Protein changes in response to lead stress of lead-tolerant and lead-sensitive industrial hemp using SWATH technology

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Hemp is a Pb-tolerant and Pb-accumulating plant, and the study of its tolerance mechanisms could facilitate the breeding of hemp with enhanced Pb tolerance and accumulation. In the present study, we took advantage of SWATH (sequential window acquisition of all theoretical mass spectra) technology to study the difference in proteomics between the Pb-tolerant seed-type hemp variety Bamahuoma (BM) and the Pb-sensitive fiber-type hemp variety Yunma 1(Y1) under Pb stress (3g/kg soil). A total of 63 and 372 proteins differentially expressed under Pb stress relative to control conditions were identified with liquid chromatography electro spray ionization tandem mass spectrometry in BM and Y1, respectively; with each of these proteins being classified into 14 categories. Hemp adapted to Pb stress through accelerating ATP metabolism; enhancing respiration, light absorption and light energy transfer; promoting assimilation of intercellular nitrogen (N) and carbon (C); eliminating reactive oxygen species; regulating stomatal development and closure; improving exchange of water and CO_2 in leaves; promoting intercellular transport; preventing aggregation of unfolded proteins; degrading misfolded proteins; and increasing the transmembrane transport of ATP in chloroplasts. Our results provide important reference protein and gene information for future molecular studies into the resistance and accumulation of Pb in hemp.

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ABSTRACT

Hemp is a Pb-tolerant and Pb-accumulating plant, and the study of its tolerance mechanisms could facilitate the breeding of hemp with enhanced Pb tolerance and accumulation. In the present study, we took advantage of SWATH (sequential window acquisition of all theoretical mass spectra) technology to study the difference in proteomics between the Pb-tolerant seed-type hemp variety Bamahuoma (BM) and the Pb-sensitive fiber-type hemp variety Yunma 1(Y1) under Pb stress (3g/kg soil). A total of 63 and 372 proteins differentially expressed under Pb stress relative to control conditions were identified with liquid chromatography electro spray ionization tandem mass spectrometry in BM and Y1, respectively; with each of these proteins being classified into

27 14 categories. Hemp adapted to Pb stress through accelerating ATP metabolism; enhancing

28 respiration, light absorption and light energy transfer; promoting assimilation of intercellular

29 nitrogen (N) and carbon (C); eliminating reactive oxygen species; regulating stomatal development

30 and closure; improving exchange of water and CO_2 in leaves; promoting intercellular transport;

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35 ***Keywords*** Pb stress; Industrial hemp (*Cannabis sativa* L.); SWATH; Pb-stress adaption

INTRODUCTION

37 The heavy metal lead (Pb) is a significant threat to human health, agricultural production, and

38 ecological safety. Soil Pb levels have been steadily increasing as a result of human activities, and

39 is now one of the most serious heavy metal pollutants. Pb pollution can persist for more than 300

40 years, with very slow degradation, and it can accumulate in soil and living organisms (Kumar et

41 al. 1995). After absorption by humans, 90% of Pb remains in the skeleton and it can be transferred

42 to the next generation through pregnancy and lactation (Iqbal 2012). Pb has a substantial negative

43 impact on the nervous system, intellectual development, renal function and physical development

44 of infants and children. Mild Pb load can cause neurophysiological damage (Iqbal 2012), but

45 severe Pb toxicity leads to genetic mutations and carcinogenesis, which are irreversible (Michalak

46 & Wierzbicka 1998). Therefore, the remediation of Pb pollution in the environment is one of the

47 priorities of current studies.

48 Pb is not required for normal plant physiology and Pb accumulation is usually toxic. Most

49 plants are susceptible to Pb pollution, and only a few can tolerate Pb accumulation, including

50 members of the Brassicaceae, Caryophyllaceae, Poaceae, Violaceae, and Fabaceae (Cappa &

51 Pilon-Smits 2014; Gallego et al. 2012; Milner & Kochian 2008), but such Pb-accumulators usually

52 exhibit slow growth and low biomass (van der Ent et al. 2013). How to develop Pb-tolerant plants

53 with high economic benefit, large biomass and, rapid growth is an urgent question to be resolved

54   (Ali et al. 2013). One feasible approach isthe development of new plant varieties with high Pb-

55   accumulationcapacity by identifying genes associated with Pb uptake and tolerance using

56   molecular technology and other biotechnologies. Several proteins have been demonstrated to play

57   roles in Pb uptake and tolerance, including NtCBP4 (*Nicotiana tabacum* calmodulin- binding

58   protein) (Arazi et al. 1999), YCF1 (a cadmium factor in yeast) (Song et al. 2003), and TaPCS1

59   (the low-affinity cation transporter 1in *Triticum aestivum*) (Gisbert et al. 2003). Further

60   investigations are needed to identify more related proteins and genes associated with Pb uptake,

61   accumulation or tolerance.

62   Because of its wide distribution, well-developed root system, rapid growth, and high

63   economic value, Industrial hemp (*Cannabis sativa* L.) plays a significant role in the textile,

64   manufacturing, and food industries (Liu et al. 2017). Hemp shows a strong Pb tolerance and

65   accumulation ability, and it is listed as one of the cultivated crops with Pb tolerance, strong

66   adaptability, and high biomass. Hemp can normally germinate in the presence of 1 g/L Pb, where

67   its germination rate is >80%   (Angelova V 2004; Jin et al. 2013; Linger et al. 2002; Xu et al.

68   2014). In addition, hemp can grow in soil with a high level of Pb pollution, and it can tolerate Pb

69   accumulation in all tissues to a level of 26.3 kg/hm^2 (Angelova V 2004; Jin et al. 2013; Linger et

70   al. 2002). The mechanism of Pb uptake, accumulation and tolerance in hemp has not been reported,

71   and the molecular mechanism of Pb tolerance and accumulation is still not clear. Thus, there is a

72   need to investigate the proteins and metabolic pathways associated with Pb tolerance and

73   accumulation. One strategy could be to use proteomics to study the physiological process of

74   adaptation to Pb stress in hemp.

75   In this study, we used high-throughput proteomics technology to analyze the proteins and

76   metabolic pathways, particularly photosynthesis, energy metabolism and stress response, related

77   to Pb stress in two hemp varieties (one Pb tolerant and one Pb sensitive). These data will provide

78   important molecular reference information for future investigations into Pb tolerance and

79   accumulation in hemp.

80

MATERIALS AND METHODS

Cultivation

We used the seed-type hemp variety Bamahuoma (BM) and fiber-type hemp variety Yunma 1 (Y1). While each variety exhibits some Pb tolerance (data not shown), BM exhibits greater tolerance than Y1, so BM and Y1 were referred to as Pb tolerant and Pb sensitive, respectively. Y1 and BM seeds were kindly provided by the Industrial Corps Institute of Yunnan Academy of Agricultural Sciences and the Guangxi Academy of Agricultural Sciences, respectively.

The pot method was used with a pot height of 24 cm, diameter of 18 cm, and holes at the bottom. Each pot was filled with 12 kg bulk material (red soil: humus soil=1: 1 with 17% moisture content) containing no Pb. Two treatments were performed for each variety: Pb stress, in which $Pb(NO_3)_2$ was applied at 3g/kg soil dry weight, and control, without Pb treatment. Soil was mixed well with the Pb, put into the pots, watered and allowed to sit for 1 week before planting. Fifteen replicate pots were prepared for each variety/ treatment combination, and each pot contained six hemp plants. A total of 60 pots were placed in a green house, and normal management practices were applied.

Determination of physiological and biochemical indexes

Obvious Pb-toxicity symptoms were observed in both varieties when the average height of the control plants was ~40 cm on average (about 40d after sowing) (Fig 1). Measurements were carried out on the third to fifth leaves from the plant base using a Li-6400 Portable Photosynthetic System (LI-COR, Lincoln, NE, USA) for photosynthetic parameters and spectrophotometry for physiological indicators [soluble protein content, chlorophyll content, malondialdehyde content (MDA) and superoxide dismutase (SOD)] (Wang 2015). In addition, 2–4g of the third to fifth fully expanded leaves from the bottom was randomly collected from hemp plants undergoing each of the four treatments, with three biological replicates. The12 samples were immediately snap-frozen and stored at −80°C prior to protein extraction.

Protein preparation

Each of the 12 samples was grinded to powder in liquid nitrogen with a mortar and pestle, then

108   dissolved with the lysis buffer(7 M urea, 2M thiourea,4% (w/v) CHAPS, 40 mMTris-HCl, 1 mM

109   phenylmethylsulfonyl fluoride,10mM dithiothreitol) and centrifuged at $25000 \times g$ for 15 min at 4

110   °C. The supernatant was precipitated with four volumes of prechilled 10% trichloroacetic acid

111   (TCA)-acetone at −20 °C overnight and centrifuged at $25000 \times g$ for 30 min at 4 °C. The precipitate

112   was washed with prechilled acetone three times. The protein was dissolved with the lysis buffer,

113   and protein concentration was determined by the Bradford assay (Bevan et al. 1998; Bradford

114   1976; Wisniewski et al. 2009). Protein digestion was performed by the filter-aided sample

115   preparation method with trypsin (Promega, Madison, WI, USA), with 0.1M triethylammonium

116   bicarbonate as the buffer solution.

117   **Fractionation by high pH reverse phase chromatography**

118   For high pH reverse phase chromatography using the LC-20AB HPLC Pump system

119   (Shimadzu, Kyoto, Japan), the SWATH -labeled peptide mixture was reconstituted with 0.1mL

120   buffer A (20mM ammonium formate, 2% acetonitrile (ACN), pH10) and loaded onto a Gemini-

121   NX 5 µ c18 110A 150 x 4.6 mm column (Phenomenex, Guangzhou, China). The peptides were

122   eluted at a flow rate of 1 mL/min with a gradient of buffer A for 10 min, 5-30% buffer B (20mM

123   ammonium formate (FA), 98%ACN, pH10) for 20 min, and 30-80% buffer B for 2 min. Elution

124   was monitored by measuring absorbance at 214 nm, and fractions were collected every 1 min. The

125   eluted peptides were pooled as 6 fractions and vacuum-dried.

126   **LC-ESI-MS/MS analysis**

127   Each fraction was resuspended in buffer A (2% ACN, 0.1%FA) and centrifuged at $20000g$

128   for 10min and was loaded onto nanoLC 425 system (Eksigent part of SCIEX Dublin, CA) with an

129   analytical C18 column (inner diameter 75 µm) packed in-house. The gradient (with a flow rate of

130   300 nL/min) was set up as follows: 5%–20% solvent B for 45 min, 20%–24% solvent B for 10

131   min; 24%–30% solvent B for 15 min; 30%–35% solvent B for 15 min; 35%–90% solvent B for 15

132   min. Data-dependent acquisition (DDA) was performed with a TripleTOF 5600 System (SCIEX,

133   Concord, ON). The source parameters were set up as follows: ion spray voltage at 2.3 kV, curtain

134   gas at 30 PSI, ion source gas at 20psi, and an interface heater temperature of 150°C. Mass

135   spectrometry (MS) was performed in a high-resolution mode (>30 000 fwhm) for TOF (Time of

136   Flight) MS scans. For DDA model, the MS range was 350 to1250 Da with ion accumulation time

137   of 250ms. Tandem MASS spectrometry (MS/MS) data were acquired in 50 ms, and as many as 40

138   product ion scans were collected if they exceeded a threshold of 125 counts per second (counts/s)

139   and with a 2^+ to 4^+ charge-state. Dynamic exclusion was set for 1/2 of peak width (15 s). For

140   SWATH model, the MS range was 350 to 1250 Da with an iron accumulation time of 50ms, with

141   57 fixed windows used at 50ms per SWATH scan with high sensitivity mode with resolution 15K

142   for *m/z* 100–1250.

143   **Library generation and SWATH™ data file process**

144   Data generated by DDA were searched by Protein Pilot 5.0 (AB SCIEX) with the Paragon

145   algorithm against the hemp transcriptome database

146   (http://genome.ccbr.utoronto.ca/downloads.html). A false discovery rate (FDR) analysis was

147   performed. The output group file was used as the reference spectral library. Then SWATH analysis

148   was processed with the SWATH Acquisition MicroApp 2.0 in PeakView® Software (SCIEX,

149   Foster City, CA) with the following parameters: five peptides/protein, five transitions/peptide,

150   peptide confidence level of >90%, FDR < 1%, excluded shared peptides, and Ion Library Mass

151   Tolerance 100 ppm. The quantitation data was normalized with the median. Three biological

152   replicates for each sample were performed for t-test analysis. The protein levels with a *P*-value

153   less than 0.05 and ratio ≥1.5 (increase) or ≤0.67 (decrease) were considered significantly different

154   in our experiment.

155   **RESULTS AND DISCUSSION**

156   **Physiological indicators under Pb stress**

157   The growth stage at which the plants were sampled was selected to coincide with rapid

158   growth. The indicators of Pb stress in BM were more stable than thoseinY1, especially the net

159   photosynthetic rate and MDA level (Table 1), indicating that Pb tolerance of BM was higher than

160   that of Y1 during rapid growth.

161   Pb stress affected all the photosynthetic indicators in hemp. Net photosynthetic rate (Photo),

162   stomatal conductance (Cond) and transpiration rate (Tromol) of Y1 under Pb stress were reduced

163   by 19%, 12.8%, and 14.4%, respectively, compared to the controls, and the decrease in net

164   photosynthetic rate was significant. However, the intracellular CO_2 (Ci) concentration was

165   increased by 5% under Pb stress. In BM, only the net photosynthetic rate was decreased, by

166   4.7%, due to Pb stress, and all other indicators were higher than those in the controls. Among

167   these, the Ci concentration was significantly elevated by 10.2%. Thus, Pb-tolerant BM exhibited

168   a reduction (albeit much less than in Y1) in photosynthetic rate upon Pb treatment, but it could

169   adapt to the stress via increasing the Ci, Cond and Tromol values.

170   The effects of Pb stress on soluble protein content, chlorophyll, SOD, and MDA showed that

171   the first three indicators decreased under Pb stress in both BM and Y1, but the MDA content of

172   Y1 was significantly elevated by 62.3% under Pb stress, whereas only a 4% non-significant

173   increase was observed in BM. MDA is one of the products of membrane lipid peroxidation, so

174   higher MDA suggests greater damage to the membranes. Therefore, it was revealed that Pb stress

175   resulted in greater damage to the membrane system of Y1 than that of BM; that is, the membrane

176   system of Y1 was more susceptible to Pb stress.

177

178   **Protein identification and analysis**

179   A total of 2131 proteins were identified in the SwissProt/UniProt database (S1 table), and 63

180   and 372 were recognized as being differentially expressed ($\geq$ 1.5-fold) under Pb stress compared

181   to the control conditions in BM (S2 table) and Y1(S3 table), respectively. Of these, 39 and 231

182   proteins in BM and Y1, respectively, were upregulated under Pb stress, which accounted for 61.9%

183   and 62.1% of the differentially expressed proteins in BM and Y1, respectively, suggesting that the

184   metabolic status and molecular physiological activities of the Pb-tolerant variety BM were more

185   stable than those of the Pb-sensitive variety Y1 under Pb stress, and the high metabolic activity in

186   Y1 resulted in Pb sensitivity.

187   The different proteins in each cultivar were functionally classified according to the method

188   proposed by Bevan et al. (1998) [20] (Figs 2–3). They were classified into 14 categories: primary

189 metabolism, energy, protein destination and storage, disease/defense, protein synthesis,

190 photosynthesis, transport, transcription, signal transduction, cell structure, secondary metabolism,

191 intracellular traffic, cell growth/division and unknown. Over 67% of proteins associated with

192 protein destination, storage, and transcription were downregulated in the differentially expressed

193 proteins of Y1, whereas >91% of proteins associated with transporters and photosynthesis in this

194 variety were upregulated. In BM, proteins associated with transporters, protein synthesis, energy

195 and secondary metabolism were upregulated under Pb stress, while proteins related to cell

196 growth/division, intracellular traffic and photosynthesis were downregulated (Figs 2–3).

197

Proteins related to energy metabolism

199 Under Pb stress, respiration is increased to provide more energy, and stored biomass is

200 consumed to produce more ATP for adapting to the stress. The ATP/ADP ratio in pea and barley

201 is increased, and their respiratory rates are elevated by >50% under Pb stress (Romanowska et al.

202 2005; Romanowska et al. 2006). We showed that many proteins associated with ATP biosynthesis

203 were upregulated in Y1 under Pb stress, namely ATP synthase subunit a (P56758, P56757), ATP

204 synthase protein MI25 (Q04613), ATP synthase protein YMF19 (P93303), nucleoside diphosphate

205 kinase III (O49203), pyruvate kinase (PKE) (Q94KE3, Q9FNN1, Q9FM97), and adenylate kinase

206 5 (ADK) (Q8VYL1). PKE is the key enzyme in the final step of glycolysis that produces pyruvic

207 acid and ATP. The resulting pyruvic acid then enters the TCA cycle to increase respiration. ADK

208 regulates the ATP content as well as the level of other adenyl phosphates (for example: ADP and

209 AMP) in the cells (Dzeja & Terzic 2009). In addition, two proteins related to the respiratory

210 electron chain, NADH dehydrogenase (ubiquinone) iron–sulfur protein (Q95748, Q42577), were

211 upregulated in Y1.This allows efficient transportation of electrons from NADH to the respiratory

212 chain to enhance respiration (Brandt 2006).

213 Other proteins associated with energy were downregulated in Pb-stressed Y1, namely *β*-1,3-

214 glucanase-like protein(Q9M2T6) for catalyzing polysaccharide degradation; apyrase 1 (APE)

215 (Q9SQG2) for catalyzing hydrolysis of anhydride bonds in nucleoside triphosphate and nucleoside

216 diphosphate; the key enzyme in the sugar degradation pathway, fructose bisphosphate aldolase

217 6(Q9SJQ9), probablyfructokinase-4 (FRK)(Q9M1B9) for decomposition of sucrose and fructose

218 in glycometabolism; and the respiratory electron transporters cytochrome C oxidase subunit 6b-1

219 (Q9S7L9) and cytochrome C oxidase subunit 5b-1 (Q9LW15). APE regulates ATP levels and

220 mediates stomatal closure (Clark et al. 2011; Wu et al. 2007). FRK affects microtubule

221 development and transportation of growth-related carbohydrates, water and minerals (Granot et al.

222 2014), and it is also upregulated under other stresses, such as salt (Zorb et al. 2010), drought (Fulda

223 et al. 2011) and anoxia (Guglielminetti et al. 2006).

224 Two energy-related proteins, aminotransferase 3 (P46644) and aldose 1-epimerase (Q9LVH6)

225 were upregulated in BM. The former is the key enzyme in the amino acid cycle and Calvin cycle,

226 which plays roles in C and N metabolism and energy metabolism to strengthen the connection

227 between photosynthesis and respiration (Granot et al. 2014). The latter is the key enzyme in

228 glycolysis (Bailey et al. 1966).

229 Pb stress induced ATP synthesis in hemp to improve energy metabolism and enhance

230 respiration and its association with photosynthesis, leading to stress adaptation. The inhibition of

231 carbohydrate catabolism and electron transfer of respiration was observed in the Pb-sensitive Y1.

232

233 **Proteins related to photosynthesis**

234 Photosynthesis disorders are one of the first symptoms of abiotic stresses (Chaves et al. 2009;

235 Flexas et al. 2004) and heavy metals have a serious impact on plant photosynthesis, resulting in

236 damage of chloroplast structure, reduction of chlorophyll content, stomatal closure and

237 interference with the electron transfer chain (Sengar et al. 2008; Xiong et al. 2006). Under Pb

238 stress, the net photosynthetic rate and chlorophyll content decreased in both varieties of hemp

239 (Table 1). Many photosynthetic proteins (87%) were upregulated in Pb-stressed Y1, whereas no

240 differentially expressed photosynthetic proteins were identified in the Pb-tolerant BM. This

241 indicates that photosynthesis of Pb-tolerant hemp is stable even under Pb stress, and that Pb-

242 sensitive hemp has the potential to increase photosynthesis, which is consistent with the

243 photosynthetic indicators in BM and Y1. The upregulated photosynthetic proteins in Pb-stressed

244 Y1 mainly consisted of photosystem I-related proteins, such as photosystem I reaction center

245 subunit V (Q9S7N7), Photosystem I reaction center subunit XI (Q9SUI4), etc, and photosystem

246 II-related proteins, such as photosystem II CP47 reaction center protein(P56777), Photosystem II

247 CP43 reaction center protein (Q9SHE8), etc, suggesting that Y1 enhances both photosystems to

248 increase light absorption and transfer (Fromme & Mathis 2004; Loll et al. 2005). In addition, the

249 chlorophyll biosynthesis-related protein, chlorophyll a-b binding protein (e.g. Q9XF88, Q9SHR7,

250 etc), was also upregulated in Y1, as well as the activity of BC1 complex kinase 1(Q8RWG1),

251 which prevents chloroplast light damage and degradation of the photosystem II core. These data

252 indicate that chlorophyll biosynthesis was increased and damage to the photosynthetic system was

253 avoided in Y1 under Pb stress(Huang et al. 2015; Yang et al. 2012). Other than photosynthesis,

254 proteins associated with photorespiration, glycerate dehydrogenase HPR (Q84VW9) and

255 ferredoxin-dependent glutamate synthase 1 (Q9ZNZ7, FDGS), were also upregulated in Y1,

256 suggesting that photorespiration is also enhanced in Y1. FDGS also plays a role in the assimilation

257 and reuse of ammonium ions in photorespiration (Coschigano et al. 1998). Due to the small effect

258 of Pb on photorespiration, enhancement of photorespiration affects C assimilation in plants

259 (Assche & Clijsters 1990).

260   Only two photosynthetic proteins, pyruvate dehydrogenase E1 component subunit β-

261 3(O64688) and ferredoxin-3(Q9ZQG8) were downregulated in Pb-stressed Y1. The former

262 converts pyruvate to acetyl-CoA and CO_2in the Kelvin cycle (Jaimes et al. 2016), while the latter

263 facilitates excitation of chlorophyll by light to accept electrons and electron transfer to ferredoxin:

264 $NADP^+$ oxidoreductase.

265   Photosynthesis and photorespiration were simultaneously strengthened in Y1 under Pb

266 stress, showing potential to increase photosynthesis, but the process of chlorophyll electron

267 transfer is inhibited.

268

269 **Primary and secondary metabolism-related proteins**

270   Under Pb stress, primary and secondary metabolism of hemp is affected, and plants produce

271   metabolites associated with Pb tolerance in order to adapt to the stress. Upregulated primary-

272   metabolism-related proteins accounted for 69% and 57% of the total upregulated proteins in Y1

273   and BM, respectively, while the proportion was 64% and 100% for the secondary-metabolism-

274   related proteins.

275   The abundance of some primary metabolites increased in response to Pb stress in Y1, namely

276   proteins related to starch metabolism [phosphoglucan phosphatase DSP4 (Q9FEB5)], proteins

277   associated with amino acid metabolism [homocysteine S-methyltransferase 1 (Q9SDL7) and

278   asparagine synthetase [glutamine-hydrolyzing] 1 (P49078, APSE)], proteins related to

279   carbohydrate metabolism [β-galactosidase 1 (A7WM73, GTE)], and proteins related to other

280   primary metabolism [pyridoxal phosphate homeostasis protein (F4JVS4, PPHP), alkaline/neutral

281   invertase E (Q9FK88) and nitrate reductase [NADH] 2 (P11035, NRD)]. The carbohydrate-

282   metabolism-related protein APSE enhances the assimilation, allocation and reactivation of N

283   through the phloem (Lam et al. 2003). GTE degrades galactose in the cell wall, thus making the

284   cell wall loose, to facilitate cell expansion (Sampedro et al. 2012). PPHP regulates intracellular

285   stability by synthesizing pyridoxal 5'-phosphate (Leasure et al. 2011). Alkaline/neutral invertase

286   E participates in the C flow between the cytoplasm and plasmids of the leaves, development of the

287   photosynthetic apparatus, and N assimilation during the seedling stage (Tan et al. 2010). NRD

288   plays roles in N absorption and assimilation in plants (Marschner & Marschner 2012).

289   The upregulated proteins in Pb-stressed BM were mainly associated with aspartate catabolism

290   [isoaspartyl peptidase/L-asparaginase 1andextensins (P50287)], and serine metabolism [peptidyl

291   serine α-galactosyltransferase (Q8VYF9)]. We showed that acid phosphatase, which is related to

292   phosphate absorption, was downregulated in Y1. Pb stress always leads to P deficiency, because

293   phosphate precipitation is triggered by Pb, and the downregulation of acid phosphatase, further

294   indicating P deficiency in Y1 under Pb stress (Cheyns et al. 2012). Therefore, metabolism in Y1

295   and BM was enhanced under Pb stress, with Y1 exhibiting improvement of N metabolism,

296   increased intercellular stability and C flow, but P deficiency may also have resulted from Pb stress.

297   Secondary metabolism proteins were all upregulated in BM, but only 69% were upregulated

298   in Y1. The upregulated secondary metabolism proteins in both varieties were all related to

299   synthesis of photosynthetic pigments and chloroplast protection, such as protein lutein deficient 5

300   (Q93VK5), lycopene epsilon cyclase (Q38932), zeta-carotene desaturase (Q38893) and probable

301   carotenoid cleavage dioxygenase 4 (O49675) of Y1, and ferrochelatase-2 (O04921) and protein

302   lutein deficient 5 (Q93VK5) of BM. However, GDSL esterase/lipase (Q9C7N4, Q9LU14),

303   isopentenyl-diphosphate delta-isomerase I (Q38929, IDPD) and membrane steroid-binding protein

304   2 (Q9M2Z4)were downregulated in Pb-stressed Y1, suggesting that the biosynthesis of

305   chlorophyll in Y1 may be suppressed by reduction of IDPD activity (Chang et al. 2013). In

306   addition, the downregulation of GDSL esterase/lipase decreases lipid synthesis, along with

307   membrane steroid-binding protein 2, so it is believed that the cell membrane structural damage in

308   Y1 caused by Pb stress lowers the stress resistant.

309

310   Protein related to disease/defense

311   Pb stress can induce a high level of reactive oxygen species (ROS), such as superoxide anion

312   radicals, hydroxyl radicals, H_2O_2 and alkoxy radicals, which react with cell membranes,

313   organelles, proteins, lipids and nucleotides to cause abnormal cell metabolism and even cell death

314   (Gupta et al. 2009; Yadav 2010). Plant tolerance of Pb is often presented in terms of increased

315   antioxidant capacity, and we found that many antioxidant proteins were upregulated in Pb-stressed

316   Y1 and BM, such as SOUL heme-binding family protein (F4K452), glutathione reductase

317   (P48641, P42770, GHR), linoleate 9S-lipoxygenase 1 (Q06327) and lipoxygenase (P38418,

318   Q9FNX8, LPX), and their upregulation facilitated scavenging of accumulated peroxide. Linoleate

319   9S-lipoxygenase 1 was differentially expressed in both varieties under Pb stress compared to non-

320   stress conditions, with up- and downregulation in BM andY1, respectively. This protein is also

321   involved in the growth and development of plants, in delaying senescence, and in regulation of

322   lateral root growth (Vellosillo et al. 2007). LPX plays an importation role in the jasmonate pathway

323   of induced disease resistance and in the prevention of lipid peroxidation to form ethylene, which

324 may lead to leaf senescence (Bannenberg et al. 2009). GHR mainly prevents conversion of

325 oxidized glutathione to glutathione, leading to reduction of H_2O_2 (Deponte 2013). Both proteins

326 were upregulated in Pb-stressed BM and Y1.

327 Probable carboxylesterase 8 (O64640), β-carbonic anhydrase 1(P27140), and Probable

328 carboxylesterase 8 (O64640), carbonic anhydrase 2(P42737) were upregulated in Pb-stressed Y1

329 and BM, respectively. These proteins are associated with H_2CO_3 metabolism, and catalyze

330 conversion of CO_2 and water to bicarbonate and water, transmitting CO_2 signals to regulate

331 stomatal development and movement, which, in turn, mediates gas exchange between plants and

332 the atmosphere (Marshall et al. 2003; Smith-Harding et al. 2017). The upregulation of these

333 proteins is consistent with the increase of Ci in BM and Y1 under Pb stress (Table 1).

334 Pb can induce stomatal closure, resulting in deficiency of CO_2. Two stomatal-closure-

335 regulating proteins were identified as being differentially expressed in Pb-stressed Y1: bifunctional

336 D-cysteine desulfhydrase/1-aminocyclopropane-1-carboxylate deaminase (F4HYF3), and

337 phospholipase D α1 (Q38882), the upregulation of which facilitates stomatal closure to allow CO_2

338 to enter the leaves (Riemenschneider et al. 2005; Zhang et al. 2012).

339 Under Pb stress, hemp is vulnerable to abiotic stresses due to damage to the cell wall and

340 membrane. Proteins related to fungal pathogen and pest resistance were upregulated in Y1,

341 including acidic endochitinase, basic endochitinase B (P19171), cinnamyl alcohol dehydrogenase

342 8 (Q02972), patatin-like protein 2 (O48723) and tetratricopeptide repeat (TPR)-like superfamily

343 protein (Q94K88). The first three are involved in the repair of the cell membrane structure (Song

344 et al. 2004).

345 Protein plant cadmium resistance 2(Q9LQU4), a protein associated with heavy metal

346 absorption, was upregulated in Y1. This protein is involved in the excretion and long distance

347 transport of zinc and cadmium in plants, and it can transport toxic substances into the xylem to

348 detoxify the epidermal cells (Song et al. 2010), so this protein may play roles in the transportation

349 and detoxification of Pb.

350

Proteins related to protein synthesis and transcription

The differentially expressed proteins associated with protein synthesis were all upregulated in BM, compared with only61% in Y1, indicating that protein synthesis plays a vital role in Pb tolerance. Of these, 34 were annotated as ribosomal proteins, accounting for 71% of the total differentially expressed proteins related to protein synthesis. Ribosomal proteins are mainly involved in the biosynthesis of ribosome proteins, plant metabolism, and multiple stress resistance (Yao et al. 2006).These proteins were associated with tolerance to Pb stress, especially in BM, and such an effect has been previously reported in hemp under salt stress (Cheng et al. 2016).The abundance of proteins related to translation, such as lysine-tRNA ligase (Q9ZPI1), serine-tRNA ligase (Q39230) and eukaryotic translation initiation factor 3 subunit A(Q9LD55)   and subunit F (O04202) was substantially reduced in Y1, but the T10F20.8 protein (Q9LMS7) was upregulated in BM. This indicated that protein synthesis in Y1 was inhibited by Pb stress, affecting cell proliferation and cell cycle progression, which might have been responsible for the weaker Pb tolerance exhibited by Y1.

The transcription proteins of Y1 exhibited a similar expression pattern to translation proteins, and downregulated proteins contributed to 75% of the differentially expressed proteins in this category, but only two transcription-related proteins were reduced in BM. However, two proteins, transcription initiation factor TFIID subunits 9 (Q9SYH2) and TFIID subunit 7 (B9DG24) were upregulated in pb-stressed plants of both BM and Y1, with these transcription factors playing indispensable roles in genetic regulation and tolerance to various abiotic stresses in plants (Nakashima et al. 2009). Thus, the increase in abundance of these two proteins ensures the transcription process in Pb-stressed hemp.

Proteins related to transport and signal transduction

Proteins related to transport were all upregulated in Pb-stressed BM, while only one of 22 transport proteins in Y1 was downregulated, indicating that transport proteins are important for Pb tolerance in hemp. The upregulated proteins in Y1 included five water transport-related aquaporins

378   (e.g. Q06611, P25818, etc), patellin (Q56ZI2, Q56Z59) for transmembrane transport of

379   hydrophobic materials, and proteins associated with transportation of organic compounds,

380   mitochondrial dicarboxylate/tricarboxylate transporter DTC (Q9C5M0), mitochondrial phosphate

381   carrier protein 3 (Q9FMU6), mitochondrial carnitine/acylcarnitine carrier-like protein (Q93XM7),

382   MD-2-related lipid recognition domain-containing protein/ML domain-containing protein

383   (F4J7G5), and ras-related protein RABA2a (O04486), all of which are responsible for the

384   intracellular transport of organic compounds and water to ensure various life processes, including

385   respiration, photosynthesis, photorespiration, N and C assimilation, and growth hormone

386   biosynthesis (Eisenhut et al. 2013; Kaldenhoff & Fischer 2006; Peterman et al. 2004; Picault et al.

387   2002; Zhu et al. 2012). The abundance of the ABC transporter E family member 2 (Q8LPJ4) was

388   increased in Pb-stressed Y1, and it has been shown to participate in the transport of cytotoxic

389   substances, including heavy metals Pb and Cd, which facilitates the detoxification of heavy metals

390   (Peters 2006; Peyro et al. 2015). Nuclear pore complex protein NUP93A (O22224) is responsible

391   for the transport of RNA and ribosomal proteins from the nucleus to the cytoplasm and the

392   transport of signal molecules and lipids into the nucleus. The abundance of this protein was

393   elevated in Pb-stressed BM, which is consistent with upregulation of ribosomal proteins in BM.

394   The abundance of all three differentially expressed proteins related to signal transduction

395   were reduced in Pb-stressed BM, compared with that of two of the three proteins in Y1, suggesting

396   that the signal transduction pathways were compromised in hemp under Pb stress. All the proteins

397   associated with Ca^{2+} signal transduction were downregulated in BM and Y1, such as Probable

398   calcium-binding protein CML13 (Q94AZ4, in Y1), CML27 (Q9LE22, in BM) and calreticulin

399   (e.g. O04151, in Y1) , indicating that Pb stress inhibits signal transmission in cell membranes (Li

400   et al. 2009; Saijo et al. 2009). Abundance of a calcium-sensing receptor (Q9FN48), which is related

401   to stomatal closure and leaf abscission (Wang et al. 2012), was increased in Y1.That is, Y1 adapted

402   to Pb stress by regulating stomatal closure and leaf abscission via this protein, and stomatal

403   conductance was reduced by 12.8% in Y1 compared to the controls (Table 1). Receptor-like

404   protein kinase FERONIA (Q9SCZ4) was downregulated in Y1, which may lead to shortened

405   vegetative growth stage of cells and suppressed root hair growth (Duan et al. 2010; Guo et al.

406   2009).

407

Proteins related to protein destination and storage

409   Over 66% of the differentially expressed proteins related to protein destination and storage

410   were upregulated in Pb-stressed BM, compared with only 33% in Y1. Abundance of the T-

411   complex protein 1 subunit (Q84WV1, P28769) and GrpE protein homolog (Q9XQC7, Q8LB47)

412   increased in BM and decreased in Y1 under Pb stress, indicating that both proteins play important

413   roles in Pb tolerance. These two proteins, as molecular chaperones, protect unfolded proteins from

414   polymerization, and degrade misfolded proteins (Hill & Hemmingsen 2001; Tan et al. 2010). The

415   abundance of other folding-related proteinswere also elevated in Y1, namely $α/β$-hydrolases

416   superfamily protein (F4K2Y3), chaperone protein dnaJ A6 (Q9SJZ7), ATP-dependent Clp

417   protease proteolytic subunit 6 (Q9SAA2), and peptidyl-prolylcis-trans isomerase FKBP16-4

418   (Q9SR70). The abundance of some proteins related to folding was reduced in Pb-stressed Y1, such

419   as chaperonin (e.g. P29197) and 26S proteasome non-ATPase regulatory subunit (e.g. Q9LNU4).

420   The abundance of two photosynthesis-related proteins, protease Do-like 2 (O82261) and serine

421   protease SPPA (Q9C9C0), was upregulated in Pb-stressed Y1. Both proteins are involved in the

422   protection of Photosystem II and degradation of light-damaged proteins, and both exhibited strong

423   light protective effects (Haussuhl et al. 2001; Wetzel et al. 2009), which is consistent with the

424   upregulation of photosynthetic proteins in photosystem II of Y1. The abundance of subtilisin-like

425   protease (Q9LVJ1, O65351) was increased in Pb-stressed BM, and this protease is associated with

426   the plant cell cycle to maintain normal cell growth (Rautengarten et al. 2005). The abundance of

427   three ubiquitin-related proteins was downregulated in Y1 (e.g. Q9SII9). Proteins tagged by

428   ubiquitin are identified and degraded rapidly, and they usually participate in DNA repair and cell-

429   cycle regulation (Pickart 2001), which also might be one of the reasons for the weaker growth of

430   Y1 compared to BM under Pb stress (Fig 1).

431

Proteins related to cell growth/division, cell structure and intracellular traffic

432

433   Only one cell growth/division protein was differentially expressed in Pb-stressed BM, but 16

434   were identified in Y1, with half of them downregulated. FRIGIDA-like protein 4b (Q940H8) was

435   increased in Y1 but reduced in BM. It is involved in flowering regulation (Michaels et al. 2003),

436   and flowering time control protein FY (Q6NLV4) was also upregulated in Y1. These data suggest

437   that the reproductive stage of Y1 is fulfilled ahead of schedule by regulating the time of flowering

438   to avoid Pb stress, and this is consistent with downregulation of the signal transduction protein

439   receptor-like protein kinase FERONIA (Q9SCZ4) in Pb-stressed Y1. The decrease in abundance

440   of two root-growth-related proteins, fasciclin-like arabinogalactanprotein (e.g. O22126) (Johnson

441   et al. 2003) and leucine-rich repeat extensin-like protein 2 (O48809) (Baumberger et al. 2003),

442   and one protein related to the growth of plant root tips and stem apex meristem, endoplasmin

443   homolog (Q9STX5) (Baumberger et al. 2003), indicates that the growth of plant roots and stem

444   tips is inhibited, which may be another reason for the compromised plant height of Y1under Pb

445   stress (Fig 1).

446   Pb cleaves chlorophyll through chlorophyll enzyme activity (Liu et al. 2008), and Protein

447   activity of BC1 complex kinase 1 (Q8RWG1) (Yang et al. 2012) associated with chlorophyll

448   degradation was upregulated in Y1. Such upregulation, along with the decrease of membrane

449   steroid-binding protein 2 (Q9M2Z4), could have reduced the chlorophyll content in Y1 under Pb

450   stress (Table 1), although the abundance of chlorophyll a-b binding protein (e.g. Q9XF88), a

451   chlorophyll biosynthesis-related protein, was elevated. Pb has comparable physical size and

452   oxidation state to other nutrient elements, therefore, it can enter cells by competing for ion

453   channels, resulting in damage of the cell membrane (Alford et al. 2010). Therefore, a protein

454   associated with chloroplast membrane structure, thylakoid luminal 15 kDa protein (O22160) (Friso

455   et al. 2004), was downregulated, as were the tubulin proteins (Q9ASR0) (Verhey & Gaertig 2007)

456   and bifunctional dTDP-4-dehydrorhamnose 3,5-epimerase/ dTDP-4-dehydrorhamnose reductase

457   (Q9LQ04) (Watt et al. 2004), which is related to cell wall structure.

458   Both differentially expressed proteins associated with intracellular traffic in BM were

459 downregulated, yet most of the corresponding proteins in Y1 were upregulated. The abundance of

460 proteins associated with transmembrane carrier protein and chloroplast transmembrane transport,

461 such as protein TIC 21(Q9SHU7), outer envelope membrane protein 7 (Q9SVC4) and outer

462 envelope pore protein 24A (Q1H5C9), was elevated in Y1, which is consistent with upregulation

463 of proteins related to ATP synthesis and photosynthesis in Y1. The abundance of other

464 transmembrane transport proteins, such as ATPase 5, plasma membrane-type(Q9SJB3),

465 transmembrane 9 superfamily member 1(Q940G0) and transmembrane protein, putative (Q94F10)

466 were also increased in Pb-stressed Y1. However, downregulation of peroxisomal membrane

467 protein PEX14 (Q9FXT6) in Y1 suggests that the transmembrane transport of peroxisomes is

468 inhibited (Singh et al. 2009), which may lower the capability of antioxidant and anti-aging

469 activities in Y1. In Pb-stressed BM, the mitochondrial import receptor subunit TOM20-2 (P82873)

470 was downregulated. This protein is responsible for the identification and transport of precursors of

471 mitochondrial synthesis, and promotion of proteins entering the mitochondrial outer membrane

472 (Lister et al. 2007).

CONCLUSIONS

474 We used high-throughput SWATH technology to investigate the tolerance mechanisms of Pb-

475 tolerant BM and Pb-sensitive Y1 under Pb stress. There were 63 and 371differentially expressed

476 proteins in Pb-stressed BM and Y1, respectively, which were classified into 14 categories. The

477 following responsive mechanisms are used by hemp to cope with Pb stress: (1) Increasing ATP

478 biosynthesis; (2) enhancing respiration; (3) strengthening the connection between respiration and

479 photosynthesis;(4)improving photosynthesis; (5) promoting N and C assimilation; (6) eliminating

480 reactive oxygen species; (7) regulating stomatal development and closure to increase air exchange;

481 (8) elevating water transportation; (9) shortening the growth period and controlling flowering time;

482 (10) protecting unfolded proteins from aggregation and degrading misfolded proteins; and (11)

483 boosting the transmembrane transport of ATP and within chloroplasts. Our results provide

484 important reference information on the proteins involved in Pb tolerance in hemp for the future

485   studies.

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Table 1(on next page)

Physiological indicators of two hemp varieties under Pb stress

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Table 1. Physiological indicators of two hemp varieties under Pb stress.

Treatment		Photo(µmol· m^{-2}·s^{-1})	Cond(mm ol·m^{-2}·s^{-1})	Ci (µl·L^{-1})	Tromol (g·m^{-2}· h^{-1})	Chlorophyll (mg/g)	Soluble sugar(mg/g)	SOD (U/g)	MDA (nmol/g)
BM	Pb	14.3± 0.46	0.51± 0.02	342.00± 6.00*	9.54± 0.29	68.31± 0.06	295.14± 0.65	193.12± 5.63	0.98± 0.38
	CK	15.0± 0.50	0.43± 0.09	310.33± 9.87	8.99± 0.32	68.75± 0.03*	304.13± 0.42*	195.00± 1.88	0.94± 0.17
	IR	-4.7%	17.6%	10.2%	6.1%	-0.6%	-3.0%	-1.0%	4.0%
Y1	Pb	14.03± 1.00	0.48± 0.02	322.67± 4.16*	8.24± 0.53	68.51± 0.11	288.47± 1.64	200.62± 3.75	0.86± 0.19*
	CK	17.33± 1.14*	0.55± 0.05	306.00± 2.65	9.74± 0.26*	68.92± 0.10*	292.94± 4.25	203.12± 9.44	0.53± 0.08
	IR	-19.0%	-12.8%	5.4%	-14.4%	-0.6%	-1.5%	-1.2%	62.3%

3   **Note:**

4   The values represent mean±SD of three replicates. *Significant difference at P<0.05. IR=(Pb-CK)/CK×100%.

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Figure 1

Comparison of hemp growth during rapid growth stage of two varieties under Pb stress.

Comparison of hemp growth during rapid growth stage of two varieties under Pb stress. a. Y1 control, b. Y1 Pb treatment, c. BM control, and d. BM Pb treatment. Soil with 3g/kg Pb and without Pb were referred to as Pb stress and control, respectively.
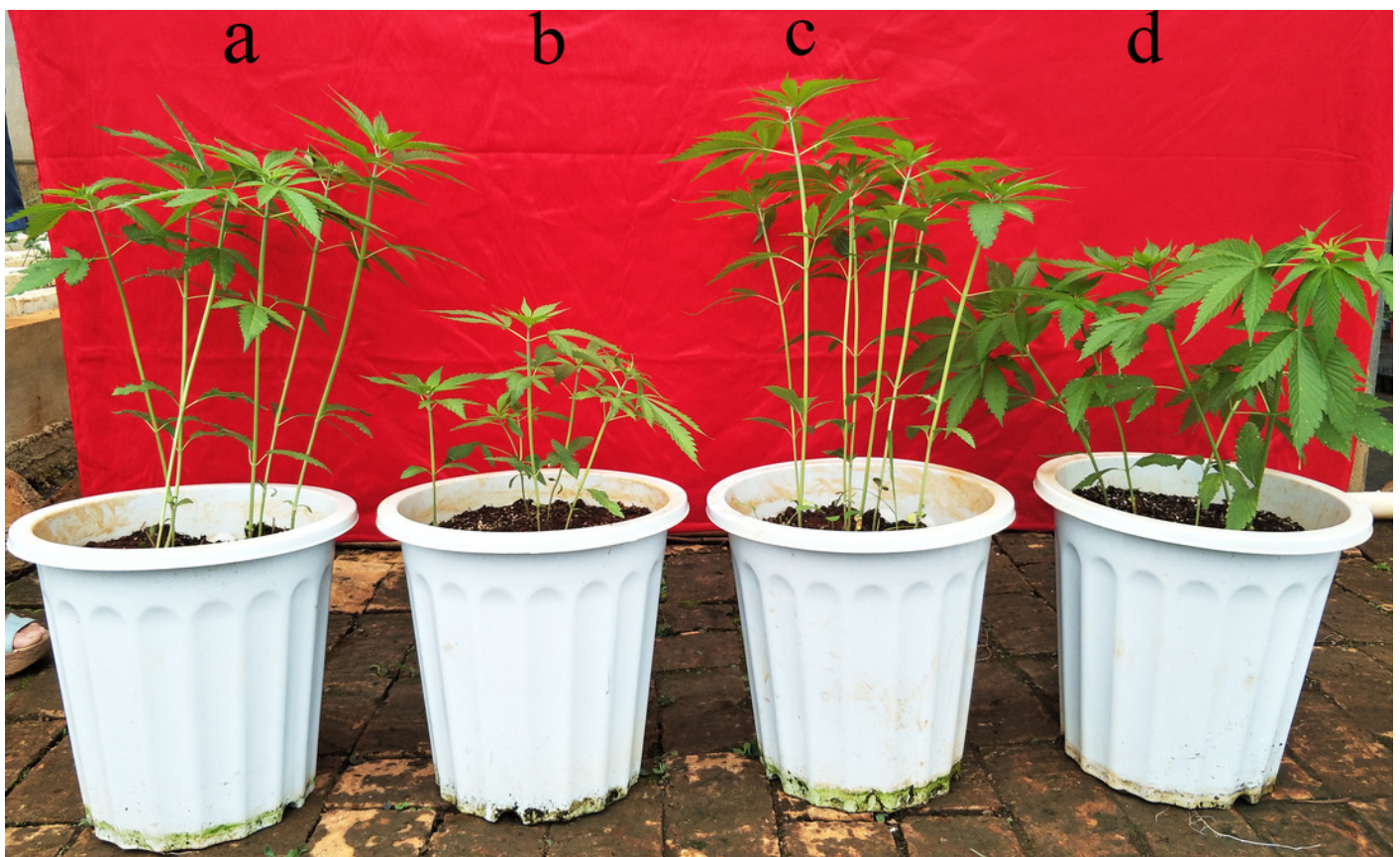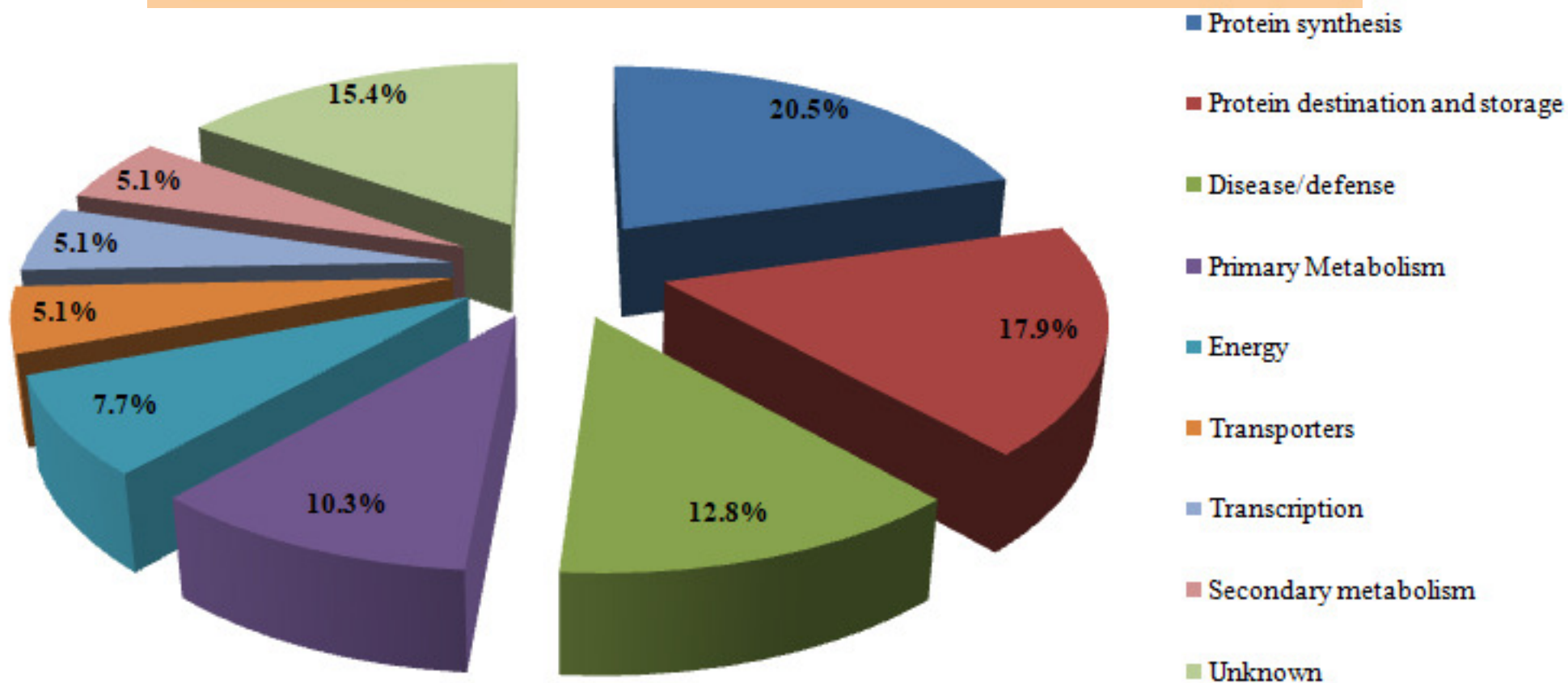
Figure 2(on next page)

Functional classification of differentially expressed proteins upregulated in hemp under Pb stress

Functional classification of differentially expressed proteins upregulated in hemp under Pb stress. a. Proteins upregulated in BM; b. proteins upregulated in Y1.

Legend (panel a):
- Protein synthesis
- Protein destination and storage
- Disease/defense
- Primary Metabolism
- Energy
- Transporters
- Transcription
- Secondary metabolism
- Unknown

Panel a percentages: 15.4%, 5.1%, 5.1%, 5.1%, 7.7%, 10.3%, 12.8%, 17.9%, 20.5%

b

Legend (panel b):
- Photosynthesis
- Disease/defense
- Primary Metabolism
- Protein synthesis
- Transporters
- Energy
- Protein destination and storage
- Intracellular traffic
- Secondary metabolism
- Cell growth/division
- Transcription
- Signal transduction
- Cell structure
- Unknown

Panel b percentages: 2.6%, 1.7%, 3.0%, 3.5%, 3.9%, 4.8%, 5.2%, 6.1%, 9.1%, 9.5%, 8.7%, 10.8%, 13.9%, 17.3%
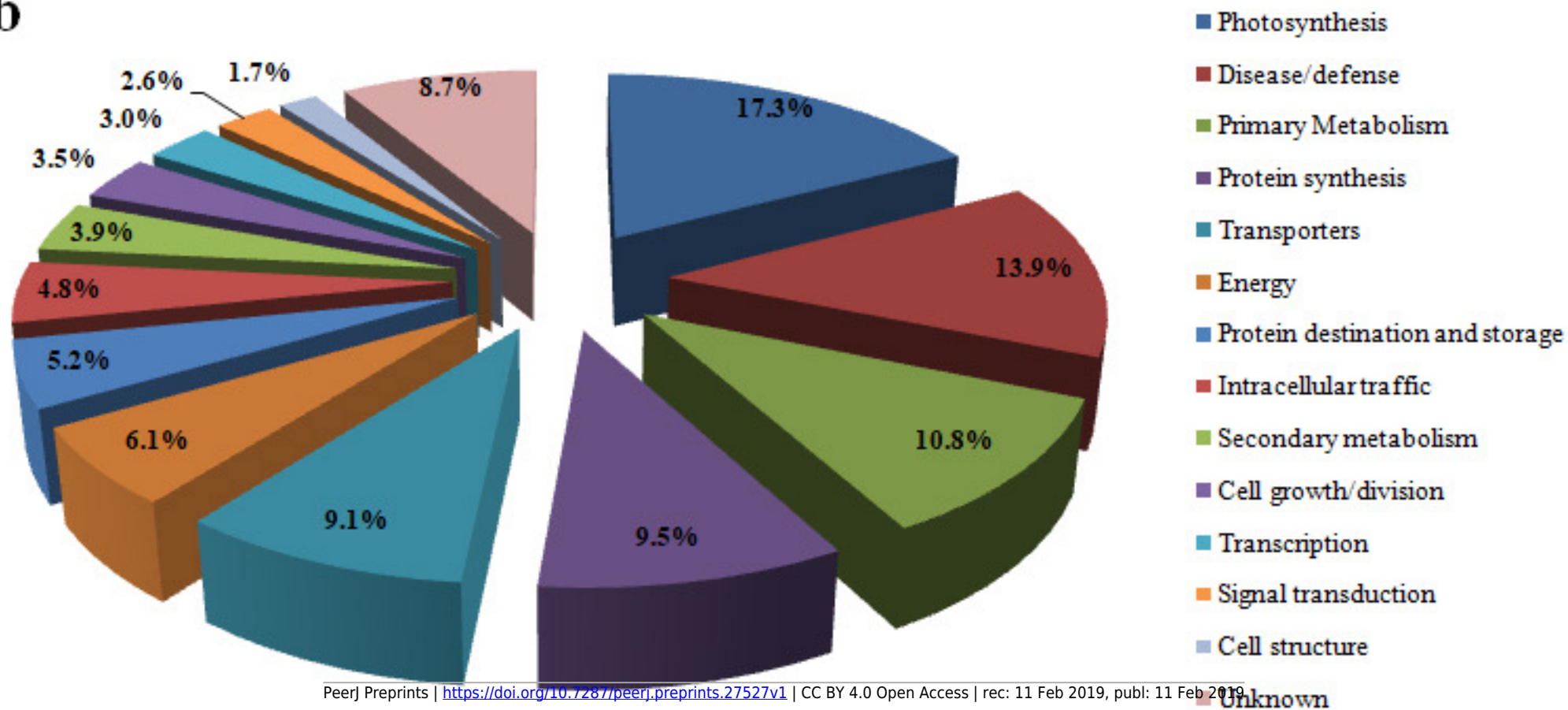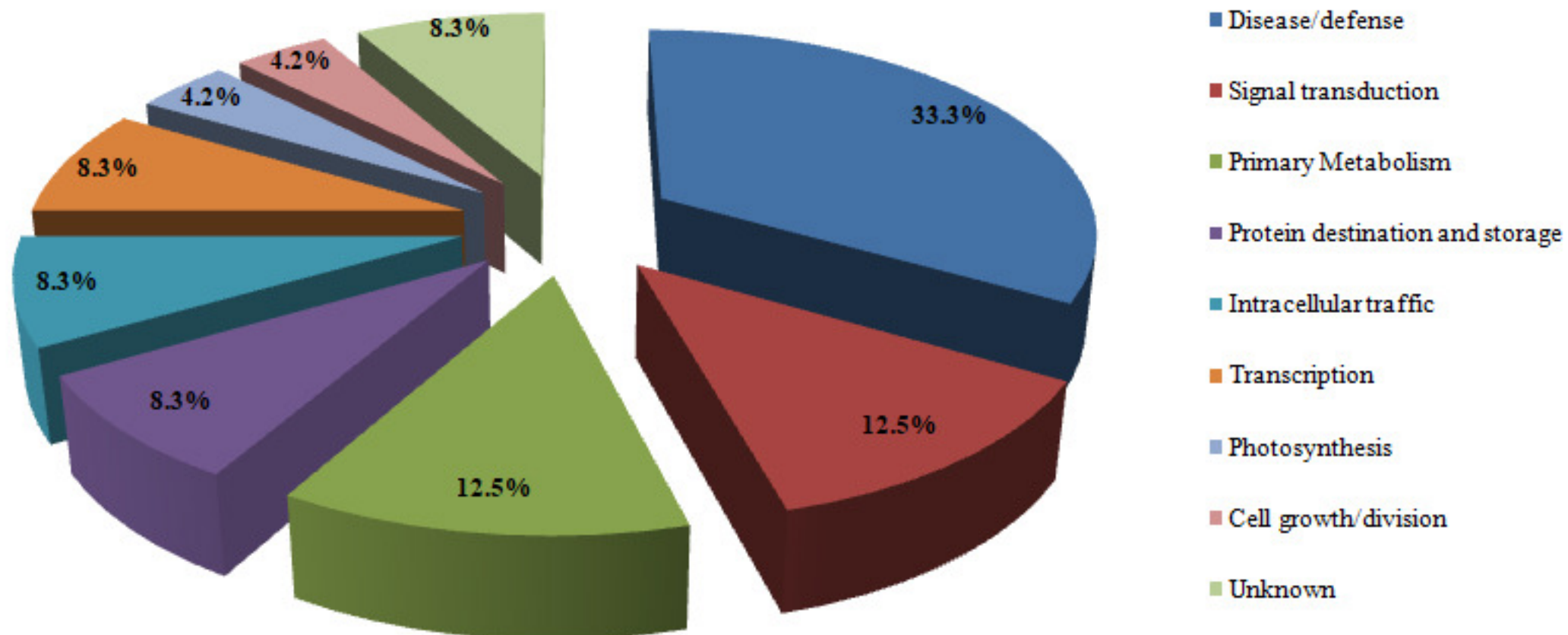
Figure 3(on next page)

Functional classification of differentially expressed proteins downregulated in hemp under Pb stress.

Functional classification of differentially expressed proteins downregulated in hemp under Pb stress. a. Proteins downregulated in BM; b. proteins downregulated in Y1.