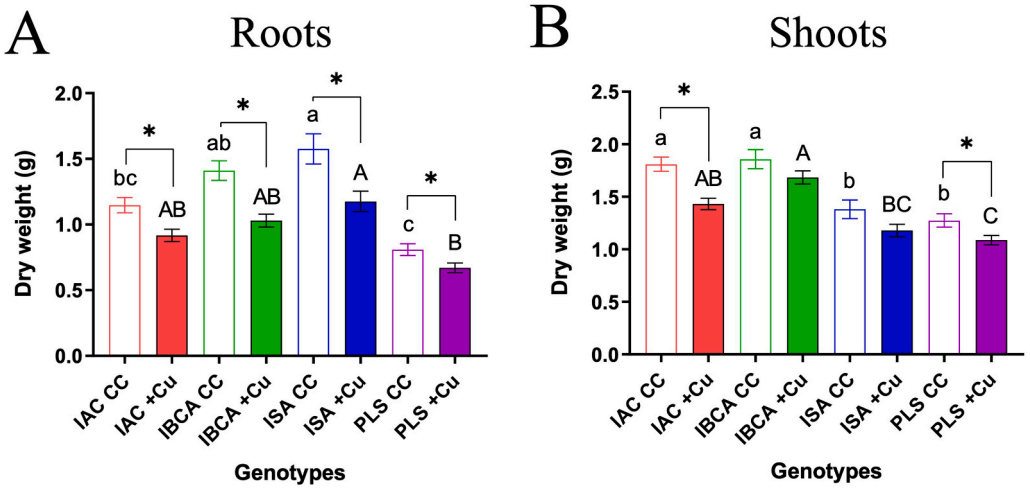

Fig. 2. Grapevine rootstock genotypes dry weight under control and copper excess conditions. (A) Root dry weight. (B) Shoot dry weight. Asterisks indicate statistically significant differences when comparing control and copper excess conditions for the same genotype (Student *t*-test, $p \leq 0.05$). Lowercase letters indicate statistically significant differences comparing genotypes under control conditions, while uppercase letters indicate differences comparing genotypes under copper excess (Two-way ANOVA and Tukey's multiple range test, $p \leq 0.05$).

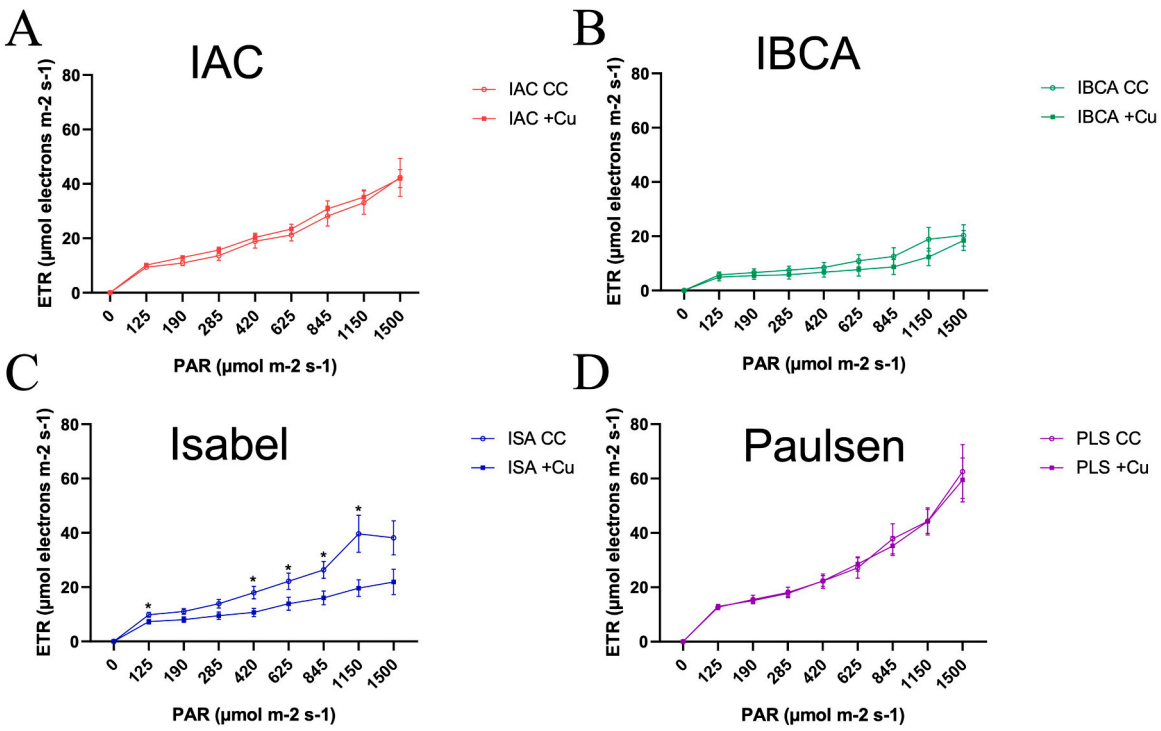

Fig. 3. Electron transport rate in leaves of rootstock genotypes under control and copper excess conditions. (A) Electron transport rate (ETR) in leaves of IAC, (B) IBCA, (C) Isabel, and (D) Paulsen plants. Asterisks indicate statistically significant differences when comparing control and copper excess conditions for the same genotype (Student *t*-test, $p \leq 0.05$).

either under control or Cu excess conditions (Fig. 4A).

Cu also accumulated in shoots in all genotypes when exposed to Cu excess (Fig. 4B). Comparing Cu concentration in control conditions and Cu excess, the increase was between 1.9-fold (Isabel) and 2.8-fold (IBCA), which is significantly lower than those observed in roots (Fig. 4A). Interestingly, IAC and IBCA showed a more pronounced increase in Cu concentration compared to Isabel and Paulsen, suggesting a distinct Cu partitioning strategy. This difference is not observed under control conditions (Fig. 4B). Altogether, the data indicates that IAC and IBCA translocated Cu to a larger extent from roots to shoots compared to Isabel and Paulsen.

The concentration of phosphorus (P) in roots was not affected by Cu excess (Fig. 4C). However, while shoot P concentration for Isabel and Paulsen did not show significant differences comparing control and Cu excess, IAC and IBCA increased P concentration under Cu excess compared to control, and values under Cu excess were higher when compared to Isabel and Paulsen (Fig. 4D). Once again, this suggests that IAC and IBCA behaved differently under Cu excess compared to the other two genotypes, and that variation in P might be linked to Cu accumulation in shoots of IAC and IBCA.

Calcium (Ca) concentrations were decreased to a similar extent in roots of all four rootstocks exposed to Cu excess when compared to control (Fig. 4E), while we found no changes in shoots except for a slight decrease in Ca concentration in Isabel (Fig. 4F). Iron (Fe) concentrations were also unchanged in roots (Fig. 4G), while IBCA showed increased Fe concentration in shoots comparing Cu excess with control (Fig. 4H). IBCA and Isabel decreased manganese (Mn) concentration in roots under Cu excess (Fig. 4I), and IBCA strongly decreased Mn concentration in shoots as well (Fig. 4J). IAC and Paulsen slightly increased Zn concentrations in roots under Cu excess (Fig. 4K), but no change was observed in shoots (Fig. 4L). Interestingly, K concentration was decreased in roots of IAC, IBCA and Isabel under Cu excess (Fig. 4M) and increased in IAC shoot tissue (Fig. 4N). On the other hand, Paulsen showed lower potassium (K) concentration in shoot tissue when plants were cultivated under Cu excess (Fig. 4N). Magnesium (Mg) concentrations were increased in roots of IAC (Fig. 4O) and in shoots of Paulsen under Cu excess (Fig. 4P). Taken together, these data show that Cu excess had an impact in both root and leaf ionomes, and that these changes can be different for each genotype.

3.4. Transcriptome responses to Cu excess in rootstock genotypes

Even though the genotypes used in this work are part of the same genus (Vitis *sp.*), they showed different responses to Cu excess, especially in the ionome of roots and shoots (Fig. 4). To understand possible differences in the transcriptional responses to this stress, we performed transcriptome analyses in roots of the four genotypes, comparing control and Cu excess-cultivated plants, after 28 days of treatment.

We sequenced three independent libraries composed of independent biological replicates for each genotype and treatment. During analyses, we removed one sample from IAC control, which showed lower quality compared to others in preliminary analyses. Clustering of our samples show that samples derived from the same genotype grouped together, as well as samples from the same treatment (Fig. 5). PCA analysis and hierarchical clustering analysis also demonstrated that genotype is the main factor separating our samples, suggesting that larger differences in transcriptome were due to genotype, not treatment, which was expected considering that we have sequence information derived from different genotypes and hybrids of *Vitis* sp. species (Fig. 5A). PCA analysis also suggested that IBCA had the most pronounced differences when comparing control and Cu excess samples, while Paulsen had the least (Fig. 5A). Moreover, hierarchical clustering showed that samples from the same genotypes and treatments cluster together (Fig. 5B), indicating that biological replicates are adequate for subsequent analyses.

Comparing control and Cu excess conditions, we found 2452 differentially expressed genes (DEGs) for IAC (1431 up-regulated and 1021 down-regulated); 2415 for IBCA (1190 up-regulated and 1225 down-regulated); 1203 for Isabel (465 up-regulated and 738 down-regulated) and 1694 for Paulsen (819 up-regulated and 875 down-regulated) (Fig. 6A and B; Supplemental Table 1). We also analyzed

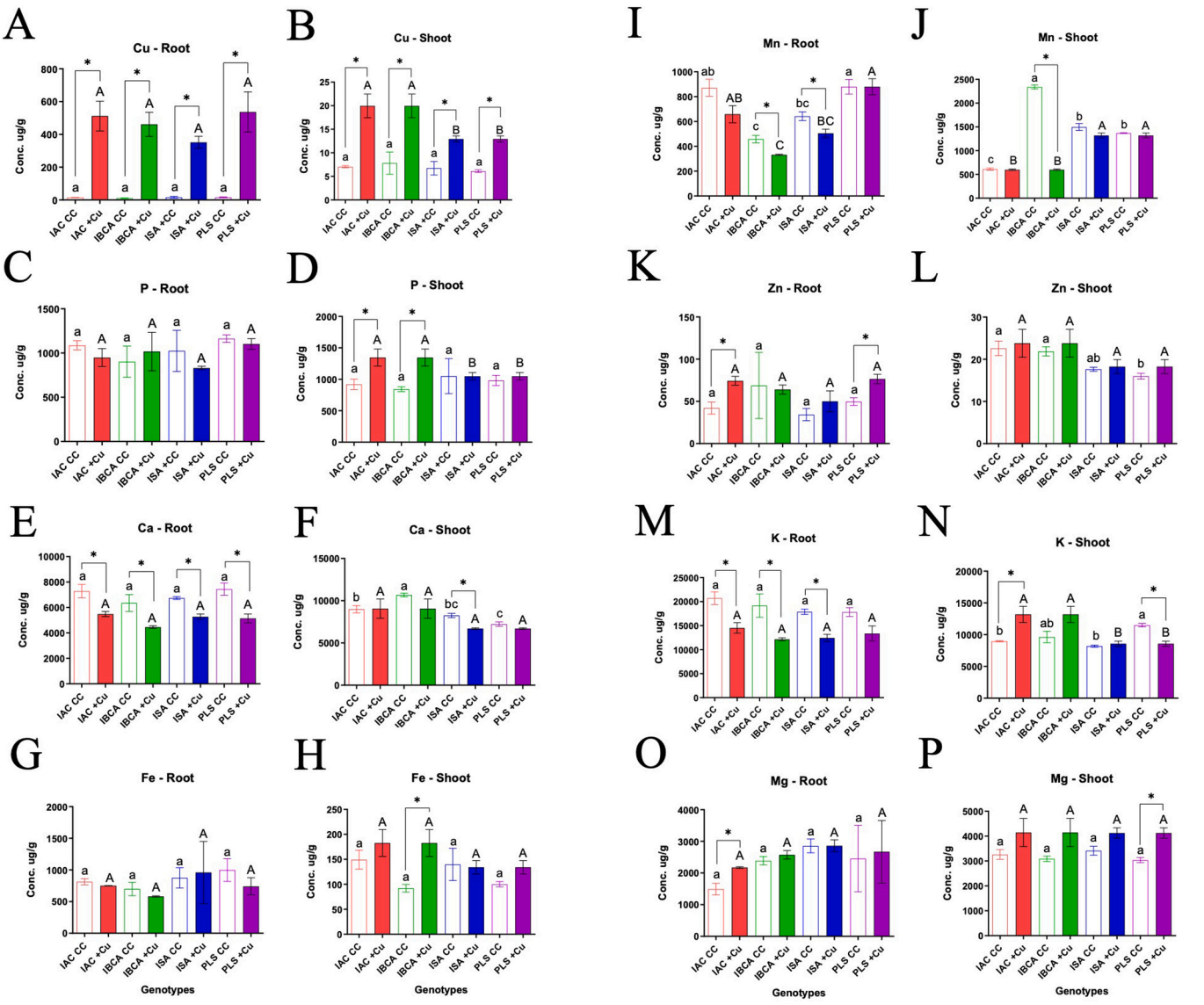

Fig. 4. Ionome changes in roots and leaves of rootstock genotypes under control and copper excess conditions. (A) Copper concentration in roots and (B) in shoots. (C) Phosphorus concentration in roots and (D) in shoots. (E) Calcium concentration in roots and (F) in shoots. (G) Iron concentration in roots and (H) in shoots. (I) Manganese concentration in roots and (J) in shoots. (K) Zinc concentration in roots and (L) in shoots. (M) Potassium concentration in roots and (N) in shoots. (O) Magnesium concentration in roots and (P) in shoots. Asterisks indicate statistically significant differences when comparing control and copper excess conditions for the same genotype (Student *t*-test, $p \leq 0.05$). Lowercase letters indicate statistically significant differences comparing genotypes under control conditions, while uppercase letters indicate differences comparing genotypes under copper excess (Two-way ANOVA and Tukey's multiple range test, $p \leq 0.05$).

the general expression pattern of all four genotypes based on fold change (Fig. 6C). The data showed that IBCA has pronounced changes in gene expression, with several up and down-regulated genes that are either not regulated or are regulated to a lower extent in the other genotypes. IAC also had several genes up-regulated to a larger extent compared to the other genotypes, whereas Isabel and Paulsen both showed less extensive changes in gene expression (Fig. 6C). Altogether, these data demonstrate that the four genotypes use different sets of genes to respond to Cu excess, as well as respond transcriptionally at different intensities.

3.5. Gene ontology analyses of differentially expressed genes

Given the large numbers of DEGs, we performed GO analyses using only genes with Log2FC > 1 for up-regulated genes, and Log2FC < –1 for down-regulated genes (Fig. 7; Supplemental Table 1). We did not find GO terms that were up-regulated in all genotypes, pointing to the largely distinct responses of the four rootstocks to Cu excess (Fig. 7). We observed that the category "Copper Ion Binding" was up-regulated in IAC and IBCA but not in Isabel and Paulsen. Interestingly, these are the two genotypes that show higher Cu concentration in shoots (Fig. 4B). Therefore, we looked for other GO categories that may be differentially regulated in both IAC and IBCA but not in Isabel and Paulsen. This search then allowed us to identify categories such as "Heme binding", "Chitin Binding", "Iron Ion Binding", "Abscisic Acid Binding", also up-regulated in both IAC and IBCA (Fig. 7). Additionally, it was observed that "Lignin Catabolic Process", "Abscisic acid−activated signaling pathway", "Hydroquinone:oxygen oxidoreductase activity", "Monooxygenase activity", "Protein phosphatase inhibitor activity", "Apoplast" and "Extracellular region" were all exclusively up-regulated in both IAC and IBCA (Fig. 7).

The GO terms "Proteolysis", "Systemic acquired resistance", "Oxidoreductase activity", "Serine−type peptidase activity", "Hydrolase activity, hydrolyzing o−glycosyl compounds" "Membrane" and "Integral component of plasma membrane" were down-regulated in all four

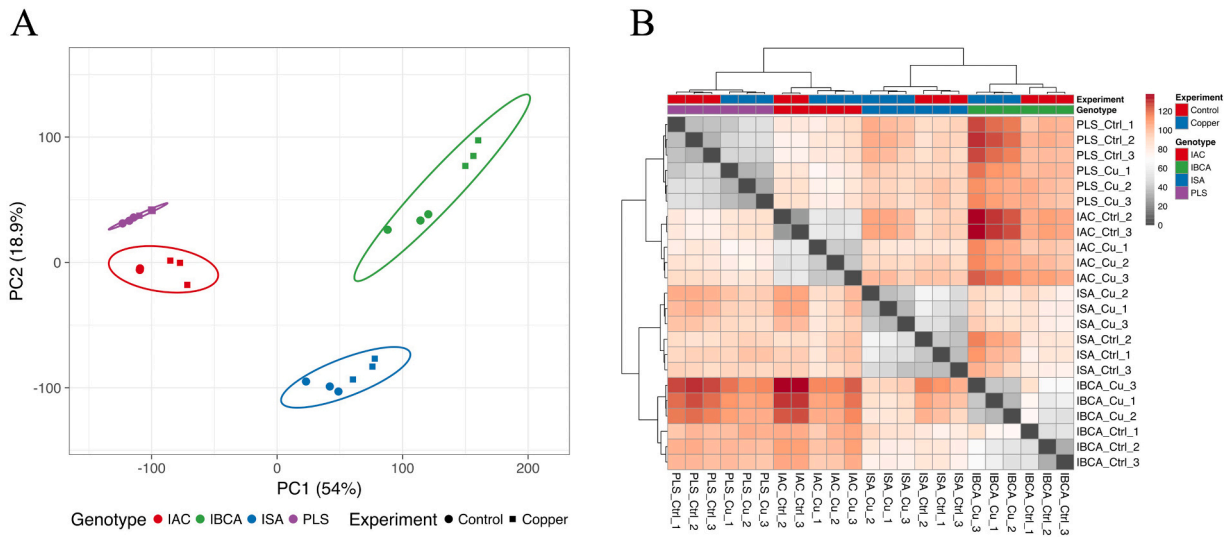

Fig. 5. Transcriptomic data quality analyses. (A) Principal component analysis of all libraries. (B) Hierarchical clustering analysis using the whole transcriptome data for each library.

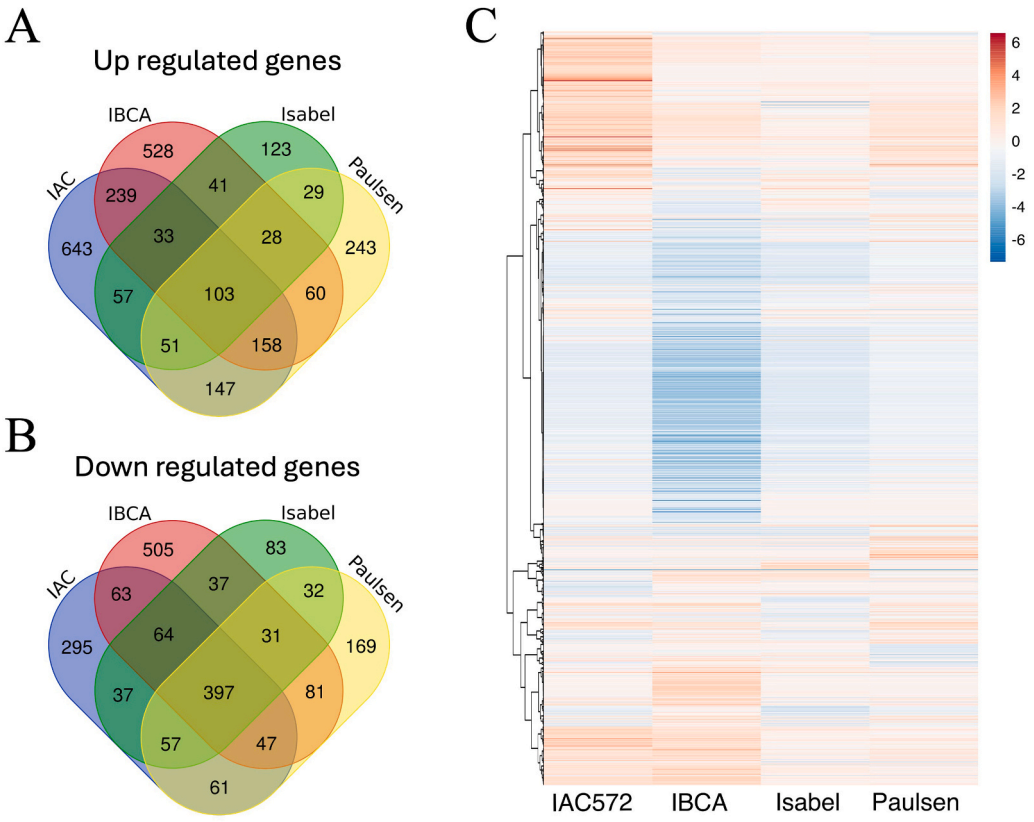

Fig. 6. General analyses of transcriptome data. (A) Venn diagrams of differentially up-regulated genes. (B) Venn diagrams of differentially down-regulated genes. (C) Heatmap for differentially expressed genes (DEG) comparing expression in roots of plants under control and Cu excess conditions, with log2 Fold Change ≥ 1.5 for up-regulated genes and ≤ 1.5 for down-regulated genes in at least one genotype.

genotypes (Fig. 7). We have also observed that all genotypes except Paulsen down-regulated five GO terms associated with "Localization and transport activity", as well as terms associated with "Binding" as "Manganese ion binding", "Iron ion binding" and "Heme Ion Binding" (Fig. 7). These observations suggest that binding metals and transport activity could be related to copper stress response. Because physiological data suggests that Paulsen might be more tolerant to Cu excess, it is possible to speculate/suggest that these GO terms are associated with Cu stress tolerance.

3.6. *The core Cu excess-responsive genes in grapevine roots*

Based on the previous results, we sought to identify what could constitute the main cluster of genes regulated under Cu excess in grapevine roots. To identify such genes, we searched for common differentially expressed genes to all four genotypes. We identified 103

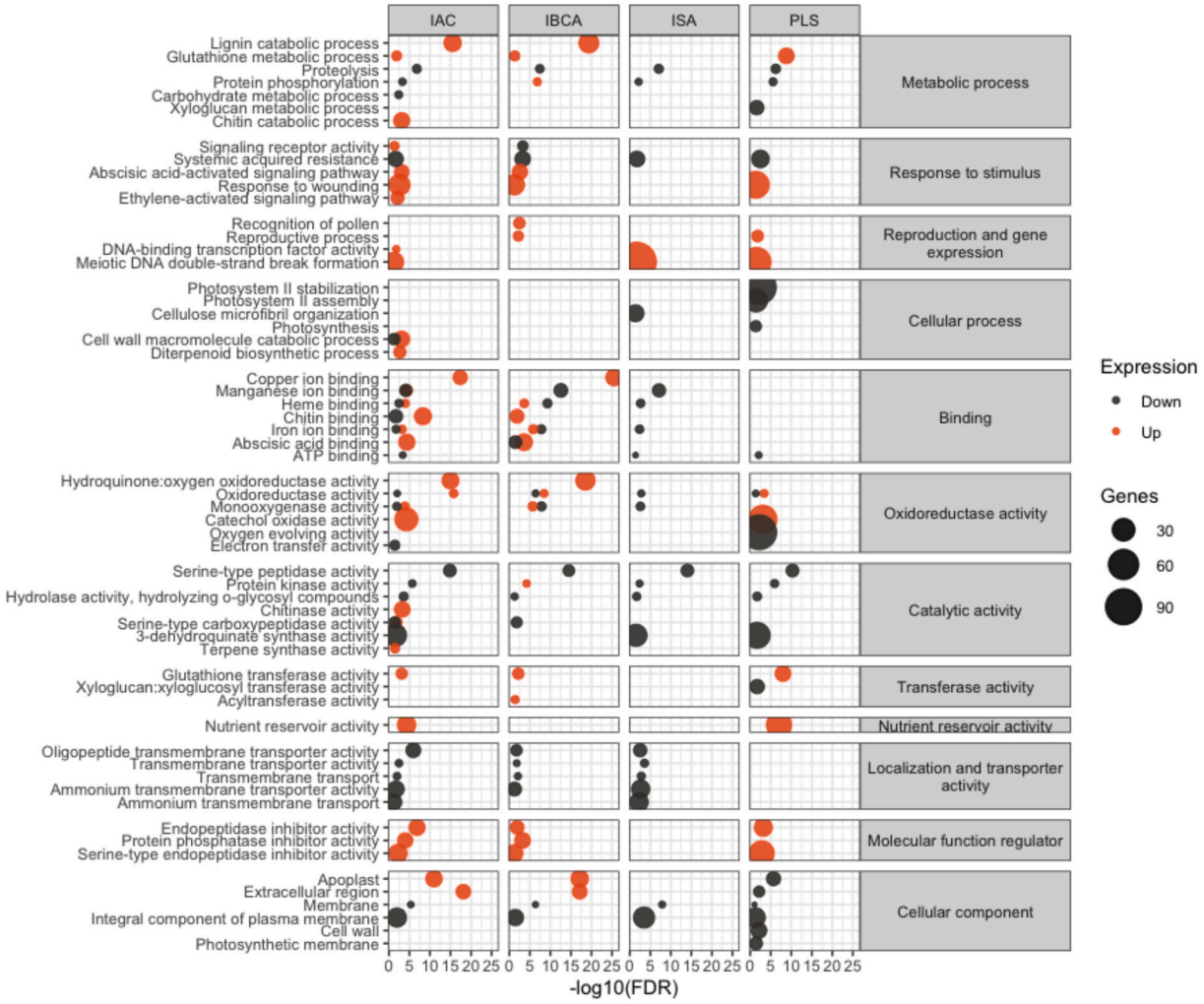

Fig. 7. Gene Ontology (GO) categories enriched in up- and down-regulated gene dataset for each genotype root transcriptomic data. General categories are shown on the right, genotypes at the top, and specific enriched GO categories on the left. Red circles (up-regulated) and black circles (down-regulated) represent significantly enriched categories in each genotype; circle size represent the number of genes in each enriched category.

genes that are up-regulated and 397 genes down-regulated in all four genotypes (Figs. 6A and 6B; Supplemental Table 1). The expression pattern analysis of all commonly regulated revealed that most genes were down-regulated, again with IBCA showing the strongest response compared to the other genotypes (Fig. 8A). We also selected a subset of genes based on their annotated function, focusing mainly on transporters and/or ionome-related gene function description based on similarity to *Arabidopsis thaliana* genes (Supplemental Table 2 and Supplemental Table 3). Thirteen (13) up-regulated and forty-two (42) down-regulated genes were selected as constituting the core responsive genes to Cu excess exposure (Fig. 8B).

Among up-regulated genes, we identified one HMA (Heavy Metal-Associated) transporter annotated as Heavy Metal ATPase 5 (Fig. 8B Supplemental Table 2). Genes from this family are known to be involved in Cu detoxification and transport to the vacuole or to the apoplast in other species [4,39,40]. We also found proteins containing Cu in their structure: a plantacyanin protein, a copper ion binding protein, a copper amine oxidase and a laccase genes (Fig. 8B; Supplemental Table 2). We found one iron/zinc-related transporter from the ZIP (Zinc-regulated/Iron-regulated Transporter Protein, which are involved in Fe, Zn and Mn transport [41,42], and two transcription factors of the bHLH family that are similar to bHLH038, known to regulate Fe deficiency responses in *A. thaliana* and to be involved in the crosstalk with Cu homeostasis [43]. Besides those, we found other transporters that could have a role in Cu excess responses (Fig. 8B, Supplemental Table 2). Altogether, the data suggest extensive regulation of the ionome in grapevine plants exposed to Cu excess.

In the group of down-regulated genes, we found a metallothionein, a protein family that is described as involved in metal binding and detoxification, including Cu (Fig. 8B, Supplemental Table 3) [44,45]. Other cupreproteins, such as Cu transport protein family and four cupredoxin superfamily proteins, were commonly down-regulated in all four genotypes (Fig. 8B, Supplemental Table 3), suggesting that these proteins might be involved in Cu excess detoxification or sequestration. We found extensive down-regulation of iron/zinc-related genes, such as a ZIP family gene [41], an iron reductase/oxidase gene of the FRO family, and a proton ATPase of the AHA family, which suggests down-regulation of Fe uptake mechanism [46,47]. Three genes from the YELLOW-STRIPE gene family were also down-regulated, as well as one Vacuolar Iron Transporter-Like (VIT) gene and two Cytochrome b561 ferric reductases (Fig. 8B, Supplemental Table 3). Therefore, it seems that Fe homeostasis is being largely affected by Cu excess.

Down-regulation of genes related to homeostasis of macro- and micro-nutrients were also observed. For example, we found a homolog of the SPX genes, known to regulate phosphorus deficiency [48], and three PHT (Phosphate Transporters) genes (Fig. 8B, Supplemental Table 3), suggesting that phosphorus uptake is also being perturbed by Cu excess. Nitrogen uptake also seems to be affected, since two nitrate

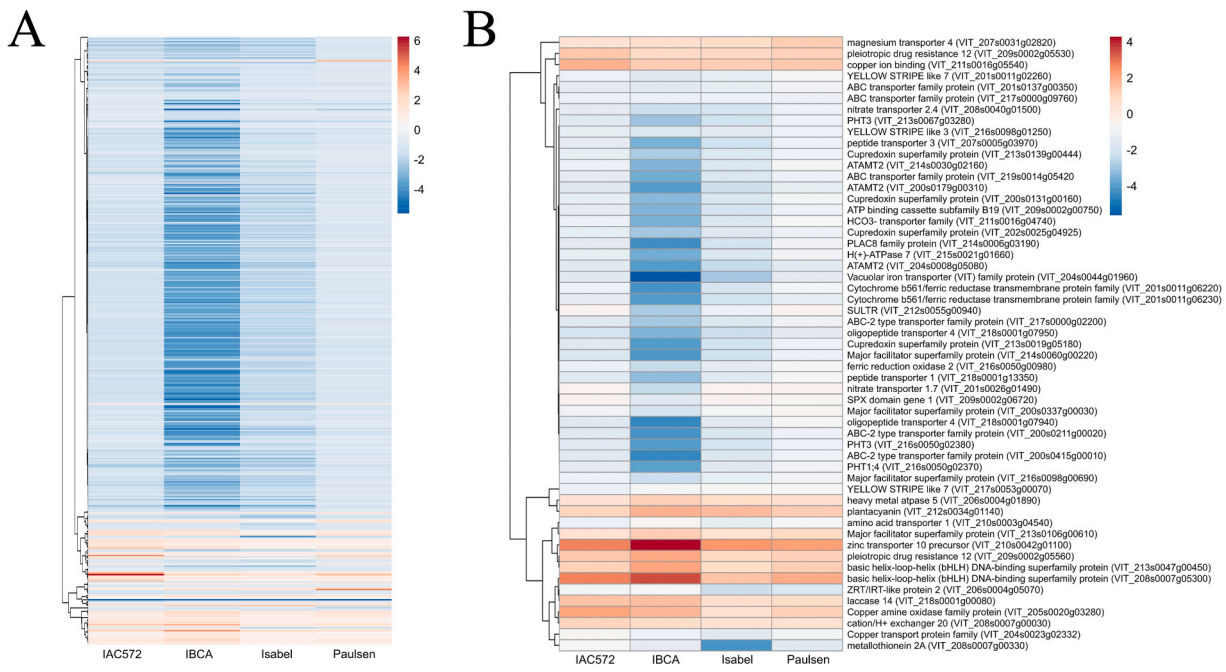

Fig. 8. Gene expression analyses of differentially expressed genes common to all four genotypes. (A) Heatmap for differentially expressed genes (DEGs) in all four genotypes comparing expression in roots of plants under control and Cu excess conditions, with log2 Fold Change $\geq$ 1.0 for up-regulated genes and $\leq$ 1.0 for down-regulated genes in at least one genotype. (B) Heatmap with selected genes from A.

and three ammonium transporters were down-regulated in all genotypes (Fig. 8B, Supplemental Table 3). A sulfate transporter and other transporters from Major Facilitator Superfamily and ABC family were found to be down-regulated consistently in all genotypes (Fig. 8B, Supplemental Table 3). Moreover, we found a protein of the PLAC8 family down-regulated, which might be involved in metal detoxification and xylem loading [49].

3.7. Differentially expressed genes in response to Cu stress when comparing IAC and IBCA rootstock genotypes

We also used the transcriptomic data to find candidate genes that could explain the distinct Cu partitioning strategies observed in IAC and IBCA compared to Isabel and Paulsen (Figs. 4A and 4B). We therefore sought to find genes commonly up- and down-regulated in IAC and IBCA

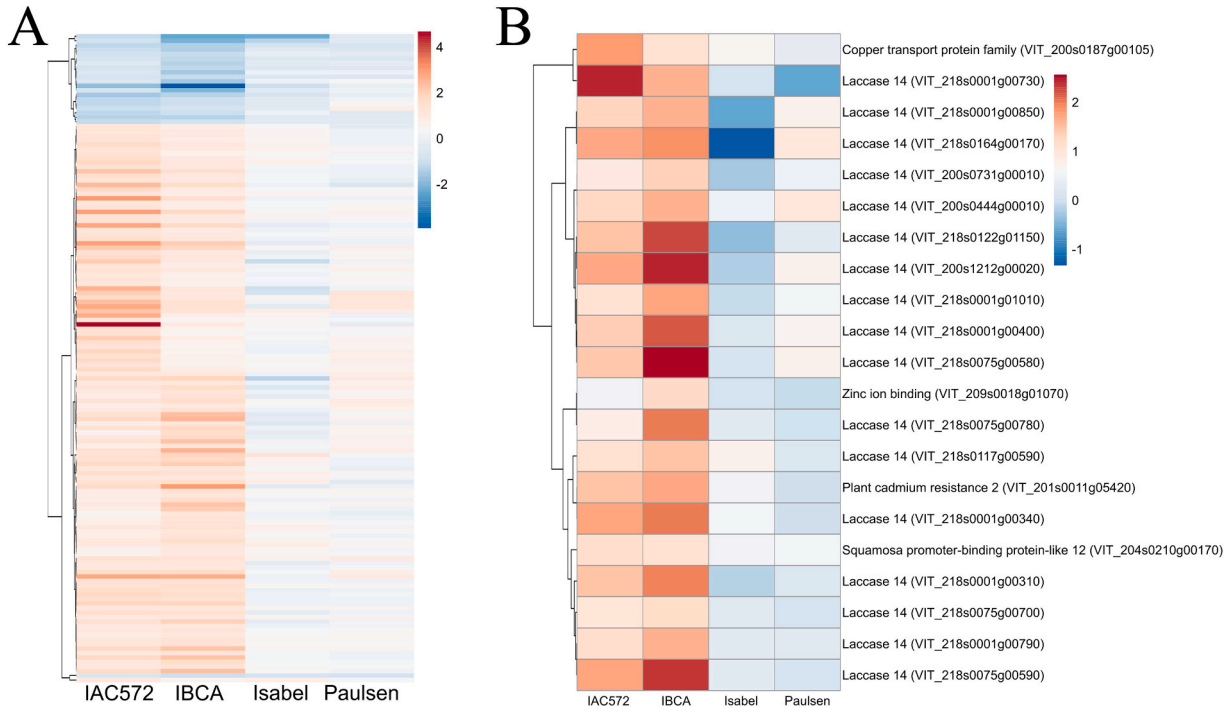

Fig. 9. Gene expression analyses of differentially expressed genes exclusive to IAC and IBCA genotypes. (A) Heatmap for differentially expressed genes (DEGs) in IAC and IBCA comparing expression in roots of plants under control and Cu excess conditions, with log2 Fold Change $\geq$ 1.0 for up-regulated genes and $\leq$ 1.0 for down-regulated genes in at least one genotype. (B) Heatmap with selected genes from A.

that were not differentially expressed in Isabel and Paulsen. We found 239 genes up-regulated, and 63 genes down-regulated only in IAC and IBCA (Figs. 6A and 6B, Fig. 9A). From these, we highlighted 21 up -regulated genes (Fig. 9B; Supplemental Table 4).

Strikingly, 17 genes annotated as laccases are up-regulated in both genotypes (Fig. 9B). Laccases are Cu oxidases/ Cu ion binding proteins which are poorly characterized but are likely to be down-regulated by Cu deficiency [1]. Therefore, the opposite regulation in Cu excess might be linked to their function in regulating Cu homeostasis. The large number of genes regulated in these two genotypes accounts at least partially for some of the GO categories that are enriched only in IAC and IBCA, such as "Copper Ion Binding", "Lignin Catabolic Process" and "Hydroquinone:oxygen oxidoreductase activity" (Fig. 7). One gene annotated as a COPT transporter is among the up-regulated [17] (Fig. 9B; Supplemental Table 4). Another interesting gene shares similarity with a well-known PLAC8 *A. thaliana* protein named Plant Cadmium Resistance 2 (PCR2), which is a known efflux excessive carrier of Cd and Zn into the rhizosphere and xylem [49]. Moreover, we also found a Zn ion binding protein (Fig. 9B; Supplemental Table 4). These genes might be linked to Cu partitioning in these two genotypes, although functional characterization would be needed to test this hypothesis.

3.8. Genes specifically regulated in Isabel

Isabel is the most Cu-sensitive rootstock in our experiments. Therefore, we analyzed which genes are regulated specifically in this genotype. There were 123 genes up-regulated and 83 down-regulated specifically in Isabel (Figs. 6A and 6B; Supplemental Table 1), of which we highlight the ones with the most pronounced fold-change (Fig. 10). Among the up-regulated genes, we identified one gene similar to auxin-responsive GH3 protein that in *A. thaliana* was described as involved in jasmonic acid conjugation with aminoacids [50] and, when overexpressed, results in decreased hypocotyl growth [51]; one Leucine-rich repeat protein kinase and one Leucine-rich repeat transmembrane protein receptor kinase, with the last sharing similarity to SCHENGEN3/-GASSHO1, which is necessary for the formation of the diffusional barrier at the endodermis, the Casparian Strip [52]; and a dirigent-like (DIR--Like) protein, which might be involved in lignin and cell wall synthesis and modification [53] (Fig. 10). Among the down-regulated genes, we found transporters such as Aquaporin NIP2.1 and an ammonium transporter of the AMT gene family [54]; a carboxylesterase that could be involved in strigolactones catabolism [55]; and one SAUR (SMALL AUXIN UPREGULATED RNA 4) (Fig. 10). Taken together, the data suggest that Isabel might specifically regulate distinct processes such diffusional barrier and phytormone signaling when exposed to Cu

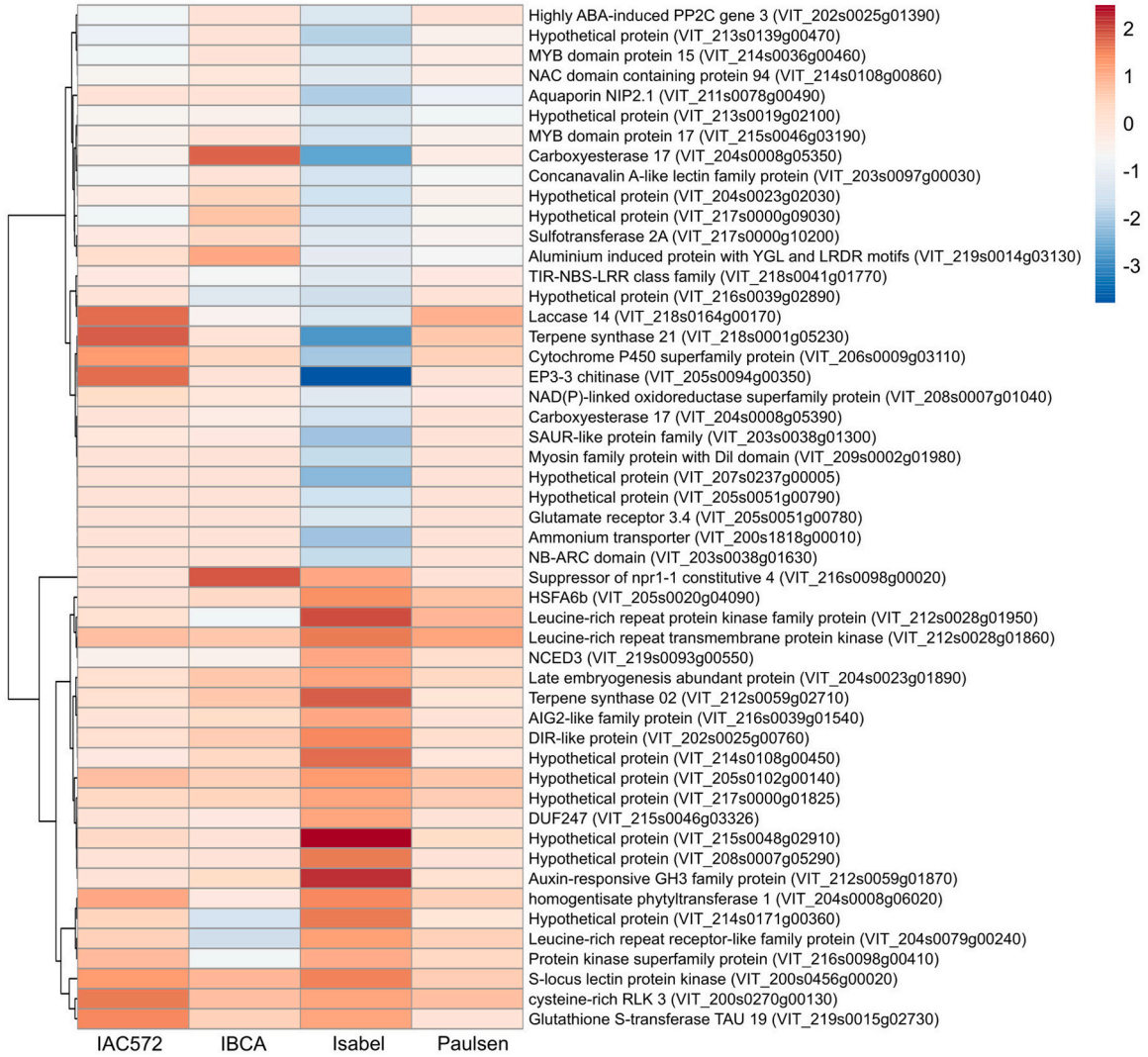

Fig. 10. Gene expression analyses of differentially expressed genes exclusive to Isabel genotype. Heatmap for differentially expressed genes (DEG) exclusive to Isabel.

excess. Whether that is related to Cu excess sensitivity will need further functional characterization.

4. Discussion

4.1. Genetic variation in tolerance and sensitivity to Cu excess in grapevine rootstocks

Cu stress has become a problem in vineyards, especially when there is a need to install new plants in old fields that have been historically grown using Cu-containing solutions to control diseases. Despite its importance, Cu homeostasis is understudied in grapevines, and little is known about Cu uptake and distribution mechanisms, as well as how plants respond to Cu stress [1,5,6]. In addition, we have only a few works available attempting to identify genetic variation in Cu responses in roots of grapevine plants, and yet we do not know which genes might be involved in such observed differences [25]. Moreover, it is important to note that Cu excess can affect plants both when a Cu solution is applied directly to leaves and when plants grow in soils containing high concentration of Cu. Responses are expected to be different, and since they are mostly impinged on scions and rootstocks, respectively, they can be considered two distinct Cu toxicity stresses that overlap. In this work, we focused on how the roots transcriptome of four rootstock genotypes respond to Cu stress. Importantly, we found that roots and shoots both accumulate higher Cu concentrations when exposed to Cu stress; however, concentrations in roots were similar in all four genotypes, while concentration in shoots were significantly higher in shoots of IAC and IBCA (Figs. 4A and 4B). Our transcriptome analyses performed on root tissues of rootstocks contribute to understand how roots might be regulating root uptake as well as root to shoot nutrient translocation.

Two previous studies provided molecular details on how grapevine plants respond to Cu excess stress [27,28]. Leng et al. [27] applied 100 μM Cu to leaves of a hybrid cultivar (*V. vinifera* × *V. labrusca*), and analyzed changes in the transcriptome after 24 h of treatment. Xia et al. [28] screened 302 cultivars for Cu tolerance, identified two contrasting hybrid cultivars (*V. vinifera* × *V. labruscana*) and compared the transcriptome changes after 48 h of 10 mM Cu treatment to leaves. Although these authors used very different concentrations, both focused on short-term leaf transcriptional responses, which largely differ from our root-focused, longer-term experiment. Moreover, these two earlier studies used scion cultivars, whereas we analyzed rootstock genotypes, except for Isabel, which despite eventually used directly rooted in soil, it is more commonly used as a scion [56].

In accordance with previous results, we identified Isabel as the most susceptible genotype to Cu excess [5]. The genotype showed reduction in root growth and decreased ETR (Figs. 2 and 3), suggesting Cu excess affects both root growth and photosynthesis. Since Isabel is not a common rootstock genotype, it was likely not selected for root traits, which may explain increased sensitivity. However, it is important to highlight that clear contrasting tolerant and sensitive genotypes were not observed in our experiments, as all genotypes had their root dry weight affected (Fig. 2A), increased Cu concentration in roots and shoots (Figs. 4A and 4B) and responded dramatically to Cu excess (Fig. 6 and Fig. 7). We do not point to one genotype as clearly more tolerant as well. Paulsen seems to be the best candidate, as it showed the least effect in root growth and no effect in ETR (Figs. 2 and 3). This is also in agreement with previous results [5]. However, IAC and IBCA, both similarly tolerant, grew more vigorously (Fig. 2), which may compensate when dealing with heterogeneous Cu distribution in the soil. Interestingly, the distinction between IAC/IBCA and Isabel/Paulsen regarding Cu partitioning, with the former genotypes translocating more Cu to shoots compared to the later (Figs. 4A and 4B), does not seem to be related to Cu tolerance. Therefore, these results suggest that *Vitis* sp. might have varying mechanisms of Cu tolerance depending on genotype, such as exclusion and tissue-based tolerance, similar as observed for Fe excess [57,58].

4.2. Effects of copper excess on the ionome

The ionome, defined as the inorganic composition of an organism, is known to be integrated, as changes in concentration of one element may affect others [59,60]. Therefore, it is expected that Cu excess may affect the homeostasis of other elements. We found evidence of that using measurements of elemental concentration (Fig. 4), which show ionomic perturbations in plants exposed to high Cu; and in the root transcriptomic data, where grapevine genes with similarity to *A. thaliana* genes known to be involved in uptake or regulatory networks of nutrients were found as differentially regulated by Cu excess.

Interaction of Cu and Fe homeostasis has been explored in several studies [1,25,61–64]. Under Cu deficiency, Cu economy response includes substitution of Cu-containing superoxide dismutases (Cu/Zn SODs, or CSDs), which are down-regulated while Fe-SODs (FSDs) are up-regulated [3]. This mechanism is well described in *A. thaliana* and seems to be conserved in *Vitis* sp. [65]. Two *Vitis* sp. CSDs up-regulated by Cu excess in both roots and shoots are post-transcriptionally down-regulated by *VvmIR398*, while FSD is transcriptionally down-regulated [65,66]. Interestingly, we did not find extensive SODs regulated in our transcriptome data. Only IBCA showed CSD DEGs, with two genes being up-regulated and one down-regulated, while no FSD genes were regulated (Supplemental Table 1). IBCA was the only genotype showing changes in Fe concentration, which increased in shoots under Cu excess (Fig. 4H).

Still, putative Fe homeostasis-related genes were regulated in our transcriptome data. Two genes with similarity with AtbHLH038, a known Fe deficiency responsive transcription factor, were up-regulated in all four genotypes Supplemental Table 2. AtbHLH038 forms a homodimer with other bHLH proteins and induces the Fe uptake genes *AtIRT1* and *AtFRO2* in *A. thaliana* roots [67]. Interestingly, it was recently shown that AtbHLH038 controls the uptake of Cu, which is induced under Fe deficiency conditions. Up-regulation of Cu^{+} uptake transporter AtCOPT2 and Cu^{2+} planta membrane reductases AtFRO4 and AtFRO5 are dependent on bHLH transcription factors when plants are in low Fe conditions [43]. Therefore, it is possible that bHLH proteins also control aspects of the crosstalk of Fe and Cu homeostasis in *Vitis* sp., although the transcriptional up-regulation does not suggest the same mechanism found in *A. thaliana*.

Besides that, one putative Fe/Zn/Mn transporter from the ZIP family was up-regulated in all genotypes, whereas transporters from ZIP, YSL and VIT were found to be down-regulated (Fig. 8B Supplemental Table 2 and 3). One ferric-chelate reductase/oxidase (FRO) and two other reductases were also down-regulated, together with an H^{+}-ATPase from the AHA family (Fig. 8B Supplemental Table 2 and 3). Both Fe and Cu need to be reduced before uptake, and proton-pumping is a common mechanism to reduce rhizospheric pH and change elemental availability [1,7,61]. These genes might be involved in rewiring Fe homeostasis in grapevine roots exposed to Cu stress. Further work will be necessary to uncover mechanisms involved in such crosstalk.

We also found down-regulated genes that share similarity with proteins that transport nitrate, ammonium, sulfate and phosphorus, again suggesting extensive effects on the ionome. However, that did not necessarily affect concentration of elements, since most changes in elemental distribution were not the same in all genotypes (Fig. 4). Interestingly, we observed that Ca concentrations decreased in roots of all four genotypes under Cu excess (Fig. 4E). Calcium ions are particularly abundant in pectins, which are part of the cell wall matrix. Pectins can have variable degree of methyl esterification, and unmethylated residues have negative charges that can interact with positively charged ions. Changes in cell wall composition have been linked to metal overload responses [3,68]. There is evidence that Cu excess hypertolerant species might exclude Cu by decreasing the total pectin and increasing the level of pectin methylation, which decreases binding sites for cations

[69,70]. Therefore, it is possible that Cu is replacing Ca in the pectin fraction under Cu excess.

4.3. Differences in copper partitioning between IAC/IBCA and Isabel/Paulsen

Grapevine plants exposed to Cu excess accumulate Cu in both roots and shoots. Roots accumulate to a much larger extent. However, we observed that IAC and IBCA had higher shoot Cu concentration under Cu excess compared to Isabel and Paulsen (Fig. 4A and B), suggesting they actively translocate more Cu from roots to shoots. Changes in metal partitioning by roots can be regulated by root-expressed proteins, which explain natural variation by changes in expression levels or transporter activity [59,71]. Interestingly, both IAC and IBCA also increased P concentration in shoots, whereas Isabel and Paulsen did not, while all four genotypes showed similar P concentrations in roots (Fig. 4C and D). These data suggest that P accumulation might be linked to higher Cu in shoots. In our root transcriptome data we found one transcript with similarity to SPX-family protein and three with PHT transporters among the down-regulated genes for all four genotypes (Fig. 8B Supplemental Table 3). SPX proteins are known to control P uptake at multiple levels, whereas PHT are phosphate uptake transporters, and both function in systemic P starvation response [46,72], suggesting that P homeostasis is being affected by Cu excess. Among the genes down-regulated only in IAC and IBCA, we identified a Purple Acid Phosphatase (PAP) and a gene with similarity to AtSPDT transporter, both down regulated at low fold change (Supplemental Table 1). PAP are important for P mobilization from the soil, and AtSPDT is involved in xylem to phloem P transfer, increasing P distribution to developing shoot tissues [73]. Therefore, IAC and IBCA shoots might be signaling high P to roots, which down-regulate P uptake machinery. Moreover, it is known that P and Fe homeostasis have antagonistic crosstalk [74], and down-regulation of P uptake could be linked to changes in Fe uptake.

We also found striking differences in IAC and IBCA root transcriptional responses regarding Cu-related genes (Fig. 9, Supplemental Table 4). We observed up-regulation of 17 laccase genes. Laccases are poorly characterized proteins containing Cu in their structure and are involved in oxidation of monolignol monomers for lignin synthesis, an activity that depends on the presence of Cu ions [3]. Laccase genes are down-regulated under Cu deprivation as part of the Cu economy response, which is partially explained by miRNAs induced by low Cu that post transcriptionally decrease laccase mRNA expression [61,75]. Under high Cu treatment, laccases are up-regulated in roots, and increase lignin content [76,77], which could be linked to metal tolerance as already observed for Fe [57].

Another interesting gene specifically up-regulated in IAC and IBCA was transcript with similarity to PCR2 (PLANT CADMIUM RESISTANCE 2) [49] (Fig. 9, Supplemental Table 4). PCR2 is a Zn and Cd efflux transporter that detoxifies Cd from the roots to the rhizosphere, as well as into the xylem. Although there is no information whether these proteins can transport Cu, it is possible that *Vitis* sp. PCR2-like protein is involved in Cu xylem loading, explaining increased Cu concentration in shoots of these two genotypes (Fig. 4B). We also identified one Copper transport protein from the HIPP gene family [78] which may functionally to be involved in Cu partitioning (Fig. 9, Supplemental Table 4). Members of this gene family were shown to be involved in Cu homeostasis in rice [79]. Transcriptional regulation of these genes in IAC and IBCA suggest that, as these two genotypes have higher Cu concentration in shoots compared to Isabel and Paulsen, up-regulation is controlled systemically, with high Cu in shoots signaling to roots to induce a particular set of genes. It will be interesting to understand how distinct partitioning of Cu affects tolerance in future experiments.

4.4. Candidate genes for engineering copper tolerance

Our root transcriptome dataset can be used to suggest genes that may be functionally characterized in detail and used in biotechnological approaches. PCR2-like and laccases are good examples (see above; Fig. 9, Supplemental Table 4, and Section 3.6). The most promising example is an HMA transporter which was up-regulated in all four genotypes, VIT_206s0004g01890 Supplemental Table 2. HMA proteins are known to be localized in the plasma membrane or tonoplast and to efflux either Zn/Cd or Cu from the cytosol or into the vacuole, but not both [59]. The gene identified in our data is similar to *A. thaliana AtHMA5*, which is involved in Cu detoxification [80]. However, AtHMA5, does not have a clearly determined subcellular localization [1]. In rice, OsHMA5 is at the plasma membrane and is involved in Cu loading from the symplast into the xylem [81]. Interestingly, rice OsHMA4 is another Cu efflux transporter localized in the vacuole, and is involved in Cu detoxification in rice roots [39]. Both genes are induced under Cu excess [81]. Therefore, it is possible that the *Vitis* sp. homologous protein might function either as a plasma membrane or as a tonoplast transporter. It would be interesting to understand the exact function of this protein, and whether manipulation of its expression could increase Cu tolerance in grapevine plants.

5. Conclusion

Here we provided the first dataset of Cu excess-regulated genes in roots of grapevine plants. We identified a cluster of genes regulated by Cu excess that may be involved in differential Cu partitioning between roots and shoots, and new candidate genes that might be explored for biotechnological approaches to increase Cu tolerance in grapevine.

Environmental Implication

Copper-based mixtures are commonly used in vineyards to control fungal diseases. However, excessive copper accumulates in the soil, becoming toxic. When vines need to be replaced, toxicity impairs plant establishment. Therefore, copper is a common contaminant in vineyard soils, and is important to identify rootstocks that can withstand copper excess. We provide physiological and transcriptional analyses of how four rootstock genotypes respond to copper excess, and how that might be related to copper tolerance and copper partitioning, and identify candidate genes that can be used in biotechnological applications.

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CRediT authorship contribution statement

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Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **George Wellington Bastos de Melo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Andriele Wairich:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Victor Hugo Rolla Fiorentini:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation.

Declaration of Competing Interest

All authors declare to have no competing interests, both financial and non-financial.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.136301.

Data availability

All data that is not publicly available can be shared upon request to the authors.

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