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Marine Messant, Umama Hani, Thaïs Hennebelle, Florence Guérard, Bertrand Gakière, Andrew Gall, Sébastien Thomine, Anja Krieger-Liszkay

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HAL Authorization

1 **Manganese excess and deficiency affect chloroplast structure and the photosynthetic**  
2 **apparatus in *Marchantia polymorpha***

3  
4 Marine Messant<sup>1</sup>, Umama Hani<sup>1</sup>, Thaïs Hennebelle<sup>1</sup>, Florence Guérard<sup>2</sup>, Bertrand Gakière<sup>2</sup>,  
5 Andrew Gall<sup>1</sup>, Sébastien Thomine<sup>1</sup>, Anja Krieger-Liszkay<sup>1,\*</sup>

6  
7 <sup>1</sup>Université Paris-Saclay, Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS,  
8 91198 Gif-sur-Yvette cedex, France

9 <sup>2</sup>Institute of Plant Sciences Paris-Saclay, CNRS, Université Paris-Sud, Institut National de la  
10 Recherche Agronomique, Université d'Evry, Université Paris-Diderot, Université Paris-  
11 Saclay, 91405 Orsay Cedex, France

12  
13 \*Corresponding author: phone: +33 1 6908 1803; E-mail: anja.liszkay@i2bc.paris-saclay.fr

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29 **Abstract**

30 Manganese is an essential metal for plant growth. The most important Mn-containing enzyme  
31 is the  $Mn_4CaO_5$  cluster that catalyses water oxidation in Photosystem II. Mn deficiency  
32 primarily affects photosynthesis, while Mn excess is generally toxic. Mn excess and  
33 deficiency were studied in the liverwort *Marchantia polymorpha*, an emerging model ideally  
34 suited for analysis of metal stress since it accumulates rapidly toxic substances due to the  
35 absence of well-developed vascular and radicular systems and a reduced cuticle. We  
36 established growth conditions for Mn excess and deficiency, performed analysis of metal  
37 content in thalli and in isolated chloroplasts. *In vivo* super-resolution fluorescence  
38 microscopy and transmission electron microscopy revealed changes in the organization of the  
39 thylakoid membrane under Mn excess and deficiency. Both, Mn excess and Mn deficiency,  
40 increased the stacking of the thylakoid membrane. We investigated photosynthetic  
41 performance by chlorophyll fluorescence at room temperature and at 77K, P700 absorption  
42 and by studying the susceptibility of thalli to photoinhibition. Non-optimal Mn concentrations  
43 changed the ratio of photosystem I to photosystem II. Upon Mn deficiency higher non-  
44 photochemical quenching was observed, electron donation to photosystem I was favoured,  
45 and photosystem II was less susceptible to photoinhibition. Mn deficiency seems to favour  
46 cyclic electron flow around photosystem I protecting thereby photosystem II in high light.  
47 The results presented here suggest an important role of Mn for the organisation of the  
48 thylakoid membrane and photosynthetic electron transport.

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## 53 **Introduction**

54 Manganese (Mn) is an essential element for plant growth. Mn homeostasis is  
55 disturbed under suboptimal or excessive Mn availability (Schmidt et al., 2016; Alejandro et  
56 al., 2020). The most important Mn-containing enzyme is the  $Mn_4CaO_5$  cluster at Photosystem  
57 II (PSII) that catalyses water oxidation. Although Mn is involved as cofactor in a range of  
58 biochemical pathways, the primary effect of Mn deficiency in photosynthetic organisms is a  
59 decrease in photosynthetic activity (Marschner, 1995). Furthermore, Mn is involved in  
60 Reactive Oxygen Species (ROS) metabolism and in the antioxidant response. Manganese is  
61 the cofactor of the Manganese Superoxide Dismutase (MnSOD) found in mitochondria and  
62 peroxisomes (Bowler et al., 1994; Corpas et al., 2017). Oxalate oxidase (OxOx) present in the  
63 apoplast also requires Mn. This enzyme catalyses the oxidation of oxalate to carbon dioxide  
64 coupled with the reduction of oxygen to hydrogen peroxide (Requena and Bornemann, 1999),  
65 the latter having an essential role in the defence against pathogens (Lane et al., 2002). When  
66 accumulated in excess, Mn can be toxic causing oxidative stress (Marschner, 1995; Pittman,  
67 2005; Delhaize et al., 2007; Peiter et al., 2007; Eroglu et al., 2016).

68 In the presence of excess Mn in the soil, there is a competition between the uptake of  
69 Mn and other metals that are also essential for the plant (Alam et al., 2005; St. Clair and  
70 Lynch, 2005; Blamey et al., 2015; Leskova et al., 2017). Indeed, at the level of the roots,  
71 plants do not have transporters that are completely selective for a single metal. Thus, a high  
72 abundance of Mn can lead to a decrease in the absorption of other essential metals such as  
73 calcium, magnesium, iron or even phosphorus, negatively affecting photosynthesis (Nable et  
74 al., 1988; Amao and Ohashi, 2008) and inhibiting chlorophyll synthesis (Clairmont et al.,  
75 1986; Subrahmanyam and Rathore, 2001). Manganese excess causes chlorosis followed by  
76 necrosis leading to plant death, but these symptoms are very variable and species-dependent  
77 (Millaleo et al., 2010). To overcome Mn toxicity, plants have developed different ways of Mn  
78 storage in vacuoles (Ducic and Polle, 2007), cell walls (Führs et al., 2010) and even Golgi  
79 vesicles (Marschner, 1995; Pittman, 2005). In the literature, plants species have been divided  
80 into tolerant and non-tolerant to Mn excess. Some species can hyperaccumulate Mn at levels  
81 above  $10\ 000\ mg.kg^{-1}$  DW (van der Ent et al., 2013).

82 The effect of Mn deficiency on photosynthetic electron transport and chloroplast  
83 structure has been studied for decades in a number of different organisms ranging from  
84 cyanobacteria to higher plants (e.g., Homann, 1967; Salomon and Keren, 2011). In general, a  
85 decline of PSII activity is observed although symptoms of Mn deficiency are species-

86 dependent (Homann, 1967). Depending on the severity of Mn deficiency the ultrastructure of  
87 chloroplasts may be perturbed. In slightly Mn-deficient spinach plants, the stroma lamellae  
88 are affected while the grana stacks are normal. Under more severe Mn deficiency, grana  
89 stacks are also disorganized (Mercer et al., 1962). More recently, similar observations were  
90 reported in the Arabidopsis knockout mutant Chloroplast Mn Transporter1 (CMT1), a  
91 transporter localized in the inner envelope membrane, where chloroplast development was  
92 abnormal and thylakoids appeared disorganized, with either hypo- or hyper-stacked grana  
93 lamellae (Eisenhut et al., 2018; Zhang et al., 2018). In this mutant, a large heterogeneity  
94 between the chloroplasts was observed with chloroplasts containing a well-structured, normal  
95 organization of the thylakoid membranes next to chloroplasts with completely disorganized  
96 architectures (Eisenhut et al., 2018). The consequences of the loss of PSII activity and the  
97 disorganization of the thylakoid membrane on the ratio of linear and cyclic photosynthetic  
98 electron transport have not been investigated in the previous studies.

99 In this present study, we investigated for the first time the consequences of both  
100 excess and deficiency of Mn on photosynthesis in the liverwort *Marchantia polymorpha*, an  
101 emerging model system. *Marchantia* has been described in the 1990s as being able to hyper-  
102 accumulate certain metals such as for example lead (Samecka-Cymerman et al., 1997). Due  
103 to the morphology of the thalli, the ventral part and hyaline parenchyma of the plant is in  
104 direct contact with the substrate allowing uptake of the metals from the medium without  
105 having to pass through the root system as occurs in higher plants. Compared to higher plants,  
106 the leaf anatomy of *Marchantia* is simpler and the tissues are thinner. Chloroplasts are less-  
107 structured than in higher plants (Tanaka et al., 2017) with smaller grana stacks, making these  
108 chloroplasts an ideal system for super-resolution fluorescence microscopy.

109 We have established conditions for cultivating *Marchantia* on plates under Mn  
110 deficiency and excess. The metal content, metabolome and antioxidant activities as well as  
111 photosynthetic activity and chloroplast structure were determined to investigate the response  
112 of *Marchantia* to non-optimal Mn supply. Manganese excess and deficiency affect these  
113 processes in different ways. Manganese excess led to a strong response of the metabolome  
114 but subtle defects in photosynthesis, while in contrast Mn deficiency affected the activity of  
115 PSII and promoted cyclic electron transport around PSI. Under Mn deficiency, an increase in  
116 non-photochemical fluorescence quenching was observed, protecting PSII against  
117 photoinhibition.

118

119

## 120 **Results**

### 121 ***Part I: Mn excess induces a stress response and affects the photosynthetic apparatus***

122 In order to determine the effect of Mn excess on *M. polymorpha*, plants were  
123 cultivated on media containing different MnCl<sub>2</sub> concentrations, ranging from 33 μM (Agar  
124 Control) to 6.5 mM (200X) manganese. Gemmae were cultured on a standard Gamborg's B5  
125 medium (33 μM MnCl<sub>2</sub>) for two weeks before being transferred for one week to the different  
126 Mn concentrations before analysis. Fig. 1A shows that thalli were able to grow normally up  
127 to 1 mM Mn. However, thalli showed reduced growth from 2 mM up to 6.6 mM Mn, with  
128 signs of stress visible as brown spots. Chlorophyll content of the thalli was significantly  
129 decreased in excess Mn conditions (SI Table 1). Determination of Mn concentration in thalli  
130 (Fig. 1B) showed that there is a positive correlation between Mn accumulation and Mn  
131 concentration in the medium. In addition to Mn, we determined Fe and Mg content since high  
132 Mn may impact negatively Fe import into the chloroplasts and thereby the assembly of FeS  
133 clusters in PSI (Millaleo et al., 2013), and Mg is important for stacking of the thylakoid  
134 membranes. Neither Fe nor Mg content were affected by the high Mn concentrations.

135 In the following, we focus on the conditions of Mn excess 30X and 200X. The first  
136 being the highest concentration at which Marchantia showed no obvious signs of stress and  
137 the second being the most stressful condition. We conducted a metabolomic analysis by GC-  
138 MS on thalli. In total, 94 metabolites have been identified and quantified (Supplemental Fig.  
139 S1; Tables S2, S3). Regarding the 200X condition, certain metabolites involved in the  
140 response to heavy metal or oxidative stresses were strongly increased while all other  
141 metabolites decreased compared to the control. A very high concentration of Mn was  
142 associated with a strong induction of polyamine synthesis with an accumulation of ornithine,  
143 citrulline and putrescine (Table 1). Among sugars and their derivatives, strong increases in  
144 trehalose, rhamnose and galacturonic acid were also observed. All these compounds are  
145 known to accumulate in plants and other organisms upon exposure to heavy metals (Sharma  
146 and Dietz, 2006 ; Hasanuzzaman et al., 2019, Liu et al., 2017; Singh et al., 2016; Alejandro et  
147 al., 2020). Most striking, N-methylalanine content was 30 and 50 times higher in 30X and  
148 200X conditions, respectively. To further investigate the response to oxidative stress, the  
149 activity of a few antioxidant enzymes was measured (Table 2). A twofold increase in SOD  
150 activity was observed at the highest Mn concentration. The activity of class III peroxidases  
151 was also slightly increased at 200X, while catalase activity gradually decreased.

152 Next, we studied the impact of Mn excess on chloroplast ultrastructure and  
153 photosynthetic activity. The concentration of Mn, Fe and Mg was measured in isolated intact

154 chloroplasts (Fig. 2A). The results show a gradual and significant increase of Mn comparable  
155 with the observation made in whole thalli. We observed an increase from 2 to 175  $\mu\text{g Mn mg}$   
156  $\text{Chl}^{-1}$  in the 200X condition compared to the Control. We determined the total chlorophyll  
157 content of the thalli to determine the proportion of Mn allocated to chloroplasts (Fig. 1B; Fig.  
158 2A; Supplemental table S1). 7-13% of the total Mn content of the thalli was detected inside  
159 the isolated chloroplasts with 13% in Control and 30X and 7% in 200X. There were no  
160 significant changes in the chloroplast Fe and Mg concentrations under Mn excess.

161 We used first bright-field and epifluorescence microscopy to see whether Mn excess  
162 changes the morphology of the cells as well as the number of chloroplasts (Fig. 2B). In  
163 Control Agar, most chloroplasts show typical plant-type spherical morphology, while in 30X,  
164 there was a divergence from this typical architecture. Some chloroplasts had a deformed  
165 shape. In 200X, this progression of deformation of the morphology was further enhanced and  
166 the chloroplasts were smaller on average (Fig. 2C, Supplemental Fig. S2). Next, we used  
167 super-resolution fluorescence nanoscopy and transmission electron microscopy to determine  
168 better the change in the organization of the thylakoid membranes triggered by excess Mn  
169 (Fig. 3). At physiological temperatures, chlorophyll fluorescence is mainly due to PSII  
170 emission, which are essentially concentrated in the grana lamellae (for reviews see Dekker  
171 and Boekema 2005; Kirchhoff, 2019). Hence, the fluorescence images partly represent the  
172 spatial organisation and relative abundance of grana stacks in *in vivo* chloroplasts. The  
173 organization of the chloroplast in the Control (Fig. 3A top panel) is similar to that of  
174 *Arabidopsis thaliana* (Iwai et al., 2018; Streckaite, 2021) with a 'round' shape and the  
175 presence of numerous grana stacks visualised as highly fluorescent yellow/orange patches in  
176 the false-colour images, albeit with less clear separation between stroma and grana lamellae.  
177 Under the 30X condition, the chloroplast is smaller, indicating a more compact spatial  
178 organization of the inter-connected granae. Furthermore, the presence of a non-fluorescent  
179 region was observed in the centre corresponding to a void in bright field images. In 200X, the  
180 smaller chloroplasts still exhibited strong chlorophyll fluorescence emissions with a well-  
181 developed distribution of patches indicative of thylakoid grana stacks. Electron microscopy  
182 images confirm clear grana stacking under control and 200X conditions, while the  
183 organisation at 30X was different with three thylakoid membranes always attached to each  
184 other concomitant with a lack of major grana stack formation (Fig. 3B, TEM insert). This  
185 uniform distribution of attached membranes under 30X Mn is in agreement with the quasi-  
186 uniform false-colour chlorophyll emission maps observed using *in vivo* fluorescence  
187 nanoscopy, attributed to mainly PSII emission.

188            Since the size of the chloroplasts and the distribution of the chlorophyll fluorescence  
189 within the chloroplasts was affected by the growth conditions, photosynthesis was studied in  
190 more detail. Table 3 shows that the Chl *a/b* ratio remained unchanged at 30X but decreased at  
191 200X Mn, indicating that the antenna size relative to reaction centre increased since Chl *b* is a  
192 major constituent of the Light Harvesting Complex II (LHCII). 77K fluorescence emission  
193 spectra (Fig. 4A) showed three characteristic peaks at 685 nm, 695 nm and 725 nm, the  
194 former two peaks are attributed to LHCII and PSII, and the latter to PSI with its antenna  
195 (Sato and Butler, 1978). For chloroplasts isolated from thalli grown at 200X, the emission  
196 attributed to PSI decreased relative to that of PSII, while there was no change when grown at  
197 30X (Fig. 4A). In line with the lower PSI fluorescence relative to PSII fluorescence in 200X,  
198 the capacity of PSI electron transport was slightly lower although the effect was not  
199 statistically significant (Table 3). However, the capacity of PSII electron transfer was  
200 significantly decreased. A damaging effect of high Mn concentration on PSII activity was  
201 confirmed by fluorescence induction curves (Fig. 4B). The appearance of the K-phase under  
202 200X condition can be attributed to a damage of the Mn<sub>4</sub>CaO<sub>5</sub> cluster at the PSII donor side  
203 according to Strasser (1997). In 200X, Fv/Fm and chlorophyll quenching fluorescence was  
204 altered during illumination with actinic light, but there was no increase in the susceptibility of  
205 PSII to photoinhibition (Table 3, Supplemental Figs S3, S4).

206

## 207 ***Part II: Mn deficiency affects the photosynthetic apparatus and may favour cyclic electron*** 208 ***flow***

209            In the following, we investigated the effect of Mn starvation on *Marchantia*  
210 *polymorpha*. Agar plates were not suitable to induce Mn deficiency since agar contains too  
211 much Mn as an impurity ( $12.8 \pm 0.4 \mu\text{M}$ ). Therefore, we substituted agar by starch as a  
212 gelling agent. Gemmae were cultured during 2 weeks on Gamborg's B5 Agar medium (33  
213  $\mu\text{M}$  MnCl<sub>2</sub>) before young thalli were transferred to Mn-free Gamborg's B5 Starch medium  
214 with or without addition of Mn (33  $\mu\text{M}$  Mn). It was not possible to culture gemmae directly  
215 on starch because they were not able to develop. Fig. 1C shows that plants exhibited no  
216 visible growth defect after one week culturing on Starch Control or without Mn. However,  
217 chlorophyll fluorescence showed a clear decrease in the quantum yield of PSII, Fv/Fm) in the  
218 developing part of the thallus when no MnCl<sub>2</sub> had been added to the medium (Supplemental,  
219 Fig. S5). The amount of metals in thalli was measured (Fig. 1D). The use of starch allowed a  
220 reduction of Mn content from 75  $\mu\text{g}$  Mn g DW<sup>-1</sup> (Starch Control) and to 20  $\mu\text{g}$  Mn g DW<sup>-1</sup>  
221 (Starch-Mn). Fe and Mg contents were not affected.

222 As for Mn excess, a metabolic analysis was carried out. No significant difference in  
223 the metabolomics profiles was observed between the two starch conditions (Supplemental  
224 Table 1) while there were clear differences between Agar Control and Starch Control  
225 (Supplemental Table 2). The activity measurements of the antioxidant enzymes showed a  
226 significant decrease in the activity of type III peroxidases (Table 3). Using a spin-trapping  
227 assay, the generation of  $\cdot\text{OH}$  derived from  $\text{H}_2\text{O}_2/\text{O}_2\cdot^-$  was detected in starch-grown plants.  
228 ROS levels were two times higher in the Starch-Mn condition.

229 Next, the impact of Mn deficiency on chloroplasts was investigated. The  
230 measurements (Fig. 2D) revealed a decrease in the Mn content by about 50% in the Starch-  
231 Mn condition compared with the Starch Control. There was a tendency that the Starch-Mn  
232 growth condition lowers slightly the Fe and Mg content, however the differences were not  
233 statistically significant. Overall, the metal content (Mn, Fe, Mg) of the chloroplasts under  
234 starch conditions was almost double compared to chloroplasts from thalli grown under Agar  
235 Control conditions. This may be due to effects on the metal uptake and transport system or  
236 also be connected to a higher intactness of the isolated chloroplasts. The epifluorescence  
237 microscopy images (Fig. 2F) showed more “horseshoe-shaped” chloroplasts under the Starch  
238 control condition. In Starch-Mn chloroplasts appeared rounder. These observations were  
239 confirmed by super-resolution nanoscopy (Fig. 3B), however the area of the chloroplasts was  
240 unaltered (Supplemental Fig. S2). In the Starch Control, the thylakoid membranes were  
241 organized in grana/lamellae-like structures. In the Starch-Mn condition, the chloroplasts had  
242 fewer voids in the 3-D volume. Compared with a standard chloroplast as represented in the  
243 Agar Control (Fig. 3A, top panel), or *Arabidopsis* (Iwai et al., 2018; Streckaite, 2021), the  
244 strongly fluorescent regions were localized more distinctly, indicating a change in the  
245 mesoscopic organization of the thylakoid membrane. This indicates smaller grana stacks with  
246 a more pronounced segregation between them and the stroma lamellae as confirmed by TEM.  
247 The TEM image shows a much higher degree of stacking of the thylakoid membranes (Fig.  
248 3B).

249 77K fluorescence (Fig. 4B) showed a decrease in the emission at 680 nm in  
250 chloroplasts isolated from Starch-Mn thalli, indicating a loss of PSII content compared with  
251 PSI. PSII activity was lower (Table 3). The quantum yield of PSII, Fv/Fm, was significantly  
252 lower upon Mn deficiency. PSI activity increased significantly, confirming the change in the  
253 PSI/PSII ratio under Mn deficiency seen in 77K fluorescence spectra. In the Starch-Mn  
254 condition, the fluorescence induction curves showed a dip phase at about 2 ms, indicative of  
255 damage to the  $\text{Mn}_4\text{CaO}_5$  cluster at the PSII donor side (Fig. 4D).

256 Figure 5 shows changes in room temperature chlorophyll fluorescence upon  
257 illumination with actinic light. Upon Mn deficiency, energy dissipation as heat was enhanced  
258 (qN; Fig. 5C). However, photochemical quenching (qP; Fig. 5D) was not affected.  
259 Furthermore, the slightly higher minimum fluorescence level during the recovery phase  
260 (Post-Illumination Fluorescence Transient, PIFT) upon Mn deficiency indicates an increase  
261 in chlororespiratory and/or cyclic electron flow. To show whether the stability of PSII or the  
262 repair of damaged PSII was affected under Mn deficiency, photoinhibition experiments were  
263 carried out with or without the protein synthesis inhibitor lincomycin (Fig. 6). Plants  
264 cultivated on Starch-Mn were more susceptible to high light in the absence of lincomycin. In  
265 the presence of lincomycin, the opposite was observed with PSII in plants cultivated on  
266 Starch-Mn being more resistant to the photoinhibitory treatment. This may be explained by  
267 the higher NPQ protecting the photosynthetic apparatus. A higher NPQ was observed also in  
268 the presence of lincomycin (Supplemental Fig. S6).

269 The higher PSI fluorescence at 77K, the higher NPQ and the protection of PSII  
270 against photoinhibition in plants cultivated on Starch-Mn may indicate that cyclic electron  
271 transport is favoured under these conditions. Absorption changes of P700 show that P700 is  
272 more slowly oxidized, both in red actinic and in far-red light, in plants cultivated on Starch-  
273 Mn indicating that more electrons are available in the electron transport chain (Fig. 7A). To  
274 show that indeed more electrons are available for P700<sup>+</sup> reduction at the luminal side, we  
275 determined the yield of donor side limitation, Y(ND) as a function of the intensity of the  
276 actinic light. Figure 7B shows less donor-side limitation of PSI over a wide range of light  
277 intensities in plants cultivated on Starch-Mn compared to those cultivated on Starch Control.  
278 These data further indicate a higher activity of cyclic electron flow under Mn-deficiency.  
279 Under both conditions, acceptor side limitation at the stromal side of PSI was not observed  
280 because *Marchantia* possesses flavodiiron proteins that use ferredoxin as electron donor to  
281 reduce oxygen to water (Shimakawa et al., 2021).

282

## 283 Discussion

284 In this study, we established growth conditions for manganese excess and deficiency in  
285 *Marchantia polymorpha*. *Marchantia* is able to absorb up to 25 mg Mn g DW<sup>-1</sup> (Fig. 1B)  
286 which is higher than the hyperaccumulator *Polygonum lapathifolium* which contains  
287 approximately 18 mg Mn g DW<sup>-1</sup> in its aerial parts (Liu et al., 2016). However, *Marchantia*  
288 shows symptoms of toxicity under the 200X conditions, and thalli only survive for a few  
289 weeks. Therefore, *Marchantia* does not qualify as hyperaccumulator despite its high Mn

290 uptake capacity. Mn excess conditions induced an increase in SOD and PRX activity while  
291 catalase activity decreased (Table 2). Increase in SOD activity could reflect a higher amount  
292 of MnSOD present under Mn excess conditions. The metabolomics analysis showed that  
293 known stress-induced metabolites accumulate upon excess Mn (Table 1; Supplemental Fig.  
294 S2, tables S2, S3), however, the metabolomics response of *Marchantia* is distinct from that of  
295 angiosperms and requires in depth functional analysis of metabolites and metabolic pathways.  
296 An excess of Mn affected the ratio between it and other metals (e.g. Fe and Mg) in both thalli  
297 and chloroplasts. Indeed, cation transporters are known not to be completely selective under  
298 stressed conditions (Barberon et al., 2011; Ducic et al., 2005; Lei et al., 2007; Millaleo et al.,  
299 2010; Vert et al., 2002); well-documented examples of excess of Mn in the soil are known to  
300 perturb the absorption and translocation of other elements including iron, magnesium and  
301 phosphorus (Millaleo et al., 2010; Ducic et al., 2005; Lei et al., 2007), causing Fe deficiency  
302 in plants. However, *Marchantia* grown at 200X does not show alterations in the Fe and Mg  
303 content inside the thalli (Figs 1B, 2A). Nevertheless, an alteration in the Mn/Fe ratio may  
304 induce Fe deficiency through competitive binding. Fe deficiency is known to affect primarily  
305 PSI that is rich in 4Fe4S clusters. As shown in Fig. 4B, PSI content is lowered in comparison  
306 to PSII in 200X. A similar reduction of PSI upon exposure to Mn excess has been reported  
307 for *Arabidopsis* (Millaleo et al., 2013). Aside PSI, the water-splitting activity of PSII seems  
308 also to be affected in the 200X condition as seen by the appearance of the K phase in  
309 fluorescence induction curves (Fig. 4C) and the decrease in PSII activity (Table 2). A toxic  
310 effect of Mn excess on PSII activity is in accordance with data on higher plants (Liang et al.,  
311 2019). 200X Mn negatively affected not only the photosynthetic apparatus but also led to a  
312 reduction in chloroplast size and an alteration of the well-separated grana stack and stroma  
313 lamellae distribution (Figs. 2 and 3).

314         Transfer of thalli from agar to starch plates allowed to establish Mn deficiency  
315 conditions. Growth on starch allowed lowering drastically the Mn content in the thalli to 21  
316  $\mu\text{g Mn g DW}^{-1}$ . According to Mengel and Kirkby (1987), the minimum Mn content required  
317 for growth of higher plants is about 20 to 40  $\text{mg Mn kg DW}^{-1}$ , and most higher plants usually  
318 contain 30 to 500  $\text{mg Mn kg DW}^{-1}$ . Manganese deficiency led to a decrease in Fv/Fm, a  
319 change in the ratio of the activity PSI/PSII (Table 3) and less PSII content relative to PSI  
320 according to 77K fluorescence (Fig. 4B). In higher plants, Mn deficiency lowers PSII activity  
321 while PSI activity remains unaffected (Homann, 1967; for review see Schmidt et al., 2016).  
322 Furthermore, the structural organization of the thylakoids was affected (Fig. 3). It is known  
323 that Mn deficiency leads to disorganization of the thylakoid membrane in higher plants

324 (Mercer et al., 1962; Homann, 1967). In *M. polymorpha*, the smaller compartmentation of the  
325 grana stacks could be partly responsible for the changes in electron transport. In addition to a  
326 change in the stoichiometry between active PSI and active PSII, a different distribution of  
327 photosynthetic complexes may favour cyclic electron flow around PSI by allowing the  
328 formation of supercomplexes likely to be required for cyclic electron flow (Iwai et al., 2010).  
329 As shown in Fig. 7, thalli grown on Starch-Mn show less limitation of electron donation to  
330 P700<sup>+</sup> than those of the Starch Control indicating a stimulation of cyclic electron flow under  
331 Mn deprivation. Cyclic electron flow is known to be induced under specific physiological  
332 conditions like anaerobiosis in *Chlamydomonas reinhardtii* (Joliot et al., 2022). Changes in  
333 the organization of PSI complexes was also observed under Mn limitation in cyanobacteria  
334 (Salomon and Keren, 2011). Increased cyclic electron flow generates a higher proton gradient  
335 across the thylakoid membrane and thereby an increase in NPQ (Fig. 5C). A higher NPQ  
336 protects photosystem II against photoinhibition as seen when thalli grown on Starch-Mn were  
337 exposed to high light in the presence of lincomycin (Fig. 6) while they were more susceptible  
338 to photoinhibition in the absence of lincomycin (Fig. 6) likely due to a slowdown of PSII  
339 repair. A slowdown of PSII repair under Mn deficiency has been observed previously in the  
340 Mn transporter mutant *nramp3nramp4* in Arabidopsis (Lanquar et al., 2010). The repair of  
341 PSII may be slowed down in the light because of the higher ROS levels in Starch-Mn (Table  
342 2) that affects the synthesis of the D1 protein (Nishiyama *et al.*, 2011).

343 In conclusion, we show here that unfavourable Mn concentrations during growth of  
344 *Marchantia* affect not only the photosynthetic apparatus but also the organisation of the  
345 thylakoid membrane. Future work is needed to explore the link between changes in the  
346 organization and stacking of the thylakoid membrane and stimulation of cyclic electron flow  
347 under Mn deficiency.

348

## 349 **Material and Methods**

### 350 **Plant growth conditions**

351 Gemmae from *Marchantia polymorpha*, Takaragaike (Tak-1) accession (male) were  
352 asexually cultured on ½ Gamborg's GB5 1% Agar medium (Gamborg et al., 1968) for two  
353 weeks under a light-dark cycle of 16 h-light, 22°C, 120 μmol quanta m<sup>-2</sup>s<sup>-1</sup>, white fluorescent  
354 lamp / 8 h-dark, 20°C. For Mn excess condition, young thalli were transferred to ½  
355 Gamborg's GB5 1% Agar medium containing 33 μM (control), 0.5 mM (15X), 1 mM (30X),  
356 2 mM (60X), 5 mM (150X) or 6.5 mM (200X) MnCl<sub>2</sub> for one week before measurements.  
357 According to the metal quantification, the control medium contained 2.52 ± 0.27 μg Mn g<sup>-1</sup>

358 fresh weight of the solid medium. For Mn deficiency, young thalli were transferred on 1/2  
359 Gamborg's GB5 6% starch medium with or without addition of 33  $\mu\text{M}$   $\text{MnCl}_2$  for one week  
360 before performing measurements. According to the metal quantification, the starch medium  
361 with  $\text{MnCl}_2$  contained  $2.03 \pm 0.13 \mu\text{g Mn g}^{-1}$  and the medium without addition of  $\text{MnCl}_2$   
362 contained  $0.26 \pm 0.08 \mu\text{g Mn g}^{-1}$  fresh weight of the solid medium.

363

#### 364 **Chloroplast and thylakoid isolation**

365 10 g thalli were collected, the media carefully mechanically removed, and ground in 100 ml  
366 of GR buffer (50 mM Hepes-KOH pH 7.5, 0.33 M sorbitol, 1 mM  $\text{MgCl}_2$ , 2 mM EDTA, 5  
367 mM Na-ascorbate) with a spatula of BSA. The slurry was filtered through two layers of  
368 Miracloth and centrifuged at 1200g (4°C) for 7 min. Pellets were resuspended in 2 mL of GR  
369 buffer then layered on top of a percoll gradient (15 ml of 30% percoll/GR buffer and 10 ml of  
370 70% Percoll/GR buffer), centrifuged at 7000g (4°C) for 17 min (no brake). Intact chloroplasts  
371 were collected and washed in 25 ml of GR buffer; centrifugation 1500g (4°C) for 5 min. The  
372 intactness was checked by light microscopy. For thylakoids, the same procedure was used  
373 without the percoll gradient.

374

#### 375 **Metal quantification**

376 Thalli were collected and dried at 70°C for 2 days before being weighted. 1 mL of nitric acid  
377 (65%) was added to 1 mg plant material. For intact chloroplasts, the chlorophyll  
378 concentration was adjusted to 100  $\mu\text{g chl mL}^{-1}$  before the addition of 2.5 volume of nitric  
379 acid. After 10-fold dilution in trace-metal-free water, the metal content of the samples was  
380 determined by atomic emission spectroscopy using a MP AES 1200 spectrometer (Agilent,  
381 USA).

382

#### 383 **Super-resolution microscopy**

384 For each sample, a small quantity of thallus was placed in a sandwich of two glass coverslips  
385 (22 mm diameter, Paul Marienfeld GmbH & Co. KG), sealed and placed inside an in-house  
386 built sample holder. The sample holder was fixed to a nano-positioning system (P-733.2CD,  
387 P-725.4CD, E-725.3CD, Physikinstrumente) coupled to an inverted microscope (Ti-U,  
388 Nikon) equipped with a CFI Apochromat Lambda 100x oil immersion objective. Room-  
389 temperature fluorescence emission was recorded by an iXon ULTRA 897 camera (Andor  
390 Technology Ltd., Belfast) coupled to the microscope with a custom-made Optomask (Cairn  
391 research Ltd, Faversham). Total chlorophyll fluorescence emission was isolated using a

392 dichroic mirror/emission filter doublet (59022BS/ET655LP, Chroma Technology  
393 Corporation). Epifluorescence images were recorded using an excitation provided by a  
394 plasma light source (HPLS245 Thorlabs Inc.) and an excitation filter (MF469-35 Thorlabs  
395 Inc.). The excitation source for the laser-scanning measurements was a 445 nm emitting laser  
396 (OBIS LX, Coherent Inc.). Laser-scanning and final image reconstruction (resolution ( $d$ )  
397  $d_{xy}=126$  nm,  $d_z=320$  nm), was as previously described (Streckaite, 2021) with a dwell time of  
398 10-30 ms, 60 nm X/Y-scan steps and 300 nm Z-scan steps.

399

#### 400 **Transmission electron microscopy (TEM)**

401 1 mm strips of thalli were cut under glutaraldehyde/paraformaldehyde fixative and  
402 impregnated by the 1% osmium and 1.5% potassium hexacyanoferrate *en bloc* staining  
403 protocol as described (Hawes et al., 1981; Juniper et al., 1982). 70 nm thick sections were cut  
404 with an EM UC6 ultramicrotome (Leica Microsystems) and deposited on to copper grids.  
405 Ultrathin sections were stained with 2% uranyl acetate (Merck) and Reynolds lead citrate  
406 according to standard procedures. Grids were examined under a JEOL 1400 TEM operating  
407 at 80 kV (JEOL, <http://www.jeol.com>). Images were acquired using a nine megapixel high-  
408 speed camera (RIO9; Gatan, <http://www.gatan.com>) and processed using Digital Micrograph  
409 (Gatan).

410

#### 411 **Pigment analysis**

412 Thalli were weighed and incubated in 100% acetone for 16 h in the dark. For complete  
413 pigment extraction, acetone incubation was repeated three times. Pigment extract was diluted  
414 to 80% acetone before measurement. Chlorophyll *a* and chlorophyll *b* contents were  
415 calculated according to Porra and Scheer (2019).

416

#### 417 **77K Chlorophyll fluorescence measurements**

418 Fluorescence spectra of intact chloroplasts diluted in GR buffer were measured with a Carry  
419 Eclipse fluorimeter (Agilent, USA); excitation wavelength: 430 nm. The spectra were  
420 normalized to the intensity of the PSI emission.

421

#### 422 **Chlorophyll fluorescence analysis at room temperature**

423 Chlorophyll fluorescence analysis was performed on 5 min dark-adapted thalli using a Dual-  
424 PAM-100 fluorimeter (Walz, Effeltrich, Germany).  $F_m$  and  $F_m'$  were determined using  
425 saturating flashes ( $10,000 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$ , duration 300 ms).  $F_o$ , minimum and  $F_m$ ,

426 maximum fluorescence in a dark-adapted sample;  $F_m'$ , maximum fluorescence and  $F'$ ,  
427 fluorescence emission from a light-adapted sample. Induction and recovery curves were  
428 measured using actinic light of  $50 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$ .  $F_v/F_m = (F_m - F_0)/F_m$ ,  $qN = (F_m -$   
429  $F_m')/(F_m - F_0')$  and  $qP = (F_m' - F)/(F_m' - F_0')$ . For induction curves (OJIP) one saturating flash  
430 was given.

431

### 432 **Photoinhibition**

433 Thalli were placed on wet filter paper and illuminated with white light ( $800 \mu\text{mol quanta m}^{-2}$   
434  $\text{s}^{-1}$ ); LED panel SL3500 (Photon Systems Instrument, Drasov, Czech Republic). For  
435 recovery, samples were placed in low light ( $10 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$ ). When indicated, thalli  
436 were incubated in lincomycin ( $1 \text{ g L}^{-1}$ ) for 4 h prior to the photoinhibition treatment.  $F_v/F_m$   
437 was measured using an Imaging-PAM (Walz, Effeltrich, Germany).

438

### 439 **P700 measurements**

440 P700 absorbance was measured using a Dual-PAM-100 fluorometer (Walz, Effeltrich,  
441 Germany). Near-infrared measuring lights (830 and 870 nm) were applied to measure the  
442 transmittance of oxidized P700. Prior to the measurements, the plants were kept in the light in  
443 the growth chamber so that the Calvin-Benson cycle enzymes were active. Five minutes dark-  
444 adapted thalli were exposed to actinic light followed by far-red light. Then, a saturating flash  
445 was given at the end of the far-red light period. To determine quantum yields of PSI donor,  
446  $Y(\text{ND})$ , and acceptor side-limitations,  $Y(\text{NA})$ , saturating pulse analysis was used  
447 (Klughammer and Schreiber, 1994). Each actinic light intensity was applied for 180 s before  
448 determining  $Y(\text{ND})$  and  $Y(\text{NA})$ .

449

### 450 **Antioxidant enzymes activity**

451 Thalli were harvested and ground in 50 mM HEPES buffer pH 6.5. The slurry was filtered  
452 through one layer of miracloth, centrifuged for 5 min at  $10.000g$  ( $4^\circ\text{C}$ ). Protein concentration  
453 in the crude extract was determined by amido black since the supernatant contained pigments  
454 (Schaffner and Weissmann, 1973).  $10 \mu\text{g mL}^{-1}$  protein was used for the enzymatic tests.  
455 Guaiacol peroxidase activity was determined spectrophotometrically by measuring the  
456 oxidation of guaiacol to tetraguaiacol at 470 nm ( $\epsilon$ :  $26.6 \text{ mM}^{-1}\text{cm}^{-1}$ ) (Chance and Maehly,  
457 1955). The reaction mixture contained 50 mM  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  pH 7.5, 3 mM  $\text{H}_2\text{O}_2$ ,  
458 0.01% (v/v) guaiacol. Catalase activity was measured polarographically at  $20^\circ\text{C}$  with a  
459 Clark-type electrode in 50 mM Tris buffer pH 8.5 and 1 mM  $\text{H}_2\text{O}_2$  as substrate. Superoxide

460 dismutase (SOD) activity was measured using a solution of 500  $\mu\text{M}$  Xanthine, 20 mM  
461 HEPES buffer pH 7; 0.2 U  $\text{mL}^{-1}$  xanthine oxidase and 100  $\mu\text{M}$  XTT (Na,3'-(1-  
462 (phenylaminocarbonyl)-3,4-tetrazolium)-bis-(4-methoxy-6-nitro) benzene sulfonic acid  
463 hydrate) as substrate. The kinetics of superoxide production were measured as increase in  
464 absorbance at 470 nm, and the SOD activity was determined by following the inhibition of  
465 the superoxide production after addition of the crude extract (Molins et al. 2013). For  $\cdot\text{OH}$   
466 detection, 15 mg thalli were incubated for 1 h in 3 ml 10 mM phosphate buffer, pH 6.0, 50  
467 mM N-tert-butyl- $\alpha$ -(4-pyridyl)nitron N'-oxide (4-POBN) and 4% ethanol (Heyno et al.,  
468 2008).

469

### 470 **Metabolite Analysis**

471 GC-MS profiling of metabolites was performed on thalli cultured on Agar Control, 30X and  
472 200X conditions and thalli grown on Starch Control and Starch-Mn. Dried and grounded  
473 samples (5 mg of dry weight) were incubated in 1 mL of H<sub>2</sub>O/ACN/isopropanol (2/3/3) with  
474 4 mg l<sup>-1</sup> of ribitol for 10 min at 4°C with shaking at 250g in an Eppendorf Thermomixer.  
475 Insoluble material was removed by centrifugation at 20 400g for 10 min. 700  $\mu\text{l}$  of  
476 supernatant were recovered, 70  $\mu\text{l}$  of a mix of H<sub>2</sub>O/MeOH/isopropanol (2/5/2) with 0.3 g L<sup>-1</sup>  
477 of myristic acid d<sub>27</sub> was added as an internal standard for retention time locking. Aliquots of  
478 each extract (100  $\mu\text{L}$ ) extracts were dried for 4 h at 30°C in a Speed-Vac and stored at -80°C.  
479 After thawing, samples were dried again in a Speed-Vac for 1 h at 30°C before adding 10  $\mu\text{L}$   
480 of 20 mg mL<sup>-1</sup> methoxyamine in pyridine to the samples. The reaction was performed for 90  
481 min at 30°C under continuous shaking in an Eppendorf thermomixer. A volume of 90  $\mu\text{L}$  of  
482 N-methyl-N-trimethylsilyl-trifluoroacetamide was then added and the reaction continued for  
483 30 min at 37°C. After cooling, 80  $\mu\text{L}$  were transferred to an Agilent vial for injection. Four  
484 hour after derivatization, 1  $\mu\text{L}$  sample was injected in splitless mode on an Agilent 7890B gas  
485 chromatograph coupled to an Agilent 5977A mass spectrometer (column: RESTEK RXI  
486 5SIL MS 30MX0.25MMX0.25UM). An injection in split mode with a ratio of 1:30 was  
487 systematically performed for quantification of saturated compounds. Raw Agilent data files  
488 were analysed with AMDIS <http://chemdata.nist.gov/mass-spc/amdis/>. The Agilent Fiehn  
489 GC-MS Metabolomics RTL Library was employed for metabolite identifications. Peak areas  
490 were determined with the Masshunter Quantitative Analysis (Agilent) in splitless and split 30  
491 modes. Peak areas were normalized to ribitol and dry weight. Metabolite contents are  
492 expressed in arbitrary units (semi-quantitative determination).

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**Author Contribution**

M.M, and A.K-L. designed the project. M.M, U.H., T.H., F.G., B.G., A.G., S.T. and A.K-L. performed the experiments and analysed the data. M.M. and A.K-L. wrote the initial version of the manuscript that was read and revised by all authors.

**Data availability**

Data will be made available on demand.

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724 **Tables**

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726 **Table 1. Increase in metabolites involved in oxidative stress response upon growth on**  
727 **high Mn concentration**

728

	<b>Ctrl</b>	<b>30X</b>	<b>200X</b>
Citrulline	0.001 ± 0	0.001 ± 0	0.003 ± 0.001
Galacturonic acid	0.004 ± 0	0.004 ± 0.001	0.008 ± 0.001
L-ornithine	0.003 ± 0.001	0.003 ± 0	0.007 ± 0.001
N-Methylalanine	0.021 ± 0.002	0.67 ± 0.237	1.101 ± 0.038
Putrescine	0.003 ± 0.001	0.002 ± 0	0.008 ± 0.001
Rhamnose	0.004 ± 0	0.004 ± 0.001	0.008 ± 0.001
Trehalose	0.053 ± 0.003	0.049 ± 0.008	0.083 ± 0.012

729

730

731 List of selected metabolites showing an increase in plants grown in 200X condition. Mean  
732 value and SD are given (N=4). P<0.05 for all values, according to two-way ANOVA test. See  
733 Supplemental Table S2 for the full list.

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737 **Table 2. Antioxidant enzymes activities in thalli from control and manganese excess and**  
738 **deficiency conditions**

	<b>Ctrl</b>	<b>30X</b>	<b>200X</b>	<b>Starch+Mn</b>	<b>Starch-Mn</b>
SOD activity	3.01 ± 0.63	4.19 <sup>**</sup> ± 0.86	6.00 <sup>***</sup> ± 1.48	5.63 ± 1.43	5.08 ± 0.63
CAT activity	1.18 ± 0.16	0.87 <sup>**</sup> ± 0.26	0.54 <sup>***</sup> ± 0.12	0.72 ± 0.16	0.70 ± 0.13
PRX activity	1.27 ± 0.38	1.17 ± 0.25	1.59 <sup>*</sup> ± 0.42	1.52 ± 0.20	1.04 <sup>**</sup> ± 0.40
ROS	-	-	-	44.4 ± 4.2	100 ± 1.4

739

740 Superoxide dismutase, Catalase and peroxidase activities ( $\mu\text{mol substrat. mg proteins}^{-1} \text{ min}^{-1}$ )  
741 were measured in crude extracts.  $\cdot\text{OH}$  derived from  $\text{H}_2\text{O}_2/\text{O}_2^{\cdot-}$  was measured using electron  
742 paramagnetic resonance (EPR) as a hydroxyethyl-N-tert-butyl- $\alpha$ -(4-pyridyl)nitron N'-oxide  
743 (4-POBN) adduct. Stars indicate significant difference compared to the control condition,  
744 based on a Mann and Whitney test (\* p<0.01, \*\* p<0.01, \*\*\* p<0.001, N=3 biological  
745 replicates).

746 **Table 3. Chl *a/b* ratio and electron transport capacities of thylakoids from thalli**  
 747 **cultured on manganese excess and deficiency.**

	<b>Ctrl</b>	<b>30X</b>	<b>200X</b>	<b>Starch+Mn</b>	<b>Starch-Mn</b>
Chl <i>a/b</i>	1.23 ± 0.09	1.30 ± 0.10	0.97 ± 0.06	0.95 ± 0.22	0.91 ± 0.20
Fv/Fm	0.775±0.005	0.773±0.003	0.765±0.004	0.772±0.012	0.751*±0.004
PSI/PSII	1.47 ± 0.02	1.42 ± 0.01	1.19* ± 0.02	1.11 ± 0.02	1.51* ± 0.01
PSII activity	48 ± 13	43 ± 17	15.0*** ± 5.1	30 ± 9	22 ± 8
PSI activity	116 ± 20	114 ± 27	100 ± 40	59 ± 8	75* ± 16

748  
 749 Chlorophyll content was determined after extraction in acetone. PSI to PSII ratio was  
 750 determined by integrating the area of the fluorescence at 77K ( $\lambda_{\max 725}/\lambda_{\max 685}$ ). PSII and PSI  
 751 activities ( $\mu\text{mol O}_2 \mu\text{g chl}^{-1} \text{h}^{-1}$ ) were measured using an O<sub>2</sub> electrode. PSII activity was  
 752 measured as oxygen evolution in the presence of 1 mM DCBQ, 10 mM NH<sub>4</sub>Cl. PSI activity  
 753 was measured as oxygen uptake in the presence of 10  $\mu\text{M}$  DCMU, 5 mM ascorbate, 30  $\mu\text{M}$   
 754 DCPIP, 500  $\mu\text{M}$  methylviologen, 10 mM NH<sub>4</sub>Cl. Stars indicate significant difference  
 755 compared to the control condition, based on a Mann and Whitney test (\* p<0.01, \*\*\*  
 756 p<0.001, N=6).  
 757

758 **Figures legends**

759

760 **Figure 1: Effect of non-optimal Mn supply on growth and metal content of *Marchantia***  
761 ***polymorpha*.**

762 **Mn excess: A:** Two-week old thalli were transferred for one week to agar plates containing  
763 33  $\mu$ M (control, Ctrl), 0.5 mM (15X), 1 mM (30X), 2 mM (60X), 5 mM (150X) and 6.5 mM  
764 (200X)  $\text{MnCl}_2$ , respectively. White scale bars represent 1 cm. **B:** Manganese, iron and  
765 magnesium concentrations were determined after one-week growth with the given Mn  
766 concentrations. **Mn deficiency: C:** Two week old thalli were transferred and grown for one  
767 week on starch plates with or without addition of 33  $\mu$ M  $\text{MnCl}_2$ . Black scale bars represent 1  
768 cm. **D:** Manganese, iron and magnesium concentrations in thalli were measured after one  
769 week of growth on starch plates with (Ctrl, hatched bars) or without  $\text{MnCl}_2$  (-Mn, white bars)  
770 in the medium. Error bars represent standard errors. Stars indicate significant differences,  
771 compared to the control condition, based on a Mann and Whitney test (\*  $p < 0.05$ , \*\*  $p < 0.01$ ,  
772 \*\*\*  $p < 0.001$ ;  $N \geq 3$  biological replicates).

773

774 **Figure 2: Chloroplast metal content and structure in *Marchantia polymorpha* grown on**  
775 **non-optimal Mn supply.**

776 **Mn excess: A:** Metal content of intact chloroplasts isolated from thalli cultured on agar  
777 (control, 33  $\mu$ M; 30X, 1 mM; 200X, 6.5 mM  $\text{MnCl}_2$ ). **B:** Microscopy images of thalli  
778 cultured on agar (a, Control; b, 30X; c, 200X). Scale bar represents 20  $\mu$ m. **C:**  
779 Epifluorescence microscopy images of chloroplast surfaces of thalli (d, Control; e, 30X; f,  
780 200X). **Mn deficiency: D:** Metal content of intact chloroplasts isolated from thalli cultured  
781 on starch (Starch+Mn, 33  $\mu$ M Mn; Starch-Mn, without Mn). **E:** Microscopy images of  
782 chloroplasts in thalli (g, Starch+Mn; h, Starch-Mn). **F:** Epifluorescence microscopy images of  
783 chloroplast surfaces of thalli (i, Starch+Mn; j, Starch-Mn). Stars indicate significant  
784 differences, compared to the control condition, based on a Mann and Whitney test (\*\*\*  
785  $p < 0.001$ ;  $N \geq 3$  biological replicates).

786

787 **Figure 3: Organisation of chloroplast membranes as evidenced by room-temperature *in***  
788  ***vivo* super-resolution chlorophyll fluorescence emission nanoscopy and by TEM.**

789 **A:** Super-resolution fluorescence microscopy. **1:** Agar Control, 30X and 200X conditions. **2:**  
790 Starch Control and Starch-Mn. For each culture condition, reading from left to right, the three  
791 reconstructed fluorescence maps are separated by 300 nm in the axial (z) direction. The false-

792 colour scale is represent by horizontal colour bar (top). Scale bar is 1 $\mu$ m. One chloroplast is  
793 represented of a total of ten chloroplasts analysed for each condition. **B:** Transmission  
794 electron microscopy images. 10-20 chloroplasts were analysed, exhibiting the same features  
795 for each culture condition. Scale bar is 1 $\mu$ m; 2.5x amplification for inset in 30X.

796

797 **Figure 4: Chlorophyll fluorescence at 77K and room temperature.**

798 **A:** 77K fluorescence emission of chloroplasts extracted from thalli grown on agar: Ctrl  
799 (black), 30X (red) and 200X (blue) (N=3, biological replicates). **B:** Same as (a) carried out on  
800 starch cultivated thalli with (Ctrl, black) or without MnCl<sub>2</sub> (-Mn, red). Spectra in **A** and **B**  
801 were normalized to the maximum emission at 725 nm. **C:** Room temperature fluorescence  
802 induction curves (OJIP) were measured on 5 min dark-adapted thalli by the application of a  
803 saturating flash with 300 ms duration. Culture conditions as in **A**. **D:** Fluorescence induction  
804 curves on thalli grown as in **B**. One representative curves is shown for each condition.

805

806 **Figure 5: Analysis of variable chlorophyll fluorescence in Mn deficiency.**

807 **A, B:** Induction and recovery fluorescence curves were measured on thalli transferred for 1  
808 week to starch medium with (black) or without added MnCl<sub>2</sub> (red). After 5 min dark  
809 adaptation, thalli were exposed to a measuring light to determine the minimum fluorescence  
810 level (dashed lines) and to a saturating flash to obtain the maximum fluorescence level.  
811 Actinic light (50  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup>) was applied for 5 min (white bar). Recovery in the  
812 dark was followed (grey bar). **C:** qN and **D:** qP parameters were calculated thanks to  
813 saturating flashes applied during the light and dark period (N=12, 3 biological replicates).

814

815 **Figure 6: Photoinhibition in Mn deficiency in the absence and presence of lincomycin.**

816 Photoinhibition was measured on starch-cultivated thalli with (black symbols) or without  
817 MnCl<sub>2</sub> (red symbols). Fv/Fm was followed during exposure to high light (800  $\mu$ mol photons  
818 m<sup>-2</sup> s<sup>-1</sup>) for 90 min (yellow bar) and during the recovery at room light (black bar). Squares:  
819 without lincomycin, triangles with lincomycin. As a control, lincomycin samples incubated in  
820 the dark are shown (stars).

821

822 **Figure 7: Activity of photosystem I in Mn deficiency.**

823 **A:** Normalized P700<sup>+</sup> signal was obtained on thalli cultivated on Starch (+Mn, black; -Mn,  
824 red). The redox state of the PSI primary donor P700 was monitored through the changes in

825 absorbance at 830 versus 875 nm. Leaves were kept in the dark for 5 min prior to the  
826 measurements. Thalli were exposed to actinic light for 15s (white bar) and to far-red light  
827 until reaching a plateau (red bar). Then, a saturating flash was applied (yellow bar) and the  
828 decay was observed in the dark (grey bar). **B:** PSI donor-side limitation Y(ND) based on  
829 saturating pulse analyses. Following the initial determination of maximal oxidation of P700,  
830 actinic light of the indicated intensities was given for 180 s.

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### 836 **Supplemental**

837 **Table S1** Chlorophyll and Mn content in thalli grown in Mn excess

838 **Table S2** List of 96 metabolites identified by GC-MS in *M. polymorpha*

839 **Table S3** Metabolites significantly decreased in Starch condition compared to Agar Control  
840 in *M. polymorpha*

841 **Figure S1** Metabolite analysis in *M. polymorpha* grown in Mn excess condition.

842 **Figure S2** Estimation of the chloroplast size under the different growth conditions

843 **Figure S3** Chlorophyll fluorescence at room temperature in *M. polymorpha* grown in control  
844 and Mn excess conditions

845 **Figure S4** Photoinhibition of photosystem II in *M. polymorpha* grown in control and Mn  
846 excess condition in the presence and absence of lincomycin

847 **Figure S5** Chlorophyll fluorescence (Fv/Fm) in *M. polymorpha* grown on starch plates in low  
848 Mn concentrations

849 **Figure S6** qN determination in the presence of lincomycin in *M. polymorpha* grown on  
850 starch in control and Mn-deficient conditions

851