Acute metal stress in Populus tremula x P. alba (717-1B4 genotype): leaf and cambial proteome changes induced by Cd2+

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1 **Acute metal stress in *Populus tremula x P. alba (717-1B4 genotype)*: leaf**
2 **and cambial proteome changes induced by Cd^{2+}**
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28 Abbreviations: DAP: DNA-binding Aspartyl Protease / PRP: proline-rich protein / PSII :

29 Photosystem 2.

30 **ABSTRACT**

31 The comprehension of metal homeostasis in plants requires the identification of

32 molecular markers linked to stress tolerance. Proteomic changes in leaves and cambial

33 zone of *Populus tremula x P. alba* (717-1B4 genotype) were analysed after 61 days of

34 exposure to Cd 360 mg.kg^{-1} soil dry weight in pot-soil cultures.

35 The treatment led to an acute Cd stress with a reduction of growth and photosynthesis. Cd

36 stress induced changes in the display of 120 spots for leaf tissue and 153 spots for the

37 cambial zone. It involved a reduced photosynthesis, resulting in a profound

38 reorganisation of carbon and carbohydrate metabolisms in both tissues. Cambial cells

39 underwent stress from the Cd actually present inside the tissue but also a deprivation of

40 photosynthates caused by leaf stress. An important tissue specificity of the response was

41 observed, according to the differences in cell structures and functions.

42   **INTRODUCTION**

43

44   Cadmium (Cd) is regarded as one of the most toxic heavy metals, responsible for human

45   and plant diseases [1,2]. When plant physiology is affected by Cd stress, symptoms are

46   generally chlorosis, necrosis, leaf rolling or drying and growth inhibition [3]. Cd toxicity

47   arises notably from competition with Zn for binding sites in biomolecules, *e.g* enzymes

48   or carriers [4,5].

49   Since a few years *Salicaceae* (*e.g.* willows and poplars) have emerged as promising

50   candidates in the search for convenient species to achieve phytoremediation of polluted

51   sites [6]. Indeed, poplar, whose small genome is now completely sequenced, is a suitable

52   model plant to support molecular studies on stress response's [7,8]. Poplar is reported to be

53   able to thrive despite metal contamination, and to accumulate metals, especially cadmium

54   [9]. Being a perennial species poplar furthermore allows the opportunity to shape an ideal

55   phytoremediator. Year after year accumulation of metals in harvestable woody tissues

56   provides a convenient means of soil clean up combined with the potential of energy

57   production. As a model-tree species poplar can also be used to study the metabolic

58   mechanisms of metal uptake and tolerance in woody species.

59   The plant responses to metal stress are mediated through modifications in gene

60   expression and protein levels. Proteins that participate in metal tolerance response are

61   reported to be involved in the induction of transcription factors, in the protection or

62   restoration of macromolecules and in detoxification activities [10].

63   Metal homeostasis and detoxification are known to be partly constitutive [2], but

64   inducible parts of these processes can be explored by a proteomic approach. Although

65   proteomic research has been conducted on the effects of metals on plants, *e.g.* on the

66   response to Cd in roots of *Oryza sativa* [11], in cell cultures of *Arabidopsis thaliana* [12], in

67   leaves of *Spinacia oleracea* [13] or in roots and shoots of *Thlaspi caerulescens* [14], a

68   recent review by Ahsan, Renaut & Komatsu [15] deplored the limited proteomic

69   investigations on this topic, especially for long-term exposition. Kieffer *et al*. [16,17]

70   recently reported proteome changes in leaves of poplar exposed to Cd up to 56 days

71   under hydroponic conditions.

72   The comprehension of the tolerance of plants to environmental constraints requires a

73   profound description of the acting molecules during the response. Typically, a constraint

3

74   will evoke a stress perception and a stress signalization leading to damage avoidance and

75   repair. For each step of the plant response, some of the involved molecules belong to a

76   generic pattern of response to all biotic/abiotic stresses, while others can be related to one

77   specific stressor. Current research mainly deals with molecules involved in the response

78   to a range of diverse constraining conditions, *e.g.* chaperones, antioxidants, proline, plant

79   growth regulators, etc. [18,19,20,21]. These molecules are responsible for the General

80   Adaptation Syndrome (GAS), initially formulated by Selye in 1951 [22,23]. However, one

81   type of stress will not equally affect the dissimilar tissues within an individual. Published

82   proteomic studies of plant stress most often concern leaf or root tissues. This is graspable

83   considering that the photosynthetic apparatus is often affected by heavy metal toxicity

84   [24], while roots are directly exposed to the contaminated environment. Yet, other tissues

85   can be considered to complement the analysis. In woody species the cambium activity

86   determines the secondary growth that represents a physical support for the extended

87   primary growth of the tree. It also shapes the structure of conductor tissues –number and

88   size of wood vessels and fibers, cellular composition of phloem. Cambium activity

89   eventually influences the production of biomass wherein heavy metals can be

90   accumulated. Despite its importance, the cambium response remains scarcely questioned.

91   Available studies on the cambial tissue generally focus on developmental questions [e.g.

92   25,26] and few deal with biotic stress [27] or abiotic stress [28].

93   A proteomics approach on the responses of plants during exposure to pollutants, *in*

94   *casus* cadmium, will point out the molecular actors supporting metal intake and also give

95   information about stress-related responses in different tissues. This knowledge will allow

96   the selection or the design of plants that prevent deleterious metal accumulation along the

97   food chain (like cadmium), or, on the contrary, crops that could solve problems of human

98   essential metal deficiencies.

99   In this study, young poplar plants were exposed to a soil contaminated by the addition

100   of 360 mg Cd.kg^{-1} soil dry weight (SDW), corresponding to a total amount of 1 mmol of

101   metal per liter of soil. Ecophysiological parameters were monitored all along the 61 days

102   of exposure to characterise the physiological state of the plant. Metal distribution in the

103   tree was also determined. A subsequent proteome analysis on leaf and cambial tissues

104   illustrated the acute stress endured by Cd and its strong effect on photosynthesis and

105   carbon metabolism.

106

107   **2. MATERIAL AND METHODS**

108

109   **2.1. Plant material and metal constraints**

110   Pot-grown *Populus tremula L. x P. alba* L. (*Populus x canescens* (Aiton) Smith)

111   genotype INRA 717-1B4 were obtained as previously described in Durand *et al.* (2009,

112   submitted). Once rooted and 3 months old, poplar plants were pruned and transplanted

113   from 0.3 L pots to 10 L pots filled with a sand - peat moss soil mixture (25:75, v/v, pH

114   6.9). Cadmium was incorporated in the 10 L soils by uniformly hand mixing.

115   Control soil contained no detectable cadmium. Cadmium contaminated soil contained

116   360 mg Cd.kg^{-1} SDW. Soil solution concentrations were determined from 100 g of fresh

117   soil after 20 min of centrifugation at 10,000 g. The supernatant was collected for

118   quantification using a Jobin-Yvon® HR-ICP-AES, as described in [29]. Soil solution of

119   metal-exposed plants contained 20.8 μM Cd^{2+}.

120   Plants were grown in culture chamber at 21°C, 70% of relative humidity, and with an

121   irradiance of 1000 μmol.m^{-2}.s^{-1} provided during 16 h per day. After 61 days of metal

122   exposure, leaf and woody tissues were collected and frozen in liquid nitrogen and stored

123   at -80°C until protein extraction.

124

125   **2.2. Protein extraction and sample preparation**

126   Four biological replicates corresponding each one to individual plants were used.

127   Leaf proteins were extracted starting from 500 mg of fresh weight collected from the

128   three last fully expanded leaves of one plant. After lyophilisation of the cutting, the bark

129   was excised. The cambial zone was then collected by softly scratching the inner face of

130   the bark with a scalpel. Proteins from the cambial zone were extracted from

131   approximately 60 mg of dry tissue of each replicate. Tissues were ground in liquid

132   nitrogen. Proteins were extracted using the TCA-acetone precipitation method described

133   by Damerval *et al* [30]. Resolubilisation of the precipitated proteins was carried out in a

134   lysis buffer (7M urea, 2M thiourea, 4% w/v 3-[(3-cholamidopropyl)-dimethylammonio]-

135   1-propane sulfonate (CHAPS), 30 mM Tris pH 8.5. The protein extracts were quantified

136   using a quantification kit (2D-Quant Kit, GE healthcare) with bovine serum albumin as

137   standard.

138   After quantification, proteins were labelled by mixing 240 pmol of fluorochromes

139   (CyDyes™, GE Healthcare) with 30 µg proteins. For each gel, Cy3 and Cy5 were used

140   for control or treated samples; Cy2 was used for the internal standard consisting of a mix

141   of equal amounts of each sample. A dye swap was used between Cy3 and Cy5 to avoid

142   problems associated with preferential labelling.

143

144   **2.3. Protein separation and relative quantification (2-D DIGE)**

145   Bidimensional electrophoresis was carried out as described in Bohler *et al.* [31], with

146   slight modifications. The isoelectric focusing was carried out on pH 3-10 IPG-strips (24

147   cm, non linear gradient; GE Healthcare, Little Chalfont, UK) using the IPGphor system

148   from GE Healthcare. Protein samples were cup loaded. The migration was performed at

149   20°C ($\leq$ 50 µA, 60V for 2 h; gradient from 60 V to 1,000 V for 3h ; hold 1,000 for 1h,

150   gradient from 1,000 to 8,000 for 3 h; hold 8,000 V until 85,000 V.h). Strips were then

151   stored at -20°C.

152   Prior to second dimension migration, the strips were equilibrated. During

153   equilibration, proteins were reduced by 1% DTT for 15 min and then alkylated by 2.5%

154   (w/v) iodoacetamide for 15 min. The SDS-PAGE was carried out on 12.5% (w/v) of

155   acrylamide-bisacrylamide (37.5/1) gels. Proteins were separated by applying 1.5 W per

156   gel for 20 min, and then 2 W per gel until the migration front reached the end of the gel.

157   After migration and fixing of proteins in the gel, images were captured using a Typhoon

158   Variable Mode Imager 9400 (GE Healthcare).

159   DeCyder v.6.05.11 software (GE Healthcare) was used to determine differentially

160   expressed proteins with a variation factor of at least 1.3 in abundance (up and down) and

161   a significant Student t-test score ($p<0.05$). The automated matching was manually

162   confirmed for all the spots that were selected for identification, and that are further

163   discussed. Spots were picked from the gel and digested by trypsin for 6 h at 37°C using

164   an Ettan Dalt Spot Handling Workstation (GE Healthcare) before acquisition of peptide

165   mass spectra with a MALDI-TOF-TOF analyser (4800 Applied Biosystems, Foster City,

166   CA, USA).

167

168   **2.4. Protein identification.**

169   As the poplar genome is not yet completely annotated and many sequences were only

170   available as ESTs, protein identification was mainly done using the EST-database on an

171   in-house mascot platform. The NCBI poplar EST database used in this study contains

172   419,944 poplar sequences and was downloaded from the NCBI database on 06/11/2009.

173   MS and MS/MS data were also submitted for analysis with a viridiplantae protein

174   database downloaded on 02/17/2009 and containing 1,214,000 sequences. All searches

175   were carried out using a mass window of 100 ppm for the precursor and 0.5 Da for the

176   MS/MS fragments. Up to 2 trypsin miscleavages were accepted. The search parameters

177   allowed for carbamidomethylation of cysteine, oxidation of methionine as well as

178   oxidation of tryptophan to kynurenine and double oxidation of tryptophan to N-

179   formylkynurenine. During automatic interrogation of databases, the MASCOT score cut-

180   off was 77 for NCBI and 65 for EST interrogations. Because all spectra and

181   identifications were manually verified, peptide scores were considered even below the

182   cut-off values for peptide scores. Identifications were validated manually with at least

183   two identified peptides at disparate sites within a protein with a score above homology.

184   Percentage of sequence coverage was given in Tables 1 & 2. Since EST's generally do

185   not represent an entire protein sequence, the authors would like to remark that this value

186   has little significance when identifications were done on EST sequences. Grouping of

187   proteins in biological process was done accordingly to KEGG and Uniprot databases

188   (http://www.genome.jp/kegg ; http://www.uniprot.org/). The functions of some proteins

189   were enlightened by the use of InterPro [32] (http://www.ebi.ac.uk/interpro/) which

190   consists in blasting the amino sequences to the integrated resources of several databases

191   like PANTHER, PROSITE and Gene3D.

192   In leaf and cambial proteomes, respectively in 2 and 7 spots, more than 1 protein was

193   identified, since this prevents any interpretation about the actual abundance of change in

194   them, biological discussion was not done on these proteins. They are nonetheless

195   presented at the end of Tables 1 & 2.

196

2.5. Statistics

198 Statistical analysis was carried out with 4 biological replicates. The variable used for
199 comparison was the log10 of standardized volume of protein spots in the gels. A Student
200 t-test analysis was carried out in the DeCyder software. The results based on the log10 of
201 standardised volume of protein spots in the gels were provided by the DeCyder software
202 by Student t-test analysis. A false discovery rate correction was applied in the software .

203

3. RESULTS

3.1. Leaf proteome profiles.

206 The 2D electrophoretic leaf pattern presented 1,023 spots (Figure 1). Among them,
207 120 spots exhibited a significant absolute variation greater than 1.3-fold between control
208 and treated conditions ($p<0.05$). The Cd treatment resulted in an increased abundance for
209 40 protein spots, and a decreased abundance for 80 protein spots. The MS analysis of
210 these spots resulted in the confident identification of 103 proteins, of which 2 were a mix
211 of at least two proteins (Table 1).

212 Briefly, the significant changes in protein abundance occurred in the following
213 functions (Table 4): photosynthesis (44 protein spots), carbon and carbohydrate
214 metabolism (21), energy metabolism (9), proteins metabolism, catabolism and folding
215 (9), citrate cycle (5), oxidoreduction (6) and glutathione metabolism (2). Other functions
216 that were influenced include an inositol-3-phosphate synthase (spot 572), a potassium
217 channel beta subunit (spot 1300), an auxin-binding protein ABP19a precursor (spot
218 1756), an alpha tubilin (spot 1192) and a translation elongation factor (spot 225).

219 When the same protein was identified in several spots, as is for instance the case for
220 Ferredoxin-NADP oxidoreductase in spot 1340, 1332, 1342, 1338, 1365 and 1359, the
221 MS spectra were compared in order to find posttranslational regulatory events. For none
222 of the proteins for which this comparison was done supplementary information was
223 obtained.

224

3.2. Cambium proteome profiles.

226   The 2D electrophoretic cambium pattern presented 1,144 spots (Figure 2). Among
227   them, 153 exhibited a significant absolute variation greater than 1.3-fold between control
228   and treated conditions (p<0.05). Compared to control conditions, the Cd treatment
229   resulted in the increased intensity of 47 proteins spots, while 106 spots were less intense.
230   The MS analysis of the differential spots resulted in the confident identification of the
231   proteins in 108 spots among which 7 presented a co-migration of more than 1 protein
232   (Table 2). The remaining 30% of the selected spots are still unidentified because of the
233   weak intensity of the spectra resulting from faintly stained spots. Furthermore, few ESTs
234   corresponding to cambial tissue are available.

235   Among the cambial proteins, 8 spots were identified as 'aspartyl protease family
236   protein' (spots number 398, 913, 996, 1140, 1146, 1157, 1488 and 1495), and 8 others as
237   'nucleoid DNA-binding protein cnd41-like protein' (spots 1106, 1115, 1142, 1148, 1149,
238   1169, 1177 and 1206). As these two groups of proteins exhibited common MS/MS
239   peptides, like 'TYTIVFDGAKER' or "ITASDYIVNVGIGTPKK", an InterProscan
240   interrogation was realised. Both of them designated proteins that possess a chloroplast
241   nucleoid DNA-binding domain as well as an aspartyl peptidase domain. Therefore, they
242   were gathered in the Tables 2 and 4 under the label "DNA-binding aspartyl peptidase".

243   Briefly, the significant changes in protein abundance specifically induced by Cd
244   treatment occurred in the following functions (Table 4): carbon and carbohydrate
245   metabolisms (22), cytoskeleton & cell wall (18), DNA binding peptidase (16), protein
246   metabolism, catabolism and folding (16), ,transcription and translation factors (8),
247   antioxidation and stress-related functions (6), lipid metabolism (5), the citrate cycle (3),
248   and others functions among which a cupin family protein (spot 1179), two beta-subunits
249   of K^+ channels (spot 1375, 1376) and two putative adenosine kinase (spots 1129 and
250   1135) were down-regulated in Cd-treated poplars whereas a Phi-1 (spot 1492), an
251   eukaryotic initiation factor 4A (spot 880) and a DEAD box RNA helicase (spot 877) were
252   up-regulated in Cd-treated poplars.

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254

255   **4. Discussion.**

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256   Young *Populus tremula x P. alba* 717-1B4 genotype plants were exposed to 360

257   mg Cd.kg^{-1} SDW, this resulted a in soil solution concentrations of 20.8 μM Cd^{2+}. An in-

258   soil experiment permitted to follow the dynamic ion partition between soil and plant,

259   which may be a determining factor in the response of the plant [33,34]. The study aimed at

260   analyzing tissues that have grown and developed under metal constraints. This allowed to

261   focus on distal equilibrium rather than on the alarm phase of stress. Thus, control and

262   stressed plants were compared after 61 days of cadmium exposure.

263   The Cd treatment resulted in a Cd accumulation in the leaves (84 mg Cd.kg^{-1}) and in

264   the cambial zone (123 mg Cd.kg^{-1}). The treatment drastically reduced the growth of the

265   plants. Net photosynthesis and stomatal conductance were inhibited. Increases in the

266   content of K$^+$, Ca^{2+}, Mg^{2+} and Zn^{2+} in tissues, especially in leaves, were observed. These

267   results are summarized in the Table 3 (adapted from [29]). The physiological data

268   demonstrate that Cd^{2+} triggered an acute stress in poplar plants from which ensues

269   important changes in leaf and cambial proteome (Figures 1 & 2).

270

4.1. Leaf proteome response to Cd

272   The proteomic data gave two levels of information that can hardly be distinguished.

273   Some changes occurred as a consequence of the stress endured, while others constitute

274   the concrete and active response of the plant defense system. The Impact Factor (IF) on

275   protein abundance was calculated as the ratio of the volume of protein spots between

276   treated and control conditions.

277   In leaf and cambial tissues the cadmium stress mainly affected the carbon metabolism.

278   In the leaf, negative IF was salient on the carbon fixation, especially on ribulose-1,5-

279   bisphosphate carboxylase/oxygenase (RuBisCO) and RuBisCO activase. Other proteins

280   involved in the Calvin Cycle were also affected (spots 284, 1116, 1120, 1124, 1127,

281   1566, 1571). Additionally 3 out of 4 spots wherein carbonic anhydrase was identified

282   decreased in intensity in Cd-treated plants (spots 1592, 1598, 1616, 1660).

283   These data are consistent with our previous results, which describe the inhibition of

284   CO$_2$ assimilation under Cd stress, which was mainly unrelated to stomata closure [29]. The

285   literature diversely reports on the stomatal response to Cd stress, although stomata

286   closure is often regarded as a typical response [35,36,37]. Shi and Cai [38] observed a Cd-

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287   induced xerophytes-like leaf morphology with more frequent but smaller stomata,

288   accompanied by a reduced stomatal conductance in peanut (*Arachis hypogaea*). On the

289   contrary Zhu, Macfie & Ding [39] reported that Cd-exposed *Brassica juncea* showed

290   fewer although larger stomata. In both cases, the reduced stomatal aperture only partly

291   explained the decreased photosynthetic activity.

292   Whatever the stomatal response, the Cd toxicity mostly arises through other processes

293   than gas exchange. In this study, the strongest negative influence of Cd on spot intensity

294   was observed for photosynthesis-related proteins. This reduced intensity was particularly

295   observed for proteins implied in the electron transport: chlorophyll a/b binding proteins

296   (10 spots down regulated, with IF from -2.08, spot 1584, to -11.02, spot 1517, Table 1),

297   Oxygen Evolving Enhancer (5OEE1 spots and 5 OEE2 spots, *i.e* 33 kDa and 23 kDa

298   subunits respectively, all down regulated, with IF from -2.75, spot 1456, to -7.03, spot

299   1441) and ferredoxin-NADP oxidoreductase (5 spots down-regulated, *e.g.* spot 1340, 2

300   other up regulated, *e.g.* spot 1338). The decrease of the photosynthetic activity, inferred

301   from the diminished CO_2 assimilation (Table 4) is further suggested by the drop in

302   abundance of a FtsH protease 8 which contributes to the turnover of the Photosystem II

303   D1 protein [40]. These results incline to show a major impact on proteins of the PSII,

304   which is contradictory with a recent study on spinach where PSI was described as more

305   sensitive to Cd^{2+} [4]. Among the 5 ATPase spots differentially expressed, 3 were down-

306   regulated (spots 620, 632 and 633), all of them chloroplastic ATPase. The 2 up-regulated

307   ATPase are localized in the mitochondrion, according to the databases (spots 594 & 599).

308   This suggests that photosynthetic processes are inhibited by Cd stress, while respiration,

309   on the contrary, is promoted. Supporting the latter, the increase in quantity of a succinate

310   dehydrogenase spot was recorded (spot 418). This enzyme takes part in the mitochondrial

311   respiratory chain. Changes in the abundance of TCA cycle enzymes, increased

312   accumulation of citrate synthase (spots 902, 903) and decreased abundance of malate

313   dehydrogenase (spots 1200, 1316), do not result in a consensus on how the respiration is

314   affected during Cd stress, but confirm that there is an effect. Consistently with an

315   increased respiration concomitant to a suppressed photosynthesis, an induction of

316   glycolysis-involved enzymes was observed: UDP-glucose pyrophosphorylase (spot 597),

317   glycosyl hydrolase family 38 protein (spot 553), 3-phosphoglycerate kinase (spot 1051).

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318   Such a relation between suppressed photosynthesis and stimulated respiration from
319   glucose catabolism in leaves was previously reported under Cd stress [41], ozone stress
320   [42], drought and heat stress [43].

321   Beside the inhibition of the carbon and carbohydrate metabolism, the analysis of the
322   leaf proteome gives clues of an inhibited protein metabolism. The proteome change
323   patterns suggest reduced protein synthesis (aminomethyltransferase, spot 1290, and
324   translation elongation factor EF-G, spot 225, inhibited), reduced protein transport (2
325   nascent polypeptide associated complex alpha chain inhibited, spots 1518 and 1752) and
326   reduced protein degradation (proteasome subunit PRGB inhibited, spot 1678).
327   Modifications of protein processes are often a way to avoid damages or to ensure
328   replacement of damaged molecules. Nevertheless, in this case, the observed changes
329   rather resulted from supply shortage due to a reduced primary metabolism in a leaf tissue
330   whose growth was heavily inhibited. Interestingly the strongest IF in the leaf proteome
331   was that of a phosphoglycerate dehydrogenase (+ 36.7, spot 548). This enzyme, involved
332   in the synthesis of several amino acids like glycine, serine or threonine, might also play a
333   role in the glutathione production.

334   Changes in the glutathione metabolism are widely reported to be part of most plant
335   response to Cd [44,45,46,16]. Direct measurement of this metabolism was not performed, but
336   indirect hints may nonetheless be considered. Two glutathione-S-transferase were up-
337   regulated (spot 1659 and 1667), as was a formate dehydrogenase (spot 1061); its role is
338   the production of reducing power (NADH). A GDP-mannose 3,5-epimerase/ NAD
339   binding protein (spot 936), implied in the ascorbate synthesis, was up-regulated.
340   Ascorbate and glutathione are coordinated in the cell by the Halliwell-Asada Cycle.
341   Interestingly, the strongly induced (IF+16.7, spot 1637) 1-cys peroxiredoxin is a
342   bifunctional enzyme with phospholipase A2 and glutathione peroxidase activities [47].
343   This denotes, as expected, an impact of Cd on the redox status of the cell, as confirmed
344   by the abundance of changes in a type 2 peroxiredoxin (IF-2.09, spot 1821). This impact
345   could explain the changes of protein folding-involved HSP 70 (IF-1.59, spot 1011, and
346   +1.6, spot 357) and chloroplast chaperonin 21 (IF-2.23, spot 1635). This could also
347   indicate an impact of Cd on the integrity of membranes, since one inositol-3-phosphate
348   synthase (IF-1.99, spot 572) is negatively affected. These changes, as well as the decrease

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349 of an alpha tubulin (IF-2.94, spot 1192) might also be connected to the leaf growth

350 inhibition in spite of a sampling realized on fully expanded leaves. Indeed, the Cd-treated

351 sampled leaves presented a significantly smaller size than control.

352 A stimulated potassium channel beta subunit (IF+1.6, spot 1300) converged with

353 previous results showing a significant alteration of potassium homeostasis under Cd

354 stress [29]. The $[K^+]_{leaves}$ increased 2.3-fold compared to control plants (Table 4). In the

355 cambial zone, on the contrary, two beta-subunit of K^+ channels decreased in quantity;

356 linked to the 48% reduced $[K^+]_{cutting}$. Only few reports on the regulation of mineral

357 homeostasis under Cd stress are available in the literature. A recently published paper

358 indicated no change in K^+ and Mg^{2+} content in leaves of Cd-tolerant mungbean (*Vigna*

359 *radiata*) under Cd exposure [48]. To our knowledge neither the mobilization of K^+ in

360 leaves in response to Cd, nor the implication of a potassium channel were reported so far.

361 It is not surprising that Cd disturbs metal homeostasis, even if proteomic data give

362 little indication on it. For instance the 2-fold increase of $[Mg^{2+}]_{leaves}$ (Table 4) can be

363 linked to the instability of photosynthesis proteins like the rubisco activase that often

364 have Mg^{2+} as cofactor. The increased $[Mg^{2+}]$ could be needed to compete with the toxic

365 Cd^{2+} in order to reduce proteins misfolding and inactivation.

366 The comparison of these results with those from a previous hydroponic poplar

367 exposure to Cd [17,16] exhibits some common traits that were underlined above, but also

368 some differences. Hydroponic and soil conditions partly affect plant physiology in

369 differential ways, notably metal bioavailability. This could account for a part of these

370 differences in the proteomic response.

371 For instance, a chitinase (spot 1552) was the sole identified pathogenesis-related (PR)

372 protein affected by Cd in leaves in this study, whereas this type of proteins was shown to

373 accumulate, especially on the long term (day 56), in Kieffer's articles [17,16]. Cd-

374 responsive proteins implied in oxidoreduction were more numerous in leaf than in

375 cambial zone, with an overall negative impact of the stress. When comparing with

376 exposure to Cd in a hydroponic system, it appears that these protein functions are more

377 responsive on a shorter time scale (7-14 days) than on 56 days.

378

379 **4.2. Cambial proteome response to Cd**

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380   As in leaf, the carbon metabolism is the principal biological process altered in cambial

381   proteome in response to Cd. The differentially expressed proteins are partly

382   corresponding to the same functions as in leaf (*e.g.* 3 malate dehydrogenase, spots 1268,

383   1290, 1336 ; 1 triosphosphate isomerase, spot 759, and 1 UDP glucose pyrophospho-

384   rylase, spot 752, all of them were inhibited in the cambial zone). In addition to these

385   proteins, the overall changes linked with carbohydrate metabolism indicate an impact on

386   glycolysis and glycogenesis. Five spots of enolase were down-regulated (e.g. spot 784,

387   798, 800, 802, 816). Three spots of UDP glucose dehydrogenase (spots 744, 752, 809),

388   one phosphofructokinase family protein (spot 851), one 2,3-bisphosphoglycerate-

389   independent phosphoglycerate mutase (spot 526) and one fructokinase (spot 1202)

390   decreased, whereas 2 alcohol dehydrogenase (spots 1069 & 1070) increased in quantity.

391   Two spots of alpha-D-xylosidase (spots 288 & 1892) were more intensively stained. This

392   enzyme is reported to be analogous to alpha glucosidase [49]. Hence the overall

393   carbohydrate metabolism was reduced.

394   In cambial zone as in the leaf, the great disturbance in the carbohydrate metabolism is

395   obviously a distal consequence of Cd stress. A previous work on poplar response to Cd

396   showed changes in sugars content [17]. Such changes, beyond stress symptomatology,

397   probably take part in the sensing and signaling of the constraint [50] even if the alarm

398   phase is very likely to have ceased before day 61. Most sugars are known to improve

399   osmoprotection during stress ; this is a role they could also play in the metal stress [51].

400   The protein metabolism is the second biological process influenced by Cd stress in the

401   cambial zone. Protein synthesis was reduced, as attested by the decreased abundance of 4

402   spots containing methionine synthase (spots 381, 383, 385, 391), 3 spots containing S-

403   adenosylmethionine synthetase (spots 881, 885, 891), an O-acetylserine (thiol)lyase (spot

404   1240), and a 60S acidic ribosomal protein P0 (spot 1302). There was also one

405   differentially displayed spot of eukaryotic initiation factor 4A (spot 902), and 3 others of

406   elongation factor 1-γ (spots 332, 892, 911). One calreticulin-1 (spot 715) and 3 protein

407   disulfide-isomerase precursors (spots 537, 541, 1161), related to protein folding exhibited

408   a reduced abundance. Proteolysis was also affected, with the decreased abundance of two

409   proteasome subunits (spots 896 & 1555) and of a Zn-dependent leucine aminopeptidase

410   (spot 796), and also 2 more abundant alpha-mannosidase (spots 390 and 433) involved in

411   the vacuolar degradation of glycoproteins. So protein metabolism, folding and catabolism

412   appeared to be impaired by Cd treatment.

413   Cd-stress also induced changes in proteins involved in cell structure and cell wall,

414   hence related to the growth function of the meristem. Two spots of rhamnose

415   biosynthesis 1 (spots 480, 487) were less abundant, one was more abundant (spot 481);

416   this enzyme participates to the production of rhamnose, an important constituent of the

417   cell wall [52]. Also in relation with cell wall synthesis, 3 spots of phenylcoumaran

418   benzylic ether reductase dropped in intensity (spots 1319, 1323, 1340). This enzyme is

419   strongly associated with phenylpropanoid biosynthesis in lignifying cells of poplar [53].

420   One cinnamoyl-CoA reductase (spot 1324), involved in the synthesis of lignin and a

421   leucine rich repeat protein (spot 1317), also implied in cell wall synthesis [54] decreased in

422   amount. Two spots of reversibly glycosylated polypeptides (spots 1168 & 1187) dropped

423   in intensity; these proteins, supposed to be plasmodesmata-associated [55] are linked to

424   cell elongation and related to cell wall formation, even if their precise role in the cell

425   remains unsolved [56]. Consistently with an inhibition of growth, 2 spots containing

426   adenosine kinase (spots 1129 & 1135), implied in nucleic acid synthesis, exhibited lower

427   content.

428   According to Garnier *et al.* [57], Cd cytotoxicity arises notably through three waves of

429   oxidative stress in tobacco cells. In the present study, in cambial tissue as it was the case

430   in leaves, the redox balance appears to be disturbed. A class III peroxidase (spot 1137)

431   was more abundant. Two 6-phosphogluconate dehydrogenase family proteins (spots 908

432   & 909), involved in the glutathione metabolism, decreased in amount, whereas a

433   glutathione-S-transferase was more abundant (spot 1657). The lower accumulation of a

434   glutamate decarboxylase (spot 796) was observed, but a putative leucine aminopeptidase

435   was identified in the same spot, preventing any interpretation concerning this calmodulin-

436   binding protein that produces GABA (γ-aminobutyric acid).

437

438   The suppression of cytoskeletal-linked proteins such as α and β chain tubulin (7 spots

439   down regulated, e.g. spot 772) or actin (spots 977, 990) in cadmium exposed poplar

440   confirms the main role of cambium as an actively growing tissue, and the different

441   growth rates between control and exposed plants. Two spots of glycine-rich RNA-

442 binding protein (spots 1836 & 1838) appeared differentially expressed. This protein

443 family of post-transcriptional regulators has been showed playing a role in cold

444 acclimation of *Arabidopsis* [58]. Cd induced antagonistic changes in 2 putative proline-

445 rich protein (PRP) (spots 926 & 1057). These cell wall structural proteins are reported to

446 accumulate under water deficit in *Phaseolus vulgaris* cells [59]. When the respective EST

447 sequences for these spots were used in BLAST analysis, the results indicated that they

448 contain a "GDSL" motif close to the N-terminus. In addition 2 GDSL-motif

449 lipase/hydrolase family protein spots decreased in amount (spots 572 & 576). These

450 proteins could have a role in the degradation of complex polysaccharides [60,61]. The

451 GDSL family has been repeatedly reported to be implied in diverse stress responses [62].

452 Among the 159 Cd-affected proteins in the cambial zone, 7 have Zn as cofactor (spots

453 796, 1069, 1070, 537, 541, 1161, 715), and 4 others potentially binds Zn (1302, 1836,

454 1838, 1179). All of them, except the 2 alcohol dehydrogenase and one of the 2 glycine-

455 rich RNA-binding protein were reduced in quantity. In the leaf proteome 7 out of 114

456 identified Cd-affected proteins needs Zn for functioning (spots : 435, 1548, 1563, 1592,

457 1598, 1616, 1660). When compared to the counted 2367 Zn-related proteins of *A.*

458 *thaliana* [63]*, out of 159,162 entries in database for this species (according to "porgn:

459 txid3702" request on http://www.ncbi.nlm.nih.gov), the present proportion of Zn-related

460 proteins affected by Cd stress did not clearly suggest a particular and quantitative

461 involvement of them in the response.

462   The quantitatively most prominent information about cambial proteome response

463 concerns plastidial DNA-binding aspartyl peptidase (DAP). Increased abundance in

464 proteolytic enzymes has already been observed in studies on poplar leaves and roots

465 under Cd exposure, but no influence on the abundance of DAP in this study under

466 hydroponic condition was observed [16]. Sixteen DAP spots showed a differential

467 expression under Cd stress (*e.g.* spot 1140), 12 were up-regulated, with IF up to 5.05

468 (spot 398). Kato *et al.* [64] reported a link between nucleoid DNA-binding protein cnd41-

469 like protein – which is a DAP – and the degradation of RuBisCO in tobacco leaves during

470 senescence. Yet no differences in the expression of DAP were observed in the leaves of

471 poplar plants. Although some cambium cells express photosynthetic genes, including at

472 least RuBisCO subunits [65], it seems unlikely that RuBisCO degradation plays such a

16

473   major role in cambial response to Cd so as to involve about 20% of the identified

474   proteome changes. Therefore, the precise role of these proteins remains intriguing.

475   The proteomic changes described in this study, when compared with the literature, are

476   implied in other constraints as part of a generic stress pattern and do not show any Cd- or

477   even metal-stress specificity [66,67,31,68]. This is not the case of the overexpression of DAP

478   which constitutes a putative Cd-specific response of cambial tissue

479   This study focused on young fully developed leaves, is it probable that other leaf

480   levels would exhibit nuances in their response profiles, for it is known that stress

481   response depends on the age of the tissue [69] , that cadmium affects more strongly basal

482   leaves of spinach [4], and that some plants achieve tolerance towards metals by

483   preferential storage in organs, tissues, cells or organelles [70,13]. We besides reported that

484   lack of compartmentalization could be linked to *P. tremula* x *P. alba* sensitivity to Cd^{2+}

485   [71].

486

487   **Conclusion**

488   In leaves containing 84 mg $Cd.kg^{-1}$, the Cd toxicity principally impaired

489   photosynthesis and primary metabolism. Consequently, primary growth was reduced. The

490   resulting loss of photosynthates in all plant tissues, like cambium, partly accounts for the

491   decreased activity of these tissues. So, cambial proteome changes resulted in part from a

492   systemic toxicity, and in part from the 123 mg $Cd.kg^{-1}$ present inside the tissue. The

493   proteomics data presented here showed contrasted responses to Cd between leaf and

494   cambial zone.

495   It now seems important to explore whether the patterns of the proteome response of

496   the different tissues persist whatever the constraint (*e.g.* under drought or heat stress). If

497   so, the General Adaptation Syndrome description for plant under stress could be enriched

498   with tissue-localized markers.

499   References
500

501   1. Cui, Y., Zhu, Y. G., Zhai, R., Huang, Y., Qiu, Y., and Liang, J., Exposure to metal
502   mixtures and human health impacts in a contaminated area in Nanning,
503   China. Environment International. 2005, *31*, 784-790.

504   2. Sanita di Toppi, L. and Gabbrielli, R., Response to cadmium in higher plants.
505   Environ exp Bot. 1999, *41*, 105-130.

506   3. Cosio, C., Vollenweider, P., and Keller, C., Localization and effects of cadmium in
507   leaves of a cadmium-tolerant willow (*Salix viminalis L.*): I.
508   Macrolocalization and phytotoxic effects of cadmium. Environ exp Bot.
509   2006, *58*, 64-74.

510   4. Fagioni, M., D'Amici, G. M., Timperio, A. M., and Zolla, L., Proteomic analysis of
511   multiprotein complexes in the thylakoid membrane upon cadmium
512   treatment. J Prot Res. 2009, *8*, 310-326.

513   5. Shute, T. and Macfie, S. M., Cadmium and zinc accumulation in soybean: A threat
514   to food safety? Science of the Total Environment. 2006, *371*, 63-73.

515   6. Unterbrunner, R., Puschenreiter, M., Sommer, P., Wieshammer, G., Tlustos, P.,
516   Zupan, M., and Wenzel, W. W., Heavy metal accumulation in trees growing
517   on contaminated sites in Central Europe. Environ Pollut. 2007, *148*, 107-
518   114.

519   7. Renaut, J., Bohler, S., Hausman, J.-F., Hoffmann, A. A., Sergeant, K., Nagib, A.,
520   Jolivet, Y., and Dizengremel, P., The impact of atmospheric composition on
521   plants. A case study of ozone and poplar. Mass Spectrom Rev. 2008, *28*,
522   495-516.

523   8. Caruso, A., Chefdor, F., Carpin, S., Depierreux, C., Delmotte, F. M., Kahlem, G.,
524   and Morabito, D., Physiological characterization and identification of genes
525   differentially expressed in response to drought induced by PEG 6000 in
526   *Populus canadensis* leaves. J Plant Physiol. 2008, *165*, 932-941.

527   9. Robinson, B. H., Mills, T. M., Petit, D., Fung, L. E., Green, S. R., and Clothier, B.
528   E., Natural and induced cadmium-accumulation in poplar and willow:
529   Implications for phytoremediation. Plant Soil. 2000, *227*, 301-306.

530   10. Hall, J. L., Cellular mechanisms for heavy metal detoxification and tolerance. J exp
531   Bot. 2002, *53*, 1-11.

532   11. Aina, R., Labra, M., Fumagalli, P., Vannini, C., Marsoni, M., Cucchi, U., Bracale,
533   M., Sgorbati, S., and Citterio, S., Thiol-peptide level and proteomic changes
534   in response to cadmium toxicity in *Oryza sativa* L. roots. Environ exp Bot.
535   2007, *59*, 381-392.

18

536   12. Sarry, J.-E., Kuhn, L., Ducruix, C., Lafaye, A., Junot, C., Hugouvieux, V., Jourdain,
537   A., Bastien, O., Fievet, J. B., Vailhen, D., Amekraz, B., Moulin, C., Ezan,
538   E., Garin, J., and Bourguignon, J., The early responses of *Arabidopsis*
539   *thaliana* cells to cadmium exposure explored by protein and metabolite
540   profiling analyses. Proteomics. 2006, *6*, 2190-2199.

541   13. Fagioni, M. and Zolla, L., Does the different proteomic profile found in apical and
542   basal leaves of spinach reveal a strategy of this plant toward cadmium
543   pollution response? J Prot Res. 2009, *8*, 2519-2529.

544   14. Tuomainen, M. H., Nunan, N., Lehesranta, S. J., Tervahauta, A. I., Hassinen, V. H.,
545   Schat, H., Koistinen, K. M., Auriola, S., McNicol, J., and Kärenlampi, S. O.,
546   Multivariate analysis of protein profiles of metal hyperaccumulator *Thlaspi*
547   *caerulescens* accessions. Proteomics. 2006, *6*, 3696-3706.

548   15. Ahsan, N., Renaut, J., and Komatsu, S., Recent developments in the application of
549   proteomics to the analysis of plant responses to heavy metals. Proteomics.
550   2009, *9*, 2602-2621.

551   16. Kieffer, P., Schröder, P., Dommes, J., Hoffmann, L., Renaut, J., and Hausman, J. F.,
552   Proteomic and enzymatic response of poplar to cadmium stress. J
553   Proteomics. 2009, *72*, 379-396.

554   17. Kieffer, P., Planchon, S., Oufir, M., Ziebel, J., Dommes, J., Hoffmann, L.,
555   Hausman, J. F., and Renaut, J., Combining proteomics and metabolite
556   analyses to unravel cadmium stress-response in poplar leaves. J Prot Res.
557   2009, *8*, 400-417.

558   18. Wang, W., Vinocur, B., Shoseyov, O., and Altman, A., Role of plant heat-shock
559   proteins and molecular chaperones in the abiotic stress response. Trends
560   Plant Sci. 2004, *9*, 244-251.

561   19. Trovato, M., Mattioli, R., and Costantino, P., Multiple roles of proline in plant
562   stress tolerance and development. Rendiconti Lincei. 2008, *19*, 325-346.

563   20. Wasternack, C., Jasmonates: an update on biosynthesis, signal transduction and
564   action in plant Stress Response, Growth and Development. Annals of
565   Botany. 2007, mcm079.

566   21. Foyer, C. H. and Noctor, G., Oxidant and antioxidant signalling in plants: a re-
567   evaluation of the concept of oxidative stress in a physiological context. Plant
568   Cell Environ. 2005, *28*, 1056.

569   22. Selye, H., The General-Adaptation-Syndrome. Ann Rev Med. 1951, *2*, 327-342.

570   23. Leshem, Y. Y., Kuiper, P. J. C., Erdei, L., Lurie, S., and Perl-Treves, R., Do Selye's
571   mammalian "GAS" concept and "co-stress" response exist in plants? Ann
572   NY Acad Sci. 1998, *851*, 199-208.

19

573 24. Küpper, H., Parameswaran, A., Leitenmaier, B., Trtílek, M., and Šetlík, I.,
574   Cadmium-induced inhibition of photosynthesis and long-term acclimation to
575   cadmium stress in the hyperaccumulator *Thlaspi caerulescens*. New Phytol.
576   2007, *175*, 655-674.

577 25. Celedon, P. A. F., de Andrade, A., Meireles, K. G. X., Costa da Cruz Gallo de
578   Carvalho, M., Gregorio Gomes Caldas, D., Moon, D. H., Carneiro, R. T.,
579   Franceschini, L. M., Oda, S., and Labate, C. A., Proteomic analysis of the
580   cambial region in juvenile *Eucalyptus grandis* at three ages. Proteomics.
581   2007, *7*, 2258-2274.

582 26. Gion, J.-M., Lalanne, C., Le Provost, G., Ferry-Dumazet, H., Paiva, J., Chaumeil,
583   P., Frigerio, J.-M., Brach, J., Barré, A., de Daruvar, A., Claverol, S.,
584   Bonneu, M., Sommerer, N., Negroni, L., and Plomion, C., The proteome of
585   maritime pine wood forming tissue. Proteomics. 2005, *5*, 3731-3751.

586 27. Lippert, D., Chowira, S., Ralph, S. G., Zhuang, J., Aeschliman, D., Ritland, C.,
587   Ritland, K., and Bohlmann, J., Conifer defense against insects: proteome
588   analysis of Sitka spruce (*Picea sitchensis*) bark induced by mechanical
589   wounding or feeding by white pine weevils (*Pissodes strobi*). Proteomics.
590   2007, *7*, 248-270.

591 28. Plomion, C., Lalanne, C., Claverol, S., Kohler, A., Bogeat-Triboulot, M.-B., Barre,
592   A., Le Provost, G., Dumazet, H., Jacob, D., Bastien, C., Dreyer, E., de
593   Daruvar, A., Guehl, J.-M., Schmitter, J.-M., Martin, F., and Bonneu, M.,
594   Mapping the proteome of poplar and application to the discovery of
595   drought-stress responsive proteins. Proteomics. 2006, *6*, 6509-6527.

596 29. Durand, T. C., Hausman, J.-F., Carpin, S., Albéric, P., Baillif, P., Label, P., and
597   Morabito, D., Zinc and cadmium effects on growth and ion distribution in
598   *Populus tremula × Populus alba*. Biol Plant. 2009, *submitted.*,

599 30. Damerval, C., De Vienne, D., Zivy, M., and Thiellement, H., Technical
600   improvement in two-dimensional electrophoresis increase the level of
601   genetic variation detected in wheat-seedling proteins. Electrophoresis. 1986,
602   *7*, 52-54.

603 31. Bohler, S., Bagard, M., Oufir, M., Planchon, S., Hoffmann, L., Jolivet, Y.,
604   Hausman, J.-F., Dizengremel, P., and Renaut, J., A DIGE analysis of
605   developing poplar leaves subjected to ozone reveals major changes in
606   carbon metabolism. Proteomics. 2007, *7*, 1584-1599.

607 32. Hunter, S., Apweiler, R., Attwood, T. K., Bairoch, A., Bateman, A., Binns, D.,
608   Bork, P., Das, U., Daugherty, L., Duquenne, L., Finn, R. D., Gough, J., Haft,
609   D., Hulo, N., Kahn, D., Kelly, E., Laugraud, A., Letunic, I., Lonsdale, D.,
610   Lopez, R., Madera, M., Maslen, J., McAnulla, C., McDowall, J., Mistry, J.,
611   Mitchell, A., Mulder, N., Natale, D., Orengo, C., Quinn, A. F., Selengut, J.
612   D., Sigrist, C. J. A., Thimma, M., Thomas, P. D., Valentin, F., Wilson, D.,

20

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613   Wu, C. H., and Yeats, C., InterPro: the integrative protein signature
614   database. Nucl Acids Res. 2009, *37*, D211-D215.

615   33. Anderson, R. H. and Basta, N. T., Application of ridge regression to quantify
616   marginal effects of collinear soil properties on phytoaccumulation of
617   arsenic, cadmium, lead, and zinc. Environ Toxicol Chem. 2009, *28*, 619-
618   628.

619   34. Ruttens, A., Mench, M., Colpaert, J. V., Boisson, J., Carleer, R., and Vangronsveld,
620   J., Phytostabilization of a metal contaminated sandy soil. I: Influence of
621   compost and/or inorganic metal immobilizing soil amendments on
622   phytotoxicity and plant availability of metals. Environ Pollut. 2006, *144*,
623   524-532.

624   35. Barcelo, J. and Poschenrieder, C., Plant water relations as affected by heavy metal
625   stress: A review. J Plant Nutr. 1990, *13*, 1-37.

626   36. Chaffei, C., Pageau, K., Suzuki, A., Gouia, H., Ghorbel, M. H., and Masclaux-
627   Daubresse, C., Cadmium toxicity induced changes in nitrogen management
628   in *Lycopersicon esculentum* leading to a metabolic safeguard through an
629   amino acid storage strategy. Plant Cell Physiol. 2004, *45*, 1681-1693.

630   37. Clemens, S., Toxic metal accumulation, responses to exposure and mechanisms of
631   tolerance in plants. Biochimie. 2006, *88*, 1707-1719.

632   38. Shi, G. and Cai, Q., Photosynthetic and anatomic responses of peanut leaves to
633   cadmium stress. Photosynthetica. 2008, *46*, 627-630.

634   39. Zhu, R., Macfie, S. M., and Ding, Z., Cadmium-induced plant stress investigated by
635   scanning electrochemical microscopy. J exp Bot. 2005, *56*, 2831-2838.

636   40. Lindahl, M., Spetea, C., Hundal, T., Oppenheim, A. B., Adam, Z., and Andersson,
637   B., The thylakoid FtsH protease plays a role in the light-induced turnover of
638   the Photosystem II D1 protein. Plant Cell. 2000, *12*, 419-432.

639   41. Kieffer, P., Dommes, J., Hoffmann, L., Hausman, J.-F., and Renaut, J., Quantitative
640   changes in protein expression of cadmium-exposed poplar plants.
641   Proteomics. 2008, *8*, 2514-2530.

642   42. He, X. Y., Fu, S. L., Chen, W., Zhao, T. H., Xu, S., and Tuba, Z., Changes in
643   effects of ozone exposure on growth, photosynthesis, and respiration of
644   Ginkgo biloba in Shenyang urban area. Photosynthetica. 2007, *45*, 555-561.

645   43. Mittler, R., Abiotic stress, the field environment and stress combination. Trends
646   Plant Sci. 2006, *11*, 15-19.

647   44. Mendoza-Cozatl, D. G., Butko, E., Springer, F., Torpey, J. W., Komives, E., Kehr,
648   J., and Schroeder, J. I., Identification of high levels of phytochelatins,

21

649   glutathione and cadmium in the phloem sap of *Brassica napus*. A role for
650   thiol-peptides in the long-distance transport of cadmium and the effect of
651   cadmium on iron translocation. Plant J. 2008, *54*, 249-259.

652 45. Semane, B., Cuypers, A., Smeets, K., Van Belleghem, F., Horemans, N., Schat, H.,
653   and Vangronsveld, J., Cadmium responses in Arabidopsis thaliana:
654   glutathione metabolism and antioxidative defence system. Physiol Plant.
655   2007, *129*, 519-528.

656 46. Dixon, D. P., Cummins, I., Cole, D. J., and Edwards, R., Glutathione-mediated
657   detoxification systems in plants. Curr Opin Plant Biol. 1998, *1*, 258-266.

658 47. Chen, J. W., Dodia, C., Feinstein, S. I., Jain, M. K., and Fisher, A. B., 1-Cys
659   peroxiredoxin, a bifunctional enzyme with glutathione peroxidase and
660   phospholipase A2 activities. J Biol Chem. 2000, *275*, 28421-28427.

661 48. Wahid, A., Ghani, A., and Javed, F., Effect of cadmium on photosynthesis, nutrition
662   and growth of mungbean. Agronomy for Sustained Development. 2008, *28*,
663   273-280.

664 49. Crombie, H., Chengappa, S., Jarman, C., Sidebottom, C., Grant, R., and Reid, J. G.,
665   Molecular characterisation of a xyloglucan oligosaccharide-acting a-D-
666   xylosidase from nasturtium (*Tropaeolum majus* L.) cotyledons that
667   resembles plant 'apoplastic' a-D-glucosidases. Planta. 2002, *214*, 406-413.

668 50. Rolland, F., Baena-Gonzalez, E., and Sheen, J., Sugar sensing and signaling in
669   plants: conserved and novel mechanisms. Annu Rev Plant Biol. 2006, *57*,
670   675-709.

671 51. Jouve, L., Hoffmann, L., and Hausman, J.-F., Polyamine, carbohydrate, and proline
672   content changes during salt stress exposure of aspen (*Populus tremula* L.):
673   involvement of oxidation and osmoregulation metabolism. Plant Biol. 2004,
674   *6*, 74-80.

675 52. Wang, J., Ji, Q., Jiang, L., Shen, S., Fan, Y., and Zhang, C., Overexpression of a
676   cytosol-localized rhamnose biosynthesis protein encoded by Arabidopsis
677   RHM1 gene increases rhamnose content in cell wall. Plant Phys Biochem.
678   2009, *47*, 86-93.

679 53. Mijnsbrugge, K. V., Meyermans, H., Van Montagu, M., Bauw, G., and Boerjan,
680   W., Wood formation in poplar: identification, characterization, and seasonal
681   variation of xylem proteins. Planta. 2000, *210*, 589-598.

682 54. Xu, S. L., Rahman, A., Baskin, T. I., and Kieber, J. J., Two Leucine-Rich Repeat
683   Receptor Kinases Mediate Signaling, Linking Cell Wall Biosynthesis and
684   ACC Synthase in Arabidopsis. Plant Cell. 2008, *20*, 3065-3079.

22

685   55.   Sagi, G., Katz, A., Guenoune-Gelbart, D., and Epel, B. L., Class 1 reversibly
686   gycosylated polypeptides are plasmodesmal-associated proteins delivered to
687   plasmodesmata via the Golgi apparatus. Plant Cell. 2005, *17*, 1788-1800.

688   56.   De Pino, V., Boran, M., Norambuena, L., Gonzalez, M., Reyes, F., Orellana, A.,
689   and Moreno, S., Complex formation regulates the glycosylation of the
690   reversibly glycosylated polypeptide. Planta. 2007, *226*, 335-345.

691   57.   Garnier, L., Simon-Plas, F., Thuleau, P., Agnel, J.-P., Blein, J.-P., Ranjeva, R., and
692   Montillet, J.-L., Cadmium affects tobacco cells by a series of three waves of
693   reactive oxygen species that contribute to cytotoxicity. Plant Cell Environ.
694   2006, *29*, 1956-1969.

695   58.   Kim, Y.-O., Kim, J. S., and Kang, H., Cold-inducible zinc finger-containing
696   glycine-rich RNA-binding protein contributes to the enhancement of
697   freezing tolerance in Arabidopsis thaliana. Plant Journal. 2005, *42*, 890-900.

698   59.   Battaglia, M., Solorzano, R., Hernandez, M., Cuellar-Ortiz, S., Garcia-Gomez, B.,
699   Marquez, J., and Covarrubias, A., Proline-rich cell wall proteins accumulate
700   in growing regions and phloem tissue in response to water deficit in
701   common bean seedlings. Planta. 2007, *225*, 1121-1133.

702   60.   Akoh, C. C., Lee, G. C., Liaw, Y. C., Huang, T. H., and Shaw, J. F., GDSL family
703   of serine esterases/lipases. Prog Lipid Res. 2004, *43*, 534-552.

704   61.   Dalrymple, B. P., Cybinski, D. H., Layton, I., McSweeney, C. S., Xue, G. P.,
705   Swadling, Y. J., and Lowry, J. B., Three *Neocallimastix patriciarum*
706   esterases associated with the degradation of complex polysaccharides are
707   members of a new family of hydrolases. Microbiology. 1997, *143*, 2605-
708   2614.

709   62.   Hong, J., Choi, H., Hwang, I., Kim, D., Kim, N., Choi, D., Kim, Y., and Hwang, B.,
710   Function of a novel GDSL-type pepper lipase gene, CaGLIP1, in disease
711   susceptibility and abiotic stress tolerance. Planta. 2008, *227*, 539-558.

712   63.   Broadley, M. R., White, P. J., Hammond, J. P., Zelko, I., and Lux, A., Zinc in
713   plants. New Phytol. 2007, *173*, 677-702.

714   64.   Kato, Y., Yamamoto, Y., Murakami, S., and Sato, F., Post-translational regulation
715   of CND41 protease activity in senescent tobacco leaves. Planta. 2005, *222*,
716   643-651.

717   65.   Goué, N., Lesage-Descauses, M.-C., Meller, E. J., Magel, E., Label, P., and
718   Sundberg, B., Microgenomic analysis reveals cell type-specific gene
719   expression patterns between ray and fusiform initials within the cambial
720   meristem of *Populus*. New Phytol. 2008, *180*, 45-56.

721 66. Xiao, X., Yang, F., Zhang, S., Korpelainen, H., and Li, C., Physiological and
722   proteomic responses of two contrasting Populus cathayana populations to
723   drought stress. Physiol Plant. 2009, *136*, 150-168.

724 67. Renaut, J., Lutts, S., Hoffmann, L., and Hausman, J.-F., Responses of poplar to
725   chilling temperatures : proteomic and physiological aspects. Plant Biol.
726   2004, *6*, 81-90.

727 68. Alvarez, S., Berla, B. M., Sheffield, J., Cahoon, R. E., Jez, J. M., and Hicks, L. M.,
728   Comprehensive analysis of the *Brassica juncea* root proteome in response to
729   cadmium exposure by complementary proteomic approaches. Proteomics.
730   2009, *9*, 2419-2431.

731 69. Bray, Elizabeth A., Bailey-Serres, Julia, and Weretilnyk, Elizabeth.
732   Response to abiotic stresses. In: Buchanan, B., Gruissem, W., and
733   Jones, R., editors. Biochemistry & Molecular Biology of
734   Plants:American Society of Plant Physiologists; 2000 p.1158-1203.

735 70. Vollenweider, P., Cosio, C., and Keller, C., Localization and effects of cadmium in
736   leaves of a cadmium-tolerant willow (Salix viminalis L.): II.
737   Microlocalization and cellular effects of cadmium. Environ exp Bot. 2006,
738   *58*, 64-74.

739 71. Durand, T. C., Baillif, P., Albéric, P., Carpin, S., Label, P., Hausman, J.-F., and
740   Morabito, D., Cd and Zn are differentially distributed in *Populus tremula* x
741   *P. alba* exposed to metal excess. Can J For Res. 2010, *submitted*,
742
743

744   **CAPTION OF THE FIGURES**

745

746   **Fig. 1.** 2D-electrophoresis gel with leaf proteins of *Populus tremula x P. alba* genotype

747   717-1B4labelled by CyDye 2. Identified proteins are indicated with their respective spot

748   number.

749

750   **Fig. 2.** 2D-electrophoresis gel with cambial proteins of *Populus tremula x P. alba*

751   genotype 717-1B4 labelled by CyDye 2. Identified proteins are indicated with their

752   respective spot number.

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755   Table 1: Differentially expressed proteins in the leaf proteome of *Populus tremula x P.alba* genotype 717-1B4 submitted during 61

756   days to a soil containing 360 mg Cd.kg^{-1} DW (n=4, *p<0.05, **p<0.01, ***p<0.001). The average ratio of the protein abundance was

757   calculated between treated and control plants.

Spot number [1]	Leaf protein identifications [2]	Protein score [3]	EST [4]	NCBI [5]	No of peptides	Sequence coverage (%)	Cd / Ctr ratio[6]	p value[7]
	Photosynthesis							
1464	RubisCO large subunit [Populus tomentosa]	283		gi|22001406	8	41	-4,3***	0.000013
1681	RubisCO large subunit	250		gi|52001641	4	29	-4.09*	0.039
677	RubisCO large subunit	260		gi|1223722	4	57	-3.99***	0.00083
1742	RubisCO large subunit	283		gi|9623109	4	32	-1.65*	0.039
1673	RubisCO large subunit	291		gi|30143303	6	29	-3.11**	0.003
1748	RubisCO large subunit	305		gi|60101630	5	25	-2.99**	0.0098
1750	RubisCO large subunit	113		gi|60101630	2	30	-6.02**	0.0025
1778	RubisCO large subunit [Malesherbia linearifolia]	123		gi|1771273	3	30	-2,91**	0.0032
1718	RubisCO large subunit	207		gi|1346967	3	36	-2,77*	0.019
1195	RubisCO large subunit	313		gi|57338574	6	40	-2.59*	0.014
1238	RubisCO large subunit	466		gi|60101630	7	36	-2.39*	0.022
694	RubisCO large subunit	138		gi|2149483	3	39	-2.36*	0.029
1686	RubisCO large subunit	114		gi|52001641	2	33	-2.12*	0.011
1737	Ribulose 1,5-bisphosphate carboxylase	279		gi|32442736	4	31	-3.41*	0.016
683	RubisCO large subunit [Populus alba]	172		gi|110227087	4	50	-1,58*	0.042
593	RubisCO large subunit [Populus alba]	129		gi|110227087	3	53	1,72***	0.00032
503	Rubisco	147		gi|47680208	3	32	4.02*	0.046
648	RubisCO large subunit	300		gi|30143315	7	42	2.03*	0.012
853	Rubisco activase precursor	137		gi|3687676	2	40	-2.22**	0.01
1043	Rubisco activase B	447		gi|7960277	6	39	-1.63*	0.025
939	Rubisco activase B	168		gi|7960277	2	33	-2.2*	0.029
969	Rubisco activase, chloroplast precursor	202		gi|10720249	2	39	-1.62*	0.022
1048	Rubisco activase 1	382 (+32)		gi|12620881	6	36	-1.83**	0.0092
912	RuBisCO activase	459	gi|52397711	gi|94549022	6		-2.02*	0.037
1578	Chlorophyll a/b binding protein(Lhcb2) [*Cicer arietinum*]	219 (+70)		gi|3928140	6	69	-10.26*	0.026
1602	Chlorophyll a/b binding protein(Lhcb2) [*Cicer arietinum*]	196 (+87)		gi|3928140	7	71	-7.67***	0.00093
1609	Chlorophyll a/b binding protein (Lhcb2)	163		gi|398599	4	18	-2.6**	0.0025
1584	Chlorophyll a/b binding protein (Lhcb2)	134 (+74)		gi|398599	5	26	-2.08*	0.029
1608	LHCII type II chlorophyll a/b-binding protein [*Vigna radiata*]	160 (+16)		gi|9587203	6	28	-4.09****	0.00083
1517	Light-harvesting complex II protein Lhcb1 [*Populus trichocarpa*]	229	gi|52533657	gi|224083006	5	65	-11,02**	0.0035
1663	Chlorophyll a/b-binding protein type III (Lhca3)	133 (+117)		gi|7271947	3	26	-4.53**	0.00236
1658	Chlorophyll a/b-binding protein type III (Lhca3)	370	gi|56824867	gi|116519121	6	63	-6.11***	0.0000043
1680	Chlorophyll-a/b binding protein Lhcb3	163	gi|50059931	gi|169124051	3	70	-3.77**	0.0017
1629	LHCB5 - chlorophyll binding	301		gi|15235029	3	46	-2.69***	0.00067

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1441	Oxygen-evolving enhancer protein 1, chloroplast precursor (OEE1)	392	gi|38580986	gi|30013657	6	51	-7.03***	0.00028
1439	Oxygen-evolving complex protein 1	250 (+60)		gi|739292	3	53	-4.42**	0.0039
1471	Oxygen-evolving enhancer protein 1, chloroplast precursor (OEE1	198		gi|12644171	2	32	-4.81**	0.0012
1480	Oxygen-evolving enhancer protein 1	83 (+62)	gi|27410837	gi|223538464	4	54	-3,65***	0.0005
1456	Oxygen evolving enhancer protein 1 precursor	203	gi|57892741	gi|119952178	4	59	-2.75**	0.0028
1692	Oxygen-evolving enhancer protein 2, chloroplast precursor (OEE2)	169		gi|131390	3	30	-5.51***	0.00019
1653	PSBP-1 (OEE PROTEIN 2); calcium ion binding	280	gi|55735291	gi|223539254	6	49	-5.38***	0.000015
1672	Oxygen-evolving enhancer protein 2, chloroplast precursor	261	gi|55734950	gi|223539254	6	52	-4,15***	0.00067
1661	Oxygen-evolving enhancer protein 2, chloroplast precursor (OEE2)	215	gi|55735137	gi|131390	5	30	-3.73**	0.0025
1699	Oxygen-evolving enhancer protein 2, chloroplast precursor	227	gi|24099140	gi|223539254	5	62	-5,88***	0.00038
	Carbohydrate metabolism							
1571	Triosephosphate isomerase, cytosolic (TIM)	304	gi|24060530	Gi : 136057	6	76	1.99**	0.0026
1566	Triosephosphate isomerase, cytosolic (TIM)	180		gi|136057	5	40	2.19**	0.0039
1120	Sedoheptulose bisphosphatase	253	gi|52530247	gi|223530064	6	53	-2.48***	0.00015
1116	Sedoheptulose bisphosphatase	292	gi|52530247	gi|118175929	6	59	-1.53*	0.03
284	Transketolase 1	256 (+68)		gi|3559814	5	20	-1.61*	0.015
1127	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	185 (+157)		gi|120671	7	32	1.91***	0.00027
1124	Glyceraldehyde 3-phosphate dehydrogenase	122	gi|57892956	gi|51703306	5	81	2.07***	0.000093
1051	3-phosphoglycerate kinase [*Populus tremuloides*]	135		gi|29124969	3	46	2.45*	0.023
1275	Phosphoglycerate kinase	112 (+16)		gi|2499497	5	30	-1,75*	0.015
501	glucose-6-phosphate isomerase [*Solanum tuberosum*]	80	gi|52620161	gi|167909863	4	60	2.22**	0.0059
597	UDP-glucose pyrophosphorylase [*Populus tremula x P. tremuloides*]	226		gi|32527831	4	37	1.49**	0.0015
553	Glycosyl hydrolase family 38 protein	106	gi|56833433	gi|186510450	3	34	1.92**	0.0099
1340	Ferredoxin-NADP oxidoreductase	314		gi|170111	6	28	-1.81*	0.014
1059	Ferredoxin-NADP oxidoreductase	314 (+83)		gi|170111	8	28	-1.67*	0.017
1365	Ferredoxin-NADP oxidoreductase	190		gi|170111	3	30	-1.55*	0.04
1342	Ferredoxin-NADP oxidoreductase	257	gi|52536292	gi|4930123	6	58	-1.38*	0.015
1359	Putative ferredoxin-NADP(H) oxidoreductase	96	gi|27415025	gi|224102711	4	62	-1,5*	0.031
1332	Ferredoxin-NADP oxidoreductase	141		gi|170111	6	28	2.01**	0.003
1338	Ferredoxin-NADP oxidoreductase	156 (+83)		gi|119905	6	49	2.84***	0.00083
884	AXS2 (UDP-D-Apiose/UDP-D-Xylose synthase 2)	182	gi|27422474	gi|18390863	4	54	2.38*	0.027
1551	Acid phosphatase [*Glycine max*]	90	gi|55734561	gi|3341443	3	54	1.78*	0.014
	Energy metabolism							
1598	Carbonic anhydrase	318		gi|1354515	6	63	-1.85*	0.014
1616	Carbonic anhydrase	570		gi|1354517	6	53	-1.43*	0.035
1592	Carbonic anhydrase	249		gi|1354515	4	40	-1.31*	0.049
1660	Carbonic anhydrase	118		gi|1354515	2	47	2.21**	0.018
620	ATPase beta subunit [*Populus tomentosa*] - chloroplast	354		gi|22094585	6	75	-4,19***	0.00085
632	ATPase beta subunit [*Populus tomentosa*] - chloroplast	295		gi|22094585	5	75	-3,91***	0.000019
633	ATPase beta subunit [*Populus tomentosa*] - chloroplast	401 (+237)		gi|22094585	7	80	-2.02**	0.0044
594	ATPase alpha subunit [*Didymeles perrieri*] - mitochondrion	240		gi|6561625	6	38	1.83*	0.015

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599	ATPase alpha subunit [*Didymeles perrieri*] - mitochondrion	137 (+71)		gi|6561625	4	33	2.51*	0.011
	Protein metabolism & catabolism							
1290	Aminomethyltransferase, mitochondrial precursor	162		gi|3334196	2	36	-1.7*	0.028
548	D-3-phosphoglycerate dehydrogenase, putative [*Ricinus communis*]	72 (+22)	gi|23995055	gi|223542068	3	29	36,78*	0.025
435	FtsH protease 8 / ATPase/Zn dependant metallopeptidase	98		gi|42561751	4	30	-1.51*	0.014
1678	PBD1 (PROTEASOME SUBUNIT PRGB); peptidase	146		gi|15228805	2	20	-1.49*	0.028
	Protein folding							
1518	Nascent polypeptide associated complex alpha chain [*Nicotiana benthamiana*]	199	gi|52502629	gi|124484511	2	60	-3,72**	0.0081
1752	Nascent polypeptide associated complex alpha chain (*Oryza sativa*)	183		gi|46575976	2	50	-5,45**	0.0024
1011	Heat shock protein 70	214		gi|48716124	3	41	-1.59**	0.0064
357	Heat shock protein 70	535 (+81)		gi|6911551	8	38	1.6*	0.014
1635	Chloroplast chaperonin 21 [*Vitis vinifera*].	188	gi|52519679	gi|50660327	5		-2.23*	0.016
427	Chaperonin, putative	81		gi|15232923	3	37	2.44	0.027
	Citrate Cycle							
1200	NAD-dependent malate dehydrogenase	87 (+57)		gi|15982948	2	37	-1,84***	0.00037
1316	Malate dehydrogenase, putative [*Ricinus communis*]	79	gi|52536153	gi|223538636	3	35	-1,75**	0.0038
902	Citrate (si)-synthase [*Populus trichocarpa*]	177	gi|53783182	gi|1648926	6	33	2.11**	0.0034
903	Citrate (si)-synthase [*Populus trichocarpa*]	119		gi|1648926	2	37	1.99**	0.0091
418	Succinate dehydrogenase	168		gi|115470493	4	25	1.45*	0.046
	Oxidoreductase							
936	GDP-mannose 3,5-epimerase/ NAD binding	294		gi|15241945	4	45	1.85**	0.0086
1202	Aldo/keto reductase family-like protein	121	gi|60216418	gi|23495741	5	63	-2.62**	0.0037
1061	Formate dehydrogenase	166 (+13)	gi|38593943	gi|224129102	5	51	2,08**	0.0043
1821	Peroxiredoxin type 2, putative	187	gi|52505836	gi|15231718	2	57	-2.09*	0.025
1637	1-cys peroxiredoxin [*Xerophyta viscosa*]	151		gi|19423862	2	10	16.7***	0.000013
	Glutathione metabolism							
1659	Glutathione S-transferase	417	gi|55734403	gi|161347485	7	48	2.11**	0.0071
1667	Glutathione S-transferase	196	gi|24102869	gi|161347485	4	55	2.12*	0.018
	Miscellaneous							
1552	Class IV chitinase [*Galega orientalis*].	285	gi|57894645	gi|33414046	3	63	1,99*	
572	Inositol-3-phosphate synthase (Myo-inositol-1-phosphate synthase)	391		gi|14548095	6	21	-1.99**	0.0082
1192	Alpha tubulin [*Nicotiana tabacum*]	224 (+74)		gi|11967906	6	38	-2,94*	0.04
1300	Potassium channel beta subunit	143 (+27)		gi|3402279	3	9	1.6**	0.0079
1756	Auxin-binding protein ABP19a precursor, putative [*Ricinus communis*]	142	gi|52530576	gi|223539406	5	14	-2,56*	0.037
225	Translation elongation factor EF-G [*Glycine max*]	354 (+47)		gi|402753	5	37	-1.64***	0.00046

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Spots presenting more than one protein identification					
1736	Glutathione S-transferase + Germin-like	84	gi|51556908	29	-3,41* 0.074
1801	Plastoquinol-plastocyanin reductase	71	gi|4586598	25	-4.93** 0.0036

758

759   1: spot number on the master gel
760   2: Protein obtained by blasting the EST against the NCBInr database
761   3: MASCOT score (protein score as given by the GPS software (Applied Biosystems))
762   4: Accession number of the EST sequence in the NCBInr database
763   5: Accession number of the corresponding protein in the NCBInr database
764   6: Average ratio of the protein abundance (Cadmium/Control). Positive values of ratio are given as such, negative values are given according to the following
765 formula : given value = -1/average ratio
766   7: p-value of Student's t-test.
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768

769 Table 2: Differentially expressed proteins in the cambial zone proteome of *Populus tremula x P.alba* 717-1B4 genotype after 61 days

770 on a soil containing 360 mg Cd.kg^{-1} DW (n=4). The average ratio of the protein abundance was calculated between treated and control

771 plants.

772

Spot number [1]	Cambial protein identifications [2]	Protein score [3]	Accession Number		Nb of peptides	Sequence coverage (%)	Cd / Ctr	
			EST [4]	NCBI [5]			ratio [6]	p value [7]
	Carbon and carbohydrate metabolism							
784	Enolase1 [*Zea mays*]	395	gi|24075693	gi|162458207	6	81	-1.39**	0.0013
798	Enolase1 [*Zea mays*]	249	gi|24102975	gi|162458207	4	61	-1.38**	0.0015
816	Enolase [*Brassica napus*]	142	gi|24020181	gi|34597332	4		-1.73***	0.00048
802	Enolase1 [*Zea mays*]	358		gi|162458207	4	42	-1.74***	0.00022
800	Os06g0136600 [*Oryza sativa* (japonica cultivar-group)]	130		gi|115466256	4	36	-2.1***	0.00035
759	Triosephosphate isomerase-like protein [*Solanum tuberosum*].	96	gi|52505071	gi|76573375	3	33	-1.59*	0.01
752	UDP-glucose pyrophosphorylase [*Populus tremula x P. tremuloides*]	239		gi|32527831	3	19	-1.52*	0.028
744	UDP-glucose dehydrogenase [*Populus tremula x P. tremuloides*]	313		gi|6164591	6	66	-1.58**	0.0015
809	UDP-glucose dehydrogenase [*Populus tremula x P. tremuloides*]	132 (+61)	gi|57892820		4	55	-1.43**	0.0011
792	UDP-glucose dehydrogenase	151 (+59)	gi|27415980	gi|6164591	4	82	-3.47**	0.0012
851	Phosphofructokinase family protein [*Arabidopsis thaliana*]	82 (+37)	gi|27411363	gi|15238818	5	44	-2.04***	0.0002
526	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	110	gi|52493827	gi|223540739	2	59	-1.82***	0.00036
288	Alpha-D-xylosidase [*Tropaeolum majus*]	123	gi|73928193	gi|5725356	2	23	1.53*	0.012
1892	Alpha-D-xylosidase precursor [*Arabidopsis thaliana*]	111	gi|28606907	gi|4163997	3		1.51*	0.01
390	Alpha-mannosidase [*Arabidopsis thaliana*]	113		gi|10177664	3	10	1.77**	0.0015
433	Alpha-mannosidase [*Arabidopsis thaliana*]	79		gi|10177664	2	12	1.44**	0.0085
480	RHM1/ROL1 (RHAMNOSE BIOSYNTHESIS1); UDP-glucose 4,6-dehydratase/ catalytic [*A. thaliana*]	123		gi|15218420	3	17	-1.4**	0.0029
481	RHM1/ROL1 (RHAMNOSE BIOSYNTHESIS1); UDP-glucose 4,6-dehydratase/ catalytic	106		gi|15218420	3	19	1.64**	0.001
487	RHM1/ROL1 (RHAMNOSE BIOSYNTHESIS1); UDP-glucose 4,6-dehydratase/ catalytic	130		gi|15218420	4	19	-1.8***	0.00013
1202	Fructokinase, putative [*Ricinus communis*]	170	gi|60697528	gi|223526803	5	62	-1,9***	

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ID	Protein	Score		gi		Coverage	Fold	p-value
1069	Alcohol dehydrogenase [*Populus tremula*]	130		gi|52851038	2	29	2.13***	0.00052
1070	Alcohol dehydrogenase [*Populus tremula*]	220		gi|52851054	5	74	1.62***	0.00071
	Protein metabolism							
381	Methionine synthase [*Solanum tuberosum*]	146 (+96)		gi|8439545	5	30	-2.58***	0.0009
385	Vitamin-b12 independent methionine synthase, [*Populus trichocarpa*]	328	gi|27414927	gi|224104961	6	67	-1.98**	0.0011
383	Methionine synthase [*Zea mays*]	170		gi|17017263	5	33	-1.99**	0.0022
391	Methionine synthase [*Carica papaya*]	138	gi|24107875	gi|151347486	3	66	-1.58**	0.0045
885	S-adenosylmethionine synthetase	98		gi|15450421	4	24	-2.02***	0.0003
891	S-adenosylmethionine synthetase [*Phaseolus lunatus*]	170	gi|24065013	gi|1346524	2	60	-1.83*	0.018
881	S-adenosylmethionine synthetase 1 [*Arabidopsis thaliana*]	100		gi|30683070	3	34	-1.88***	0.000026
1240	O-acetylserine (thiol)lyase [*Populus alba x Populus tremula*]	121		gi|34099833	3	34	-1.38***	0.00075
1302	60S acidic ribosomal protein P0 [*Zea mays*]	167		gi|162460698	3	32	-1.63*	0.039
	Protein catabolism							
896	26S proteasome ATPase subunit [*Pisum sativum*]	243		gi|49175787	5	82	-1.82***	0.00016
1555	Proteasome subunit alpha type, putative [*Ricinus communis*]	154	gi|24076896	gi|223529618	3	76	-1,49*	0.034
796	Leucine aminopeptidase, putative [*Ricinus communis*]	140	gi|73877305	gi|223531128	2	62	-1,47**	0.0037
	Protein folding							
537	Protein disulfide-isomerase precursor (PDI)	387	gi|24065613	gi|11133775	5	63	-1.69*	0.012
541	Protein disulfide-isomerase precursor (PDI)	433	gi|24065613	gi|11133775	5	63	-1.99***	0.00056
1161	Protein disulfide isomerase, putative [*Ricinus communis*]	101 (+18)	gi|23994033	gi|223545789	3	65	-1,71***	0.0015
715	Calreticulin-1 [*Glycine max*]	156	gi|60214695	gi|117165712	5	47	-1.88**	0.0045
	Transcription and translation factors							
892	Elongation factor 1-gamma [*Glycine max*]	116	gi|24069167	gi|18958499	2	20	-1.83***	0.00015
911	Elongation factor 1-gamma [*Glycine max*]	103		gi|18958499	2	20	-1.55*	0.012
332	Elongation factor 1-gamma [*Oryza sativa* Japonica Group]	114	gi|24103246	gi|169244489	3	60	1.65	0.073
877	DEAD box RNA helicase [*Pisum sativum*]	105		gi|25809054	3	33	1.45**	0.0023
920	Eukaryotic initiation factor 4A [*Oryza sativa* (japonica cultivar-group)]	156 (+51)		gi|303844	3	53	-1.85*	0.01
1830	*RNA-binding glycine-rich protein-1a [Nicotiana sylvestris]*	118	gi|52512794	gi|469070	2	52	-1.62*	0.037
1836	Glycine-rich RNA-binding protein [*Euphorbia esula*]	126 (+26)	gi|56819713	gi|2674201	2	48	6.41**	0.0021
1838	Glycine-rich RNA-binding protein [*Euphorbia esula*]	137	gi|24008735	gi|2674201	4	50	-1.89**	0.0095
	Cytoskeleton & cell wall							
772	Alpha tubulin 1 [*Pseudotsuga menziesii var. menziesii*]	341		gi|56481443	6	71	-1.49**	0.0045
773	Alpha-tubulin [*Trifolium repens*]	366		gi|37789885	6	71	-1.53**	0.0042
782	Alpha-tubulin 1 [*Populus tremuloides*]	342		gi|29124983	5	52	-1.71***	0.0009

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708	Beta-6 tubulin [*Zea mays*]	451		gi|162459800	8	67	-1.77***	0.00051
695	Beta-6 tubulin [*Zea mays*]	370		gi|162462765	7	59	-1.68**	0.0017
702	Tubulin beta-5 chain (Beta-5-tubulin)	456		gi|8928427	7	58	-1.5**	0.0026
710	Tubulin beta-5 chain (Beta-5-tubulin)	383		gi|8928427	7	59	-1.62**	0.0036
1319	Phenylcoumaran benzylic ether reductase [*Populus trichocarpa*]	603	gi|33453463	gi|3114901	7	84	-1.57**	0.0046
1323	Phenylcoumaran benzylic ether reductase [*Populus trichocarpa*]	122	gi|33453463	gi|224066197	5	65	-1.62*	0.03
1340	Phenylcoumaran benzylic ether reductase [*Populus trichocarpa*]	415	gi|33453463	gi|224066197	5	56	-1.67*	0.012
1168	Reversibly glycosylated polypeptide	112 (+38)	gi|23978092	gi|2317729	2	11	-2.72***	0.000046
1187	Reversibly glycosylated polypeptide-1	170	gi|57894464	gi|2317729	2	76	-3.93***	0.000017
1317	Leucine rich repeat protein [*Populus tremula*]	134 (+26)	gi|33451847	gi|190897438	4	52	-1,6***	0.00065
977	Actin [Stevia rebaudiana]	133		gi|23955912	3	58	2.15***	0.00048
990	Actin [*Gossypium hirsutum*]	650		gi|32186906	8	77	-1.54**	0.0024
1057	Putative proline-rich protein [*Oryza sativa* Japonica Group]	193	gi|38594177	gi|14209541	2	16	-2.13*	0.027
926	Putative proline-rich protein [*Oryza sativa* Japonica Group]	83 (+46)	gi|38594177	gi|14209541	3	22	2.08**	0.0094
1324	Cinnamoyl-CoA reductase [*Jatropha curcas*]	247	gi|73869335	gi|239909311	5	27	-1.46*	0.02
	Stress response - defense							
1179	Cupin family protein	140	gi|24106291	gi|15226926	6	30	-1.54***	0.00016
	Citrate Cycle							
1268	NAD-dependent malate dehydrogenase [*Prunus persica*]	157	gi|52521122	gi|15982948	4	33	-1.41**	0.0037
1290	Malate dehydrogenase precursor [*Medicago sativa*]	132	gi|24062191	gi|2827080	3	54	-1.58*	0.036
1336	Malate dehydrogenase [*Glycine max]*	133	gi|73871484	gi|5929964	5	47	-1.61**	0.0062
	Oxidoreductase							
1137	Peroxidase [*Nicotiana tabacum*]	147	gi|57894628	gi|14031049	4	42	2.93*	0.015
	Glutathione metabolism							
1657	Glutathione-s-transferase theta, gst, putative [*Ricinus communis*]	120	gi|24100781	gi|223551315	3	57	1,66*	0.027
908	6-phosphogluconate dehydrogenase, putative [*Ricinus communis*]	146 (+32)	gi|50069101	gi|223529624	4	70	-1.54***	0.00033
909	6-phosphogluconate dehydrogenase family protein	149 (+39)	gi|50069101	gi|223529624	3	70	-1.51**	0.0012
	Lipid metabolism							
1059	Acetylcholinesterase [*Macroptilium atropurpureum*]	128	gi|24064109	gi|168274274	6	38	1.46**	0.0029
1064	Acetylcholinesterase [*Macroptilium atropurpureum*]	212	gi|24064109	gi|168274274	5	38	1.59***	0.00077
1066	Acetylcholinesterase [*Macroptilium atropurpureum*]	323	gi|24064109	gi|168274274	5	39	1.89***	0.00019
572	GDSL-motif lipase/hydrolase family protein [*Arabidopsis thaliana*]	227	gi|38594604	gi|15242458	2	33	-1.73*	0.023

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576	GDSL-motif lipase/hydrolase family protein [*Arabidopsis thaliana*]	218	gi|23976058	gi|21553940	3	42	-1.63** 0.0042
	DNA-binding aspartyl peptidase						
1140	Aspartyl protease family protein [*Arabidopsis thaliana*]	92	gi|38572690	gi|18412482	2	17	-1.66*** 0.00027
913	Aspartyl protease family protein [*Arabidopsis thaliana*]	228	gi|33184151	gi|15232503	5	30	-1.46** 0.0037
996	Aspartyl protease family protein [*Arabidopsis thaliana*]	191	gi|33450735	gi|15232503	5	48	-1.64* 0.01
1157	Aspartyl protease family protein [*Arabidopsis thaliana*]	93	gi|38572403	gi|15238250	2	25	-1.34* 0.016
1146	Aspartyl protease family protein [*Arabidopsis thaliana*]	201	gi|23994041	gi|15238250	3	44	2.15*** 0.00015
398	Aspartyl protease family protein [*Arabidopsis thaliana*]	148	gi|33453642	gi|15232503	5	52	5.05* 0.041
1488	Aspartyl protease family protein [*Arabidopsis thaliana*]	78 (+19)	gi|52514491	gi|15232503	4	27	2.59* 0.026
1495	Aspartyl protease family protein [*Arabidopsis thaliana*]	120	gi|27421141	gi|15232503	4	24	2.94*** 0.00063
1106	41 kD chloroplast nucleoid DNA binding protein (CND41	133	gi|23994041	gi|24430421	3	31	1.58*** 0.00021
1115	Nucleoid DNA-binding protein cnd41-like protein	153	gi|38572403	gi|110740049	2	30	1.78*** 0.00033
1206	Nucleoid DNA-binding protein cnd41-like protein [*A. thaliana*]	171	gi|38572403	gi|110740049	3	26	1.67* 0.012
1142	Nucleoid DNA-binding protein cnd41-like protein	226	gi|23989413	gi|8979711	2	75	2.13*** 0.00029
1148	Nucleoid DNA-binding protein cnd41-like protein	113 (+71)	gi|38572403	gi|110740049	4	30	1.85** 0.0076
1149	Nucleoid DNA-binding protein cnd41-like protein	170	gi|38572403	gi|110740049	2	30	2.09** 0.0048
1169	Nucleoid DNA-binding protein cnd41-like protein	202	gi|23989413	gi|8979711	2	59	2.01** 0.0058
1177	Nucleoid DNA-binding protein cnd41-like protein	163	gi|38572403	gi|110740049	3	26	1.7* 0.04
	Nucleotides metabolism						
1129	Putative adenosine kinase [*Populus alba x Populus tremula*]	66 (+54)		gi|41350585	3	55	-1,34** 0.0071
1135	Putative adenosine kinase [*Populus alba x Populus tremula*]	129		gi|41350585	3	56	-1.41** 0.0023
	Miscellaneous						
1375	Putative beta-subunit of K+ channels [*Solanum tuberosum*]	180 (+32)	gi|57892112	gi|223542738	4	51	-1.69** 0.0014
1376	potassium channel beta, putative [*Ricinus communis*].	118 (+14)	gi|57892112	gi|223542738	3	51	-1,78** 0.0012
1492	Phi-1 [*Nicotiana tabacum*]	120 (+36)	gi|18014334	gi|3759184	3	59	1.99* 0.035
491	Vacuolar ATP synthase catalytic subunit A (V-ATPase subunit A)	306		gi|137460	6	55	-1.8*** 0.00022
519	Vacuolar proton pump subunit alpha	220	gi|56834889	gi|137460	6	61	-1.48** 0.0028
1411	predicted protein [*Populus trichocarpa*]	150	gi|90190382	gi|224144967	3	43	-1,64* 0.012
	Spots presenting more than one protein identification						
880	Eukaryotic initiation factor 4A + predicted protein	115		gi|303844			2.69*** 0.00077
1112	aspartyl protease family protein + acid alpha galactosidase 1	116	gi|23989413	gi|8979711			2.58*** 0.000013
1122	Glutamine synthatase + Unidentified	116		gi|37956277			-1.43** 0.0067

33

1	2	3	4	5	6	
1118	Alcohol dehydrogenase *[Populus tremula]* + Aspartic proteinase nepenthesin-2 precursor, putative	82		gi|52851028	2,64**	0.003
1236	Aspartyl protease family protein + glycosylated protein	88	gi|38572690	gi|15238250	-1.89**	0.0012
785	Adenosylhomocysteinase + Enolase + 20S proteasome subunit alpha-1	98	gi|60206618		-2.51**	0.0023
796	Glutamate decarboxylase + leucine aminopeptidase, putative	80 (+67)	gi|38581604	gi|21536919	-1.47**	0.0037

773
774   1: spot number on the master gel
775   2: Protein obtained by blasting the EST against the NCBInr database
776   3: MASCOT score (protein score as given by the GPS software (Applied Biosystems))
777   4: Accession number of the EST sequence in the NCBInr database
778   5: Accession number of the corresponding protein in the NCBInr database
779   6: Average ratio of the protein abundance (Cadmium/control). Positive values of ratio are given as such, negative values are given according to the following
780   formula : given value = -1/average ratio

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781

782   Table 3 : Effect of 61 days of exposure to 360 mg $Cd.kg^{-1}$ SDW on different

783 physiological parameters of *Populus tremula* x *P. alba*, genotype 717-1B4.

784

Physiological parameter	Effect of Cd treatment
Stem height	-64%
Radial growth	-95%
CO_2 assimilation	-94%
Stomatal conductance	-56%
Cd^{2+} content in leaves	84.0 $mg.kg^{-1}$
Cd^{2+} content in cambial zone	123 $mg.kg^{-1}$
Zn^{2+} content in leaves	+127%
Mg^{2+} content in leaves	+91%
Ca^{2+} in leaves	+49%
K^+ content in leaves	+132%
K^+ content in cutting	-48%

785

786 Table 4 : Summary of the principle proteome changes in the leaf and in the cambial zone
787 of *Populus tremula x P.alba,* genotype 717-1B4 exposed 61 days to 360 mg Cd.kg$^{-1}_{SDW}$.

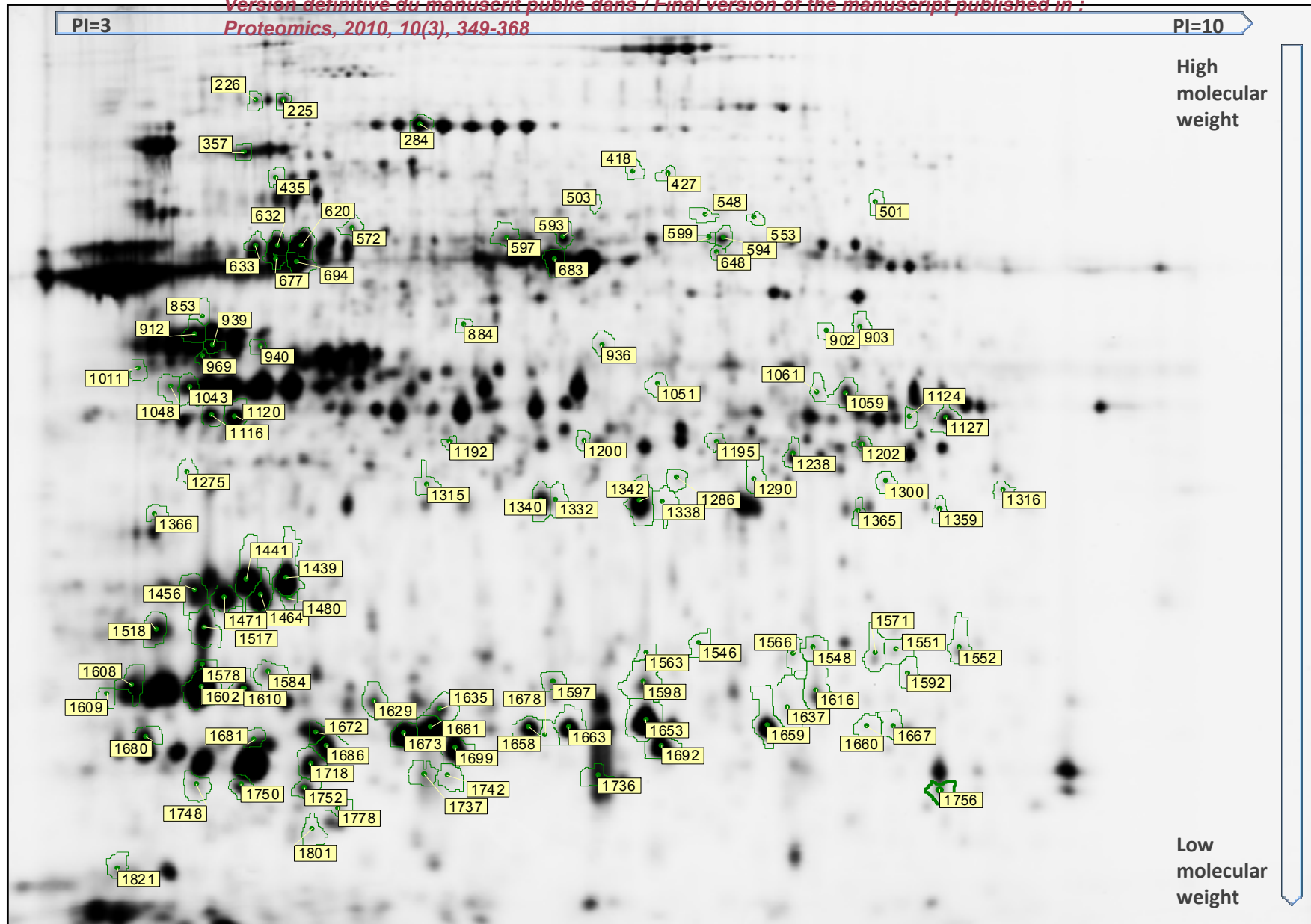| Metabolic functions	Leaf	Cambial zone
Photosynthesis	**3↑** (593, 503, 648)	0
	41↓ (e.g. 1464, 1043)	0
Carbon and carbohydrate metabolism	**12↑** (e.g. 501, 1127, 1571)	**7↑** (*e.g.* 288, 1892, 390, 481, 1070)
	9↓ (e.g. 284, 1120, 1200)	**15↓** (e.g. 480, 802, 851)
Energy metabolism	**3↑**(1660, 594, 599)	0
	6↓(620, 632, 633, 1598, 1616, 1592)	0
Protein metabolism	**1↑** (548)	0
	1↓(1290	**8↓** (e.g. 381, 885, 1302, 1240)
Protein catabolism	0	0
	2↓ (1678, 435)	**3↓** (796, 896, 1555)
Protein folding	**1↑** (357)	0
	4↓ (1011, 1635, 1518, 1752)	**4↓** (537, 541, 715, 1161)
Transcription & translation factors	0	**2↑** (1836, 877)
	1↓ (225)	**6↓** (332, 920, 892, 911, 1830, 1838)
Cytoskeleton & cell wall	0	**2↑** (926, 977)
	1↓ (1192)	**16↓** (*e.g.* 772, 1168, 990)
Stress Response – defense	**1↑** (1552)	0
	0	**1↓** (1179)
Citrate Cycle	**3↑** (418, 902, 903)	0
	2↓ (1200, 1316)	**3↓** (1268, 1290, 1336)
Oxidoreduction	**3↑**(1061, 936, 1637)	**1↑** (1137)
	2↓ (1202, 1821)	0
Glutathione metabolism	**2↑**(1659, 1667)	**2↑** (1657, 1744)
	0	**2↓** (908, 909)
Lipid metabolism	0	**3↑** (1064, 1066, 1059)
	0	**2↓** (572, 576)
DNA-binding Aspartyl Peptidase	0	**4↑** (1140, 913, 996, 1157)
	0	**12↓** (*e.g.* 1146, 1106, 1169)
Nucleotide metabolism	0	0
	0	**2↓** (1129, 1135)

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Figure 1. 2D-electrophoresis gel with **Leaf** proteins of *Populus tremula x P. alba* genotype 717-1B4 colored by CyDye 2. Identified proteins are labelled with their respective spot number.
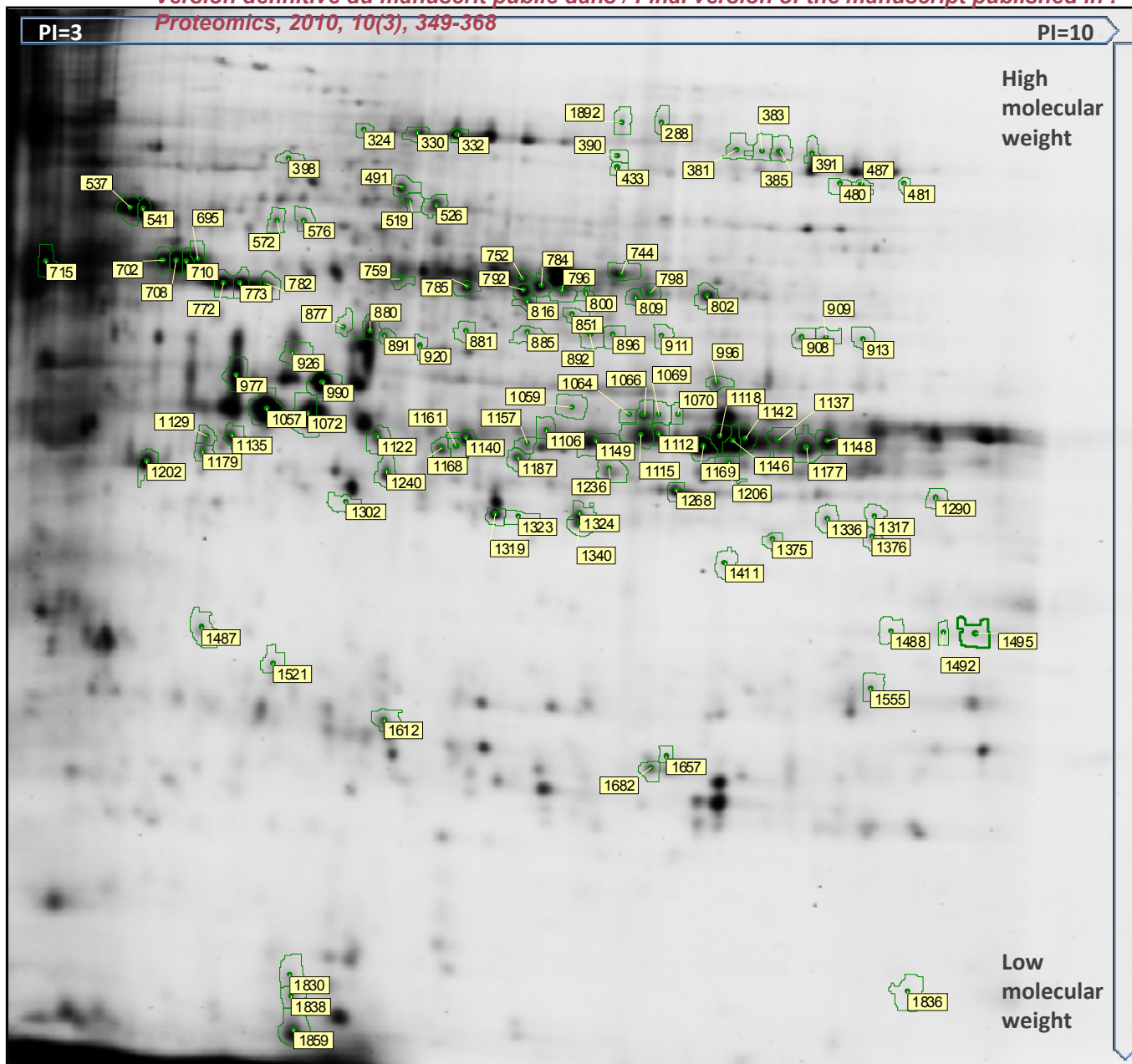
Figure 2. 2D-electrophoresis gel with **Cambial** proteins of *Populus tremula x P. alba* genotype 717-1B4 colored by CyDye 2. Identified proteins are labelled with their respective spot number.