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

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Keywords : Cadmium, Cambium, Fluorescence two-dimensional difference gel electrophoresis, Mass spectrometry, Metal stress, Phytoremediation.

Abbreviations: DAP: DNA-binding Aspartyl Protease / PRP: proline-rich protein / PSII : 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.

INTRODUCTION

Cadmium (Cd) is regarded as one of the most toxic heavy metals, responsible for human and plant diseases ^[1,2]. When plant physiology is affected by Cd stress, symptoms are generally chlorosis, necrosis, leaf rolling or drying and growth inhibition ^[3]. Cd toxicity arises notably from competition with Zn for binding sites in biomolecules, *e.g.* enzymes or carriers ^[4,5].

Since a few years *Salicaceae* (*e.g.* willows and poplars) have emerged as promising candidates in the search for convenient species to achieve phytoremediation of polluted sites ^[6]. Indeed, poplar, whose small genome is now completely sequenced, is a suitable model plant to support molecular studies on stress response's ^[7,8]. Poplar is reported to be able to thrive despite metal contamination, and to accumulate metals, especially cadmium ^[9]. Being a perennial species poplar furthermore allows the opportunity to shape an ideal phytoremediator. Year after year accumulation of metals in harvestable woody tissues provides a convenient means of soil clean up combined with the potential of energy production. As a model-tree species poplar can also be used to study the metabolic mechanisms of metal uptake and tolerance in woody species.

The plant responses to metal stress are mediated through modifications in gene expression and protein levels. Proteins that participate in metal tolerance response are reported to be involved in the induction of transcription factors, in the protection or restoration of macromolecules and in detoxification activities ^[10].

Metal homeostasis and detoxification are known to be partly constitutive ^[2], but inducible parts of these processes can be explored by a proteomic approach. Although proteomic research has been conducted on the effects of metals on plants, *e.g.* on the response to Cd in roots of *Oryza sativa* ^[11], in cell cultures of *Arabidopsis thaliana* ^[12], in leaves of *Spinacia oleracea* ^[13] or in roots and shoots of *Thlaspi caerulescens* ^[14], a recent review by Ahsan, Renaut & Komatsu ^[15] deplored the limited proteomic investigations on this topic, especially for long-term exposition. Kieffer *et al.* ^[16,17] recently reported proteome changes in leaves of poplar exposed to Cd up to 56 days under hydroponic conditions.

The comprehension of the tolerance of plants to environmental constraints requires a profound description of the acting molecules during the response. Typically, a constraint

will evoke a stress perception and a stress signalization leading to damage avoidance and repair. For each step of the plant response, some of the involved molecules belong to a generic pattern of response to all biotic/abiotic stresses, while others can be related to one specific stressor. Current research mainly deals with molecules involved in the response to a range of diverse constraining conditions, *e.g.* chaperones, antioxidants, proline, plant growth regulators, etc. ^[18,19,20,21]. These molecules are responsible for the General Adaptation Syndrome (GAS), initially formulated by Selye in 1951 ^[22,23]. However, one type of stress will not equally affect the dissimilar tissues within an individual. Published proteomic studies of plant stress most often concern leaf or root tissues. This is graspable considering that the photosynthetic apparatus is often affected by heavy metal toxicity ^[24], while roots are directly exposed to the contaminated environment. Yet, other tissues can be considered to complement the analysis. In woody species the cambium activity determines the secondary growth that represents a physical support for the extended primary growth of the tree. It also shapes the structure of conductor tissues –number and size of wood vessels and fibers, cellular composition of phloem. Cambium activity eventually influences the production of biomass wherein heavy metals can be accumulated. Despite its importance, the cambium response remains scarcely questioned. Available studies on the cambial tissue generally focus on developmental questions ^[e.g. 25,26] and few deal with biotic stress ^[27] or abiotic stress ^[28].

A proteomics approach on the responses of plants during exposure to pollutants, *in casus* cadmium, will point out the molecular actors supporting metal intake and also give information about stress-related responses in different tissues. This knowledge will allow the selection or the design of plants that prevent deleterious metal accumulation along the food chain (like cadmium), or, on the contrary, crops that could solve problems of human essential metal deficiencies.

In this study, young poplar plants were exposed to a soil contaminated by the addition of 360 mg Cd.kg⁻¹ soil dry weight (SDW), corresponding to a total amount of 1 mmol of metal per liter of soil. Ecophysiological parameters were monitored all along the 61 days of exposure to characterise the physiological state of the plant. Metal distribution in the tree was also determined. A subsequent proteome analysis on leaf and cambial tissues

illustrated the acute stress endured by Cd and its strong effect on photosynthesis and carbon metabolism.

2. MATERIAL AND METHODS

2.1. Plant material and metal constraints

Pot-grown *Populus tremula* L. x *P. alba* L. (*Populus x canescens* (Aiton) Smith) genotype INRA 717-1B4 were obtained as previously described in Durand *et al.* (2009, submitted). Once rooted and 3 months old, poplar plants were pruned and transplanted from 0.3 L pots to 10 L pots filled with a sand - peat moss soil mixture (25:75, v/v, pH 6.9). Cadmium was incorporated in the 10 L soils by uniformly hand mixing.

Control soil contained no detectable cadmium. Cadmium contaminated soil contained 360 mg Cd.kg⁻¹ SDW. Soil solution concentrations were determined from 100 g of fresh soil after 20 min of centrifugation at 10,000 g. The supernatant was collected for quantification using a Jobin-Yvon® HR-ICP-AES, as described in [29]. Soil solution of metal-exposed plants contained 20.8 µM Cd²⁺.

Plants were grown in culture chamber at 21°C, 70% of relative humidity, and with an irradiance of 1000 µmol.m⁻².s⁻¹ provided during 16 h per day. After 61 days of metal exposure, leaf and woody tissues were collected and frozen in liquid nitrogen and stored at -80°C until protein extraction.

2.2. Protein extraction and sample preparation

Four biological replicates corresponding each one to individual plants were used. Leaf proteins were extracted starting from 500 mg of fresh weight collected from the three last fully expanded leaves of one plant. After lyophilisation of the cutting, the bark was excised. The cambial zone was then collected by softly scratching the inner face of the bark with a scalpel. Proteins from the cambial zone were extracted from approximately 60 mg of dry tissue of each replicate. Tissues were ground in liquid nitrogen. Proteins were extracted using the TCA-acetone precipitation method described by Damerval *et al* [30]. Resolubilisation of the precipitated proteins was carried out in a lysis buffer (7M urea, 2M thiourea, 4% w/v 3-[(3-cholamidopropyl)-dimethylammonio]-

After quantification, proteins were labelled by mixing 240 pmol of fluorochromes (CyDyes™, GE Healthcare) with 30 µg proteins. For each gel, Cy3 and Cy5 were used for control or treated samples; Cy2 was used for the internal standard consisting of a mix of equal amounts of each sample. A dye swap was used between Cy3 and Cy5 to avoid problems associated with preferential labelling.

Bidimensional electrophoresis was carried out as described in Bohler *et al.* [31], with slight modifications. The isoelectric focusing was carried out on pH 3-10 IPG-strips (24 cm, non linear gradient; GE Healthcare, Little Chalfont, UK) using the IPGphor system from GE Healthcare. Protein samples were cup loaded. The migration was performed at 20°C ($\leq 50 \mu\text{A}$, 60V for 2 h; gradient from 60 V to 1,000 V for 3 h ; hold 1,000 for 1h, gradient from 1,000 to 8,000 for 3 h; hold 8,000 V until 85,000 V.h). Strips were then stored at -20°C.

DeCyder v.6.05.11 software (GE Healthcare) was used to determine differentially expressed proteins with a variation factor of at least 1.3 in abundance (up and down) and a significant Student t-test score ($p < 0.05$). The automated matching was manually confirmed for all the spots that were selected for identification, and that are further discussed. Spots were picked from the gel and digested by trypsin for 6 h at 37°C using an Ettan Dalt Spot Handling Workstation (GE Healthcare) before acquisition of peptide

mass spectra with a MALDI-TOF-TOF analyser (4800 Applied Biosystems, Foster City, CA, USA).

2.4. Protein identification.

As the poplar genome is not yet completely annotated and many sequences were only available as ESTs, protein identification was mainly done using the EST-database on an in-house mascot platform. The NCBI poplar EST database used in this study contains 419,944 poplar sequences and was downloaded from the NCBI database on 06/11/2009. MS and MS/MS data were also submitted for analysis with a viridiplantae protein database downloaded on 02/17/2009 and containing 1,214,000 sequences. All searches were carried out using a mass window of 100 ppm for the precursor and 0.5 Da for the MS/MS fragments. Up to 2 trypsin miscleavages were accepted. The search parameters allowed for carbamidomethylation of cysteine, oxidation of methionine as well as oxidation of tryptophan to kynurenine and double oxidation of tryptophan to N-formylkynurenine. During automatic interrogation of databases, the MASCOT score cut-off was 77 for NCBI and 65 for EST interrogations. Because all spectra and identifications were manually verified, peptide scores were considered even below the cut-off values for peptide scores. Identifications were validated manually with at least two identified peptides at disparate sites within a protein with a score above homology.

Percentage of sequence coverage was given in Tables 1 & 2. Since EST's generally do not represent an entire protein sequence, the authors would like to remark that this value has little significance when identifications were done on EST sequences. Grouping of proteins in biological process was done accordingly to KEGG and Uniprot databases (<http://www.genome.jp/kegg> ; <http://www.uniprot.org/>). The functions of some proteins were enlightened by the use of InterPro ^[32] (<http://www.ebi.ac.uk/interpro/>) which consists in blasting the amino sequences to the integrated resources of several databases like PANTHER, PROSITE and Gene3D.

In leaf and cambial proteomes, respectively in 2 and 7 spots, more than 1 protein was identified, since this prevents any interpretation about the actual abundance of change in them, biological discussion was not done on these proteins. They are nonetheless presented at the end of Tables 1 & 2.

2.5. Statistics

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

3. RESULTS

3.1. Leaf proteome profiles.

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

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

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

3.2. Cambium proteome profiles.

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

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

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

4. Discussion.

Young *Populus tremula* x *P. alba* 717-1B4 genotype plants were exposed to 360 mg Cd.kg⁻¹ SDW, this resulted a in soil solution concentrations of 20.8 µM Cd²⁺. An in-soil experiment permitted to follow the dynamic ion partition between soil and plant, which may be a determining factor in the response of the plant [33,34]. The study aimed at analyzing tissues that have grown and developed under metal constraints. This allowed to focus on distal equilibrium rather than on the alarm phase of stress. Thus, control and stressed plants were compared after 61 days of cadmium exposure.

The Cd treatment resulted in a Cd accumulation in the leaves (84 mg Cd.kg⁻¹) and in the cambial zone (123 mg Cd.kg⁻¹). The treatment drastically reduced the growth of the plants. Net photosynthesis and stomatal conductance were inhibited. Increases in the content of K⁺, Ca²⁺, Mg²⁺ and Zn²⁺ in tissues, especially in leaves, were observed. These results are summarized in the Table 3 (adapted from [29]). The physiological data demonstrate that Cd²⁺ triggered an acute stress in poplar plants from which ensues important changes in leaf and cambial proteome (Figures 1 & 2).

4.1. Leaf proteome response to Cd

The proteomic data gave two levels of information that can hardly be distinguished. Some changes occurred as a consequence of the stress endured, while others constitute the concrete and active response of the plant defense system. The Impact Factor (IF) on protein abundance was calculated as the ratio of the volume of protein spots between treated and control conditions.

In leaf and cambial tissues the cadmium stress mainly affected the carbon metabolism. In the leaf, negative IF was salient on the carbon fixation, especially on ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and RuBisCO activase. Other proteins involved in the Calvin Cycle were also affected (spots 284, 1116, 1120, 1124, 1127, 1566, 1571). Additionally 3 out of 4 spots wherein carbonic anhydrase was identified decreased in intensity in Cd-treated plants (spots 1592, 1598, 1616, 1660).

These data are consistent with our previous results, which describe the inhibition of CO₂ assimilation under Cd stress, which was mainly unrelated to stomata closure [29]. The literature diversely reports on the stomatal response to Cd stress, although stomata closure is often regarded as a typical response [35,36,37]. Shi and Cai [38] observed a Cd-

induced xerophytes-like leaf morphology with more frequent but smaller stomata, accompanied by a reduced stomatal conductance in peanut (*Arachis hypogaea*). On the contrary Zhu, Macfie & Ding ^[39] reported that Cd-exposed *Brassica juncea* showed fewer although larger stomata. In both cases, the reduced stomatal aperture only partly explained the decreased photosynthetic activity.

Whatever the stomatal response, the Cd toxicity mostly arises through other processes than gas exchange. In this study, the strongest negative influence of Cd on spot intensity was observed for photosynthesis-related proteins. This reduced intensity was particularly observed for proteins implied in the electron transport: chlorophyll a/b binding proteins (10 spots down regulated, with IF from -2.08, spot 1584, to -11.02, spot 1517, Table 1), Oxygen Evolving Enhancer (5OEE1 spots and 5 OEE2 spots, *i.e* 33 kDa and 23 kDa subunits respectively, all down regulated, with IF from -2.75, spot 1456, to -7.03, spot 1441) and ferredoxin-NADP oxidoreductase (5 spots down-regulated, *e.g.* spot 1340, 2 other up regulated, *e.g.* spot 1338). The decrease of the photosynthetic activity, inferred from the diminished CO₂ assimilation (Table 4) is further suggested by the drop in abundance of a FtsH protease 8 which contributes to the turnover of the Photosystem II D1 protein ^[40]. These results incline to show a major impact on proteins of the PSII, which is contradictory with a recent study on spinach where PSI was described as more sensitive to Cd²⁺ ^[4]. Among the 5 ATPase spots differentially expressed, 3 were down-regulated (spots 620, 632 and 633), all of them chloroplasmic ATPase. The 2 up-regulated ATPase are localized in the mitochondrion, according to the databases (spots 594 & 599). This suggests that photosynthetic processes are inhibited by Cd stress, while respiration, on the contrary, is promoted. Supporting the latter, the increase in quantity of a succinate dehydrogenase spot was recorded (spot 418). This enzyme takes part in the mitochondrial respiratory chain. Changes in the abundance of TCA cycle enzymes, increased accumulation of citrate synthase (spots 902, 903) and decreased abundance of malate dehydrogenase (spots 1200, 1316), do not result in a consensus on how the respiration is affected during Cd stress, but confirm that there is an effect. Consistently with an increased respiration concomitant to a suppressed photosynthesis, an induction of glycolysis-involved enzymes was observed: UDP-glucose pyrophosphorylase (spot 597), glycosyl hydrolase family 38 protein (spot 553), 3-phosphoglycerate kinase (spot 1051).

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

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

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

of an alpha tubulin (IF-2.94, spot 1192) might also be connected to the leaf growth inhibition in spite of a sampling realized on fully expanded leaves. Indeed, the Cd-treated sampled leaves presented a significantly smaller size than control.

A stimulated potassium channel beta subunit (IF+1.6, spot 1300) converged with previous results showing a significant alteration of potassium homeostasis under Cd stress^[29]. The $[K^+]_{\text{leaves}}$ increased 2.3-fold compared to control plants (Table 4). In the cambial zone, on the contrary, two beta-subunit of K^+ channels decreased in quantity; linked to the 48% reduced $[K^+]_{\text{cutting}}$. Only few reports on the regulation of mineral homeostasis under Cd stress are available in the literature. A recently published paper indicated no change in K^+ and Mg^{2+} content in leaves of Cd-tolerant mungbean (*Vigna radiata*) under Cd exposure^[48]. To our knowledge neither the mobilization of K^+ in leaves in response to Cd, nor the implication of a potassium channel were reported so far.

It is not surprising that Cd disturbs metal homeostasis, even if proteomic data give little indication on it. For instance the 2-fold increase of $[Mg^{2+}]_{\text{leaves}}$ (Table 4) can be linked to the instability of photosynthesis proteins like the rubisco activase that often have Mg^{2+} as cofactor. The increased $[Mg^{2+}]$ could be needed to compete with the toxic Cd^{2+} in order to reduce proteins misfolding and inactivation.

The comparison of these results with those from a previous hydroponic poplar exposure to Cd^[17,16] exhibits some common traits that were underlined above, but also some differences. Hydroponic and soil conditions partly affect plant physiology in differential ways, notably metal bioavailability. This could account for a part of these differences in the proteomic response.

For instance, a chitinase (spot 1552) was the sole identified pathogenesis-related (PR) protein affected by Cd in leaves in this study, whereas this type of proteins was shown to accumulate, especially on the long term (day 56), in Kieffer's articles^[17,16]. Cd-responsive proteins implied in oxidoreduction were more numerous in leaf than in cambial zone, with an overall negative impact of the stress. When comparing with exposure to Cd in a hydroponic system, it appears that these protein functions are more responsive on a shorter time scale (7-14 days) than on 56 days.

4.2. Cambial proteome response to Cd

As in leaf, the carbon metabolism is the principal biological process altered in cambial proteome in response to Cd. The differentially expressed proteins are partly corresponding to the same functions as in leaf (e.g. 3 malate dehydrogenase, spots 1268, 1290, 1336 ; 1 triosphosphate isomerase, spot 759, and 1 UDP glucose pyrophosphorylase, spot 752, all of them were inhibited in the cambial zone). In addition to these proteins, the overall changes linked with carbohydrate metabolism indicate an impact on glycolysis and glycogenesis. Five spots of enolase were down-regulated (e.g. spot 784, 798, 800, 802, 816). Three spots of UDP glucose dehydrogenase (spots 744, 752, 809), one phosphofructokinase family protein (spot 851), one 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (spot 526) and one fructokinase (spot 1202) decreased, whereas 2 alcohol dehydrogenase (spots 1069 & 1070) increased in quantity. Two spots of alpha-D-xylosidase (spots 288 & 1892) were more intensively stained. This enzyme is reported to be analogous to alpha glucosidase ^[49]. Hence the overall carbohydrate metabolism was reduced.

In cambial zone as in the leaf, the great disturbance in the carbohydrate metabolism is obviously a distal consequence of Cd stress. A previous work on poplar response to Cd showed changes in sugars content ^[17]. Such changes, beyond stress symptomatology, probably take part in the sensing and signaling of the constraint ^[50] even if the alarm phase is very likely to have ceased before day 61. Most sugars are known to improve osmoprotection during stress ; this is a role they could also play in the metal stress ^[51].

The protein metabolism is the second biological process influenced by Cd stress in the cambial zone. Protein synthesis was reduced, as attested by the decreased abundance of 4 spots containing methionine synthase (spots 381, 383, 385, 391), 3 spots containing S-adenosylmethionine synthetase (spots 881, 885, 891), an O-acetylserine (thiol)lyase (spot 1240), and a 60S acidic ribosomal protein P0 (spot 1302). There was also one differentially displayed spot of eukaryotic initiation factor 4A (spot 902), and 3 others of elongation factor 1- γ (spots 332, 892, 911). One calreticulin-1 (spot 715) and 3 protein disulfide-isomerase precursors (spots 537, 541, 1161), related to protein folding exhibited a reduced abundance. Proteolysis was also affected, with the decreased abundance of two proteasome subunits (spots 896 & 1555) and of a Zn-dependent leucine aminopeptidase (spot 796), and also 2 more abundant alpha-mannosidase (spots 390 and 433) involved in

the vacuolar degradation of glycoproteins. So protein metabolism, folding and catabolism appeared to be impaired by Cd treatment.

Cd-stress also induced changes in proteins involved in cell structure and cell wall, hence related to the growth function of the meristem. Two spots of rhamnose biosynthesis 1 (spots 480, 487) were less abundant, one was more abundant (spot 481); this enzyme participates to the production of rhamnose, an important constituent of the cell wall ^[52]. Also in relation with cell wall synthesis, 3 spots of phenylcoumaran benzylic ether reductase dropped in intensity (spots 1319, 1323, 1340). This enzyme is strongly associated with phenylpropanoid biosynthesis in lignifying cells of poplar ^[53]. One cinnamoyl-CoA reductase (spot 1324), involved in the synthesis of lignin and a leucine rich repeat protein (spot 1317), also implied in cell wall synthesis ^[54] decreased in amount. Two spots of reversibly glycosylated polypeptides (spots 1168 & 1187) dropped in intensity; these proteins, supposed to be plasmodesmata-associated ^[55] are linked to cell elongation and related to cell wall formation, even if their precise role in the cell remains unsolved ^[56]. Consistently with an inhibition of growth, 2 spots containing adenosine kinase (spots 1129 & 1135), implied in nucleic acid synthesis, exhibited lower content.

According to Garnier *et al.* ^[57], Cd cytotoxicity arises notably through three waves of oxidative stress in tobacco cells. In the present study, in cambial tissue as it was the case in leaves, the redox balance appears to be disturbed. A class III peroxidase (spot 1137) was more abundant. Two 6-phosphogluconate dehydrogenase family proteins (spots 908 & 909), involved in the glutathione metabolism, decreased in amount, whereas a glutathione-S-transferase was more abundant (spot 1657). The lower accumulation of a glutamate decarboxylase (spot 796) was observed, but a putative leucine aminopeptidase was identified in the same spot, preventing any interpretation concerning this calmodulin-binding protein that produces GABA (γ -aminobutyric acid).

437

The suppression of cytoskeletal-linked proteins such as α and β chain tubulin (7 spots down regulated, e.g. spot 772) or actin (spots 977, 990) in cadmium exposed poplar confirms the main role of cambium as an actively growing tissue, and the different growth rates between control and exposed plants. Two spots of glycine-rich RNA-

binding protein (spots 1836 & 1838) appeared differentially expressed. This protein family of post-transcriptional regulators has been showed playing a role in cold acclimation of *Arabidopsis* ^[58]. Cd induced antagonistic changes in 2 putative proline-rich protein (PRP) (spots 926 & 1057). These cell wall structural proteins are reported to accumulate under water deficit in *Phaseolus vulgaris* cells ^[59]. When the respective EST sequences for these spots were used in BLAST analysis, the results indicated that they contain a "GDSL" motif close to the N-terminus. In addition 2 GDSL-motif lipase/hydrolase family protein spots decreased in amount (spots 572 & 576). These proteins could have a role in the degradation of complex polysaccharides ^[60,61]. The GDSL family has been repeatedly reported to be implied in diverse stress responses ^[62]. Among the 159 Cd-affected proteins in the cambial zone, 7 have Zn as cofactor (spots 796, 1069, 1070, 537, 541, 1161, 715), and 4 others potentially binds Zn (1302, 1836, 1838, 1179). All of them, except the 2 alcohol dehydrogenase and one of the 2 glycine-rich RNA-binding protein were reduced in quantity. In the leaf proteome 7 out of 114 identified Cd-affected proteins needs Zn for functioning (spots : 435, 1548, 1563, 1592, 1598, 1616, 1660). When compared to the counted 2367 Zn-related proteins of *A. thaliana* ^[63], out of 159,162 entries in database for this species (according to "porgn:txid3702" request on <http://www.ncbi.nlm.nih.gov>), the present proportion of Zn-related proteins affected by Cd stress did not clearly suggest a particular and quantitative involvement of them in the response.

The quantitatively most prominent information about cambial proteome response concerns plastidial DNA-binding aspartyl peptidase (DAP). Increased abundance in proteolytic enzymes has already been observed in studies on poplar leaves and roots under Cd exposure, but no influence on the abundance of DAP in this study under hydroponic condition was observed ^[16]. Sixteen DAP spots showed a differential expression under Cd stress (e.g. spot 1140), 12 were up-regulated, with IF up to 5.05 (spot 398). Kato *et al.* ^[64] reported a link between nucleoid DNA-binding protein cnd41-like protein – which is a DAP – and the degradation of RuBisCO in tobacco leaves during senescence. Yet no differences in the expression of DAP were observed in the leaves of poplar plants. Although some cambium cells express photosynthetic genes, including at least RuBisCO subunits ^[65], it seems unlikely that RuBisCO degradation plays such a

major role in cambial response to Cd so as to involve about 20% of the identified proteome changes. Therefore, the precise role of these proteins remains intriguing.

The proteomic changes described in this study, when compared with the literature, are implied in other constraints as part of a generic stress pattern and do not show any Cd- or even metal-stress specificity^[66,67,31,68]. This is not the case of the overexpression of DAP which constitutes a putative Cd-specific response of cambial tissue

This study focused on young fully developed leaves, is it probable that other leaf levels would exhibit nuances in their response profiles, for it is known that stress response depends on the age of the tissue^[69], that cadmium affects more strongly basal leaves of spinach^[4], and that some plants achieve tolerance towards metals by preferential storage in organs, tissues, cells or organelles^[70,13]. We besides reported that lack of compartmentalization could be linked to *P. tremula* x *P. alba* sensitivity to Cd²⁺^[71].

Conclusion

In leaves containing 84 mg Cd.kg⁻¹, the Cd toxicity principally impaired photosynthesis and primary metabolism. Consequently, primary growth was reduced. The resulting loss of photosynthates in all plant tissues, like cambium, partly accounts for the decreased activity of these tissues. So, cambial proteome changes resulted in part from a systemic toxicity, and in part from the 123 mg Cd.kg⁻¹ present inside the tissue. The proteomics data presented here showed contrasted responses to Cd between leaf and cambial zone.

It now seems important to explore whether the patterns of the proteome response of the different tissues persist whatever the constraint (e.g. under drought or heat stress). If so, the General Adaptation Syndrome description for plant under stress could be enriched with tissue-localized markers.

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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.

753

754

Table 1: Differentially expressed proteins in the leaf proteome of *Populus tremula* x *P.alba* genotype 717-1B4 submitted during 61 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 calculated between treated and control plants.

Spot number ¹	Leaf protein identifications ²	Protein score ³	Accession Number EST ⁴	NCBI ⁵	No of peptides	Sequence coverage (%)	Cd / Ctr ratio ⁶	p value ⁷
	Photosynthesis							
1464	RubisCO large subunit [<i>Populus tomentosa</i>]	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 [<i>Malesherbia linearifolia</i>]	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 [<i>Populus alba</i>]	172		gi 110227087	4	50	-1.58*	0.042
593	RubisCO large subunit [<i>Populus alba</i>]	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 I	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) [<i>Cicer arietinum</i>]	219 (+70)		gi 3928140	6	69	-10.26*	0.026
1602	Chlorophyll a/b binding protein(Lhcb2) [<i>Cicer arietinum</i>]	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	LHCI1 type II chlorophyll a/b-binding protein [<i>Vigna radiata</i>]	160 (+16)		gi 9587203	6	28	-4.09****	0.00083
1517	Light-harvesting complex II protein Lhcb1 [<i>Populus trichocarpa</i>]	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	LHCBS - chlorophyll binding	301		gi 15235029	3	46	-2.69***	0.00067

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 biphosphatase	253	gi 52530247	gi 223530064	6	53	-2.48***	0.00015
1116	Sedoheptulose biphosphatase	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 [<i>Populus tremuloides</i>]	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 [<i>Solanum tuberosum</i>]	80	gi 52620161	gi 167909863	4	60	2.22**	0.0059
597	UDP-glucose pyrophosphorylase [<i>Populus tremula</i> x <i>P. tremuloides</i>]	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 [<i>Glycine max</i>]	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 [<i>Populus tomentosa</i>] - chloroplast	354		gi 22094585	6	75	-4.19***	0.00085
632	ATPase beta subunit [<i>Populus tomentosa</i>] - chloroplast	295		gi 22094585	5	75	-3.91***	0.000019
633	ATPase beta subunit [<i>Populus tomentosa</i>] - chloroplast	401 (+237)		gi 22094585	7	80	-2.02**	0.0044
594	ATPase alpha subunit [<i>Didymosporium perrieri</i>] - mitochondrion	240		gi 6561625	6	38	1.83*	0.015

599	ATPase alpha subunit [<i>Didymeles perrieri</i>] - 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 [<i>Ricinus communis</i>]	72 (+22)	gi 23995055	gi 223542068	3	29	36.78*	0.025
435	FtsH protease 8 / ATPase/Zn dependant metalloprotease	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 [<i>Nicotiana benthamiana</i>]	199	gi 52502629	gi 124484511	2	60	-3.72**	0.0081
1752	Nascent polypeptide associated complex alpha chain [<i>Oryza sativa</i>]	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 [<i>Vitis vinifera</i>].	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 [<i>Ricinus communis</i>]	79	gi 52536153	gi 223538636	3	35	-1.75**	0.0038
902	Citrate (si)-synthase [<i>Populus trichocarpa</i>]	177	gi 53783182	gi 1648926	6	33	2.11**	0.0034
903	Citrate (si)-synthase [<i>Populus trichocarpa</i>]	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	Peroxisome type 2, putative	187	gi 52505836	gi 15231718	2	57	-2.09*	0.025
1637	1-cys peroxiredoxin [<i>Xerophyta viscosa</i>]	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 [<i>Galega orientalis</i>].	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 [<i>Nicotiana tabacum</i>]	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 [<i>Ricinus communis</i>]	142	gi 52530576	gi 223539406	5	14	-2.56*	0.037
225	Translation elongation factor EF-G [<i>Glycine max</i>]	354 (+47)		gi 402753	5	37	-1.64***	0.00046

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

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1: spot number on the master gel

2: Protein obtained by blasting the EST against the NCBI database

3: MASCOT score (protein score as given by the GPS software (Applied Biosystems))

4: Accession number of the EST sequence in the NCBI database

5: Accession number of the corresponding protein in the NCBI database

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 formula : given value = -1/average ratio

7: p-value of Student's t-test.

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

Spot number ¹	Cambial protein identifications ²	Protein score ³	Accession Number EST ⁴	NCBI ⁵	Nb of peptides	Sequence coverage (%)	Cd / Ctr ratio ⁶	p value ⁷
Carbon and carbohydrate metabolism								
784	Enolase1 [<i>Zea mays</i>]	395	gi 24075693	gi 162458207	6	81	-1.39**	0.0013
798	Enolase1 [<i>Zea mays</i>]	249	gi 24102975	gi 162458207	4	61	-1.38**	0.0015
816	Enolase [<i>Brassica napus</i>]	142	gi 24020181	gi 34597332	4		-1.73***	0.00048
802	Enolase1 [<i>Zea mays</i>]	358		gi 162458207	4	42	-1.74***	0.00022
800	Os06g0136600 [<i>Oryza sativa</i> (japonica cultivar-group)]	130		gi 115466256	4	36	-2.1***	0.00035
759	Triosephosphate isomerase-like protein [<i>Solanum tuberosum</i>]	96	gi 52505071	gi 76573375	3	33	-1.59*	0.01
752	UDP-glucose pyrophosphorylase [<i>Populus tremula</i> x <i>P. tremuloides</i>]	239		gi 32527831	3	19	-1.52*	0.028
744	UDP-glucose dehydrogenase [<i>Populus tremula</i> x <i>P. tremuloides</i>]	313		gi 6164591	6	66	-1.58**	0.0015
809	UDP-glucose dehydrogenase [<i>Populus tremula</i> x <i>P. tremuloides</i>]	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 [<i>Arabidopsis thaliana</i>]	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 [<i>Tropaeolum majus</i>]	123	gi 73928193	gi 5725356	2	23	1.53*	0.012
1892	Alpha-D-xylosidase precursor [<i>Arabidopsis thaliana</i>]	111	gi 28606907	gi 4163997	3		1.51*	0.01
390	Alpha-mannosidase [<i>Arabidopsis thaliana</i>]	113		gi 10177664	3	10	1.77**	0.0015
433	Alpha-mannosidase [<i>Arabidopsis thaliana</i>]	79		gi 10177664	2	12	1.44**	0.0085
480	RHM1/ROL1 (RHAMNOSE BIOSYNTHESIS1); UDP-glucose 4,6-dehydratase/ catalytic [<i>A. thaliana</i>]	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 [<i>Ricinus communis</i>]	170	gi 60697528	gi 223526803	5	62	-1.9***	

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

708	Beta-6 tubulin [<i>Zea mays</i>]	451		gi 162459800	8	67	-1.77***	0.00051
695	Beta-6 tubulin [<i>Zea mays</i>]	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 [<i>Populus trichocarpa</i>]	603	gi 33453463	gi 3114901	7	84	-1.57**	0.0046
1323	Phenylcoumaran benzylic ether reductase [<i>Populus trichocarpa</i>]	122	gi 33453463	gi 224066197	5	65	-1.62*	0.03
1340	Phenylcoumaran benzylic ether reductase [<i>Populus trichocarpa</i>]	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 [<i>Populus tremula</i>]	134 (+26)	gi 33451847	gi 190897438	4	52	-1.6***	0.00065
977	Actin [<i>Stevia rebaudiana</i>]	133		gi 23955912	3	58	2.15***	0.00048
990	Actin [<i>Gossypium hirsutum</i>]	650		gi 32186906	8	77	-1.54**	0.0024
1057	Putative proline-rich protein [<i>Oryza sativa</i> Japonica Group]	193	gi 38594177	gi 14209541	2	16	-2.13*	0.027
926	Putative proline-rich protein [<i>Oryza sativa</i> Japonica Group]	83 (+46)	gi 38594177	gi 14209541	3	22	2.08**	0.0094
1324	Cinnamoyl-CoA reductase [<i>Jatropha curcas</i>]	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 [<i>Prunus persica</i>]	157	gi 52521122	gi 15982948	4	33	-1.41**	0.0037
1290	Malate dehydrogenase precursor [<i>Medicago sativa</i>]	132	gi 24062191	gi 2827080	3	54	-1.58*	0.036
1336	Malate dehydrogenase [<i>Glycine max</i>]	133	gi 73871484	gi 5929964	5	47	-1.61**	0.0062
Oxidoreductase								
1137	Peroxidase [<i>Nicotiana tabacum</i>]	147	gi 57894628	gi 14031049	4	42	2.93*	0.015
Glutathione metabolism								
1657	Glutathione-s-transferase theta, gst, putative [<i>Ricinus communis</i>]	120	gi 24100781	gi 223551315	3	57	1.66*	0.027
908	6-phosphogluconate dehydrogenase, putative [<i>Ricinus communis</i>]	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 [<i>Macroptilium atropurpureum</i>]	128	gi 24064109	gi 168274274	6	38	1.46**	0.0029
1064	Acetylcholinesterase [<i>Macroptilium atropurpureum</i>]	212	gi 24064109	gi 168274274	5	38	1.59***	0.00077
1066	Acetylcholinesterase [<i>Macroptilium atropurpureum</i>]	323	gi 24064109	gi 168274274	5	39	1.89***	0.00019
572	GDSL-motif lipase/hydrolase family protein [<i>Arabidopsis thaliana</i>]	227	gi 38594604	gi 15242458	2	33	-1.73*	0.023

576	GDSL-motif lipase/hydrolase family protein [<i>Arabidopsis thaliana</i>]	218	gi 23976058	gi 21553940	3	42	-1.63**	0.0042
DNA-binding aspartyl peptidase								
1140	Aspartyl protease family protein [<i>Arabidopsis thaliana</i>]	92	gi 38572690	gi 18412482	2	17	-1.66***	0.00027
913	Aspartyl protease family protein [<i>Arabidopsis thaliana</i>]	228	gi 33184151	gi 15232503	5	30	-1.46**	0.0037
996	Aspartyl protease family protein [<i>Arabidopsis thaliana</i>]	191	gi 33450735	gi 15232503	5	48	-1.64*	0.01
1157	Aspartyl protease family protein [<i>Arabidopsis thaliana</i>]	93	gi 38572403	gi 15238250	2	25	-1.34*	0.016
1146	Aspartyl protease family protein [<i>Arabidopsis thaliana</i>]	201	gi 23994041	gi 15238250	3	44	2.15***	0.00015
398	Aspartyl protease family protein [<i>Arabidopsis thaliana</i>]	148	gi 33453642	gi 15232503	5	52	5.05*	0.041
1488	Aspartyl protease family protein [<i>Arabidopsis thaliana</i>]	78 (+19)	gi 52514491	gi 15232503	4	27	2.59*	0.026
1495	Aspartyl protease family protein [<i>Arabidopsis thaliana</i>]	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 [<i>A. thaliana</i>]	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 [<i>Populus alba</i> x <i>Populus tremula</i>]	66 (+54)		gi 41350585	3	55	-1.34**	0.0071
1135	Putative adenosine kinase [<i>Populus alba</i> x <i>Populus tremula</i>]	129		gi 41350585	3	56	-1.41**	0.0023
Miscellaneous								
1375	Putative beta-subunit of K ⁺ channels [<i>Solanum tuberosum</i>]	180 (+32)	gi 57892112	gi 223542738	4	51	-1.69**	0.0014
1376	potassium channel beta, putative [<i>Ricinus communis</i>].	118 (+14)	gi 57892112	gi 223542738	3	51	-1.78**	0.0012
1492	Phi-1 [<i>Nicotiana tabacum</i>]	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 [<i>Populus trichocarpa</i>]	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 synthetase + Unidentified	116		gi 37956277			-1.43**	0.0067

1118	Alcohol dehydrogenase [<i>Populus tremula</i>] + 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

1: spot number on the master gel

2: Protein obtained by blasting the EST against the NCBI database

3: MASCOT score (protein score as given by the GPS software (Applied Biosystems))

4: Accession number of the EST sequence in the NCBI database

5: Accession number of the corresponding protein in the NCBI database

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 formula : given value = -1/average ratio

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

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

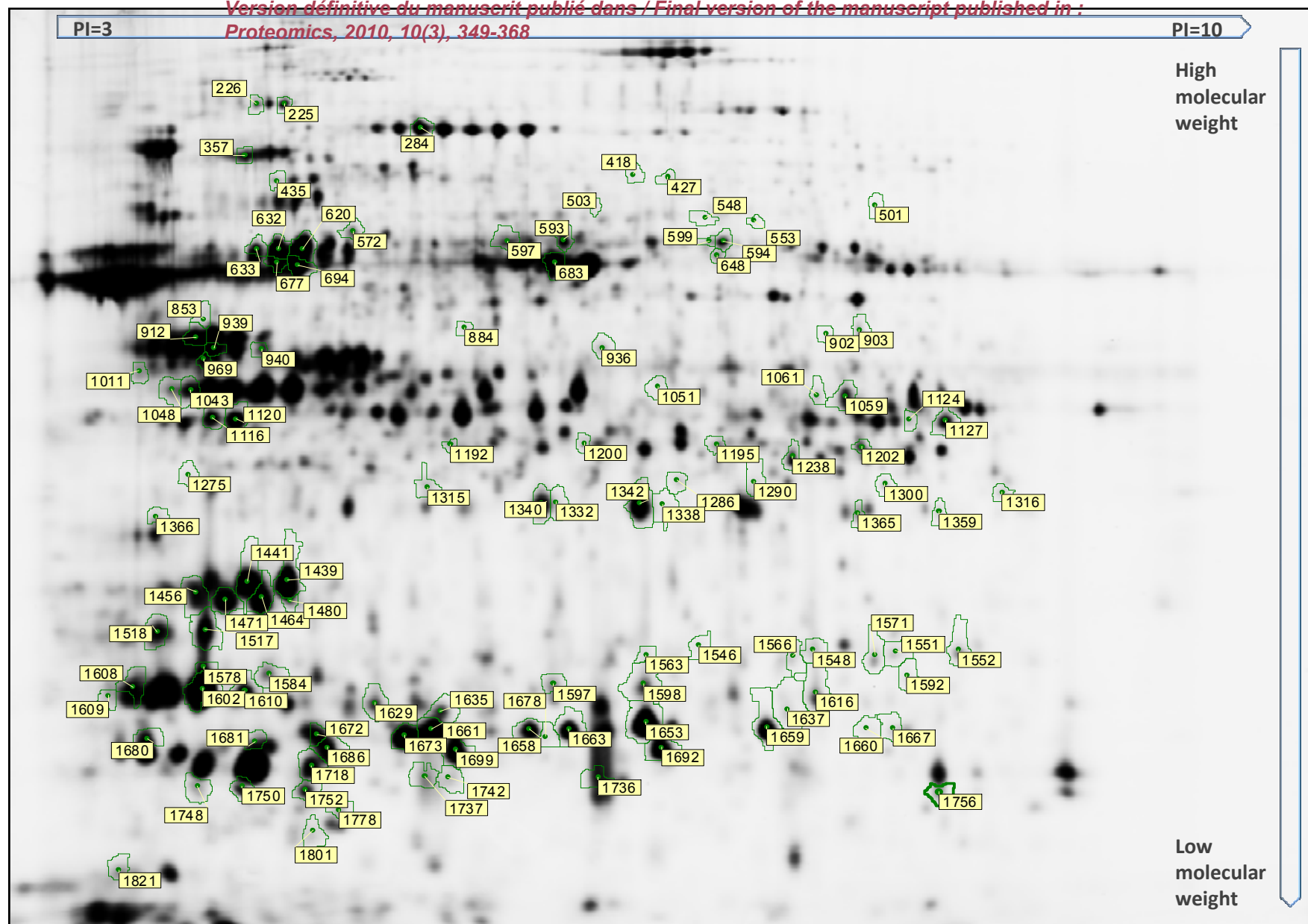


Figure 1. 2D-electrophoresis gel with **Leaf** proteins of *Populus tremula* x *P. alba* genotype 717-1B4 colored by CyDye 2. Identified proteins are labelled with their respective spot number.

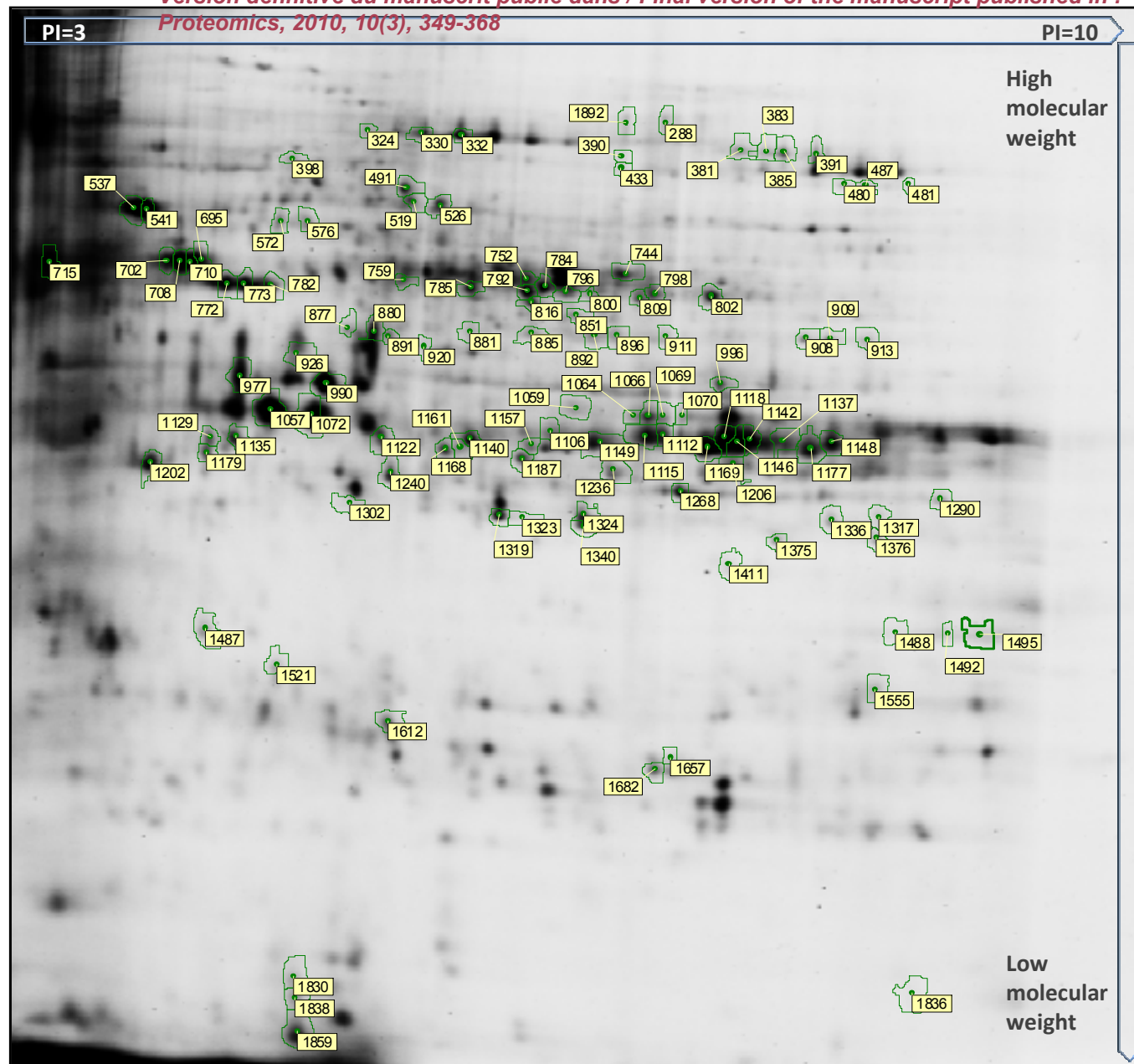


Figure 2. 2D-electrophoresis gel with **Cambial** proteins of *Populus tremula* x *P. alba* genotype 717-1B4 colored by CyDye 2. Identified proteins are labelled with their respective spot number.