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Bioremediation potential of Cd by transgenic yeast expressing a metallothionein gene from *Populus trichocarpa*

Authors: Vinicius Henrique **DE OLIVEIRA**[a,1], Ihsan **ULLAH**[a], Jim M. **DUNWELL**[a], Mark **TIBBETT**[a,b*]

[a] School of Agriculture, Policy and Development, University of Reading, Earley Gate, Reading, RG6 6AR, UK.

[b] Department of Sustainable Land Management & Soil Research Centre, University of Reading, RG6 6AR, UK

[1] Present address: Department of Plant Biology, Institute of Biology, University of Campinas, Campinas, Sao Paulo 13083-970, Brazil

*__Corresponding author__: Mark Tibbett m.tibbett@reading.ac.uk

Address: School of Agriculture, Policy and Development, University of Reading, Whiteknights, PO Box 237, Reading, RG6 6AR.

21 **Abstract**

22 Cadmium (Cd) is an extremely toxic environmental pollutant with high mobility in soils, which
23 can contaminate groundwater, increasing its risk of entering the food chain. Yeast biosorption
24 can be a low-cost and effective method for removing Cd from contaminated aqueous solutions.
25 We transformed wild-type *Saccharomyces cerevisiae* (WT) with two versions of a *Populus*
26 *trichocarpa* gene (*PtMT2b*) coding for a metallothionein: one with the original sequence
27 (*PtMT2b* 'C') and the other with a mutated sequence, with an amino acid substitution (C3Y,
28 named here: *PtMT2b* 'Y'). WT and both transformed yeasts were grown under Cd stress, in
29 agar (0; 10; 20; 50 µM Cd) and liquid medium (0; 10; 20 µM Cd). Yeast growth was assessed
30 visually and by spectrometry OD_{600}. Cd removal from contaminated media and intracellular
31 accumulation were also quantified. *PtMT2b* 'Y' was also inserted into mutant strains: *fet3fet4*,
32 *zrt1zrt2* and *smf1*, and grown under Fe-, Zn- and Mn-deficient media, respectively. Yeast
33 strains had similar growth under 0 µM, but differed under 20 µM Cd, the order of tolerance
34 was: WT < *PtMT2b* 'C' < *PtMT2b* 'Y', the latter presenting 37% higher growth than the strain
35 with *PtMT2b* 'C'. It also extracted ~80% of the Cd in solution, and had higher intracellular Cd
36 than WT. Mutant yeasts carrying *PtMT2b* 'Y' had slightly higher growth in Mn- and Fe-
37 deficient media than their non-transgenic counterparts, suggesting the transgenic protein may
38 chelate these metals. *S. cerevisiae* carrying the altered poplar gene offers potential for
39 bioremediation of Cd from wastewaters or other contaminated liquids.

40

41 **Keywords**: biosorption; environmental biotechnology; functional expression; heavy metals;
42 transgenic yeast; waste treatment

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47 **1. Introduction**

48 Cadmium (Cd) is an element that lacks a known biological function. It is one of the
49 most hazardous metals in the environment, because it can affect animals, plants and
50 microorganisms at relatively low concentrations (Alloway, 2013). Several anthropogenic
51 activities are responsible for Cd addition into the environment, such as: atmospheric deposition,
52 industrial and municipal wastes, mining activities, smelting and metal ore processing, battery
53 production, soil fertilisation and sewage sludge application (Mirlean and Roisenberg, 2006;
54 Smolders and Mertens, 2013; He et al., 2015; Khan et al., 2017). Sewage sludge is an inevitable
55 by-product from industrial or domestic wastewater processing, and is commonly used as an
56 organic amendment in soils; however if wastewater is not pre-treated for metal removal, it can
57 lead to high metal contents being added into agricultural soils and crops (Chen and Wang,
58 2008; Jamali et al., 2009).

59 Cd is also highly mobile in soils (Lei et al., 2010) with a potential risk of contaminating
60 the groundwater. Estimated leaching of Cd from European soils is between 100 to 5,700 mg
61 Cd ha^{-1} $year^{-1}$ (Smolders and Mertens, 2013). Cd is readily taken up by plant roots and poses a
62 risk when entering the food chain, possibly causing biomagnification, in which a low Cd
63 concentration can increase and become even more toxic through different trophic levels
64 (Janssen et al., 1993).

65 A low-cost and effective method of removing heavy metals from wastewater or aqueous
66 solutions is by using natural materials of biological origin (algae, fungi, bacteria, yeast) in a
67 process known as biosorption (Goksungur et al., 2005; Bulgariu and Bulgariu, 2016; Beni and
68 Esmaeili, 2020). This process has many advantages, such as low operating costs, decreased
69 volume of the sludge generated and high efficiency in detoxifying very dilute effluents
70 (Marques et al., 2000). The yeast *Saccharomyces cerevisiae* has been frequently studied as a
71 biosorbent for several heavy metals, such as Pb, Cr, Zn, Cu and Cd (Oliveira et al., 2012;

72 Vijayaraghavan and Balasubramanian, 2015). Although biosorption is a term commonly used
73 for non-living biomaterials that bind and concentrate contaminants, this process occurs in both
74 living and dead organisms (Amirnia et al., 2015).

75 Employing living microorganisms for metal biosorption has an advantage of
76 simultaneously exploiting their inherent ability of absorbing and accumulating heavy metals
77 intracellularly, a process known as bioaccumulation (Pankiewicz et al., 2015). Recently, a
78 system of continuous growth of *S. cerevisiae* was demonstrated to be an efficient method of
79 removing copper and lead ions from water (Amirnia et al., 2015). *S. cerevisiae* is a promising
80 candidate for bioremediation of metal-contaminated waters or other liquids for several reasons,
81 such as: (1) its reproduction by budding (asexual) or spore formation (sexual) (Wang and Chen,
82 2009); (2) it is easy to cultivate and available from various food and beverage industries (Wang
83 and Chen, 2006); (3) it has high adsorbent capacity even in dead cells (Goksungur et al., 2005),
84 (4) it can accumulate high intracellular amounts of heavy metals (Brady and Duncan, 1994;
85 Joutey et al., 2013), (5) it can flocculate easily in metal solutions and sediment, which facilitates
86 the separation process after remediation (Machado et al., 2008, Soares, 2011), and, finally, (6)
87 *S. cerevisiae* is a model system in biology and can be easily manipulated genetically and
88 morphologically for numerous purposes (Karathia et al., 2011; Farcasanu and Ruta, 2017).

89 Genetically engineered microorganisms appear to be the next frontier in terms of
90 bioremediation and biodegradation of contaminants, in which remediation pathways are
91 enhanced by inserting foreign genes of specific interest (Joutey et al., 2013; Kulshreshtha,
92 2013). Genes coding for phytochelatins (PCs) and metallothioneins (MT) are frequently the
93 focus for engineering microorganisms for heavy metal remediation (Sriprang et al., 2003;
94 Singh et al., 2008; Ruiz et al., 2011).

95 Metallothioneins are low-molecular weight proteins rich in Cys (usually 9-16 Cys
96 residues), which are able to bind metals in metal-thiolate clusters (Cobbett and Goldsbrough,

97 2002; Sheoran et al., 2011), such as Zn^{2+} and Cu^{2+} (Bulgarelli et al., 2016). Most MT proteins
98 belong to the sub-family MT2 of plants, which is known for binding divalent cations, such as
99 Cd^{2+} (Cobbett and Goldsbrough, 2002), or some nutrients like Fe^{2+}, Zn^{2+} and Cu^{2+} (Jin et al.,
100 2014). The MT2 sub-family has already been demonstrated to increase Cd tolerance through
101 heterologous expression in yeast (Kohler et al., 2004) and *Arabidopsis thaliana* (Gu et al.,
102 2012).

103 Similarly, we have recently demonstrated that the gene *PtMT2b* from tree species
104 *Populus trichocarpa* cv 'Trichobel' was able to reduce Cd toxicity when expressed in *S.*
105 *cerevisiae* (De Oliveira et al., 2020). This poplar clone is particularly tolerant to elevated Cd
106 concentrations (De Oliveira and Tibbett, 2018), whose high expression of MT2b in roots was
107 shown to be correlated to enhanced Cd sequestration (De Oliveira et al., 2020). Moreover, those
108 yeasts expressing poplar MT2b may effectively remove Cd from contaminated water by
109 preventing the excretion of metals back to the medium through chelation (Ruta et al., 2017).

110 Considering the role of MTs in binding divalent cations and micronutrients (e.g. Zn,
111 Cu) (Jin et al., 2014), it is possible that MT2b in involved in binding other metals besides Cd,
112 such as Fe, Mn and Zn. In this sense, the use of mutant *S. cerevisiae* strains, lacking a particular
113 metal transporter, can help unveil these roles. For instance, DEY1453 is defective for low and
114 high-affinity Fe^{2+} uptake systems, while ZHY3 lacks two Zn^{2+} transporters (ZRT1 and ZRT2)
115 and SMF1 strain lacks a high affinity Mn^{2+} uptake gene (SMF1) (Ullah et al., 2018). If under
116 nutrient deficiency these mutants (carrying MT2b) display growth improvement, it could mean
117 that MT2b is also involved in binding those nutrients.

118 Therefore, our main objectives were to verify the effect of two versions of the poplar
119 gene *PtMT2b* (the original gene and a mutated version) in Cd tolerance, accumulation and
120 bioremediation potential of *S. cerevisiae*. We hypothesised that: i) *PtMT2b* increases Cd
121 tolerance in transformed yeast; ii) a mutated version of the gene *PtMT2b* 'Y' (encoding a C3Y

122 substitution) is not as efficient in conferring Cd tolerance in yeast due to the lack of one cysteine
123 in the peptide sequence; and iii) if transformed yeasts are more tolerant to Cd, they can also
124 effectively bioremediate Cd from aqueous solutions (by surface biosorption or intracellular
125 accumulation). Considering the role of MTs in binding divalent cations, it was also
126 hypothesised that this metallothionein could improve the growth of mutant *S. cerevisiae* strains
127 in nutrient depleted media (Fe, Mn or Zn), possibly by containing a larger internal metal storage
128 than the non-transformed yeast.

129

130 2. Materials and Methods

131 2.1 Cloning of poplar's *PtMT2b*

132 2.1.1 RNA extraction and cDNA synthesis

133 DNA was extracted from *Populus trichocarpa* cv. 'Trichobel' roots and leaves with
134 DNeasy Plant Mini Kit (Qiagen, UK), following the manufacturer's instructions. Total RNA
135 was extracted from approximately 100 g of fresh weight material (roots) macerated in liquid
136 nitrogen via TissueLyser II (Qiagen®). Extraction was performed by a modified version of the
137 CTAB method (Jaakola et al., 2001): macerated samples were incubated with CTAB buffer
138 (hexadecyltrimethylammonium bromide) for 25 min at 65°C (instead of 10 min), while LiCl
139 addition was 1/3 of total extract volume (instead of 1/4). After overnight precipitation at 4°C,
140 extract was centrifuged for 60 min (instead of 20 min); supernatant was then discarded and
141 RNA pellets were purified with the RNeasy Plant Mini kit (Qiagen, UK), including a DNAse
142 treatment (Qiagen, UK) for 20 min.

143 The extracted RNA was converted into cDNA using the SensiFAST cDNA synthesis
144 kit (Bioline, UK) following the manufacturer's instructions. The full coding sequence was then
145 amplified with a *PtMT2b* primer set containing attB overhang (annealing temperature: 58°C),
146 with sequences (5' – 3'), according to De Oliveira et al., (2020):

147 F - GGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCTTGCTGTGGAGGAAA;

148 R - GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATTTGCAGGAGCATGGAT.

149 *PtMT2b* sequence has been deposited in GenBank (accession number: MN974475).

150

151 **2.1.2 Amino acid substitution (*PtMT2b* 'Y')**

152 During the cloning process, two different *PtMT2b* sequences were obtained due to a
153 probable error during DNA amplification (Fig. 1). This was later confirmed by sequencing the
154 *MT2b* gene directly from the genomic DNA extracted. One codon had a single nonsynonymous
155 nucleotide substitution, from the original 'TGC' to 'TAC', which consequently changed the
156 correspondent amino acid from a cysteine (C) to a tyrosine (Y) at the third position (C3Y).
157 Considering that cysteine is responsible for the divalent cation binding ability in MTs, it was
158 possible that the C3Y substitution would lead to a different Cd tolerance phenotype in yeast.
159 Therefore, these two versions of the same gene were used in yeast transformation, the original
160 (*PtMT2b* 'C') and the mutated (*PtMT2b* 'Y').

161

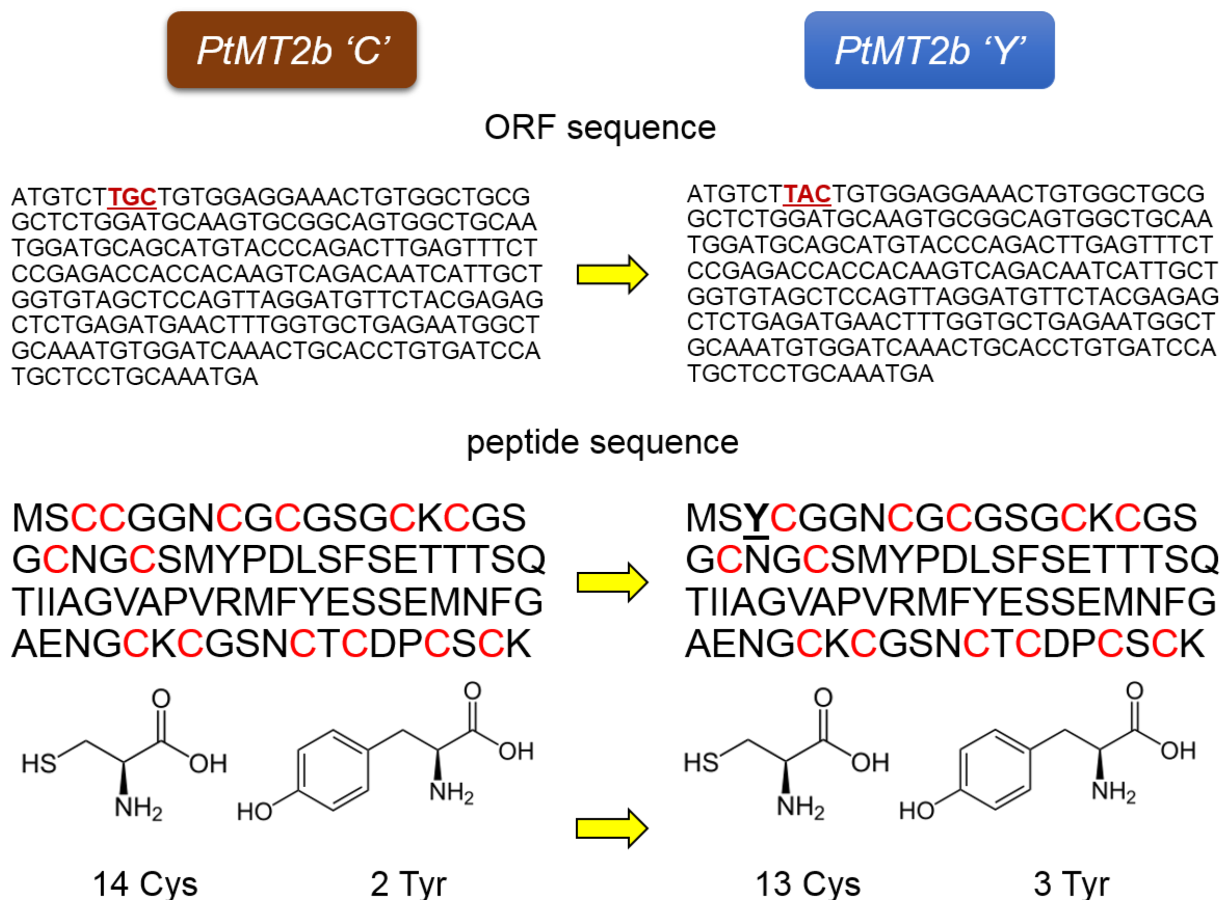

Fig. 1. Substitution of one single nucleotide in *Populus trichocarpa* MT2b gene during amplification (from TGC to TAC), leading to the cysteine in the third position being replaced by a tyrosine (C3Y). Left: original open reading frame (ORF) and peptide sequences. Right: altered sequences.

162

163 **2.2 Yeast transformation**

164 The wild-type *S. cerevisiae* strain DY1457 (WT) was used for transformations. The
165 genes were introduced into a Gateway® donor vector pDONR221 (containing the kanamycin
166 resistance gene – Fig. S1; Sup. Files) using Gateway® BP Clonase® II enzyme mix.
167 Chemically competent *Escherichia coli* cells (TOP10) were transformed with the entry clones
168 and grown overnight in LB agar + Kanamycin medium at 37ºC. Plasmids were isolated from
169 transformed *E. coli* and introduced into destination vector pDR195 (Fig. S2; Sup. Files) using

170 the Gateway® LR Clonase® II enzyme mix. *E. coli* cells were transformed with the expression
171 vectors and grown in LB agar + Ampicillin, same parameters as before. WT yeast was
172 transformed with either the expression vector containing *PtMT2b* 'C', *PtMT2b* 'Y' or an empty
173 vector (pDR195) as control, the latter strain will be referred to as simply "WT" throughout the
174 manuscript. The transformants were selected on synthetic complete (SC) drop-out medium
175 without uracil [1 g/L drop out medium Y1501 Sigma® + 6.7 g/L yeast nitrogen base
176 Invitrogen™] + 2% dextrose (v/v). Plasmids were restricted (entry vector: *Sac*I and *Ssp*I;
177 expression vector: *Sac*I and *Hin*dIII) and sequenced at every stage to confirm ORF integrity
178 and direction.
179

180 **2.3 Expression of PtMT2b (C and Y) in yeast under Cd stress**

181 Yeast cells were grown overnight at 30°C (250 rpm) in SC liquid media (5 mL; pH:
182 5.5). Cells were then pelleted by centrifugation, and re-suspended in 5 mL of sterile water.
183 Optical density at 600 nm of wavelength (OD_{600}) was recorded using SpectraMax i3x
184 (Molecular Devices) microplate reader. Cultures were diluted in sterile water to reach OD_{600}
185 of 0.1, which were used for serial dilutions (1:10 v/v). All dilutions of transformed (*PtMT2b*
186 'C' and 'Y') and empty vector yeast ('WT') were spotted (5 μL) into SC agar plates at 0; 10;
187 20; and 50 μM Cd (in the form of $CdCl_2$), then grown at 30°C for 72 hours in the dark (three
188 replicates). In order to quantify yeast growth under Cd stress, all strains were grown in liquid
189 SC media (initial OD_{600}: 0.01), containing either 0; 10 or 20 μM Cd (three replicates) for 48
190 hours (30°C, 250 rpm; dark), after which the OD_{600} was recorded. The concentration of 50 μM
191 Cd was not used in any liquid media assays due to high toxicity.
192

193 **2.4 Cd accumulation and extraction in yeast containing PtMT2b 'Y'**

194 The *PtMT2b* 'Y' and WT (empty vector) yeasts were grown in 5 mL of SC liquid media
195 + 2% dextrose, containing 0; 10 and 30 µM Cd at 30ºC in the dark with constant shaking (initial
196 OD_{600}: 0.01; four replicates). After 72 h, OD_{600} was recorded and cells were pelleted by
197 centrifugation (10 min, 4000 rpm). All contaminated media were transferred to new tubes
198 without disturbing the pellet, these were denominated Left Over (LO) and were later analysed
199 by ICP-MS to determine the remaining Cd concentration after yeast growth. Pelleted cells were
200 re-suspended in 10 mL of EDTA (20 mM) and washed for 10 minutes (by inverting tubes) in
201 order to remove adhering Cd ions from yeast surface (Ullah et al., 2018). Cells were pelleted
202 again and washed twice with 10 mL of deionised water. Yeasts were oven-dried at 80ºC for 48
203 hours. Dried cells were digested in 5 mL of 69% nitric acid (TraceSELECT™ grade) in closed
204 glass vessels for 8 h at 110ºC (in duplicates). Pure acid was used as blank and 0.05 g of
205 reference material (IAEA-359 cabbage leaves) was digested in the same manner for quality
206 control. Cd accumulation in cells and the remaining Cd in Left Over media were determined
207 via ICP-MS (Thermo Scientific™ iCAP™ Q). Cd extraction potential was calculated by the
208 following equation:

209 $$(1) \quad Cd\ extracted\ (\%) = 100 - \frac{LO\ Cd \times 100}{Initial\ Cd}$$

210

211 In which *"LO Cd"* is the Cd concentration determined in the Left Over media solution
212 after yeast growth (mg L^{-1}); and "*Initial Cd"* the concentration of Cd added in the growth media
213 before yeast inoculation, also determined via ICP-MS (mg L^{-1}).

214

215 **2.5 Cell Dry Weight vs OD_{600}**

216 In order to estimate Cd concentration in terms of cell dry weight (CDW), transformed
217 (*PtMT2b* 'Y') yeast was grown in conical flasks (three replicates), containing 60 mL of
218 uncontaminated SC media, with OD_{600} starting at 0.01. Every 3 h an aliquot of 10 mL from

219 each flask had its OD_{600} determined, cells were pelleted and washed with deionised water and
220 dried in previously weighed glass vials at 80ºC. After 72 h, dry weight was recorded. The
221 relationship between CDW and OD_{600} was determined by linear regression model ($\alpha = 0.05$;
222 15 samples).
223

224 **2.6 PtMT2b 'Y' expression in mutant yeast under nutrient deficiency**

225 In order to verify the specificity of this gene, transgenic mutant yeast were subjected to
226 nutrient deficient conditions (Fe, Mn and Zn). If MT2b 'Y' proteins also bind these nutrients,
227 these yeast strains would be able to grow under deficiency due to a higher nutrient storage
228 capacity in their cells. Strains used for transformation were the single mutant SMF1 (*smf1*),
229 and the double mutants DEY1453 (*fet3fet4*) and ZHY3 (*zrt1zrt2*), as well as the corresponding
230 parental wild type strain DY1457. All strains were transformed either with *PtMT2b* 'Y' or an
231 empty vector (e.v.) as control. Mutant yeasts were also transformed with *TcNramp5,* a metal
232 transporter gene from cocoa trees known to increase Cd^{2+} and Zn^{2+} uptake in yeast (Ullah et
233 al., 2018), and were used as a positive control. Transformations were carried out as described
234 previously.

235 Primary cultures were stablished from a single colony, and grown in 10 mL SC media
236 supplemented with either 0.4% (v/v) Fe, 0.2% Mn or 0.4% Zn; for DEY1453, SMF1 and ZHY3
237 strains, respectively (30ºC, 72h, 250 rpm, dark). Initial growth in a rich media was carried out
238 to promote a nutrient stock in yeast cells before being transferred to deficient media (pre-
239 growth stage). Afterwards, cultures were serial diluted and spotted (5 µL) into SC + agar plates,
240 with or without chelating agents to decrease nutrient availability: 10 µM BPS
241 (Bathophenanthrolinedisulfonic acid) for creating iron deficient plates (- Fe); 12.5 mM EGTA
242 (Ethylene glycol-bis(2-aminoethylether)-N,N,N′) for Mn deficiency (- Mn); and 100 µM
243 EDTA (Ethylenediamine tetraacetic acid) for Zn deficiency (- Zn).

244

245 **2.7 Statistical analyses**

246 ANOVA and Tukey test were performed for all datasets that met ANOVA's
247 assumptions. Some variables needed transformation (x^2) to attain normality and
248 homoscedasticity, i.e. OD_{600} values for *SMF1* strains (with and without MT2b) grown under
249 Mn deficiency. After being unable to transform the data for Cd content ($\mu g\ g^{-1}$) to attain
250 normality, the non-parametric Kruskal-Wallis test was performed. Linear regression analysis
251 was used for obtaining the CDW ($mg\ mL^{-1}$) and OD_{600} relationship, in which the Min/Max
252 accuracy and MAPE (mean absolute percent error) were used to assess the model accuracy. All
253 statistical analyses were performed using R software.

254

255 **3. Results**

256 **3.1 Amino acid substitution in MT2b increased Cd tolerance in yeast**

257 The spot assay clearly showed that the strains transformed with both versions of the
258 *PtMT2b* gene were able to cope with higher Cd concentrations than the strain transformed with
259 the empty vector only, especially at 50 µM, in which its growth was completely suppressed
260 (Fig. 2).

261 In liquid media contaminated by Cd, yeast strains had similar growth under 0 µM, but
262 differed under 10 and 20 µM Cd (ANOVA: $p < 0.001$). Under the highest Cd concentration the
263 order of tolerance was WT < *PtMT2b* 'C' < *PtMT2b* 'Y'; determined after Tukey test (variation
264 coefficient = 6.5%), in which the growth of yeasts carrying the tyrosine-replaced MT2b was
265 around 37% higher than strain expressing the original gene sequence (*PtMT2b* 'C') (Table 1).

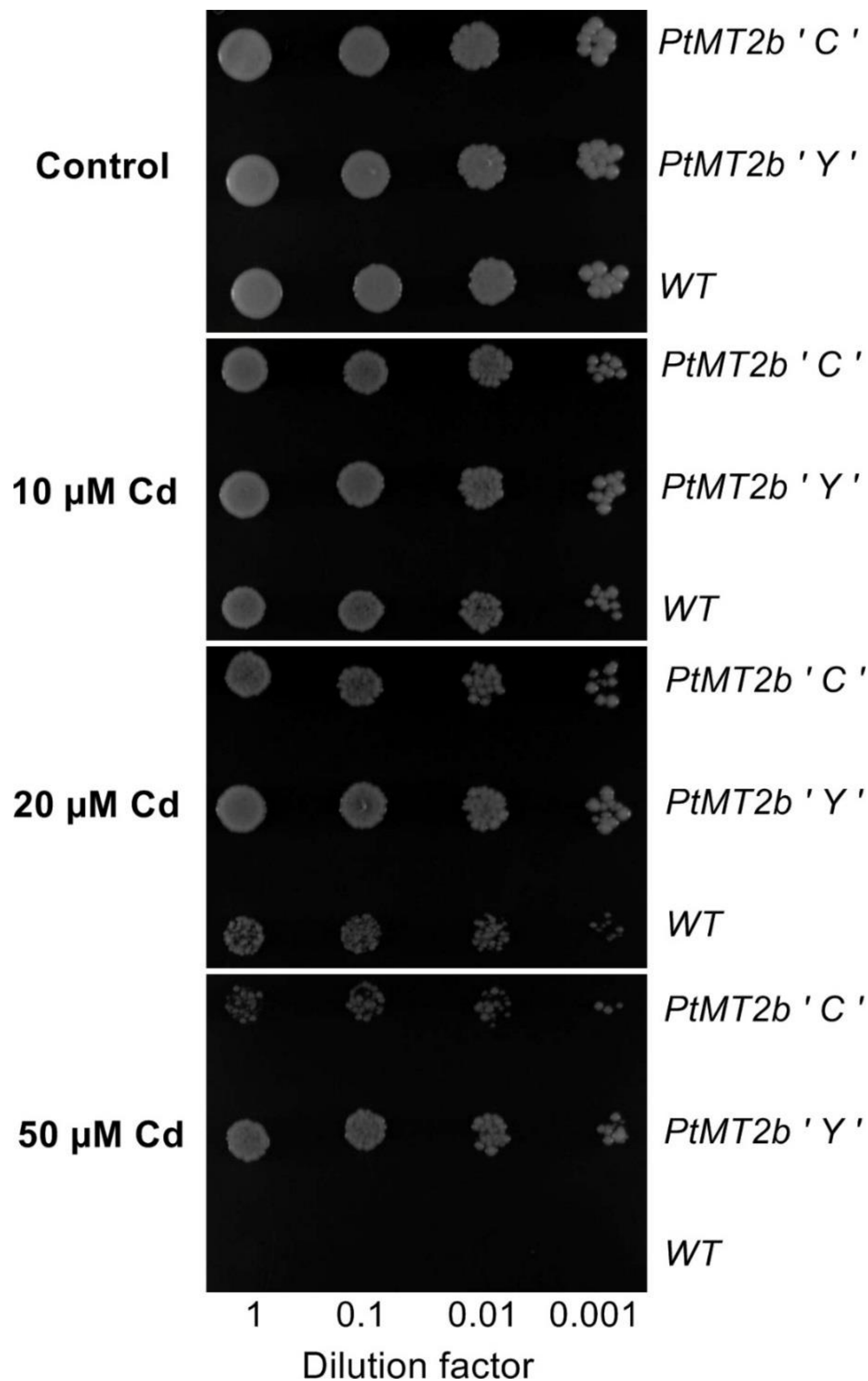

Fig. 2. Heterologous expression of *PtMT2b* in *S. cerevisiae* growing under increasing Cd concentrations. WT: Wild type (DY1457) strain with empty vector; PtMT2b 'C': wild type yeast transformed with the original *PtMT2b*; PtMT2b 'Y': transformed yeast expressing the modified *PtMT2b* gene, with cysteine to tyrosine replacement (C3Y).

266

Table 1 – Growth of transformed *S. cerevisiae* strains under Cd stress, determined by OD_{600} after 48 hours (mean ± st. error)[1]. WT: wild type with empty vector; *PtMT2b* 'C': gene with original sequence; and *PtMT2b* 'Y': gene with cysteine to tyrosine replacement (C3Y).

Strain	Media Cd concentration	
	10 µM	20 µM
WT	0.20 ± 0.009 a	0.15 ± 0.009 a
WT + *PtMT2b* 'C'	0.31 ± 0.005 b	0.25 ± 0.003 b
WT + *PtMT2b* 'Y'	0.29 ± 0.005 b	0.35 ± 0.012 c

[1]- Different letters correspond to significant differences among strains within columns (same Cd concentrations), as determined by Tukey test after ANOVA ($p < 0.001$).

267

268 **3.2 Mutated PtMT2b gene increased Cd accumulation and removal by yeast**

269 Since yeast carrying the mutated gene sequence (*PtMT2b* 'Y') were more tolerant than
270 the strains expressing the original gene, they were ultimately selected for Cd bioremediation
271 trials. Results showed that WT strain (empty vector) was significantly affected by Cd toxicity,
272 while growth of transformed strain was unaffected by Cd additions (Fig. 3).

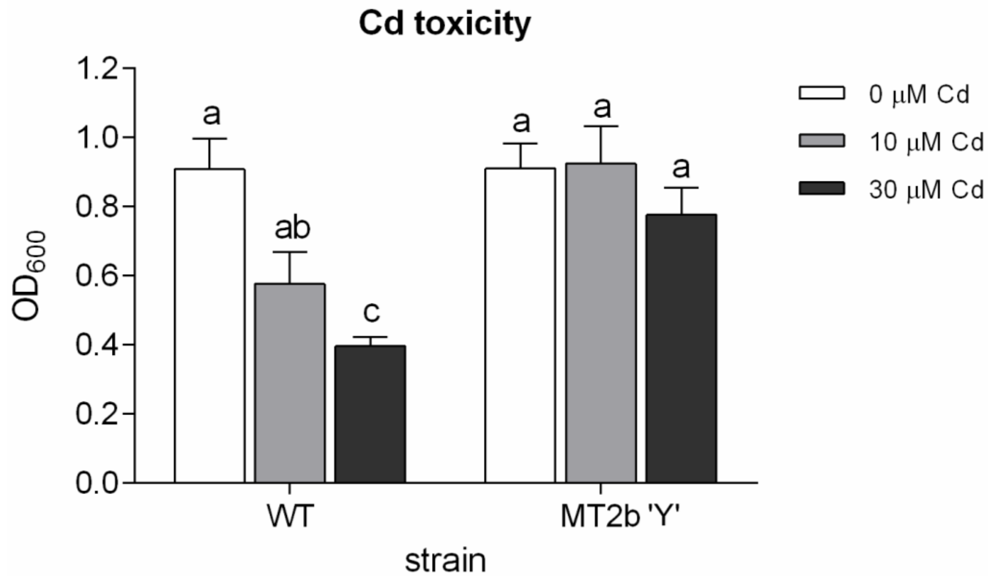

Fig. 3. *S. cerevisiae* growth under three Cd concentrations, as determined by OD_{600} in liquid SC media after 72h. WT: Wild type (DY1457) with empty vector; MT2b 'Y': transformed yeast carrying mutated *PtMT2b* gene, with an amino acid substitution (C3Y). Different letters represent significant differences among treatments by Tukey test (ANOVA; $p = 0.00085$)

273

274 Transformed yeast accumulated high contents of Cd within cells, with concentrations
275 at least 30 times higher than the strains carrying empty vectors only (Fig. 4a). In the WT yeast,
276 internal Cd uptake was similar regardless of media concentration, but in transformed yeast,
277 accumulation significantly increased under the highest Cd dose (30 µM).

278

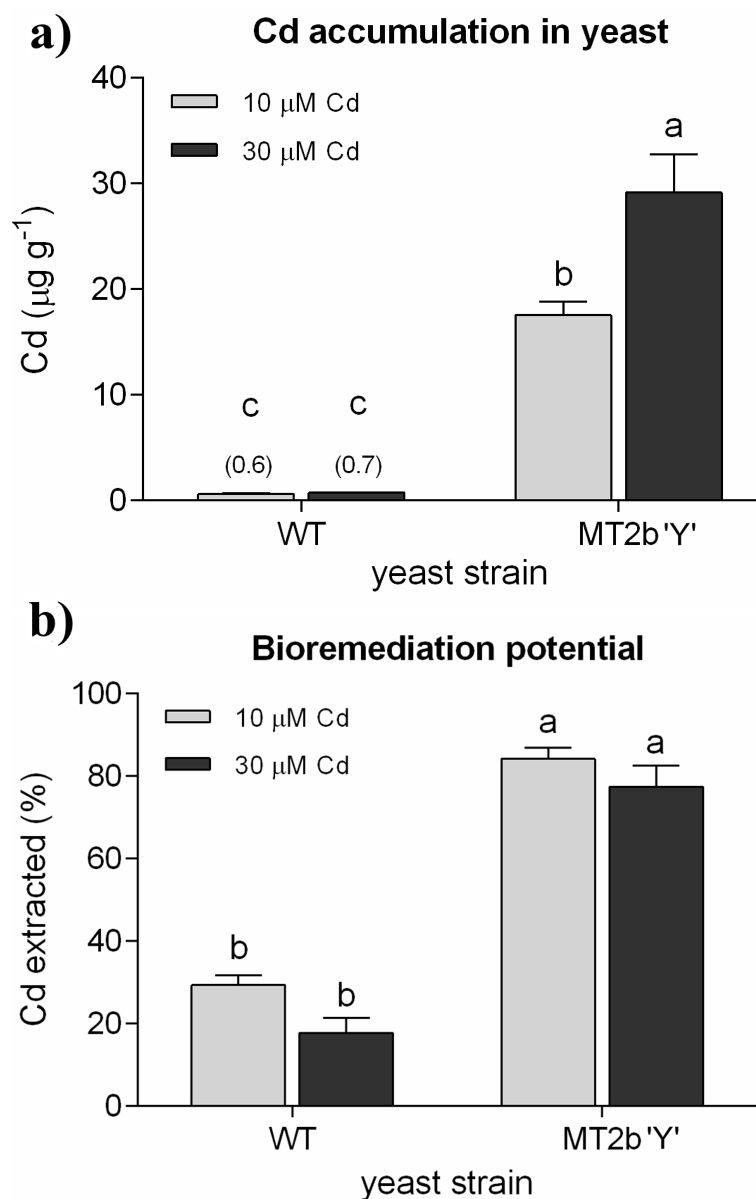

Fig. 4. Cd accumulation in *S. cerevisiae* strains after 72 hours of growth. WT: Wild type + empty vector (DY1457); MT2b 'Y': transformed yeast carrying mutated *PtMT2b* gene, with an amino acid substitution (C3Y). a) Amount of Cd in dried yeast cells (μg g^{-1}) after EDTA washing and acid digestion. b) Percentage of Cd removal from liquid media after yeast growth (72 h). Different letters represent significant differences among treatments by Kruskal-Wallis and Dunn test ($p = 0.004$) in a); and by Tukey test (ANOVA; $p < 0.001$) in b)

279

280 In order to convert the OD_{600} values into CDW (cell dry weight) and express the results
281 in µg of Cd per g CDW, the following equation was used:
282
283 $$CDW_{(mg/ml)} = 2.496 \times OD_{600} + 0.0303$$
284
285 This equation was obtained by a linear regression analysis between CDW (mg mL^{-1})
286 and OD_{600} values of 15 samples at different growth stages ($R^2 = 0.974$; $p < 0.001$); with 94.8%
287 of Min/Max accuracy and 5.6% of MAPE (mean absolute percent error) (Fig. S3; Sup. Files).
288 In terms of Cd removal from the media (%), which includes internal Cd accumulation,
289 cell wall binding and sorption processes; the transformed yeast removed around 84% and 77%
290 of the total Cd concentration initially added (10 µM and 30 µM, respectively) in a 72 h period,
291 while in WT strain those values were on average under 30% (Fig. 4b).
292

293 3.3 Mutated PtMT2b gene slightly increases yeast growth under Fe and Mn deficiency

294 Spot assay of transformed mutant yeasts under deficient conditions showed that
295 *PtMT2b* 'Y' could not recover the growth of double mutant *zrt1zrt2* under Zn deficiency, but
296 slightly promoted growth in *SMF1* and *fet3fet4* strains in Mn and Fe deficient plates,
297 respectively (Fig. 5). From those strains, the *PtMT2b* 'Y' effect appeared to be more
298 pronounced only in *SMF1* (Fig. 5b). For quantification purposes, this mutant strain was
299 cultivated in liquid media under Mn deficiency, under which conditions *SMF1* + *PtMT2b* 'Y'
300 had on average 71% higher growth (OD_{600}: 0.90 ± 0.01) than when carrying an empty vector
301 (OD_{600}: 0.52 ± 0.13) (ANOVA; $p = 0.008$).

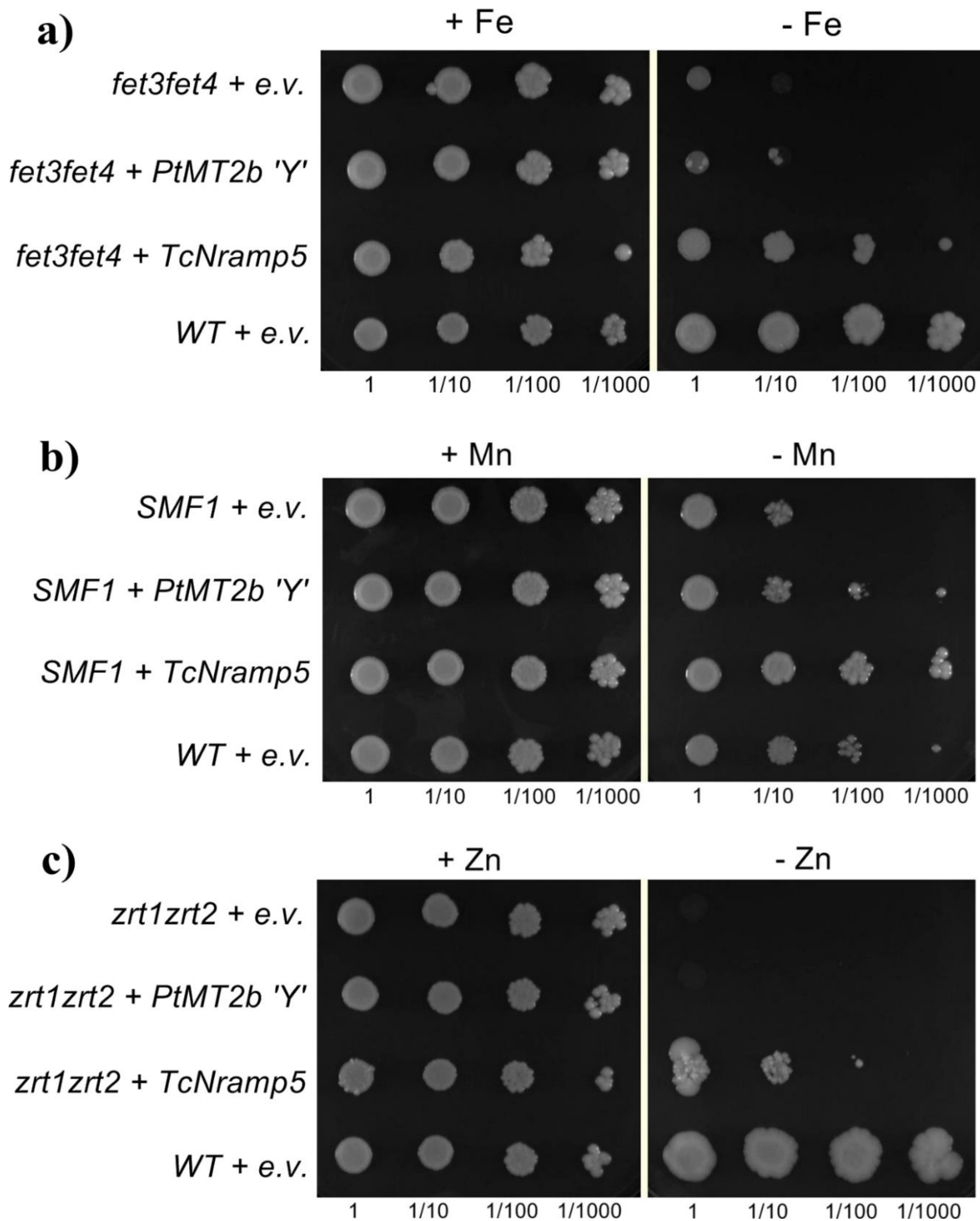

Fig. 5. Growth of mutant *S. cerevisiae* strains in nutrient sufficient (+ X) and nutrient deficient (- X) plates for 72 hours. Yeast strains were: *fet3fet4*, with double mutations for Fe uptake (a); *SMF1*, with single mutation for Mn uptake (b); and *zrt1zrt2*, with double mutations for Zn uptake (c). WT: wild type; e. v.: empty vector (DY1457); *PtMT2b* 'Y': poplar metallothionein with cysteine to tyrosine replacement (C3Y); and *TcNramp5*: cocoa tree metal transporter Nramp5. Dilution 1 = 0.1 OD_{600}

303 ## 4. Discussion

304 ### 4.1 Poplar's metallothionein confers Cd tolerance in yeast

305 As demonstrated previously (Fig. 2), *P. trichocarpa* metallothionein MT2b is indeed
306 able to increase Cd tolerance in transformed *S. cerevisiae* (De Oliveira et al., 2020). Besides
307 chelating and inactivating metals in their toxic forms, such as Cd^{2+}, MTs have a role in
308 scavenging reactive oxygen species (ROS) from cells under stress (Wong et al., 2004; Ruttkay-
309 Nedecky et al., 2013). Genes for ROS tolerance are highly expressed in wild type *S. cerevisiae*
310 exposed to Cd (Thorsen et al., 2009); therefore it is clear that the addition of *PtMT2b* would
311 enhance Cd tolerance by producing even more ROS-scavenging proteins than a WT strain.

312 Heterologous expression of other plant metallothionein genes in yeast have been
313 assessed under heavy metal stress, with similar results, but mostly from herbaceous plant
314 species (Zhou and Goldsbrough, 1994; Guo et al., 2008; Zhang et al., 2014a; Zhang et al.,
315 2014b). For Cd, metallothioneins from sunflower, rice, *Arabidopsis*, *Noccaea caerulescens*,
316 and even mycorrhizal fungi *Rhizophagus irregularis* and *Hebeloma cylindrosporum* were
317 shown to complement Cd sensitivity in mutant yeast (Farcasanu and Ruta, 2017). In the present
318 study, the non-transgenic strain (WT + empty vector) had a decrease in biomass of around 50%
319 under 10 μM Cd in liquid media (Fig. 3), which is in accordance with the results from Hosiner
320 et al., (2014), who reported an EC50 (half maximal effective concentration) of 10 μM $CdCl_2$
321 for *S. cerevisiae*. In the transgenic strain, however, growth was barely affected even at 30 μM
322 Cd, confirming our initial hypothesis that *PtMT2b* increases Cd tolerance.

323

324 ### 4.2 Amino acid substitution further enhanced Cd tolerance

325 Metallothioneins are characterised by their high content of Cys residues - generally 10
326 to 17 in plants – which are able to bind divalent metal cations in their sulfhydryl (R–SH) group,
327 thus forming thiolate bonds (Hassinen et al., 2011; Nguyen et al., 2017) and, in the case of the

328 type II sub-family, their amino-terminal portion has a highly conserved domain, starting with
329 Cys-Cys arrangement (Bulgarelli et al., 2016). Because of this obvious role of the cysteine
330 content in providing metal binding sites in these proteins, it was interesting to observe that
331 *PtMT2b* 'Y', a gene encoding a MT with one fewer Cys residue (replaced by one tyrosine -
332 Tyr), not only did not lose its function as we hypothesised, but in fact enhanced Cd tolerance
333 in transformed yeast. We could speculate two main reasons for this: 1) the tyrosine aromatic
334 ring; 2) the position in which the substitution took place (C3Y).

335 Despite lacking the characteristic sulfhydryl group from Cys, Tyr has a phenolic
336 aromatic ring that can also effectively bind divalent cations such as Cd^{2+} in their aromatic
337 structure forming tyrosine-metal complexes (Hu et al., 1995), from which different
338 conformations have been proposed (Fig. S4; Sup. Files). In this sense, Vandenbossche et al.,
339 (2015) developed a synthetic material enriched with tyrosine molecules that was able to
340 efficiently remove copper from contaminated waters.

341 Another reason for increased Cd tolerance is also related to the aromatic group in Tyr,
342 which can form a non-covalent bond with cationic metals, known as cation-π interactions. This
343 interaction is essentially electrostatic, in which a cation is attracted to the negatively charged
344 cloud of electrons from aromatic groups (π systems), and is considered one of the strongest
345 noncovalent interactions (Ma and Dougherty, 1997; Mahadevi and Sastry, 2013). Although
346 mostly reported for monovalent cations, cation-π can also happen with divalent metal ions,
347 such as seen with Mg^{2+} (Stewart et al., 2013).

348 The position in which the substitution took place may possibly have influenced the
349 results observed. Plant MTs have two short cysteine-rich terminal domains linked by a long
350 spacer, devoid of Cys, and of around 40 amino acids (Domenech et al., 2006). These Cys
351 domains in opposite ends can interact with each other and bind metals, forming a cluster,
352 conferring the *hairpin* structure model typical of MT2 proteins (Hassinen et al., 2011). In the

353 present work, *PtMT2b* 'Y' had only the third amino acid of the peptide chain (Cys) replaced
354 by a Tyr (C3Y; Fig. 1), which means that it is unlikely for it to have affected the overall protein
355 folding, considering that this domain had another seven Cys residues to interact with the six
356 Cys from the opposite domain. Moreover, the domain in which this substitution occurred may
357 also explain why there was no loss of protein function. For instance, Cismowski et al. (1991)
358 observed that yeast carrying a mutated mammalian MT gene (Cys to Tyr substitution) had a
359 markedly lower resistance to Cd when it occurred in one domain (C50Y), but no effects when
360 this mutation was present in another domain (C13Y). Nevertheless, our results have shown for
361 the first time that the Cys to Tyr (C3Y) substitution in a plant metallothionein gene can in fact
362 increase Cd tolerance and accumulation in yeast. This suggests that metallothionein
363 manipulation and editing could be further explored to enhance bioremediation capacity in
364 microorganisms.

365

366 **4.3 Bioaccumulation and removal of Cd by transgenic yeast**

367 Yeast can remove metals from solutions by mainly two mechanisms, one is passive and
368 requires no energy expenditure (e.g. cell wall binding and metal diffusion) and the other active,
369 metabolism-dependent and being carried out only by living cells, involving
370 compartmentalisation in subcellular organelles such as vacuole or mitochondria (Vijver et al.,
371 2004; Wang and Chen, 2009). Metal binding by metallothioneins is one of the most important
372 strategies for metal accumulation (or toxicity avoidance) in living cells, a process seen in
373 almost all eukaryotic organisms, such as animals, plants, yeast and ectomycorrhizal fungi
374 (Vijver et al., 2004; Nguyen et al., 2017). Although in *S. cerevisiae* the induction of MT
375 production seems to occur mainly through exposure to Cu (Wang and Chen, 2006) or Ag
376 (Hosiner et al., 2014).

377 Linear regression resulted in a good prediction model for converting OD_{600}
378 measurements into cell dry weight (CDW) and allowed converting Cd concentrations in yeast
379 to µg of Cd per gram of biomass. It should be noted, however, that those predictions should be
380 applied only under the experimental conditions of the present work (strain type, growth period,
381 temperature etc.), as well as the equipment use for OD_{600} determination, since it can vary
382 according to the device used (Ude et al., 2014).

383 Transgenic strains carrying the mutated *PtMT2b* gene were not only highly tolerant but
384 also effectively accumulated more Cd (in µg g^{-1}) than wild type yeast, with Cd contents at least
385 10 times higher, which supports our hypothesis that Cd tolerance can lead to enhanced Cd
386 accumulation. Ruta et al., (2017) recently showed that *S. cerevisiae* transformed with *NctMT2a*
387 and *NcMT2b* (from *Noccaea caerulescens*) had a 5-fold and a 4-fold increase in Cd
388 accumulation, respectively, compared to the non-transformed strain. Yeast expressing *SaMT2*
389 from hyperaccumulator *Sedum alfredii* also had a 50% increase in Cd accumulation in relation
390 to the control (Zhang et al., 2014b). However enhanced Cd accumulation is not always
391 observed, such as the case of the *S. cerevisiae* strains transformed with a range of MTs from *A.*
392 *thaliana* (Guo et al., 2008). Bacteria may also display similar effects, such as the *E. coli*
393 expressing a metallothionein from mice (*mt-1*), in which the gene promoted higher tolerance
394 and accumulation of mercury from contaminated media (Ruiz et al., 2011), and the *CeMT2b*
395 gene from tolerant weed species *Colocasia esculenta*, that doubled Cd accumulation in *E. coli*
396 (Kim et al., 2011).

397 Due to their biosorption characteristics, yeast cell walls can remove heavy metals from
398 aqueous wastes even if the cells are no longer alive. Machado et al., (2008) verified that after
399 applying dead *S. cerevisiae* biomass (12 mg mL^{-1}) in nickel contaminated water, almost 80%
400 of the Ni^{2+} in solution was removed after only 30 minutes. By using the OD_{600} to CDW (mg
401 mL^{-1}) conversion equation previously determined, we were able to estimate that despite

402 removing around 80% of Cd^{2+} from the growth media, this amount would represent a
403 biosorption capacity of 1.5 mg g^{-1} of dried yeast. Even though this assay ran for only 72 hours
404 and did not reach saturation, the result is quite low compared to other biosorbent materials,
405 such as dried chestnut burr, which is able to remove 16.2 mg of Cd per gram, pinecones (4.3
406 mg g^{-1}) or the breakthrough biosorbent known as MMBB (a mix of tea wastes, mandarin peels
407 and maple leaves), which can absorb 31.7 mg g^{-1} of Cd from solution (Kim et al., 2015;
408 Abdolali et al., 2016). However, those are dead materials, and are not susceptible to metal
409 toxicity effects. Living yeasts provide a constant source of biosorbent material, which is also
410 able to actively accumulate metals within cells, removing metals continuously through internal
411 detoxification mechanisms (Wang and Chen, 2006). In this sense, Amirnia et al., (2015)
412 developed a continuous bioreactor-biosorption system, which is efficient for simultaneous
413 production of *S. cerevisiae* and removal of Cu^{2+} and Pb^{2+} from liquid waste without requiring
414 much nutritional input for yeast growth. The authors also suggested that this process is
415 facilitated by using flocculant strains that are easily able to decant and separate from the growth
416 solution (Soares, 2011), a feature that was observed in the WT strains in the present work.
417

418 **4.4 *PtMT2b* 'Y' possible role in binding Mn and Fe in mutant yeast**

419 Considering the evidence that metals such as Zn and Cu can affect the expression of
420 MT2b in white poplar (Cicatelli et al., 2010), and that both Cd and Zn concentrations were
421 verified to be highly correlated to *MT2b* expression in leaves of *P. tremula* x *P. tremuloides*
422 (Hassinen et al., 2009), we hypothesised that the double mutant strain *zrt1zrt2*, lacking two Zn
423 transporters, would have increased growth if carrying the *PtMT2b* gene. This was based on the
424 concept that prior to yeast inoculation into the Zn-depleted media, during pre-growth stage,
425 transgenic yeast would have built up a larger nutrient storage capacity within their cells by
426 forming MT-Metal chelates, which could then be accessed under nutrient deficiency. The same

427 was tested for Fe and Mn, using their respective mutant strains, for it is known that MTs are
428 also able to bind these metals (Zhang et al., 2014a; Farcasanu and Ruta, 2017).

429 In our work, the spot assay showed that mutant strain *zrt1zrt2* had no effects from
430 *PtMT2b* 'Y' transformation under Zn deficiency, showing virtually no growth. One reason
431 could be that the double mutation did not allow enough Zn to penetrate the yeast cells during
432 pre-growth. *S. cerevisiae* acquires Zn via mainly three transporters: Zrt1 (high affinity), Zrt2
433 (low affinity) and Fet4 (non-specific), therefore, this mutation severely hinders Zn acquisition
434 pathways (Zhao and Eide, 1996; Schothorst et al., 2017). The *PtMT2b* gene was also shown to
435 have slightly higher expression in poplars under high Zn concentrations (De Oliveira et al.,
436 2020), so it is probably involved in Zn binding; however it is possible that the amino acid
437 substitution (C3Y) in this gene could have led to a protein with lower Zn affinity, resulting in
438 poor Zn storage.

439 However, yeasts carrying *PtMT2b* 'Y' were able to grow, to some extent, in Mn- and
440 Fe- agar deficient media, confirming in part our initial hypothesis, although only by verifying
441 metal contents intracellularly could we reach a more empirical conclusion. When grown in
442 liquid media, the transgenic *SMF1* strain had a 71% increase in growth under Mn deficiency,
443 suggesting that this gene is involved in Mn binding. The involvement of MTs in Mn
444 homeostasis has not been thoroughly explored in plants thus far, except for a few studies with
445 MTs from animals or plants (Kobayashi et al., 2007; Benatti et al., 2014).

446

447 ## 5. Conclusions

448 Heterologous expression of the metallothionein gene (*PtMT2b*) from the Cd tolerant
449 tree *Populus trichocarpa* is able to confer tolerance to *S. cerevisiae* under Cd concentrations
450 up to 50 μM. Contrary to our hypothesis, replacement of Cys by Tyr (C3Y) in the amino acid
451 sequence did not affect protein function, and, in fact, increased yeast growth under Cd stress.

The transgenic strains carrying the mutated gene were able to extract up to 80% of Cd from contaminated media solution, mostly due to continuous growth and constant metal biosorption. This specific strain offers great potential for bioremediation of Cd from waters or effluents, possibly in a bioreactor system, and further studies should be carried out to assess its potential use in a mixture of cationic metals, such as Zn, Mn or Cu, as well as tested on different bioreactors.

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7. Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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