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1 Transcriptome assembly and abiotic related gene expression analysis of Date
2 palm reveal candidate genes involved in response to cadmium stress

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27 A B S T R A C T

28 Date palm in Tunisia are of major economic importance but are also factors of social,
29 environmental and economic stability. An annotated assembly of the transcriptome of cultivar
30 Deglet Nour was reported. RNA was isolated from plant Cd-contaminated leaves, and 37,049
31 unique Illumina RNA-seq reads were used in a transcriptome assembly. The draft transcriptome
32 assembly consists of 6789 contigs and 17,285 singletons with a means length of 858 bp and
33 1,042 bp, respectively. The final assembly was functionally annotated using Blast2GO
34 software, allowing the identification of putative genes controlling important agronomic traits.
35 The annotated transcriptome data sets were used to query all known Kyoto Encyclopedia of
36 Genes and Genomes pathways. The most represented molecular functions and biological
37 processes were nucleotide binding and transcription, transport and response to stress and abiotic
38 and biotic stimuli. A prediction of the genes interaction network was proposed by selecting
39 corresponding functionally similar genes from *Arabidopsis* datasets, downloaded by
40 GeneMANIA version 2.1. Several Cd-responsive genes expression was monitored in *in vitro*
41 isolated explant of Cd stressed Deglet Nour. Some chelators encoding genes were upregulated
42 confirming *in silico* findings. Genes encoding HMs transporters in date palm showed
43 expression enhancement more pronounced after 20 days of exposure. *P. dactylifera*
44 transcriptome provides a valuable resource for future functional analysis of candidate genes
45 involved in metal stress response.

46 *Keywords:* Cd-stress, date palm, genes expression, Illumina RNA-seq, transcriptome

47 1. Introduction

48 The date palm (*Phoenix dactylifera* L.) is a woody plant belonging to the *Arecaceae* family,
49 cultivated in the Middle East and North Africa (El-juhany, 2010). *Phoenix dactylifera* is a
50 valuable tree due to very high sugar content (50 % of the total weight of mesocarp) of the fruit
51 (dates; Cao et al. 2011). This species plays an important role in locations where it is planted. Its
52 fruits are considered as food, their trunks were used as architectural materials and palm trees
53 are also ornamental plants. The arid desert climate is very beneficial for *P. dactylifera*. We
54 assumed that this species contains genes for resistance to biotic and abiotic stresses.

55 One of the major abiotic stresses agriculture has to face is the accumulation of heavy metal
56 ions which are very toxic to cells. According to their effect on plant growth, they can be divided
57 in two categories. 1- Essential minerals; trace amounts of which are necessary for growth. The
58 excess of these ions causes toxicity. 2- Non essential ions with no biological function in higher

59 organisms such as cadmium (Cd) and lead (Pb) cause toxicity. For instance, it has been shown
60 that Cd can be absorbed by the roots and transported to the shoot and thereby adversely affected
61 the absorption of nutrients and homeostasis in plants even at low concentrations (Nazar et al.,
62 2012). Cd²⁺ has adverse impacts on various biochemical and physiological processes (Krämer
63 et al., 2007). They include changes in the transcriptome and proteome of plants, resulting in the
64 limitation of the process of seed germination and development (Thapa et al., 2012), the
65 inhibition of the growth of shoots and roots, and ultimately the overall performance reduction
66 of the plant (Clemens et al., 2002; Roth et al., 2006; Zhang et al., 2015). Because of its harmful
67 effects, the use of Cd has been strictly banned in material such as plastic since December 2011
68 (the Regulation of the European Commission REACH No 1907/2006 of 18 December 2006).
69 Despite these restrictions and prohibitions, another 96 tons of Cd were released into the
70 atmosphere (EEA, 2013) in Europe. The metal continues to exist in arid countries soils such as
71 Tunisia soil due to the emergence of heavy metals (HMs) contamination in mining wastes, the
72 use of phosphate fertilizers and Cd-rich irrigation water. The metal persistent in soil is a matter
73 of concern for soil fertility (Tanase et al., 2012). It increases the danger for plants, seed grains
74 and human health. In this context, it is important to study the mechanisms of plant defenses to
75 cope with Cd²⁺ exposure and toxicity. This can be made through the delineation of the
76 transcriptomic responses of plants able to resist to abiotic stresses. An initial sensory activation
77 phase, followed by a physiological response, is usually induced by abiotic stresses in plants
78 (Mahajan and Tuteja, 2005; Zhu et al., 2003). Little is known about stress responses to Cd of
79 non-model plants especially *Phoenix dactylifera* which showed a great potential for Cd
80 accumulation (Chaâbene et al., 2013). However, information may be obtained from stress
81 response studies, which have been performed in *Arabidopsis thaliana* (Matsui et al., 2008;
82 Zeller et al., 2009).

83 In this study, the first analysis of the transcriptome of *P. dactylifera* L. cv Deglet Nour using
84 the Illumina GA IIX platform is described. The generated large EST dataset enables us to
85 broaden our knowledge of the genomic resources of the plant and to investigate gene activities
86 in diverse tissues and different stages under Cd-stressful conditions. Here, RNA-seq technology
87 has been used to explore the transcriptional profiles as well as to identify putative candidate
88 genes and functional elements. Metabolic pathways associated with resistance to Cd stress have
89 been proposed. Various differentially regulated genes were identified then validated using real
90 time polymerase chain reaction (qPCR). Based on the monitoring of gene expression variation
91 along time exposure to different Cd concentrations, we represent a first comprehensive

92 transcriptome-based characterization of Cd responsive gene regulatory networks in date palm.
93 These genes could be used to strength efforts for the genetic improvement of date palm.

94 **2. Materials and methods**

95 **2.1. Plant material**

96 Leaves of *Phoenix dactylifera* cv Deglet Nour were used in this study. Sampled trees were
97 from contaminated site of Sfax region in Tunisia. This area is submitted to strong heavy metals
98 pollution in which date palm leaves accumulate Cd of more than 1,5 mg kg⁻¹ (Chaâbene et al.,
99 2019) while, the standard limits of WHO (1996) who are 0.02 mg kg⁻¹. After thorough washing
100 with double distilled water, samples were immediately frozen in liquid nitrogen and transported
101 to the laboratory on dry ice. The samples were stored at -80 °C until use.

102 **2.2. Preparation of cDNA library for transcriptome sequencing**

103 The CTAB method of Zhao et al. (2002) was used for the extraction of total RNA from leaf
104 tissues of *Phoenix dactylifera* CV Deglet Nour. RNA integrity was analyzed by capillary
105 electrophoresis on an Agilent BioAnalyzer 2100. Polyadenylated RNA was selected using
106 oligo(dT) purification and reverse-transcribed to cDNA. These cDNAs were fragmented, blunt-
107 ended, and ligated to the Illumina TruSeq Adaptor Index 3 (Illumina Inc.,
108 <http://www.illumina.com>). The library was size-selected for an insert size of 250bp, and
109 quantified using the Invitrogen Pico Green dsDNA assay (Life Technologies).

110 **2.3. Illumina sequencing**

111 From both 5' and 3' ends, the cDNA library was sequenced on the Illumina GA IIxplatform
112 according to the manufacturer's instructions. The fluorescent images processing to sequences,
113 base-calling and quality values calculation were performed by the Illumina data processing
114 pipeline (version 1.4), in which 75 bp paired-end reads were generated.

115 **2.4. Bioinformatics and Data mining**

116 The CLC Genomics Workbench 8.1 de novo assembly tool was used to assemble the reads
117 into contigs. Functional annotations and gene ontologies were predicted for each contig using
118 Blast2GO software (Conesa et al., 2005; Götz et al., 2008) with the following parameters:
119 BLASTx against the NCBI non redundant protein database and TAIR10 release of the

120 Arabidopsis transcriptome and proteome (<http://www.arabidopsis.org> ; Lamesch et al. 2012),
121 BLAST E-value = 0.001 and reporting the top 20 hits. After getting GO annotations for each
122 transcript, WEGO software (Ye et al., 2006) was used to conduct GO functional classification.
123 Furthermore, for all date palm sequences, the KEGG Automatic Annotation Server
124 (<http://www.genome.jp/tools/kaas/>) was used to identify KEGG orthologs with default
125 parameters. Using Geneious V5.6.2 (Biomatters; <http://www.geneious.com>), the underlying
126 sequences for each contigs and singletons were trimmed and mapped to the respective mRNA
127 of *Phoenix dactylifera* cv khalas. Transcription factors (TF) and protein kinases in the cDNA
128 library were identified and classified into different groups using the iTAK pipeline
129 (<http://bioinfo.bti.cornell.edu/tool/itak/> ; Pérez-Rodríguez et al., 2010).

130 **2.5. Interaction Network Exploration**

131 The genes interaction network was predicted by using selected corresponding functionally
132 similar genes from *Arabidopsis* datasets, downloaded by GeneMANIA version 2.1 (Warde-
133 farley et al., 2010; Zuberi et al., 2013 ; <http://www.genemania.org/>) and visualized in Cytoscape
134 (Montejo et al., 2010).

135 **2.6. In vitro experimentation**

136 Isolated explants of date palm cultivar Deglet Nour were obtained from the laboratory of
137 plant biotechnology applied on the amelioration of culture, university of Sfax, Tunisia. Explants
138 were transferred under sterile conditions into the Murashige and Skoog (MS) medium (1962)
139 supplemented with different concentrations of CdCl₂ (0.02, 0.2 and 1 mM) and incubated in
140 16h photoperiod at 24 °C during 60 days of exposure. Date shoot clusters of 20, 40 and 60 days
141 of development were used for total RNAs extraction.

142 **2.7. Total RNA isolation, cDNA synthesis and gene identification**

143 RNAs extraction and cDNA synthesis were performed as per Chaâbene et al. (2018). From
144 the previously constructed Deglet Nour date palm cDNA bank, we looked for ABC transporter
145 C family membre 2 like (*abcc*), ATPase family (*Pdhma*), Multi-antimicrobial extrusion protein
146 *mate*, natural resistance-associated macrophage protein (*Nramp*) coding genes. Homologous
147 sequences of genes from various organisms recognized in NCBI
148 (<http://www.ncbi.nlm.nih.gov/>) using BLASTn search has been aligned against clones
149 nucleotide sequences from our date palm cDNA bank using BioEdit version 7.0.5.3. Some

150 clones revealed partial homology with selected genes. Flowing methods described by
151 (Chaâbene et al., 2018) enabling retrieval of gene sequences coding for phytochelatin synthase
152 (*Pdpcs*) and metallothionein (*Pdmt*), we have identified *Pdabcc*, *Pdhma*, *Pdmate* and *PdNramp*.

153 **2.8. Real time qPCR amplification**

154 Using candidate gene specific primers designed by Primer3Plus on line software (Rozen and
155 Skaletsky 2000; <http://frodo.wi.mit.edu/>) and verified using Net Primer and Beacon Designer
156 programs (Table 1), quantitative real-time polymerase chain reaction (qPCR) amplification
157 method was performed according to Brulle et al (2014). The expression levels and the relative
158 fold expression (RFE) were determined according to previously described procedures (Brulle
159 et al., 2006). The geometric mean of the 3 most stable reference genes in control and Cd-
160 stressful condition identified by Chaâbene et al. (2018) was used to calculate expression of
161 target gene levels according to Brulle et al. (2014). Absolute quantification of genes expression
162 levels are in log₂.

163 **2.9. Statistical analysis**

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

167 **3. Results**

168 **3.1. Sequencing and Data analysis**

169 Three out of four Illumina GA IIxrun on one normalized cDNA library constructed from
170 *Phoenix dactylifera* cv Deglet Nour leaves were performed generating approximately 37.84
171 Mbp of sequence data from 37,049 reads with an average length of 1,021 bp. De novo
172 transcriptome assembly was realized using CLC Genomics Workbench 8 program. This has
173 been shown to perform better than a number of other commonly used assemblers. Table 2 shows
174 the transcriptome sequencing and assembly statistics: 19.54 % of the reads were assembled into
175 6,789 contigs, with an average assembled length of 858 bp and 17,285 singletons with an
176 average length of 1,042 bp. The size distribution of transcripts was shown in Fig. S1.

177 **3.2. Functional annotation of the *P. dactylifera* transcriptome**

178 The 24,074 transcripts (contigs and singletons) were blasted against the NCBI non-
179 redundant (nr) and *Arabidopsis* databases. A total of 23,717 (98, 52 %) transcripts matched a
180 homolog in the nr database using BLASTx program (E-value threshold of 1e-5). The top-hit
181 species distribution of nr BLAST matches was shown in Fig. S2A. Approximately 90.3 % of
182 the sequences had significant matches with genes from *P. dactylifera* cv Khalas, followed by
183 *Musa acuminata* (2.29 %) and *Vitis vinifera* (0.74 %). E-value distribution of the top hits in the
184 databases had shown 12,3 % of matched sequences with strong homology ($< 1 \text{ e-}180$; Fig. S2B).
185 To study the level of global sequence conservation between *P. dactylifera* and *A. thaliana*, we
186 realized a BLASTx comparison of the *P. dactylifera* transcriptome assembly to the *Arabidopsis*
187 TAIR10 peptide database (Table S1). The homology of each predicted peptide to the most
188 similar *Arabidopsis* protein was measured by the percentage of similarity (Fig. 1a) and
189 percentage of coverage (Fig. 1b). A smooth scatter plot representing the percentage similarity
190 and percentage coverage for each *P. dactylifera* sequence compared to the closest *Arabidopsis*
191 peptide sequence was shown in Fig. 1c. A large proportion of transcripts ($> 85 \%$) showed at
192 least 60 % similarity to an *Arabidopsis* proteins. A total of 12,354 of *P. dactylifera* predicted
193 peptides had at least one match to an *Arabidopsis* gene with $>60 \%$ similarity $>30 \%$ coverage
194 (Fig. 1c, boxes).

195 The sequences with a best match from *Arabidopsis* database were further assigned with GO
196 terms and Enzyme Commission (EC) numbers using the Blast2GO platform. A total of 38,379
197 GO terms were assigned to 21,655 cDNAs (including contigs and singletons; Table 3). Among
198 all the GO terms extracted, 2391 GO term (6.22 %) belong to the Molecular Function (MF)
199 class, 4,272 (11,13 %) to Biological Process (BP) class and 773 (2 %) to Cellular Component
200 (CC) class. There are 12,519 cDNAs assigned to multiple GO terms (Fig. 2). 16,179 sequences
201 associated with metabolic processes and 17,753 sequences associated with Cellular Process
202 (GO level 2) were found (Fig. S3a). GO terms of primary metabolites (sugars, amino acids,
203 nucleotides, lipids) and energy sources that are essential for plant survival were also identified.
204 GO terms associated with secondary metabolites that play key roles in maintaining plant
205 wellness and the protection of plants against metallic stress were found. In the molecular
206 function (MF) category, 11,768 transcripts haven high similarity to proteins with binding
207 activity (nucleotide binding, ion binding and oxidoreductase binding; Fig. S3b) and 9,815
208 transcripts for catalytic activity have been identified.

209 **3.3. Functional classification by Kyoto encyclopedia of genes and genomes (KEGG)**

210 By mapping EC numbers to the referral canonical pathways, 10,632 transcripts (44.16 %)
211 had important matches and were appointed to 140 KEGG pathways (Table S2, Table 4, Fig.
212 S4). “Metabolism” is the biggest class (9,341 sequences, 96.5 %), followed by Environmental
213 Information Processing (338, 2.11 %) containing solely a pair of sub-units (“mTOR signaling
214 pathway” (51 sequences) and a second set of “mTOR signaling pathway” (154 sequences)).
215 “Genetic Information Processing” (77, 0.8 %) was the smallest class. In metabolism pathways,
216 polypeptides involved in amino acids, carbohydrates and lipid metabolism consisted of about
217 65 % of the whole sequences. The relative quantity of proteins belonging to metabolism of
218 cofactors and vitamins (MCV), xenobiotics biodegradation and secondary metabolites
219 (BGS), and nucleotide metabolism are conjointly represented by a sizable amount of
220 sequences (Table S2). Additionally, 221 transcripts involved in phenyl propanoid biosynthesis,
221 152 sequences related to glutathione metabolism and 128 sequences assigned to TCA cycle
222 were found.

223 ***3.4. Candidate genes for cadmium transporters, signal sensing and transduction proteins***

224 Using keywords "cadmium ion Transport: GO: 0015691", 53 genes were identified as
225 candidate genes involved as members of different metal transporter families. Using the program
226 GeneMania, interactions between these proteins which were mainly related to ABC (PDR and
227 ABCG43), ZIP families, CAX and Nramp proteins, were predicted (Fig. 4, Table S4).

228 Using keywords "regulation of metal ion Transport: GO: 0010959" for our search in the
229 annotation results, we found 9 cDNAs calcineurin B-like protein (CBL), 30 cDNAs CBL-
230 interacting protein kinase (CIPK), 19 cDNAs highly homologous to genes encoding calcium-
231 dependent protein kinase (CPK or CDPK) were identified and 13 cDNAs histidine kinase
232 (AHK) have a very important role in the regulation of transport of metal ions and transduction
233 signals. The interactions between the proteins were predicted using the program GeneMania
234 (Fig. 4 and Table S5).

235 ***3.5. Identification of Candidate Genes Involved in Biosynthesis of Metal-chelating*** 236 ***Compounds and Glutathione Metabolism***

237 In the present analysis of sequences, six transcripts corresponding to homologous genes
238 encoding metal chelators; MT2 and MT3 have been evidenced (Table S6). Four sequences
239 encoding the glutathione synthase (EC 6.3.2.3) have been annotated (Table S7). Moreover,
240 based on the KEGG pathway assignment, 152 transcript sequences were found to encode 16
241 putative enzymes involved in glutathione metabolism from the present *de novo* assembled

242 transcriptome (Fig. 5). Note that more than one transcript sequence was annotated as the same
243 enzyme, implying that such transcript sequences may represent different fragments of a single
244 transcript, or different members of a gene family (Hyun et al., 2012). We noticed that there
245 were some enzymes that are induced, for example L-ascorbate peroxidase (APX, EC 1.11.1.11,
246 19 sequences), glutathione S-transferase (GST, EC 2.5.1.18, 29 sequences), gamma-
247 glutamylcysteinylsynthetase or PCS (GGS, EC 6.3.2.2,23 sequences), and glutathione
248 peroxidase (GP, EC 1.11.1.9).

249 **3.6. Gene expression variations of Cd-related candidates**

250 *In vitro* study has been performed to monitor Cd-responsive gene expression variations
251 during two months of treatment of *P. dactylifera* explant *in vitro* generated leaves. Candidate
252 genes were expressed in a specific manner along metal concentration. There are not only
253 present but fully operational in *P. dactylifera* (Table 5). Their expressions were altered during
254 abiotic stress. Both Cd concentrations and time exposure influences significantly ($p < 0.000$)
255 gene expression in vitroplant tissues. The mean relative expression values of biomarkers were
256 presented after scaling with values of non-treated samples. The relative fold expression of
257 candidate genes appeared differentially expressed (Table 5). The Cd treatment induced the
258 expression of HM chelator encoding genes after 20 days of exposure and began at the lowest
259 Cd stress. However, *Pdpcs1* induction factor (more than 15 folds) in date palm explant
260 exposed to 0.02 Cd during 20 days of exposure is more important than in date palm hypocotyl
261 tissues issued from seeds (more than 4 folds; Chaâbene et al. 2018). *Pdmt3* expression begins
262 only at high-dose metal treatment after 20 days of exposure.

263 Significantly highly induction of *Pdabcc* has been obtained under 0.02 and 0.2 mM Cd
264 during 40 days of treatment. They encoded ABCC-type transporters transporting PCs and PC-
265 HM complexes (Kretzschmar et al., 2011). *Pdabcc* expression reached a maximum ($9.07 \pm$
266 0.1) at 0.02 mM. However, the expression level of the other HMs transporters (*Pdhma2*,
267 *PdNramp6* and *Pdmaet5*) expressed a slightly but significant increase. At high Cd
268 concentration (1 mM Cd), negative values of absolute quantification of *Pdabcc* was obtained
269 after 40 days of metal stress (Table 5). Whereas, decrease in *Pdmate5* and *PdNramp6* has been
270 shown. Although they were still induced, *Pdmate5* and *PdNramp6* remained lower than the
271 control at high metal concentration after 40 days of stress. In contrast, under these conditions,
272 *Pdhma2* showed more resistant capability to metal stress (Table 5). 60 days of higher Cd

273 treatment suppressed target genes expression in date palm explant especially *Pdmt3*, *Pdabcc*
274 and *PdNramp6*.

275 **4. Discussion**

276 After Illumina sequencing and cDNA library construction from date palm Phoenix
277 dactylifera leaves, several complementary approaches were adopted to extensively analyse
278 and annotate the transcription. Firstly, we used the Gene Ontology (GO) classification system
279 in Blast2GO software to annotate the possible functions of identified genes. The creation of
280 the consortium GO is a joint project of many databases from different organizations with a
281 structured and controlled vocabulary to describe gene functions and gene products in specific
282 organizations (Ashburner et al., 2000). In our cDNA banq, the most representative categories
283 with more than 11 % of extracted GO terms, belong to Biological Process (BP) category that
284 comprised different types of metabolic processes. BP class was followed respectively by the
285 Molecular Function class (6.22 %) and Cellular Component class (2 %; Fig. 2). This result is
286 in agreement with the metabolic network in plants which is more extensive compared to other
287 organisms (Aharoni, 2010). From BP category, GO terms of primary and secondary
288 metabolites were identified. The genes associated with pathways of secondary metabolite
289 biosynthesis represent a large number of annotated enzymes (Torales et al., 2013). Another
290 category worthy to mention is “response to stimulus” (BP category, level 2). Indeed, 13,216
291 sequences associated with this category, which include candidate genes for resistance to biotic
292 and abiotic stressor, were found (fig. 2). Focusing on plant resistance mecanismes, KEGG
293 pathway constructed database contains pathway maps for The phosphatidyl inositol signaling
294 system comprised kinase activity (99 sequences, Fig. S5) and phosphatase activity (55
295 sequences) and alternative enzymes (Table S3). Many studies illustrated that the phosphatidyl
296 inositol signaling system provide a vital role in resistance to abiotic stresses (Levine, 2002;
297 Zhu et al., 2003). Furthermore, pathways belonging to xenobiotics biodegradation, secondary
298 metabolites, phenyl propanoid biosynthesis and glutathione metabolism were found. These
299 pathways, better-known to induce defense response in plants through the salicylic acid and
300 Ca²⁺-related signaling pathways (Ahn et al., 2005; Goyer, 2010), that might play roles in biotic
301 or abiotic stresses (Korkina, 2007). Moreover, secondary metabolites like phenyl propanoid
302 derivatives play vital roles in resistance to pathogens and were recently used in health issues
303 given their antioxidants, anti-tumor and anti-inflammatory activities (Korkina, 2007;
304 Ramadan et al., 2010).

305 The evolutionary relationship of DNA sequences between *P. dactylifera* and *Arabidopsis*
306 enabled identification of potential date palm orthologs responsible for controlling metal
307 transport and stress response. For each *P. dactylifera* transcript, the top 20 BLASTx hits
308 against the *Arabidopsis* peptide database were mined for hits to *Arabidopsis* genes known to
309 control these traits. Metal carriers could play a very important role in reducing the intracellular
310 concentration of heavy metals through the transport of metal ions out of the cell or sequestering
311 them in the vacuole. There are several metal transporters families that can contribute to the
312 resistance of plants to heavy metals (Hall, 2002) including ATP binding cassette (ABC),
313 Natural resistance-associated macrophage proteins (Nramp), Zrt- and IRT-related proteins
314 (ZIP), Cation Exchanger (CAX) and Pleiotropic Drug Resistance protein family (PDR). To
315 investigate the conservation of the metal transport pathway, we attempted to reconstruct the
316 transport and signal transduction pathways in date palm using predicted peptides from the
317 transcriptome assembly. GeneMania program helped us to predict interactions between
318 peptides haven very important role in the regulation of transport of metal ions and transduction
319 signals. Peptides like calcineurin B-like protein (CBL), CBL- interacting protein kinase
320 (CIPK), calcium-dependent protein kinase (CPK or CDPK) were identified (Fig. 4 and Table
321 S5). Plants are often exposed to changing environmental stresses to which they must
322 accumulate. For instance, many biotic or abiotic extracellular stimuli can change the
323 intracellular concentration of Ca^{2+} (Knight et al., 2014). These variations are responsible for
324 the over expression of the various calcium-binding proteins and protein kinases (DeFalco et
325 al., 2010). Cellular responses to Ca^{2+} signals are mediated by different families of genes in
326 plants, such as Ca^{2+} dependent protein kinases (CDPKs) and CDPK-related kinases (CRKs),
327 by calmodulins (CAMs) and calmodulin-dependent protein kinases (CaMKs), by calcineurin
328 B-like proteins (CBLs) and CBL interacting protein kinases (CIPKs, also called SnRK3s), and
329 by Ca^{2+} and calmodulin-dependent protein kinases (CCaMKs). CCaMKs are present as single
330 gene in a number of plant species having symbiotic interactions, but not in *Arabidopsis*
331 (Harper et al., 2004).

332 Another mechanism used by plants for resistance against heavy metals is the synthesis of
333 chelating metal compounds (Lin and Aarts, 2012). Numbers of transcripts corresponding to
334 genes encoding metallothionein (MTs) and phytochelatin synthase (PCS) has been predicted.
335 MTs are low molecular weight cysteine rich proteins able to bind metals. They are usually
336 classified into four groups (MT1-4). Currently, MT genes have been identified in a number of
337 higher plants such as *Arabidopsis* and *Brassica juncea* (Cobbett and Goldsbrough, 2002).
338 Phytochelatins (PC) is another important class of heavy metal-binding ligands, which can bind

339 metal ions via thiolate coordination (Cobbett, 2000). Its synthesis is controlled by PCS. Many
340 studies have confirmed that glutathione (GSH) is the substrate for the biosynthesis of PC
341 (Anjum et al., 2016) and that this conversion of GSH to PC is catalyzed by the enzyme
342 phytochelatin synthase (PCS; Yadav, 2010).

343 Among identified metal transporter and chelator genes, the level of expression of
344 orthologous genes known to respond to elevated metal concentrations in other plants and
345 identified in the present cDNA bank of *P. dactylifera* was monitored by qPCR. In order to
346 uncover the molecular basis involved in Cd²⁺ storage and tolerance capabilities, gene transcript
347 were measured in *in vitro* generated explants of date palm cultivar Deglet Nour (genetically
348 homogenous tissues) at different metal concentrations and various periods of exposure.
349 Candidate genes of Cd-chelators; *Pdpcs1* and *Pdmt3* are not only present but fully operational
350 in *P. dactylifera* (Table 5). Their expressions were altered especially, at high Cd
351 concentrations and after a long time exposure. However, *Pdpcs1* induction factor in date palm
352 vitroplants remains more important than in date palm hypocotyl tissues (genetically
353 heterogenous tissues) issued from seeds (Chaâbene et al., 2018). This may be due to genetic
354 diversity of date palm seeds tissues which may produce less tolerant varieties than Deglet Nour
355 cultivar. In the other hand, *Pdmt3* was found to be the less sensitive gene. Interestingly, this
356 latter showed non-significant induction during explant development in standard conditions
357 (Table 5). Metallothionein are cysteine (Cys)-rich proteins that detoxify heavy metals,
358 participate in the regulation of the homeostasis of essential metals like zinc and copper
359 (Dabrowska et al., 2012) containing in Murashige and Skoog (MS) medium and in controlling
360 the concentration of ROS by activating defenses, e.g. via the mitogen-activated protein kinase
361 MAPK cascade (Finatto et al., 2015).

362 Increasing metal time exposure further induced Cd-transporters; *Pdabcc*, *Pdhma2*,
363 *Pdmtate5* and *PdNramp6* expression under 0.2 mM Cd (Table 5). *Pdabcc* showed to be more
364 sensitive to stress than the other Cd-transporter genes. It encoded ABCC which are known to
365 be involved in transport and detoxification of glutathione-conjugates (Bhati et al., 2015). High
366 enhancement of ABC encoding genes by Cd stress in wheat; *Taabcc3*, *Taabcc4*, *Taabcc11* is
367 shown (Bhati et al., 2015). Indirectly, abcc may be induced by the accumulation of H₂O₂ in
368 Cd-stressed cells. Whereas, some of the wheat ABCC genes showed suppression in the
369 expression under Cd stress (Bhati et al., 2015). *Pdabcc* was more sensitive to Cd stress than
370 *Pdmtate5*, *PdNramp6* and *Pdhma2*. This later has important Cd transport activity in cells.
371 However, it lacks high specificity because it is involved in Zn transport in addition to Cd
372 (Ueno et al., 2010). Likewise, Nramp family lack Cd transport specificity and acts as an efflux

373 pump of elements in the membrane controlling metal availability (Bereczky et al., 2003).
374 Furthermore, comprising the multidrug transporter superfamily (MTS), MATEs are
375 responsible for secondary metabolite transport (Thompson et al., 2010). It has been shown that
376 MATE transporters mediate the citrate efflux to confer plant tolerance to Al toxicity
377 (Magalhaes, 2010). Further protein functions maintained related genes expression even at high
378 Cd amount. However, increasing metal exposure further decreased gene expression indicating
379 the progression of Cd stress severity. This linear regression analysis was in correlation with
380 phenotypic disturbances, toxicity signs occurrence and *Phoenix* explant growth and
381 development inhibition showed during experiment (data not shown).

382 **5. Conclusions**

383 Our study generated (i) 24,074 transcripts from *P. dactylifera*, (ii) identified putative
384 function in 21,655 transcripts for the species and several genes related to numerous metabolic
385 and biochemical pathways and (iii) predicted pathways to detoxify Cd such glutathione
386 pathway. These markers could facilitate the detection of functional variations and could be the
387 signature of selection in genome scans in association with genetic approaches. Multiple
388 candidate genes involved in defense and detoxification were successfully predicted in response
389 to Cd stress. *In vitro* study has been realised to monitor the expression pattern of gene involved
390 in cultivar Deglet Nour response to Cd. Genes encoding HM-chelators and HM-transporters
391 showed gene expression variation to different metal amount during different phase of explant
392 development. The molecular basis of the response to Cd stress in *P. dactylifera* provides a solid
393 foundation for the investigation of the molecular regulation mechanisms of heavy metal
394 accumulation and tolerance in leaves and roots.

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398 Kanehisa Laboratories for granting the permission to publish the Kyoto Encyclopedia of Genes
399 and Genomes (KEGG) pathways map image.

400 **Author Contribution statement**

401 Imen Rekik Hakim and Zayneb Chaâbene performed research, analyzed the data and wrote
402 the paper; Amine Elleuch, Franck Vandenbulcke, Hafedh Mejdoub supervised development of

403 work, helped in data interpretation and manuscript evaluation and designed the research. Walid
404 Kriaa Helped to evaluate and edit the manuscript. All authors read and approved the final
405 manuscript.

406 **Competing interests**

407 The authors declare that they have no competing interests.

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513 **Figure captions**

514 **Fig. 1.** Similarity and coverage of *Phoenix dactylifera* transcripts versus *Arabidopsis* genes. (a)
515 Histogram showing frequency versus percentage similarity (positive amino acid identity) of *P.*

516 *dactylifera* contigs versus an *Arabidopsis* peptide. (b) Histogram showing frequency versus
517 percentage coverage (longest positive hit/peptide length) of *P. dactylifera* contigs versus an
518 *Arabidopsis* peptide. (c) Smoothed color density representation of the percentage similarity (x
519 axis) of each *P. dactylifera* transcript plotted against the percent coverage of the *Arabidopsis*
520 protein similarity (y axis). The plot was produced using the ‘smooth Scatter’ function in R (R
521 Development Core Team, 2008), which produces a smoothed density representation of the
522 scatter plot using a kernel density estimate (nbin= 100). Darker color indicates a higher density
523 of transcripts in a given position, with the darkest ‘bin’ containing over 3000 transcripts. Boxes
524 encompassing transcripts encoding peptides with 60 % sequence similarity and 30 % coverage
525 are shown in the upper right corner.

526 **Fig. 2.** Gene ontology classification of assembled transcripts.

527 **Fig. 3.** Catalytic activity distribution in annotated *P. dactylifera* transcripts.

528 **Fig.4.** Interaction network of cadmium transporters and regulation proteins in *P. dactylifera*.
529 Protein interactions of metal-responsive genes were predicted by protein homologs in
530 *Arabidopsis* by GeneMANIA analysis.

531 **Fig.5.** Prediction of genes involved in glutathione metabolism in *P. dactylifera* transcripts. The
532 colored enzymes are identified in the *P. dactylifera* library.

533 **Supplemented Materiel**

534 **Fig. S1.** A. Length distribution of *P. dactylifera* Singletons. B. Length distribution of *P.*
535 *dactylifera* contigs

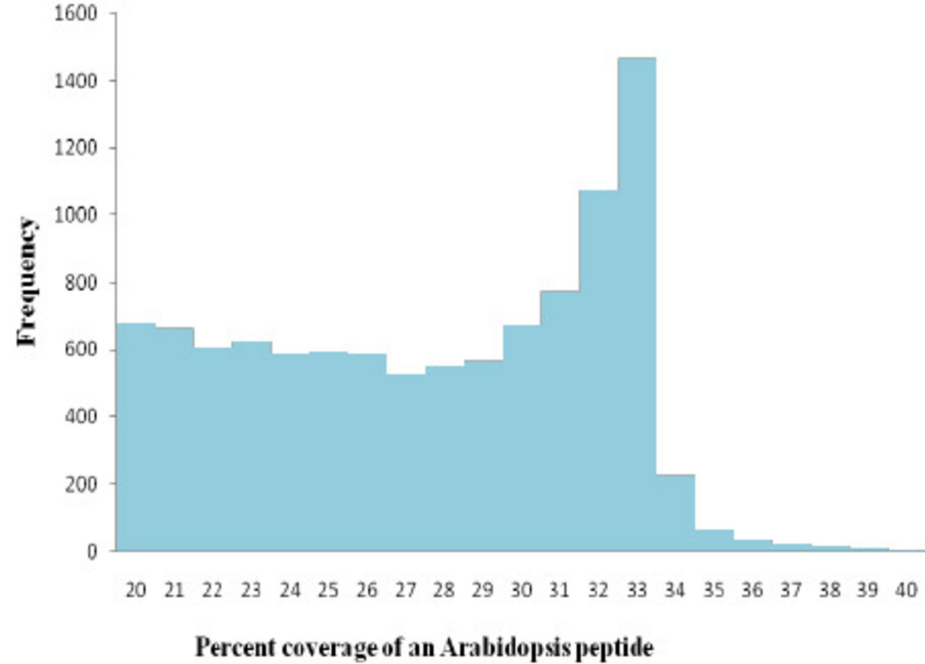
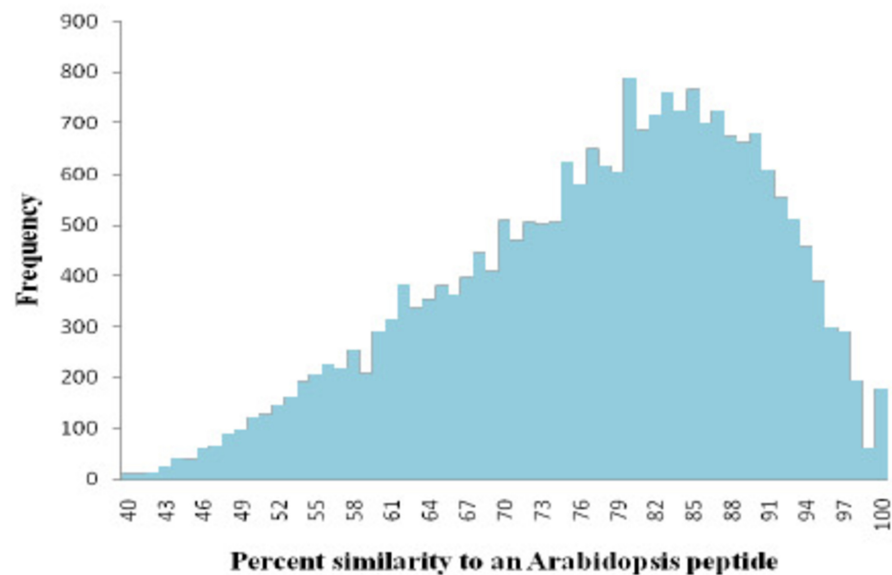
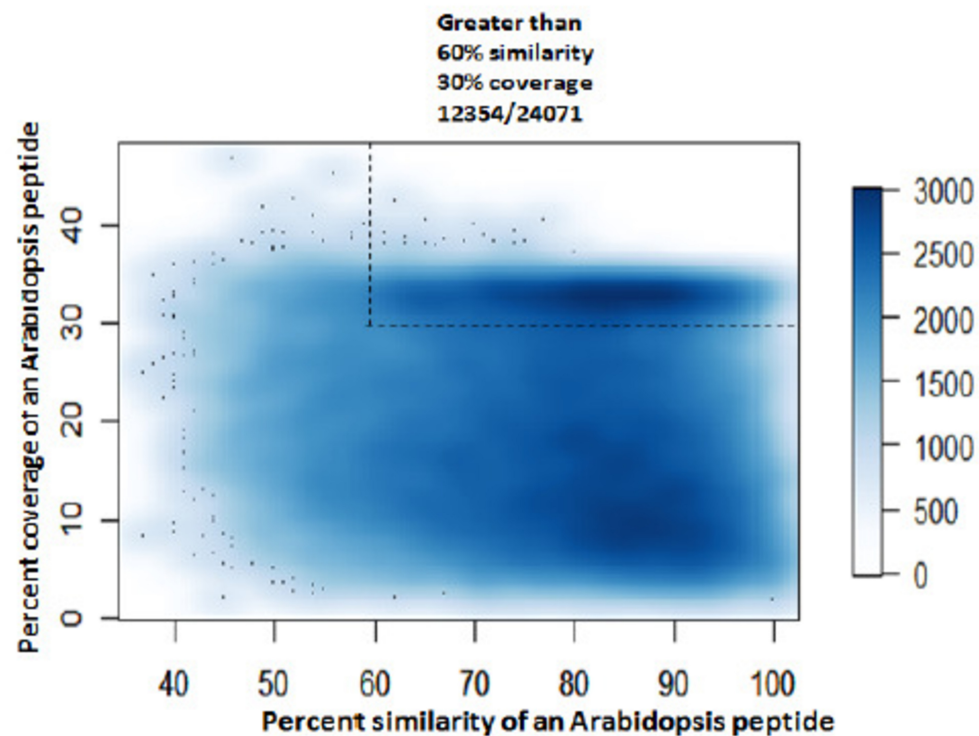
536 **Fig. S2.** A. Species distribution is shown as the percentage of the total homologous sequences
537 (with an E-value $\leq 10^{-5}$). We searched the NCBI nr database by using BLASTx and extracted
538 the best hit of each sequence for analysis. B. (A) E-value distribution of BLAST hits for each
539 transcript with a cutoff E-value of $1.0E^{-5}$ in database.

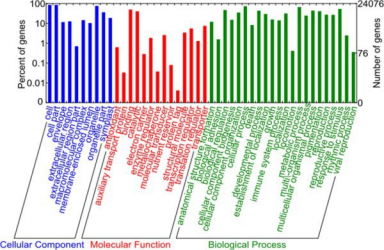
540 **Fig. S3.** Functional classification of *P. dactylifera* library.

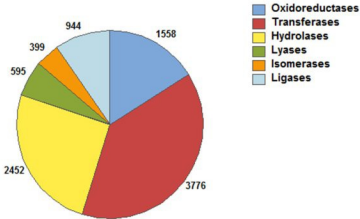
541 **Fig. S4.** Pathway assignment based on KEGG. Classification based on metabolism categories.

542 **Fig. S5.** Prediction of genes involved in phosphatidylinositol signaling system in *P. dactylifera*
543 transcripts. The colored enzymes are identified in the *P. dactylifera* library.

544

b**a****c**







Functions legends

- metal ion transport
- calmodulin-dependent protein kinase activity
- query genes

Networks legends

- Co-expression
- Co-localization
- Genetic interactions
- Physical interactions
- Predicted
- Shared protein domains

GLUTATHIONE METABOLISM

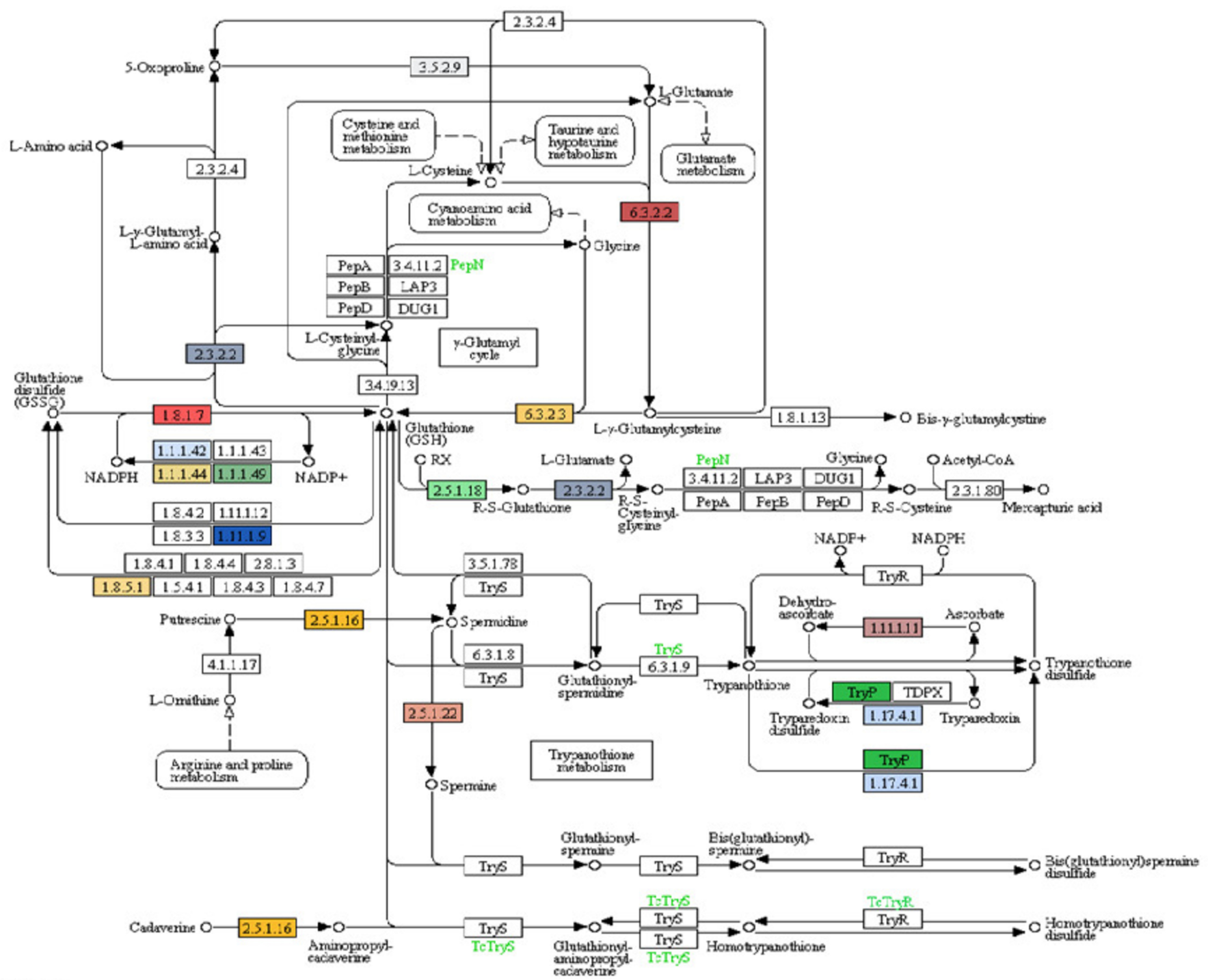


Table 1

Bioinformatic softwares results

Illumina RNA-seq reads and de novo assembly statistics

Statistics	Singletons	All Contigs
Number of sequences	17.285	6789
Min Length (bp)	130	105
Average Length (bp)	1.042	858
Max Length (bp)	7.410	5505
N50 size (bp)	2376	1254
<i>Annotation summary of P. dactylifera transcripts</i>		
Category	Number of transcripts	Percentage
TAIR annotated transcripts	21655	89.95 %
KOG classified	15020	62.39 %
KEGG classified	10632	44.16 %
<i>KEGG pathway mapping results</i>		
KEGG pathways	sequences number	
Metabolism pathway	9341	
Carbohydrate metabolism	2419	
Energy metabolism	424	
Lipid metabolism	1098	
Nucleotide metabolism	714	
Amino acid metabolism	712	
Metabolism of other amino acids	1850	
Metabolism of cofactors and vitamins	508	
Metabolism of terpenoids and polyketides	272	
Biosynthesis of other secondary metabolites	584	
Xenobiotics biodegradation and metabolism	500	
Glycan biosynthesis and metabolism	260	
Environmental Information Processing	338	
Genetic Information Processing	77	

Table 2

Overview of relative expression pattern (log₂) results in explant of *P. dactylifera* exposed to different Cd concentrations for 60 days. Data presented are means \pm standard error of three independent experiments. Two-way ANOVA main effects shown as *p* values. Differences between groups are shown as results of one-way ANOVA post-hoc Tukey's test, where small letters show differences between concentrations (in columns) and major letters show differences in time exposure (in rows). Means not showing the same letter are statically different ($p < 0.05$). Genes relative fold expression (RFE, gene expression in exposed samples compared to gene expression in control samples) is presented by arrows; \nearrow non-significant upregulation ($1.25 < \text{RFE} < 2.00$), \uparrow significant upregulation ($\text{RFE} > 2.00$), \searrow non-significant downregulation ($-2.00 < \text{RFE} < -1.25$), \downarrow significant downregulation ($\text{RFE} < -2.00$).

		20 d	40 d	60 d
<i>Pdpcs1</i>	0 mM	0.42 \pm 0.04 aA	0.54 \pm 0.02 aA	0.71 \pm 0.06 aA
	0.02 mM	6.57 \pm 0.10 \uparrow bA	4.67 \pm 0.10 \uparrow bB	2.47 \pm 0.04 \uparrow bC
	0.2 mM	4.18 \pm 0.05 \uparrow cA	4.33 \pm 0.12 \uparrow bA	2.00 \pm 0.00 \uparrow cB
	1 mM	4.44 \pm 0.20 \uparrow cA	2.01 \pm 0.10 \uparrow cB	1.19 \pm 0.04 \nearrow cC
<i>Pdmt3</i>	0 mM	2.58 \pm 0.00 aA	2.67 \pm 0.04 aB	2.55 \pm 0.00 aC
	0.02 mM	1.71 \pm 0.01 bA	3.57 \pm 0.09 \nearrow bB	3.29 \pm 0.01 \nearrow bC
	0.2 mM	2.89 \pm 0.06 cA	5.23 \pm 0.71 \nearrow cB	-3.53 \pm 0.63 \downarrow cC
	1 mM	3.28 \pm 0.30 \nearrow cA	-0.44 \pm 0.07 aB	-1.76 \pm 0.66 \downarrow cB
<i>Pdabcc</i>	0 mM	0.94 \pm 0.05 aA	1.09 \pm 0.01 aB	1.30 \pm 0.00 aC
	0.02 mM	2.8 \pm 0.12 \uparrow bA	9.07 \pm 0.1 \uparrow bB	-0.36 \pm 0.15 \searrow bC
	0.2 mM	1.43 \pm 0.04 \uparrow cA	0.33 \pm 0.01 \uparrow aB	0.72 \pm 0.03 cC
	1 mM	2.07 \pm 0.1 \uparrow dA	-1.64 \pm 0.21 \searrow cB	2.24 \pm 0.14 \uparrow dA
<i>Pdhma2</i>	0 mM	4.34 \pm 0.07 aA	2.71 \pm 0.12 aB	3.34 \pm 0.07 aC
	0.02 mM	6.1 \pm 0.15 \uparrow bA	4.93 \pm 0.09 \uparrow bB	1.93 \pm 0.08 bC
	0.2 mM	4.63 \pm 0.19 \nearrow aA	3.43 \pm 0.09 \uparrow cB	1.64 \pm 0.36 bC
	1 mM	6.72 \pm 0.24 \uparrow bA	3.85 \pm 0.06 \nearrow cB	0.82 \pm 0.1 cC

<i>Pdmate5</i>	0 mM	4.27 ± 0.1 aA	3.45 ± 0.17 aB	4.8 ± 0.14 aA ↑
	0.02 mM	6.87 ± 0.04 ↑	8.05 ± 0.07 ↑	6.3 ± 0.12 bC
	0.2 mM	5.92 ± 0.1 ↗	6.55 ± 0.08 ↑	4.8 ± 0.13 aC
	1 mM	6.01 ± 0.02 ↑	2.02 ± 0.03 dB	1.59 ± 0.13 cC
<i>PdNramp6</i>	0 mM	5.26 ± 0.09 aA	5.78 ± 0.01 aB	5.57 ± 0.11 aAB
	0.02 mM	6.6 ± 0.15 ↑	9.29 ± 0.13 ↑	6.01 ± 0.02 ↑
	0.2 mM	7.92 ± 0.09 ↑	6.79 ± 0.13 ↗	-0.16 ± 0.04 bB
	1 mM	9.09 ± 0.14 ↑	4.07 ± 0.11 dB	2.65 ± 0.92 cC



Dust emission rich in Cd

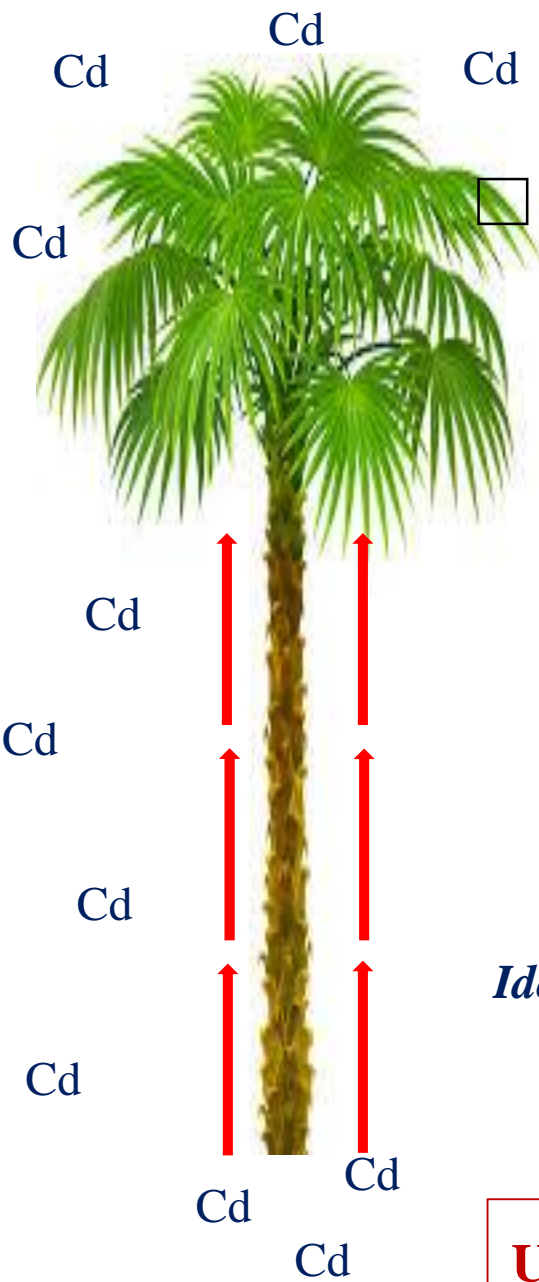


Cd rich waste storage

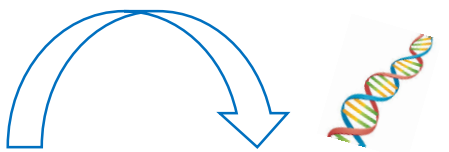
Cd emission



Metallic element release into the environment



Cd absorption



cDNA bank construction

Functional annotation

Cd related genes identification

Identification of the gene pathways responding to Cd

Understanding the acclimation responses to Cd exposure