ISSN: 2639-7455

Research Article

Earth & Environmental Science Research & Reviews

# Novel Transcriptome Study Provides New Insights on Date Palm Response to Copper Stress

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**Submitted:** 2026, Mar 10; **Accepted:** 2026, Apr 01; **Published:** 2026, Apr 13

**Citation:** Chaâbene, Z., Rekik, I. H., Elleuch, A., Mejdoub, H., Vandenbulcke, F. (2026). Novel Transcriptome Study Provides New Insights on Date Palm Response to Copper Stress. *Earth & Envi Scie Res & Rev, 9*(1), 01-12.

## Highlights

- Copper exposed homogeneous tissus of deglet nour variety are used to reveal plant response to metal stress.
- Bio informatics tools combined with molecular analysis reveal key Cu-responsive genes.
- Transcriptomic and Molecular response to copper stress in date palm.

## Abstract

*To disclose the transcriptomic basis of date palm Phoenix dactylifera exposed to copper, massive reprogramming of gene expression, with differentially expressed genes identified in cv Deglet Nour cDNA library was predicted using GeneMANIA program. Cu-transcription factors (TFs) were well-characterized including SQUAMOSA promoter binding protein-like (SPL). Co-regulation between genes of the same family like fsd (coding FeSOD) and of different families, fsd and csd (Cu/Zn SOD) were predicted. Other Co-expression links between Cu-transporters responsible for metal uptake such as copt (for COPper Transporter) or for metal transport between vacuole and cytoplasm like Nramp (for natural resistance associated macrophage proteins) or for Cu-efflux to the vacuole like abcc (ATP-Binding Cassette) were predicted. Monitoring of selected candidate genes expressions was performed in in vitro generated genetically homogeneous tissues: explant of Deglet Nour, exposed to Cu stress. Pdpcs1, Pdmt3, Pdabcc, Pdhma2, Pdmate5 and PdNramp6 were unregulated significantly by metal concentrations and time exposure except for Pdpcs1. This later gene did not decrease by increasing metal stress during experience showing its crucial involvement in metal tolerance. However, for the other genes amounts of transcript levels significantly decreased by increasing metal and exposure time which induced signs of alteration in explants.*

**Keywords:** Cu-Transporters, Cu-Responsive Genes, Genemania, Qpcr, Transcriptome Analysis, Venn Diagram.

## 1. Introduction

The socio-economic importance of the date palm *Phoenix dactylifera L.* in arid and semi-arid regions is due to its ecological capabilities. It exhibits high capacity to survive under extremophile environment such as drought and salinity [1,2]. Furthermore, with the long taproot of the Arecaceae family, date palm is used as a potential absorbent of unwanted materials (dyes, and phenolic compounds…) from wastewater [3]. It may also tolerate and accumulate heavy metals (HMs;) [4]. Thus, the plant is of great interest for ecological and ecotoxicological studies focusing on molecular mechanisms allowing to cope with harsh conditions [5]. Date palm seeds of Deglet Nour variety, exhibit high levels

of tolerance and accumulation of cadmium (Cd); copper (Cu) and chromium (Cr;) [4,6,7]. However, being a cross-pollinating species, date palm maintains genetic variability, differences in phenotypic characters between cultivars, and high seeds heterogeneity [8]. This genetic diversity has important consequences on the long-term viability of populations and functioning of ecosystems and complicates the identification of the underlying mechanisms involved in detoxification of HMs.

The use of *in vitro* culture of plant explant to explore molecular genetics has opened new avenues in plant study and improvement [9]. Tissue culture is a powerful tool that gives physiological information about plant cells behavior under stress conditions [10]. It consists of a collection of experimental procedures for aseptic culture of isolated plant callus on nutrient media under controlled environmental conditions. Growth and morphogenesis of explant are greatly influenced by the composition of the culture medium, which has often been modified according to purposes. For instance, cell lines tolerant to elevated levels of salt and elements concentrations such as aluminum have been studied [11].

Being a redox-active transition microelement, copper (Cu) has many functions for plant growth and development [12]. As a cofactor for many enzymes in the processes of respiration, electrons transport and photosynthesis [13]. It has been shown that optimum Cu concentrations in the medium enhance morphogenetic potential of explants and usually Cu have positive effects on development of explants of many *in vitro* cultivated species [14]. However, as a redox metal, copper can also be toxic and may lead to various disorders in metals uptake leading to explant growth troubles.

Understanding the transcriptomic basis of date palm cv Deglet Nour under Cu stress is a fundamental challenge. Thus, with the rapid progress of transcriptome RNA sequencing (RNA-seq), the new generation sequencing technologies produce large amounts of sequence data [15]. It made new possibilities for creating genomic resources with reduced cost and without having a reference genome. A first analysis of the transcriptome of *P. dactylifera* L. cv Deglet Nour using the Illumina GA IIx platform as well as the main transcriptional pathways associated with resistance to Cd stress have been very recently published [5]. However, there is still no investigation of the transcriptional profiles of the date palm under Cu stress.

To fully exploit the potential of RNA-seq data, tools for *in silico* transcriptome analysis is necessary. In this study we aim to

i. Identify the key regulatory network(s) of transcriptome of the plant subjected to Cu stress,
ii. Characterize and investigate functional categories of responsive genes to Cu stress by mean of current Gene Ontology annotations, and
iii. Validate or invalidate the *in silico* findings by monitoring the expression of candidate-genes using qPCR in vitro plants exposed to copper stress.

## 2. Materials and Methods

### 2.1. Copper Related Genes Interaction Network

The first cDNA library construction and transcriptome sequencing of date palm cv Deglet Nour was obtained by Rekik et al. [6]. In summary, authors used highly advanced techniques for library sequencing (Illumina GA IIxplatform), De novo transcriptome assembly using RNA-seq program and annotation based on similarity with known genes from NCBI using BLASTx and BLASTn algorithms. The unigenes were aligned by BLASTx search against NCBI protein databases including non-redundant sequences database (Nr), KEGG and Swiss-Prot. The best alignment results were used to predict the coding DNA sequences. The complex biological behavior of the gene was analyzed by pathways annotation based on the KEGG database. Functional annotation was conducted using GO terms that were analyzed using the Blast2GO software. Thus, based on this previously constructed cDNA library of the Deglet Nour variety, the interaction network of genes involved in copper detoxification mechanisms was predicted by using selected corresponding functionally similar genes from Arabidopsis datasets, downloaded by GeneMANIA version 2.1 and visualized in Cytoscape [16-18]. Interest was given to Cu-transporter genes identified and functionally predicted as described above.

### 2.2. Comparative Analysis of Gene Expression Profiling of Date Palm Exposed to Different Metal Stress Using Orthovenn Software

Transcripts of the date palm plant submitted to two types of metallic stress; Cu or Cd, were translated and used for comparisons of orthologous clusters with the OrthoVenn software [19]. Using previous analysis of Deglet Nour cDNA banq exposed to Cd stress, the software OrthoVenn was used as default parameters for clustering analysis (threshold: $<e^{-10}$) [6].

### 2.3. In Vitro Experimentation, Genes Identification and QPCR Measurement

Isolated explants of date palm cultivar Deglet Nour were obtained from the laboratory of vegetable biotechnology applied on the amelioration of culture, university of Sfax, Tunisia. Explants were transferred under sterile conditions into the Murashige and Skoog (MS; 1962) medium supplemented with different concentrations of CuSO4 (0.02, 0.2 and 2 mM) and incubated in 16h photoperiod at 24 °C during 60 days of exposure. Date shoot clusters of 20, 40 and 60 days of development were used for total RNAs extraction. Interest was given to genes encoding phytochelatin synthase type-1 (*Pdpcs1*), metallothionein type-3 (*Pdmt3*) ABC transporter C family member 2 like (*Pdabcc*), ATPase (*Pdhma2*), Multi-antimicrobial extrusion protein MATE type 5 (*Pdmate5*), and natural resistance-associated macrophage protein 6-like (*PdNramp6*). Using candidate gene specific primers designed by Primer3Plus on line software (Rozen and Skaletsky 2000; http://frodo.wi.mit.edu/) and verified using Net Primer and Beacon Designer programs (Table 1), quantitative real-time polymerase chain reaction (qPCR) amplification were performed according to Brulle et al. [20].

| Name | Amplification length (bp) | qPCR specific Primer sequence (5'→3') | Length (bp) | Tm | GC% | PCR Efficiency ±SD |
|---|---|---|---|---|---|---|
| *Pdabcc* | 104 | F : GATTTGCTTCCAGGAGGTGA | 20 | 60.2 | 55.0 | 1.97±0.01 |
| | | R : TCCGAATATACTGCCCTTGC | 20 | 60.1 | 50.0 | |
| *Pdhma2* | 162 | F : CATGCAACCACAAGCAAGAC | 20 | 60.3 | 55.0 | 2±0.01 |
| | | R : TCGACGTTCAAACATAGAGGAG | 20 | 59.4 | 54.5 | |
| *Pdmate5* | 103 | F : CTTGCTCTCAAAGCGAAAGG | 20 | 60.3 | 50.0 | 1.98±0.05 |
| | | R : TTCCGTCGCCAACACATAG | 19 | 60.7 | 52.6 | |
| *PdNramp6* | 154 | F : CGGAAGCTCTGGTCTCACA | 19 | 60.1 | 57.9 | 1.95±0.04 |
| | | R : CGACAGCAACTGGATCAGAA | 20 | 60 | 50.0 | |

**Table 1: Qpcr Primer Sequences of Candidate Genes Used in this Study**

The expression levels and the relative fold expression (RFE) were determined according to previously described procedures [21]. The geometric mean of the 3 most stable reference genes in control and Cd-stressful condition identified by Chaâbene et al. [5] was used to calculate expression of target gene levels according to Brulle et al. [20]. Absolute quantification of genes expression levels are in log2. The results were expressed as "Induction factor" were obtained by scaling the absolute quantification of genes in stressful conditions with those in control conditions.

### 2.4. Statistical Analysis

The relative expression ratios between control and treated plants were transformed and subjected to a one-way and two-ways ANOVA. Student test has been used to analyze significance were $p<0.05$ was considered to be significant.

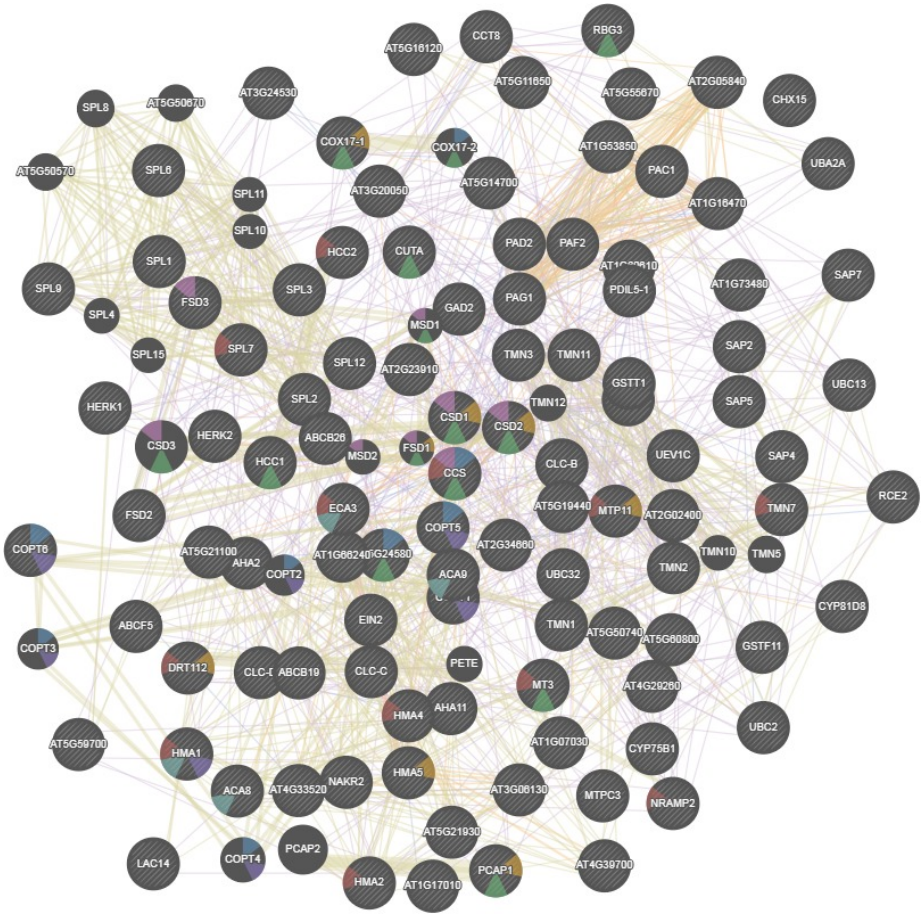
## 3. Results and Discussion

### 3.1. Cu-Signaling Genes Functional Partners' Prediction

To further disclose Cu-signaling gene's link predictions, we examined a subset of the inferred network related to A. thaliana copper-responsive genes available in NCBI. The high degree of conservation among organisms of most of the gene sequences helps for the draw of the diagram of the inferred copper regulatory network showed in figure 1.

# GeneMANIA report

Created on : 5 March 2019 13:24:38
Last database update : 13 March 2017 00:00:00
Application version : 3.6.0




## Networks

- Co-expression
- Predicted
- Shared protein domains
- Physical Interactions
- Co-localization

## Functions

- copper ion transport
- response to copper ion
- copper ion transmembrane transporter activity
- copper ion binding
- calcium ion transmembrane transporter activity
- metal ion homeostasis
- cellular response to oxidative stress

**Figure 1:** Copper-Controlled Regulatory Network in *P. Dactylifera* Cells Inferred By the Genemania

GeneMANIA recovered well-characterized Cu-transcription factors (TFs). They describe large classes of regulators that control gene expression at the transcriptional level. Among TFs, the SQUAMOSA promoter binding protein-like (SPL) is encoded by a large gene family in plants. Twelve classes of *spl* were found in the present cDNA library of *P. Dactylifera* (Figure 1). The *spl7*, which interacts with high number of gene sequences, is expressed under Cu stress in date palm. With a SBP domain (SQUAMOSA promoter binding protein domain), SPL7 is proved to be required for the expression of multiple microRNAs, including miR397, miR398, miR408 and miR857. It may, also, activates the transcription of multiple copper-responsive genes such as copper transporters, and copper chaperone (HCC; the first metallochaperone described in plant) [22]. In *Arabidopsis*, Yamasaki et al. proposed that SPL7

is a master regulator involved in copper-sensing and homeostasis. It was found to mediate the copper-dependent switching of SOD between Cu/Zn SOD and FeSOD controlled by *fsd1* (FeSOD). In figure 1, co-expression between *fsd2* and *fsd3* was shown. They respond to oxidative stress signals especially to ROS (reactive oxygen species) accumulation. The formation of hetero-complexes between FSD2 and FSD3 in chloroplast nucleosides enhanced transgenic Arabidopsis tolerance to oxidative stress induced by $Cu^{2+}$ ions [23]. The regulation of genes encoding chloroplast antioxidant enzymes is further improved by the expression of CuZnSOD encoding genes (*csd1* and *csd2*; Figure 1).

Gene's link predictions between *fsd 1* and *csd1* and 2 were shown in figure 1. The overexpression of *fsd2* and *fsd3* resulted in high csd2 transcription level in Arabidopsis [23]. A strong copper mediated link between *csd1, csd2* and plastocyanin expression was found [24]. As the plastocyanin is the most prominent Cu-binding protein in plants, that controlled the expression of *csd1* and *csd2*, evidence may be concluded of copper regulation pathways adopted by *P. dactylifera* plant cells. Additionally, Cu-insertion into CuZnSOD was controlled by copper chaperone for SOD (CCS). Direct link and co-expression between ccs and *csd* genes was predicted (Figure 1). These results proved previous funding which suggest that *csd1-3* were activated by *ccs* in Arabidopsis. On the other hand, intracellular copper distribution and its uptake through the plasma membrane are performed by Cu-transporters that contain GTAC binding sites for the transcription factor SPL7 in their promoter [25]. With high affinity copper uptake, six members of COPT (COPper Transporter) encoding genes has been identified in date palm similarly to *Arabidopsis* genome [26]. This CTR-like sequences of Cu transporters helps $Cu^{2+}$ enters eukaryotic cells [26]. Co-expression of *copt3, copt4* and *copt6* has been predicted in *P. dactylifera* (Figure 1). However, only, COPT1 and COPT5, have been characterized for their functions in Cu transport in rice [27]. Yet only *copt1* has been shown in figure 1. COPT1 has been characterized as high-affinity copper transport protein [28]. Furthermore, the transcript coding for COX (mitochondrial Cytochrome c Oxidase) have been validated as miR398 targets [29]. *Cox17* has been identified in *P. dactylifera* to be involved in copper tolerance. Since COX17 is a highly conserved protein, enzyme may mediate the delivery of Cu to mitochondria [30]. Besides, date palm copper metabolism requires other $Cu^{2+}$ chelaters. Not less than 32 Members of UBC genes including *ubc13* have been identified in the plant. Phytochelatin synthase type 1 (*Pdpcs1*) and Metallothionein type 3 (*Pdmt3*) have been present in figure 1 and have been previously identified in date palm cv Deglet Nour [31]. Gene's up-regulation under Cu-stress proved the involvement of *Pdmt3* and of *Pdpcs1* in copper detoxification mechanisms. Described as the third major copper homeostasis proteins, MTs may sequester until 12 copper ions and are involved in copper transport in the phloem. Likewise, suggested that *pcs* was the most active gene involved in copper regulation in *A. germinans leaves* [32].

From more than 30 members of *ubc* family identified in *P. Davtylifera*, gene coding UBC2/13/32 were predicted to be linked to each other and to *mmz* (AT5G19440) and are involved in copper mobilization in the plant. The UBC 13 catalyze non-canonical Lys63-linked ubiquitin chains and plays important roles in signal transduction among eukaryotes. However, MMZ3 play a role in DNA damage responses and error-free post-replicative DNA repair by participating in lysine-63-based poly-ubiquitination reactions [33]. The MMZ3 gene is known to interact with UBC35/36, while, in our plant model, *mmz3* were showed to co-expressed especially to *ubc32* [33]. It was demonstrated that MMZ3 could form di-ubiquitin and tri-ubiquitin chains in combination with UBC13A/UBC35. This interaction was predicted to be controlled at transcriptional level.

### 3.2. Cu-Transporter Genes Regulatory Network

Because Cu-transporters have primary importance in maintenance of physiological limits of Cu homeostasis in plant cells, figure 2 put the accent on the transporter gene's links in date palm exposed to metal stress. Different categories of transporters are identified in organisms. Important process of Cu-transport between chloroplast and cytoplasm is controlled by Heavy Metal ATPase (HMA) family of transporters [34]. There are necessary to maintain a concentration gradient for free Cu ions over the plasma membrane with a relatively low free Cu concentration in the cytosol [26]. Although its transmembrane metal binding site is different from that of Cu-transporting ATPase, HMA1 have been implicated in the reverse process. It can efflux nutrients out of the chloroplast. Gene coding for the third ATP-driven metal transporter in the chloroplast; *Pdhma1*, has been identified in the present cDNA library [26]. Besides, other members of *hma* family encoding *hma2/4/5/6* (*PAA1*) and 8 (*PAA2*) have been identified and are highly expressed (Figure 2). HMA2 to 4 were shown to be also involved in tolerance to excess zinc in *Arabidopsis* [35]. Whereas HMA5-8 described Cu-ATPases. The HMA5, primary expressed in *A. thaliana* in the plasma membrane, removes Cu from the cell to allow xylem loading in the roots and prevent cellular Cu overload. Functional links between *hma5* and *Nramp2* (for natural resistance associated macrophage proteins) were predicted describing co-expression relationship (Figure 2). The Nramp family described a group of divalent metal cation transporters which regulates nutrient transport between vacuole and cytoplasm. With EIN2 (Ethylene Insensitive 2) domain shared between Nramp2/3 and 5 (Figure 2), Nramps play a central role in ethylene signaling and may be involved in sensing metal, which could be related with copper requirement of the ethylene receptor. The Nramp proteins have been isolated and characterized from plant-metal hyperaccumulator like *A. halleri* and *T. japonicum*. In yeast, NRAMP1 was involved in iron uptake and was further participated in manganese transport in leaves of *Arabidopsis* [36].

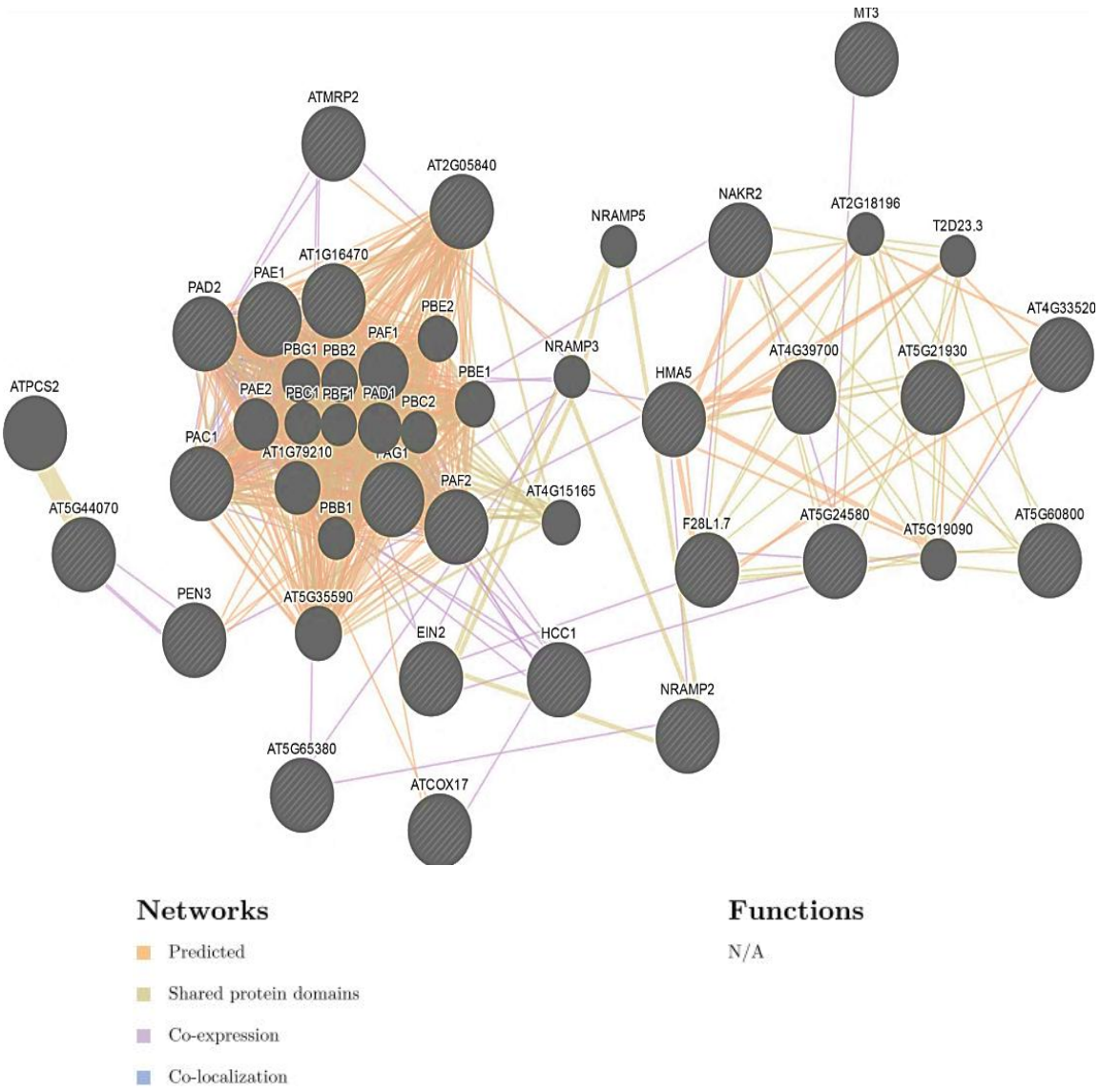



**Figure 2:** Copper-Transporters Genes Links in *p. Dactylifera* by the Genemania

Across membranes, membrane ATP-Binding Cassette (ABC) transporters are divided into two subfamilies (MRP and PDR) and are responsible for transporting various solutes such as heavy metal including $Cu^{2+}$ ions. As multidrug resistance-related proteins, the ABC, especially the subgroup ABCC, efflux heavy metal into the vacuole and may have key roles to sequester or exclude chelated forms of Cu or other toxic adducts [37]. In date palm cDNA library, co-expression between *abcc* and *Nramp3* was predicted (Figure 2).

No genes functional links have been shown before. However, co-expression has been shown only between abcc encoding genes of *A. Thaliana* which exhibit overlapping expression patterns due to duplicated regulatory DNA motifs in their promoters indicating its involvement in similar process. Also, AtPCS1 and AtABCC1 are shown to be vacuolar PC transporters exhibiting an increase in arsenic resistance in *A. thaliana* [38]. Like ABC, MATE family of transporters are important multidrug transporters superfamily. However, despite its importance to reduce ROS production in mitochondria, GeneMANIA program did not found implication of MATE (Multi antimicrobial extrusion protein) in Cu tolerance in date palm unlike what it was shown for Cu in the brown algae and for aluminuim (Al) in rice. According to Ritter et al, mate up-regulation is related to the secretion of Cu-conjugates [39]. While, these secondary active transporters, function as proton-dependent efflux transporters and were up-regulated under Cu stress playing important role in Cu-detoxification in grapevine [40].

### 3.3. Venn Diagram Generation from Cluster File of Cu and Cd Date Palm Responses

Gene expression patterns change in response to different toxic elements. More than 50 specific proteins were identified to be involved in date palm response to copper, while only 28 of Cd-

specific protein were found (Table 2). Comparison of inferred proteins among Deglet Nour responses to metals stress in OrthoVenn resolved 11 specific protein clusters of Cu stress including SPL protein. More than 45 % of Cu-protein clusters (5 specific protein clusters) shared with 9 specific clusters of Cd stress including ABC transporter protein family and MATE efflux family protein (Figure 3). The number of unique clusters was a little different among plant responses to metal stress (Figure 3). Six specific Cu-responded protein clusters have been found (Figure 3). This analysis suggested common terms were expressed in response to both metal stresses, while some metal transcriptome-specific response existed. The transcriptomic changes upon Cu or Cd stress have been widely investigated in several plant species, including Arabidopsis and barley [41].

| Species | Proteins | Clusters | Singletons |
|---|---|---|---|
| Copper | 51 | 11 | 26 |
| Cadmium | 28 | 9 | 7 |

**Table 2: Orthovenn Summary Report**

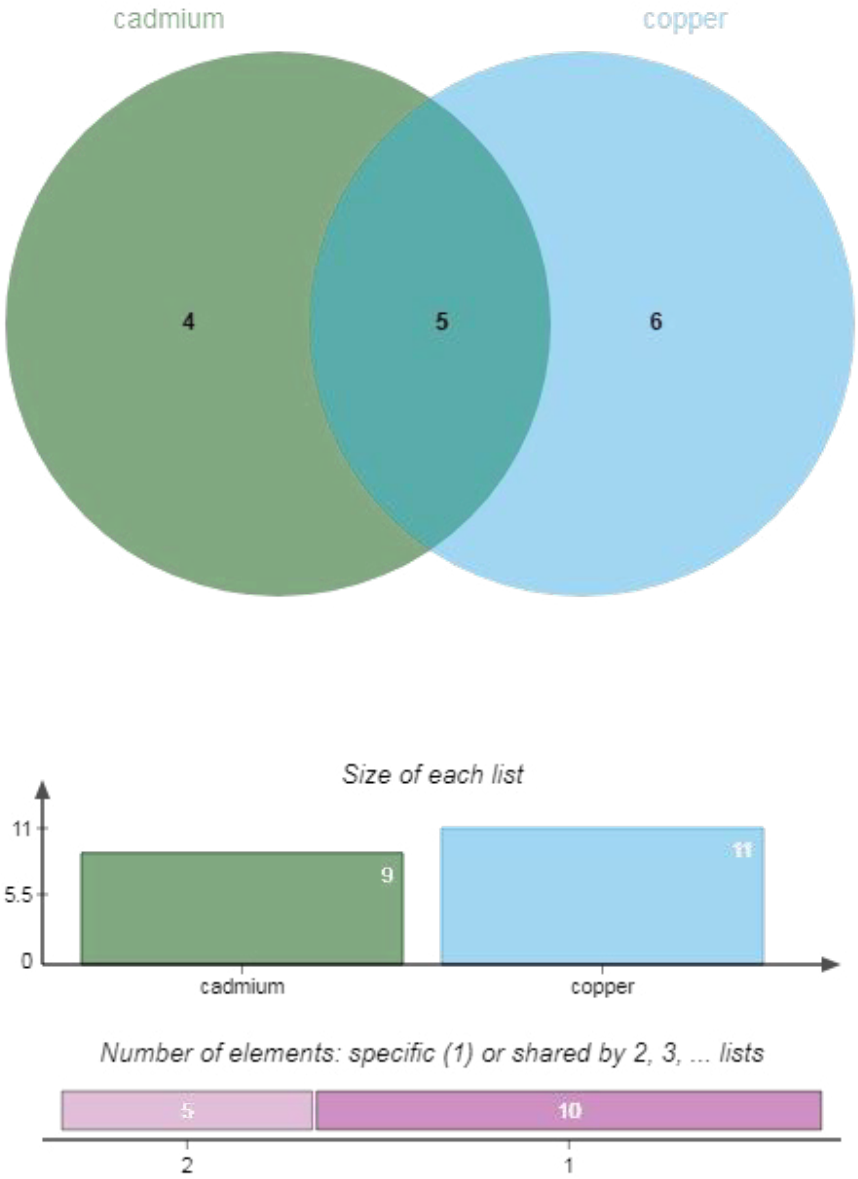



**Figure 3:** Orthovenn Producing Venn diagram Of Inferred Protein Clusters Involved in Date Palm Responses to Cu or Cd Stress. Total Numbers of Inferred Protein Clusters for Each Plant Response Are Given in the Bar Plot

### 3.4. Gene's Expression Responding to Cu Stress

To study the molecular mechanism underlying responding genes to Cu-stress, monitoring of gene's expression in *in vitro* generated explant of *P. dactylifera* cv Deglet Nour has been made along two months of metal exposure. *In vitro* generated Deglet Nour tissues allowed as avoiding the genetic diversity of *P. dactylifera* that may influence genes expression. qPCR expression analysis allowing to study multiple durations of the treatment of selected two chelators and four transporters' genes sensitive to Cu stress (Figure 4). Their expressions were altered during Cu-stress.

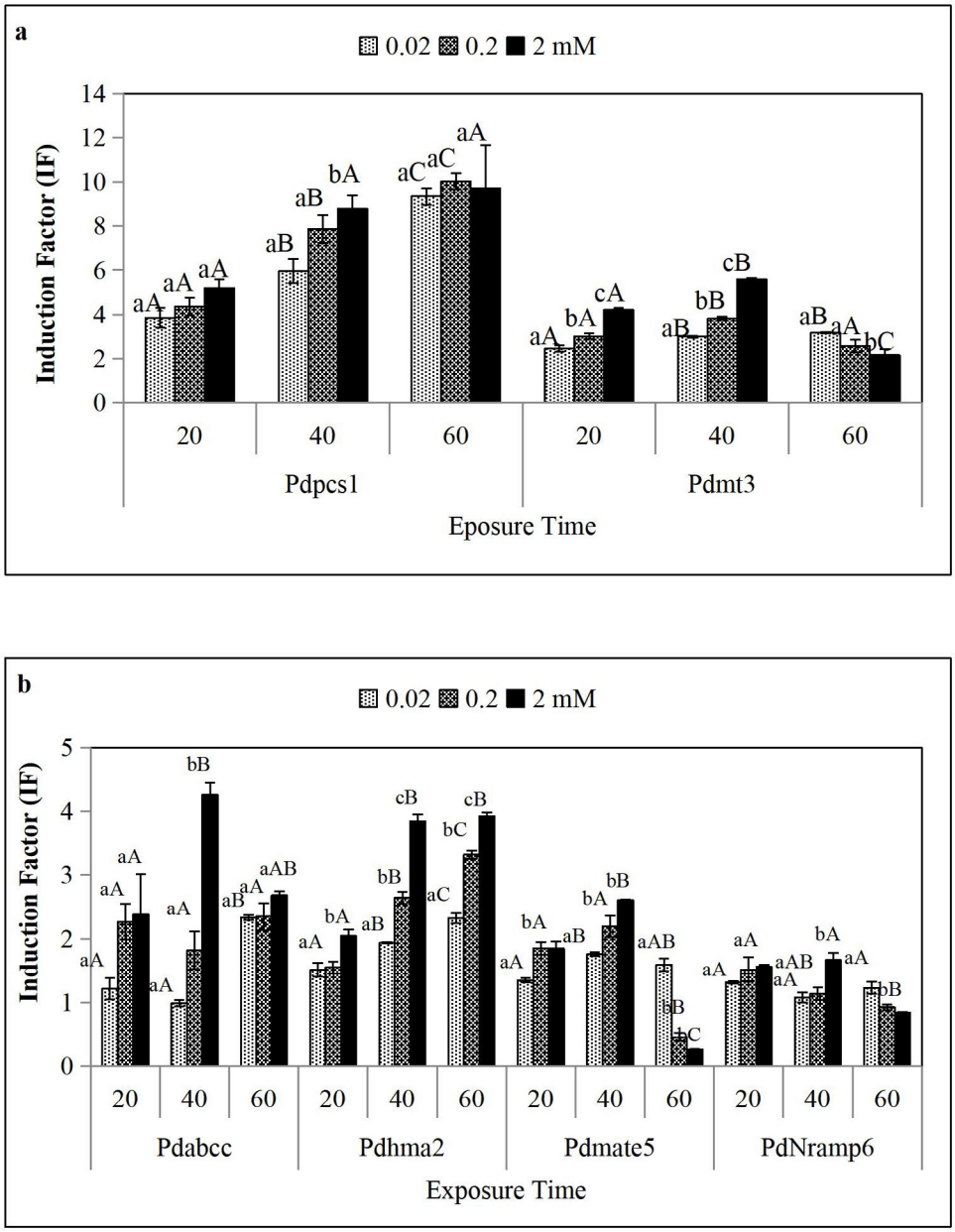



**Figure 4:** Transcription Factor of a Cu Chelators (*pdpcs1* and *pdmt3*) and b cu-transporters genes (*pdabbc, pdhma2, pdmate5 and pdnramp6*). Data presented are means ± standard error of three independent experiments. Differences between groups are shown as results of one-way anova post-hoc tukey's test; where small letters show differences between concentrations. Means not showing the same letter are statically different

Both metal concentration and time exposure influenced significantly ($p < 0.000$) gene expression in the vitroplant tissues except for *Pdpcs1* which showed the best mRNA production (Table 3). The gene induction factor continued to increase with increasing metal stress and time exposure. *Pdpcs1* reached its maximum (10.03) after 60d of containing 0.2 mM Cu in the MS medium (Figure 4). The gene overexpression in *E. coli* cells transformed with pGEX-5X-pcs ameliorated its growth under high temperature, NaCl (6 %

w/v), $CdCl_2$ (4mM), $CuCl_2$ (1 mM), and UV-B (10 min) exposure. It revealed its role in tolerance against different abiotic stressors.

Important non-significant enhancement of *Pdpcs1* has been observed since the first 20d (Figure 4).

| Two way ANOVA main effects | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Pdpcs1* | *Pdmt3* | *Pdabcc* | *Pdhma2* | *PdNramp* | *Pdmate5* |
| Cu | Concentration | 0.023* | 0.000* | 0.000* | 0.000* | 0.353 | 0.024* |
| | Exposure Time | 0.000* | 0.000* | 0.028* | 0.000* | 0.000* | 0.000* |
| | Concentration x Exposure time | 0.313 | 0.000* | 0.000* | 0.000* | 0.000* | 0.000* |

**Table 3: Significant Differences between Gene's Expressions Were Tested Using Tukey's Test HSD Test after One-Way and Two-Ways Anova with Exposure Time and Metal Concentrations as the Two Factors.**

However, PdPCS1 mRNA production decreased from the first 40d of exposure with 1 mM Cd-stress of explant of Deglet Nour [5]. Long exposure stress influenced gene expression event at low Cd amount (0.02 mM). Similarly, long time exposure reduced *Pdmt3* expression although it is still enhanced by 0.2 and 2 mM Cu (Figure 4a). *Pdmt3* transcription factor described a biphasic curve at 2 mM Cu, attending a maximum by 5.5 after 40d before it declined by a half at the end of the experience. In *Silene vulgaris*, both copies of *Svmt3* have been identified and were functional. While, high Cu and Cd concentrations down-regulated gene expression in *S. vulgaris* ecotypes and Deglet Nour explants respectively [5] . *pcs1* and *mt3* offers a key non-translationally phytochelatins (PCs) and translationally metallothionein (MTs) chelators respectively scavenging free $Cu^{2+}$ and controlling copper homeostasis in plants [42] . However, overexpressing AtPCS1 alone did not result in an increased metal tolerance [38]. The Cu-conjugates are transported and compartmentalized in vacuoles. ABCC and MATE proteins are particularly active in the sequestration of chelated $Cu^{2+}$ [39].

Thereby, *abcc* expression increased in parallel to *pcs1* and *mt3* expression (Figure 4b). It maintained linear increase throw time exposure by 0.02 and 0.2 mM Cu. Members of *abcc* family has been identified in *A. thaliana*. ABCC1 and ABCC2 was identified in *A. thaliana* exhibiting redundant function in the translocation of plant vacuolar phytochelatin [38].

Besides, *Atpcs1* and *Atabcc1* exhibited a consistent increase as result in plants with enhanced arsenic tolerance [38]. AtABCC1 and AtABCC2 are not synthesized de novo but constitutively present in a plant cell to rapidly respond to toxic metals and xenobiotic stresses in a way similar to PCS1. Yet, similarly to *mt3, abcc* expression has fallen by more than 50% after 60d of exposure with 2 mM (Figure 4b). However, Cd stress badly influenced ABCC mRNA production more than Cu since abcc down-expression has been shown from the first 40d of Deglet Nour explant exposed to 1 mM Cd [5]. Although *pcs1* was highly expressed under copper stress especially with 0.2 mM concentration in the medium, *mate5* transcript levels were significantly decreased after 2 months of treatment by more than 75 % and 90 % with respectively 0.2- and 2mM Cu. Long exposure time affected gene expression (Figure 4b). Yet, in brown algae, MATE stress-related transporters were specifically up-regulated by Cu stress [39]. Also, *mate* was described as the candidate gene for the export of toxic cations such as Al in soybean and rice respectively [43,44]. It may be involved in the transport of secondary metabolites and the detoxification of xenobiotics [45,46]. Other transporters involved in copper tolerance in date palm have been monitored (Figure 4b). In the same way as *mt3, abcc* and *mate5*, *Nramp6* transcript levels, with the lowest mRNA production, described a biphasic curve (Figure. 4b). Maximum transcript factor exceeding 1.6 was accrued by 2 mM Cu after 40d of exposure. After that period, only 0.02 mM Cu continued to improve gene expression, while it decreased for 0.2 mM Cu and especially 2 mM Cu (Figure 4b). Member 6 of Nramp family encoding gene is not much studied before. Based on this finding, we suggest a novel gene expressed in date palm in response to Cu stress; *PdNramp6*. It was also involved in Cd tolerance in the plant [31]. Also, Ishimaru et al. highlighted the importance of OsNramp5 for Cd phytoremediation were OsNramp5 may contributes to manganese (Mn), Cd, and Fe transport in rice [47]. *AtNramp1* plays a pivotal role in Fe uptake and transport in the plant [48]. However, the overexpression of *nramp3* in *A. Thaliana* increased Cd sensitivity. On the other hand, HMA2 is a $Zn^{2+}$-dependent ATPase that is activated especially by $Cd^{2+}$ but also by other divalent heavy metals like $Cu^{2+}$, $Pb^{2+}$, $Ni^{2+}$ and cobalt ($Co^{2+}$;) [49]. In our experience, *Pdhma2* is the unique gene, from the tested genes that expressed significant increase even at high copper level and after long time of treatment (Figure 4b). However, the gene may act differently against metal stress and between plant types. In fact, at the same conditions, *P. dactylifera* explants decreased *hma2* after 2 months of Cd-containing medium [7]. In addition, *hma2*, characterized as efflux transporter genes responsible for the transport of $Cd^{2+}$ from pericycle parenchyma cells to xylem, was up-regulated in a Cd cultivar Baiyewuyueman and down-regulated in Kuishan'aijiaoheiye in Cd treatments compared with the controls [50].

## 4. Conclusion

GeneMania analysis showed abundance of members of *spl* and *ubc* family in date palm cv Deglet Nour cDNA library. Genes of UBC2/13/32 were predicted to be linked to each other and to *mmz3* and were involved in copper mobilization in the plant. Co-regulation relationships between *fsd2* and *fsd3* followed by

gene functional link predictions between fsd and csd as well as between *fsd* and *csd* were shown. To further disclose copper regulation pathways adopted by *P. dactylifera* cells, specific research on transporter genes was made. Members of *hma* family encoding *hma2/4/5/6* (*PAA1*) and 8 (*PAA2*) have been identified and are highly expressed. Functional links between *hma5* and *Nramp2* as well as co-expression between *abcc* and *Nramp3* were predicted. Among the high number of Cu-responsive genes, qPCR amplification of six genes in in vitro culture of explant of date palm cv Deglet Nour exposed to increasing Cu concentration was used. In general, all tested genes, *Pdpcs1*, *Pdmt3*, *Pdabcc*, *Pdhma2*, *Pdmate5* and *PdNramp6*, were up-regulated. However, except for *Pdpcs1*, the best induced gene, amounts of transcript levels significantly decreased by increasing metal and exposure time. Thus, event most genes still overexpressed, there were not strong enough to confer increased Cu-tolerance in which explants showed necrosis and growth alteration that may probably linked to Cu-repression of some related genes at the highest concentration and after two months of exposure.


## Acknowledgements

We are thankful to Pr. Fki Lotfi who provided expertise which improved the manuscript significantly. Also, we express our appreciation for technical assistance to Ayadi Mohamed who shared his wisdom with us during this research.


## Author's Contribution

Franck Vandenbulcke monitor the research, Amine Elleuch designed the research; Zayneb Chaâbene and Imen Rekik Hakim performed research and analyzed the data; Zayneb Chaâbene wrote the paper. All authors read and approved the final manuscript.


## Funding

This work was supported by grants from the Tunisian minister of Higher Education and Scientific Research and the University of Lille Sciences and technologies.


## Compliance with Ethical Standards

**Conflicts of Interest:** The authors declare no conflicts of interests.

**Ethics Approval:** Ethics approval was not required for this study.

**Disclosure:** The authors report no disclosures or financial support

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