TITLE; Manganese toxicity disrupts indole acetic acid homeostasis and suppresses CO₂ assimilation reaction in rice plants

RUNNING TITLE; Mechanism of symplastic Mn toxicity in rice

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29 **HIGHLIGHT (18 words)**

30 Increased Mn concentration lowers auxin concentrations in rice leaves, which suppresses
31 photosynthesis by changing stomatal function and development.

32

33 **ABSTRACT (176 words)**

34 Despite the essentiality of Mn in terrestrial plants, its excessive accumulation in plant tissues
35 causes growth defects, known as Mn toxicity. Mn toxicity can be divided into apoplastic and
36 symplastic types depending on its onset. For growth defects, symplastic rather than
37 apoplastic Mn toxicity is hypothesised to be more critical. However, details of the
38 relationship between growth defects and symplastic Mn toxicity remains elusive. In this
39 study, we aimed to elucidate the molecular mechanisms of symplastic Mn toxicity in rice
40 plants. We found that under excess Mn conditions, CO₂ assimilation was inhibited by
41 stomatal closure, and both carbon anabolic and catabolic activities were decreased. In
42 addition to stomatal dysfunction, stomatal and leaf anatomical development were also
43 altered by excess Mn accumulation. Furthermore, the indole acetic acid (IAA) concentration
44 was decreased, and auxin-responsive gene expression analyses showed IAA-deficient
45 symptoms in leaves due to excess Mn accumulation. These results suggest that excessive
46 Mn accumulation causes IAA deficiency, and low IAA concentrations suppress plant growth
47 by suppressing stomatal opening and leaf anatomical development for efficient CO₂
48 assimilation in leaves.

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52 **KEYWORDS**

53 amino acid metabolism, auxin, bundle-sheath cell, leaf anatomy, manganese,
54 photorespiration, photosynthesis, rice, respiration, stomata,

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57 ABBREVIATIONS

58 AOX, alternative oxidase; ARF, auxin response factor; AUX/IAA, auxin/indoleacetic acid; Cc,
59 chloroplastic CO₂ partial pressure; COX, cytochrome c oxidase; Ci, internal CO₂
60 concentration in leaves; DAO, dioxygenase for auxin oxidation; D.W., dry weight; F.W., fresh
61 weight; Fv/Fm, maximum quantum yield of photosystem II; GC-MS, gas
62 chromatography-mass spectrometry; g_s, stomatal conductance; IAA, indole acetic acid;
63 ICP-OES, inductively coupled plasma optical emission spectroscopy; HPLC,
64 high-performance liquid chromatography; PODs, class III peroxidases; PS, photosystem;
65 ROS, reactive oxygen species; SEM, scanning electron microscope; SOD, superoxide
66 dismutase; TCA, tricarboxylic acid; Y(II), quantum yield of photosystem II; Y(I), quantum
67 yield of photosystem I; Y(NPQ), quantum yield of non-photochemical quenching; Y(NO),
68 quantum yield of non-radiative decay; Y(ND), quantum yield of non-photochemical
69 quenching at the donor-side of photosystem I; Y(NA), quantum yield of non-photochemical
70 quenching at the acceptor-side of photosystem I

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85 INTRODUCTION

86 Mn is an essential nutrient for both terrestrial plants and animals (Shao *et al.*, 2017). With
87 regard to terrestrial plants, Mn was first discovered in their ash, and McHargue (1922)
88 proved that Mn is an essential nutrient. Manganese possesses a wide variety of
89 physiological functions in plant cells. For example, Mn activates more than 35 different
90 enzymes such as chloroplast RNA-polymerase, and several enzymes involved in the
91 tricarboxylic acid (TCA) cycle and shikimic acid pathway (Broadley *et al.*, 2012). Mn is also
92 directly involved in the physiological function of Mn-superoxide dismutase (SOD) as a
93 cofactor to detoxify reactive oxygen species (ROS) and oxalate oxidase (Broadley *et al.*,
94 2012). Among these physiological functions, the Mn cluster within the oxygen-evolving
95 complex of photosystem II (PSII) is crucial for driving photosynthesis in terrestrial plants
96 (Suga *et al.*, 2015).

97 Although Mn is indispensable for terrestrial plants, excess Mn accumulation in
98 leaves reduces their growth and crop yield, through what is known as Mn toxicity (Foy *et al.*,
99 1978; Li *et al.*, 2019). The toxic effects of Mn are observed in various terrestrial plants, but
100 the critical concentration for expressing toxicity varies depending on plant species and
101 genotype (Foy *et al.*, 1978; Horst *et al.*, 1999; Horiguchi, 1987; Millaleo *et al.*, 2010). For
102 example, *Zea mays* L. shows signs of Mn toxicity at an accumulation of 200 $\mu\text{g Mn g}^{-1}$ (dry
103 weight; D.W.) in the leaves; in contrast, *Lupinus albus* L. or woody Mn hyperaccumulator
104 species, such as *Gossia bidwillii*, can accumulate more than 10,000 $\mu\text{g Mn g}^{-1}$ (D.W.)
105 without Mn toxicity symptoms (Blamey *et al.*, 1986, 2015; Fernando *et al.*, 2006). These
106 differences in the critical concentration for Mn toxicity are derived from the different
107 capacities of Mn compartments within cells (Blamey *et al.*, 2015). Excessive Mn is
108 sequestered to vacuoles in a chelated form with organic acids (malate/citrate) to maintain
109 the Mn concentration within cells (Blamey *et al.*, 2015). However, when the vacuole capacity
110 for storing Mn reaches the upper limit, the Mn concentration increases in the symplastic and
111 apoplastic regions, where Mn toxicity occurs (Horiguchi, 1987; Führes *et al.*, 2009). In fact,
112 Mn-sensitive plants show a smaller vacuole capacity for retaining Mn than do Mn-tolerant

113 plants (Horst *et al.*, 1999; Blamey *et al.*, 2015). This interpretation has been validated by
114 loss-of-function and overexpression mutants of tonoplast-localised Mn transporter proteins
115 such as metal tolerant protein (MTP) 8 and calcium exchanger (CAX) 2, which show a
116 susceptible and tolerant phenotype to Mn toxicity, respectively (Hirschi *et al.*, 2000; Delhaize
117 *et al.*, 2003; Chen *et al.*, 2013; Eroglu *et al.*, 2016). In addition to these mechanisms,
118 Mn-tolerant plants, such as *Helianthus annuus* L., use trichomes as Mn storage tissue to
119 avoid an excessive increase in Mn concentration within cells (Blamey *et al.*, 1986, 2015).
120 However, this mechanism depends on the plant species and is not a general strategy to
121 prevent Mn toxicity (Blamey *et al.*, 2015).

122 Mn toxicity can be divided into two categories depending on the cell part of its
123 onset: apoplastic and symplastic (Fernando *et al.*, 2016). The primary symptoms of
124 apoplastic Mn toxicity in terrestrial plants are brown spots containing oxidised phenolics,
125 oxidised Mn [Mn^{3+} and Mn^{4+}], and callose in the leaves (Kenten and Mann, 1957; Horiguchi,
126 1987; Wissemeir and Horst, 1987, 1992; Horst *et al.*, 1999; Fecht-Christoffers *et al.*, 2003a,
127 b; Blamey *et al.*, 2015). Mn accumulation in the apoplast stimulates the expression of class
128 III peroxidases (PODs), which undertake both H_2O_2 production through NADH-peroxidase
129 activity and H_2O_2 consumption through guaiacol-peroxidase activity (Fecht-Christoffers *et al.*,
130 2003a, b; Führes *et al.*, 2009). Furthermore, the concentrations of phenolics, which suppress
131 the NADH-peroxidase activity, are lowered in the apoplast and, as a consequence, H_2O_2
132 production is accelerated in the apoplast (Fecht-Christoffers *et al.*, 2006; Führes *et al.*, 2009).
133 The H_2O_2 consumption reaction by the guaiacol activity of PODs also proceeds by utilising
134 phenolics in the apoplast; subsequently, the intermediate phenolic oxidation product,
135 phenoxyl radicals ($PhO^\bullet$), oxidises Mn^{2+} to Mn^{3+} (Takahama, 2004). Because $PhO^\bullet$ is
136 regenerated to phenolics after the reaction with Mn^{2+} , the continuous reactions of
137 H_2O_2 -production/consumption by the class III PODs accumulate the oxidised phenolics and
138 oxidised Mn in the apoplast (Kenten and Mann, 1957).

139 Although brownish spots are an indicator of Mn toxicity in terrestrial plants,
140 attenuation of plant growth by Mn toxicity is not always accompanied by the expression of

141 brown spots in leaves (Blamey *et al.*, 1986, 2015; Horiguchi, 1987; Wissemeier and Horst,
142 1992; González *et al.*, 1998; Chen *et al.*, 2013; Tsunemitsu *et al.*, 2018). Under excess Mn
143 accumulation in the symplast, leaves show lower photosynthetic activity and lower Chl
144 content (Nable *et al.*, 1988; Macfie and Taylor, 1992; González and Lynch, 1997; Kitao *et al.*,
145 1997; Fernando and Lynch, 2015). Houtz *et al.* (1988) demonstrated a decrease in
146 photosynthetic activity despite the absence of brown necrotic spots on leaves grown under
147 high Mn concentrations, implying that a decrease in photosynthetic activity occurred
148 independently of apoplastic Mn toxicity. These characteristics are termed symplastic Mn
149 toxicity (Fernando *et al.*, 2016). The decreases in Chl concentration and photosynthetic
150 activities have been hypothesised to be caused by photoinhibition by ROS; the decrease in
151 photosystem I (PSI) content or the disturbance of Chl synthesis due to the inhibition of Fe
152 absorption; Mn binding to Rubisco instead of Mg; or stomatal dysfunction (Clairmont *et al.*,
153 1986; Suresh *et al.*, 1987; Houtz *et al.*, 1988; McDaniel and Toman, 1994; Millaleo *et al.*,
154 2013). However, these hypotheses are often independently discussed at both *in vivo* and *in*
155 *vitro* levels; therefore, the mechanisms by which symplastic Mn toxicity suppresses
156 photosynthesis are less evident. Elucidating Mn toxicity including both its apoplastic and
157 symplastic mechanisms would contribute to establishing a strategy for protecting terrestrial
158 plants against Mn toxicity in agricultural land (Fernando and Lynch, 2015). Currently, the
159 strategy for preventing Mn toxicity depends on two mechanisms. One is limiting Mn
160 absorption and transport, and the other is Mn sequestration from the cytosol to the vacuole
161 (Li *et al.*, 2019). These strategies are important in preventing Mn toxicity. However,
162 identifying the expression mechanisms of symplastic Mn toxicity would be informative for
163 manipulating Mn toxicity in terrestrial plants without limiting the Mn transportation system.
164 This knowledge would be applicable to plant engineering processes such as Mn
165 phytoremediation (Lei *et al.*, 2007).

166 In this study, we investigated the mechanisms of symplastic Mn toxicity to suppress
167 photosynthetic activity in rice leaves. Rice shows high tolerance toward Mn toxicity, which
168 make it easy to investigate the effect of excess Mn concentrations without severe necrosis

169 and chlorosis (Fühns *et al.*, 2010). Additionally, complete genetic information is available for
 170 investigating gene expression. Previous studies have indicated that photosynthetic electron
 171 transport activities on the thylakoid membrane and Rubisco isolated from leaves grown
 172 under high Mn concentrations were robust, although CO₂ assimilation was substantially
 173 suppressed in leaves (Houtz *et al.*, 1988; Nable *et al.*, 1988; Chatterjee *et al.*, 1994). Based
 174 on these observations, we hypothesised that chloroplast proteins are not the primary targets
 175 of Mn toxicity, but factors that modulate CO₂ assimilation are targeted under Mn toxicity.
 176 Here, we discuss how photosynthetic activity is limited under Mn toxicity conditions from a
 177 wide range of physiological responses in plant cells.

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197 MATERIALS AND METHODS

198 Plant materials and plant growth conditions

199 *Oryza sativa* L. 'Nipponbare' wild-type (WT) plants were grown in hydroponic culture, as
200 reported in a previous study (Makino *et al.*, 1994). We employed two Mn concentration
201 conditions by changing the MnSO₄ application to 0.5 µM (control conditions) and 200 µM
202 (Mn-toxic conditions) (Tsunemitsu *et al.*, 2018). The pH of the hydroponic culture was
203 adjusted to 5.2 using HCl, and the solution was replenished twice a week. The chamber was
204 maintained at 60% relative humidity with a 14 h light (28 °C) and 10 h dark (25 °C)
205 photoperiod. The light intensity was 500–600 µmol photons m⁻² s⁻¹. All physiological,
206 structural, and genetic analyses were performed for fully and newly expanded leaves after
207 70 d of germination, and the analyses were completed before heading.

209 Leaf photosynthesis and respiration measurements in rice plants

210 The gas exchange analysis, Chl fluorescence, and oxidised reaction centre Chl in PSI
211 (P700⁺) were simultaneously measured using a combined system of Li-6400 (Li-COR Inc.,
212 Lincoln, USA), Mini-PAM, and PAM-101 equipped with a dual-wavelength emitter-detector
213 unit (ED-P700DW) (Heinz Walz GmbH, Effeltrich, Germany) with a cold halogen lamp.
214 Ambient air (40 Pa CO₂ and 21 kPa O₂) and pure CO₂ gas were mixed to maintain the CO₂
215 concentration during measurements. The gases were saturated with water vapour at 18.0 ±
216 0.1 °C, and the leaf temperature was maintained at 28 °C. The Chl fluorescence parameters
217 Y(II), Y(NPQ), and Y(NO) were calculated as described by Baker (2008), and a measuring
218 light (0.1 µmol photons m⁻² s⁻¹) and a saturated pulse (10,000 µmol photons m⁻² s⁻¹, 600
219 ms) were employed to calculate the photosynthetic parameters of PSII. The
220 oxidation-reduction state of P700 in PSI was determined according to the method described
221 by Klughammer and Schreiber (1994). The maximum oxidation level of P700 (Pm) was
222 obtained using a saturated pulse under far-red light illumination, and PSI photosynthetic
223 parameters [Y(I), Y(ND), and Y(NA)] were calculated using the oxidation-reduction state of
224 P700 as determined by a saturated pulse method.

The CO₂ emission rate was measured using a Li-6400 in the gaseous phase. The O₂ absorption rate was measured using an O₂-electrode (CB1D; Hansatech Instruments Ltd., King's Lynn, UK) in the aqueous phase at 25 °C (Hachiya *et al.*, 2010). For efficient absorption of the chemical reagent into leaves in the aqueous phase, the leaf surface was washed with 10% (v/v) dimethyl sulfoxide, and the leaves were finely cut up. The O₂ absorption rate was measured in the reaction buffer [50 mM *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid (HEPES)-KOH (pH 6.6), 10 mM 2-(*N*-morpholino)ethanesulfonic acid, 0.2 mM CaCl₂, 50 mM sucrose]. To evaluate the uncoupled respiration rate and alternative oxidase (AOX) and cytochrome *c* oxidase (COX) activities in the mitochondria, 10 µM carbonyl cyanide *m*-chlorophenyl hydrazine, 2 mM *n*-propyl gallate, and 2 mM KCN were added sequentially.

Quantification of leaf mineral elements and chlorophyll content

Leaf mineral elements were analysed according to Takagi *et al.* (2020). Briefly, the leaf blades were dried at 70 °C and ground using a homogeniser. To determine the mineral content, except for that of N, the dry matter was digested overnight in an acid mixture (HNO₃:H₂SO₄:HClO₄ = 5:1:2). Subsequently, the solution was heated at 150 °C for 30 min in a heat block. After cooling, the solutions were heated again at 200 °C for 1 h. After decomposition, the mineral contents were quantified by inductively coupled plasma optical emission spectroscopy (ICP-OES) (iCAP™ 7200; Thermo Fisher Scientific Inc., Waltham, USA). To measure the total N concentration in the leaves, dried leaves were decomposed using 60% (v/v) H₂SO₄ with 30% (v/v) H₂O₂. The N concentration was determined using Nessler's reagent in a decomposed solution after the addition of 10% (w/v) potassium sodium tartrate and 2.5 M NaOH, and the absorbance was measured at 420 nm (Takagi *et al.*, 2017). The leaf Chl content was quantified using fresh samples with *N,N*-dimethylformamide, as described previously (Porra *et al.*, 1989).

Carbohydrates quantification in leaves

253 Leaves were sampled at the end of the day (1 h before the start of the dark period) and at
254 the end of the night (1 h before the start of the light period); subsequently, leaves were
255 frozen using liquid nitrogen. Glucose, sucrose, and starch were extracted from leaves
256 according to Morita *et al.* (2015) and quantified using the Enzytech™ D-Glucose/Sucrose kit
257 (R-Biopharm AG, Darmstadt, Germany).

258

259 **Quantification of leaf amino acid contents**

260 The leaves were homogenised using 10 mM HCl with liquid nitrogen. After centrifugation
261 (15,000 × g, 4 °C, 5 min), the supernatant was applied to a centrifugation filter (Amicon Ultra
262 0.5 mL 3 K device; Merck KGaA, Darmstadt, Germany), and the fraction containing free
263 amino acids was obtained by centrifugation (15,000 × g, 4 °C, 10 min). The amino acid was
264 labelled using AccQ-Tag Ultra Derivatization Kit (Waters Co., Milford, USA) according to the
265 manufacture's instructions, and the free amino acids were separated using an HPLC system.
266 The HPLC system consisted of a 305 Piston Pump as the system controller and a 306 Pump
267 as a multi-pump application equipped with an 811D Dynamic Mixer and 805 Manometric
268 Module, using the Trilution LC software (Gilson, Villiers le Bel, France). The sample solution
269 was manually injected into the HPLC system. The derivative amino acids were separated on
270 a Sepax Bio-C18 (φ4.6 × 250 mm) (Sepax Technologies, Inc., Newark, USA) and eluted by
271 100% solvent A [5% (v/v) methanol in 20 mM CH₃COONa (pH 6.5)] for 4 min after injection
272 and by a linear gradient of 94% solvent A to 30% solvent B (100% acetonitrile) for another
273 65 min. The column was washed with 100 % solvent B for 20 min and equilibrated with
274 100% solvent A for 16 min. The flow rate was 0.6 mL min⁻¹, and the column temperature was
275 maintained at 25 °C by incubation in a hot pocket (Thermo Fisher Scientific Inc., Waltham,
276 USA). The peaks derived from derivatised amino acids were detected using UV/VIS-155
277 (Gilson, Villiers le Bel, France).

278

279 **TCA cycle enzyme activities**

280 The TCA cycle enzyme activities in the leaf blades were determined according to Noguchi

281 and Terashima (2006). Briefly, the leaves were homogenised with the extraction buffer [100
282 mM HEPES-KOH (pH 7.5), 10 mM KH₂PO₄, 0.5 mM EDTA-Na, 10 mM dithiothreitol, 0.05%
283 (v/v) TritonX-100, and 20% (v/v) glycerol] with polyvinylpyrrolidone. The reaction medium
284 was prepared for each enzyme according to the previous study, and the enzyme activity was
285 determined at 30 °C using UV-160A equipped with a temperature control system (Shimadzu,
286 Kyoto, Japan).

287

288 **Structural analysis of stomata complex and leaf cross-section**

289 For scanning electron microscopic analysis, the leaves were fixed with half-strength
290 Karnovsky's solution [50 mM phosphate buffer (pH 7.2), 2.5% (v/v) glutaraldehyde, 2% (v/v)
291 paraformaldehyde] with vacuum infiltration for 3 h. After fixation, the leaves were washed
292 with the phosphate buffer and dehydrated using a dilution series of ethanol, followed by
293 t-butanol. Leaves were dried using a t-butanol freeze drier (Vacuum Device Inc., Ibaraki,
294 Japan) and coated with Pt (JEC-300FC; JEOL Ltd., Tokyo, Japan). The stomatal complex
295 structure was monitored using a JSM-IT200 scanning electron microscope (JEOL Ltd.,
296 Tokyo, Japan). The size of the stomatal complex, comprising the paired guard cells, the
297 paired subsidiary cells, and the pore itself, and the stomatal density were analysed using
298 SEM Operation software (JEOL Ltd., Tokyo, Japan). Under each growth condition, the size
299 of the stomata complex and the stomatal density were analysed on an independent section
300 of the leaves as indicated in figure legends of six biologically independent plants. To analyse
301 the vertical and horizontal leaf sections, leaves were sectioned and fixed with
302 formalin-acetate-alcohol (FAA) [63% (v/v) ethanol, 5% (v/v) acetic acid, 5% (v/v) formalin]
303 with vacuum infiltration for 1 h, followed by incubation at 4 °C for 2 d. The leaves were
304 dehydrated using a dilution series of ethanol, followed by n-butanol. The dehydrated leaves
305 were embedded in paraffin and were sectioned at 10 µm thickness using an RX-860
306 microtome (Yamato Kohki Industrial Co., Ltd., Saitama. Japan). The FAA-fixed leaf sections
307 were stained with 0.05% (w/v) toluidine blue O. To investigate the intact leaf sections, leaves
308 were embedded in 50 mM phosphate buffer (pH 7.2) containing 5% (w/v) agar. The

309 agar-embedded leaves were sectioned at a thickness of 30 µm using DTL-1000 vibratome
310 (Dosaka EM Co., Ltd., Kyoto, Japan). Intact leaf sections and FAA-fixed leaf sections were
311 analysed using an optical microscope (BX53; Olympus Co., Japan).

312

313 **Indole acetic acid quantification**

314 The IAA content in leaves was quantified using a GC-MS system, according to Nghi *et al.*
315 (2021). Briefly, the leaves were homogenised in 70% (v/v) cold acetone. After centrifugation,
316 the supernatant was reduced to the aqueous phase by vacuum centrifugation equipped with
317 a cold trap. Subsequently, HCl (0.1 mM) was added to adjust the pH to 2.8, and diethyl ether
318 was added to dissolve IAA into the diethyl ether fraction. The diethyl ether fraction was dried
319 under vacuum and subsequently dissolved in *N,O*-bis(trimethylsilyl) tri-fluoroacetamide
320 (BSFTA) containing 1% trimethylchlorosilane. IAA was silylated at 70 °C for 1 h. The
321 silylated IAA was identified using a GC-MS-QP2010 SE (Shimadzu, Kyoto, Japan) with a
322 DB-WAX column. The conditions for GC-MS analysis were the same as those described
323 previously (Fujii *et al.*, 2010). The silylated IAA was identified by fragment ion 202 (*m/z*) and
324 molecular ion 319 (*m/z*) (Ljung *et al.*, 2005; Gutierrez *et al.*, 2009; Nghi *et al.*, 2021).

325

326 **Gene expression analysis**

327 To investigate gene expression in rice leaves, mRNA was isolated from the leaf blades
328 according to Suzuki *et al.* (2004). After mRNA isolation, cDNA was synthesised using
329 PrimeScript™ RT Reagent Kit (Takara Bio Inc., Shiga, Japan), and the mRNA content was
330 quantified by real-time PCR using the LightCycler® 96 System (Roche Diagnostics K.K.,
331 Tokyo, Japan) with a KAPA SYBR FAST One-Step qRT-PCR Kit (Nippon Genetics Co., Ltd,
332 Tokyo, Japan). The primer sequences used for real-time PCR analyses are listed in
333 Supplementary Table S1.

334

335 **Simulation of CO₂ fixation rate according to biochemical photosynthetic model**

336 Rubisco limited CO₂ fixation rate was calculated from the equation of von Caemmerer and

Table S1

337 Farquhar (1981):

$$338 A = EV_c(C_c - \Gamma^*) / [C_c + K_c(1 + O / K_o)] - R_d, (1)$$

339 Where E is the amount of Rubisco protein (assumed to be 5.3 $\mu\text{mol m}^{-2}$, from rice grown
340 with hydroponic solution same as in the present study described by Suganami et al. 2021),
341 V_c is the Rubisco activity of carboxylation, C_c is the chloroplastic CO_2 partial pressure, O is
342 the partial pressure of O_2 in the chloroplast (assumed to be the same as in the atmosphere,
343 21 kPa), K_c and K_o are the Michaelis-Menten constants for CO_2 and O_2 , and R_d is the day
344 respiration (assumed to be 1.0 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Γ^* is the CO_2 compensation point of
345 photosynthesis in the absence of R_d as defined as follows:

$$346 \Gamma^* = 0.5V_oK_o / V_cK_o, (2)$$

347 where V_o is the Rubisco activity of oxygenation. Mg^{2+} -binding Rubisco kinetics were taken
348 from Makino et al., (1988). Mn^{2+} -binding Rubisco kinetics were calculated by multiplying the
349 average ratio of $\text{Mn}^{2+}/\text{Mg}^{2+}$ -binding Rubisco kinetics and Mg^{2+} -binding Rubisco kinetics. The
350 average ratio of $\text{Mg}^{2+}/\text{Mn}^{2+}$ -binding Rubisco kinetics were calculated by the data from Bloom
351 and Kameritsch (2017). Rubisco kinetics were summarized in Supplementary Table S2.
352 Photorespiration rate were was calculated from the equation of von Caemmerer and
353 Farquhar (1981):

$$354 F = 0.5EV_oO / (O + K_o(1 + C_c / K_c), (3)$$

355

356 **Statistical analysis**

357 All measurement data are expressed as means $\pm$ standard deviation (SD), or box plots with
358 bars indicating the 1.5 $\times$ interquartile range (IQR) and with squares indicating the mean
359 value of at least three independent biological analyses. Significant differences in
360 physiological and biological parameters between the control- and Mn-toxic conditions were
361 detected by the non-parametric Kruskal-Wallis test. All statistical analyses were performed
362 using Origin Pro 2020 (LightStone Corp., Tokyo, Japan).

363

364

Table S2

365 RESULTS

366 Phenotypes of rice grown under Mn-toxic conditions

367 To study the effects of Mn toxicity, *Oryza sativa* L. 'Nipponbare' was grown under high Mn
368 concentrations (200 μ M; Mn-toxic conditions) in hydroponic culture for 70 d after
369 germination. Under Mn-toxic conditions, the leaves showed pale green and brown-coloured
370 sections, especially at the tips (Fig. 1A, B). These observations correspond with typical Mn
371 toxicity symptoms in rice leaves (Vlamis and Williams, 1964; Chen *et al.*, 2013). Compared
372 with the control conditions, Mn-toxic conditions decreased the total dry weight of rice,
373 especially the dry matter of the leaf sheath and root (Fig. 1C, D).

Fig. 1

374 To study the effects of high Mn application on leaf mineral composition, the leaf
375 mineral concentrations were quantified. The leaf Mn concentration was significantly
376 increased under Mn-toxic conditions (Fig. 2). This concentration was comparable to that of
377 WT rice, which showed growth inhibition when grown at a concentration of 1,000 μ M Mn for
378 3 weeks (Sasaki *et al.*, 2011). The K, Ca, and Fe concentrations were similar under the
379 control and Mn-toxic conditions (Fig. 2). In contrast, the Mg, Zn, and Cu concentrations
380 increased under Mn-toxic conditions (Fig. 2). These results demonstrated decreased growth
381 and the Mn toxicity symptoms accompanied by Mn accumulation in leaves grown under
382 Mn-toxic conditions.

Fig. 2

384 Mn-toxic conditions suppressed CO₂ assimilation by limiting stomatal conductance 385 in leaves

386 Next, the steady-state photosynthetic activities were measured under ambient air conditions
387 (40 Pa CO₂ and 21 kPa O₂). The CO₂ assimilation rate was significantly decreased under
388 the high light irradiance by the Mn-toxic treatments (Fig. 3A). The Mn-toxic treatments
389 showed lower stomatal conductance (g_s) than did the control plants, and the change in g_s
390 with increasing light intensity was also suppressed (Fig. 3B). These stomatal responses to
391 the increase in Mn concentration were consistent with previous studies (Suresh *et al.*, 1987;
392 Nable *et al.*, 1988; González and Lynch, 1997; Lidon *et al.*, 2004; Santos *et al.*, 2017). The

Fig. 3

393 internal CO₂ concentration in leaves (C_i) was lower under the Mn-toxic conditions than
394 under control conditions (Fig. 3C). These results indicated that, under Mn-toxic conditions,
395 the stomata limit CO₂ diffusion into leaves and CO₂ assimilation is suppressed, particularly
396 under high light conditions.

397 In addition to the CO₂ assimilation rate, the photosynthetic electron transport
398 activities in both PSII and PSI were analysed by chlorophyll fluorescence and
399 P700⁺-dependent absorbance changes in the leaves. The quantum yield of PSII [Y(II)] was
400 lower under Mn-toxic conditions than under control conditions at the high light irradiance
401 (Fig. 3D). This result indicated that the electron transport activity in PSII was suppressed at
402 the high light irradiance, but compared with the CO₂ assimilation rate, the change in Y(II)
403 was marginal. The quantum yield of non-photochemical quenching [Y(NPQ)] increased
404 similarly with the increase in light intensity under both control and Mn-toxic conditions (Fig.
405 3E). The quantum yield of non-radiative energy loss [Y(NO)] was different between the
406 control and Mn-toxic conditions under low light irradiance; however, this difference was
407 masked under high light irradiance (Fig. 3F). These results showed that the photoprotective
408 mechanisms in PSII are robust, but the redox state in PSII can be perturbed under Mn
409 toxicity. The quantum yield of PSI [Y(I)] showed similar kinetics to that of Y(II), and no
410 significant differences were observed between the control and Mn toxic condition (Fig. 3G).
411 The quantum yield of non-photochemical quenching at the donor side of PSI [Y(ND)] was
412 higher under Mn-toxic conditions than under the control conditions at high light irradiance
413 (Fig. 3H). In contrast, the quantum yield of non-photochemical quenching at the acceptor
414 side of PSI [Y(NA)] was lower under Mn-toxic conditions than under the control conditions at
415 high light irradiance (Fig. 3I). These results indicated that the whole-chain photosynthetic
416 electron transport rate was less affected under Mn-toxic conditions than under the control
417 conditions; however, the photosynthetic electron transport reaction was limited to the donor
418 side of PSI, and PSI was more oxidised under Mn-toxic conditions than under the control
419 conditions.

420 The maximum quantum yield of PSII (F_v/F_m) was significantly, but marginally

421 decreased under Mn-toxic conditions (Fig. 2J). The total Chl content in leaves was also
422 decreased under Mn-toxic conditions (Fig. 2K). These results indicated that Mn toxicity
423 stimulates PSII photoinhibition. In contrast, the total N concentration was not significantly
424 different between the control and Mn-toxic conditions (Fig. 2L). Based on the linear function
425 between the N and Rubisco concentrations in the leaves (Nakano *et al.*, 1997), this result
426 indicated that the quantity of Rubisco was less affected by Mn toxicity.

427

428 **Carbohydrate metabolism in leaves grown under Mn-toxic conditions**

429 To examine whether the suppression of photosynthesis by excessive Mn accumulation
430 affects carbon acquisition during growth, the major carbohydrate concentrations were
431 quantified in the leaves. Leaves were sampled at both the end of the day and the end of the
432 night to visualise the CO₂ assimilation activity during the day (Takagi *et al.*, 2016a). At the
433 end of the day, Mn-toxicity treatments showed lower sucrose concentrations in the leaves
434 than did the control plants (Fig. 4). In contrast, at the end of the night, the sucrose
435 concentration was similar under both control and Mn-toxic conditions (Fig. 4). The glucose
436 and starch concentrations were lower than the sucrose concentration under both control and
437 Mn-toxic conditions, and significant differences were not detected between the growth
438 conditions at the end of the day and the end of the night (Fig. 4). The sum of the sucrose,
439 glucose, and starch concentrations was lower in leaves grown under Mn-toxic conditions at
440 the end of the day, but the sum of these carbohydrate concentrations was similar between
441 the growth conditions at the end of the night (Fig. 4). These results indicated that carbon
442 acquisition is suppressed, corresponding to lower CO₂ assimilation activities, in leaves
443 grown under Mn-toxic conditions.

444

445 **Effects of Mn toxicity on mitochondrial respiration in the leaves**

446 The carbohydrates fixed during the day are metabolised at night and consumed at a
447 constant rate from the beginning of the night to the dawn (Moraes *et al.*, 2019). Therefore,
448 the difference in carbohydrate concentrations between the end of the day and end of the

Fig. 4

449 night indicates the carbon catabolic activity during the night (Takagi *et al.*, 2016a). To
 450 confirm whether carbon catabolism is suppressed under Mn-toxic conditions (Fig. 4), leaf
 451 respiration was examined under control and Mn-toxic conditions. The respiratory CO₂
 452 emission rate was decreased under Mn-toxic conditions (Fig. 5A). To examine the effect of
 453 the change in respiration activities on carbon catabolism, the leaf amino acid content was
 454 quantified during the night (Sulpice *et al.*, 2014). Subsequently, the Asp, Glu, and Gly
 455 contents were significantly decreased, and the Ser content was increased under Mn-toxic
 456 conditions (Fig. 5B). These results indicated that carbon catabolism is affected by Mn-toxic
 457 conditions during the night.

Fig. 5

458 Next, we investigated the effects of Mn toxicity on the mitochondrial enzymes
 459 involved in respiration. First, the respiratory electron transport activities were measured in
 460 the presence of sucrose and mitochondrial electron transport inhibitors *in vivo* using an
 461 aqueous phase O₂-electrode (Hachiya *et al.*, 2010). In the absence of inhibitors, the O₂
 462 consumption rate did not differ between the control and Mn-toxic conditions (Fig. 5C). CCCP
 463 dissipated mitochondrial membrane potential ($\Delta\psi_m$) and similarly stimulated O₂
 464 consumption rate in both leaves grown under the control and Mn-toxic conditions (Fig. 5C).
 465 The COX and AOX activities in the presence of CCCP and sucrose were determined by
 466 adding n-propyl gallate and KCN. Subsequently, the control and Mn-toxic conditions showed
 467 similar O₂ consumption rates depending on the COX and AOX activities (Fig. 5C). The effect
 468 of Mn toxicity on mitochondrial respiratory electron transport activity was reproduced when
 469 these activities were determined on a leaf fresh-weight basis (Supplementary Fig. S1).
 470 These results indicated that under sucrose feeding, the mitochondrial respiratory electron
 471 transport activities were similar between the control and Mn-toxic conditions.

Fig. S1

472 To understand the effects of Mn toxicity on the enzyme activities of the TCA-cycle,
 473 the six types of TCA cycle enzyme activities were determined on both leaf area and
 474 fresh-weight basis. NADP-isocitrate dehydrogenase, NAD-isocitrate dehydrogenase,
 475 NAD-malate dehydrogenase, and aconitase showed similar activities under the control and
 476 Mn-toxic conditions (Fig. 5D-G, I, Supplementary Fig. S2). The NAD-malic enzyme activity

Fig. S2

477 decreased under Mn-toxic conditions (Fig. 5H, Supplementary Fig. S2), whereas the
478 fumarase activity increased (Fig. 5I, Supplementary Fig. S2). These results indicated that
479 Mn toxicity did not completely suppress the enzyme activities of the TCA cycle, but
480 specifically stimulated fumarase and suppressed NAD-malic enzyme activities.

481

482 **Difference in stomatal development in the leaves between the control and Mn-toxic** 483 **conditions**

484 To examine whether the decrease in g_s under Mn-toxic conditions was related to the change
485 in stomatal structure and development, the stomatal complex on the adaxial side of a leaf
486 was monitored. The stomatal complex, consisting of guard and subsidiary cells, was located
487 linearly to the vein under the control conditions (Fig. 6A). However, under Mn-toxic
488 conditions, the stomatal complex showed a more scattered distribution (Fig. 6B). The
489 stomatal density increased under Mn-toxic conditions (Fig. 6C). In contrast, the size of the
490 stomatal complex evaluated by the maximum major and minor axis was smaller under
491 Mn-toxic conditions than under control conditions (Fig. 6A, B, D, E). The changes in
492 stomatal size were consistent with a previous study evaluating rice stomata structure under
493 excess Mn application (Lidon, 2002). These results indicated that Mn-toxic conditions
494 change stomatal development in the leaves.

Fig. 6

495

496 **Anatomical changes within a leaf blade grown under Mn-toxic conditions**

497 Following the change in stomatal development on the leaf epidermis, the leaf anatomy was
498 also examined. Figure 7A shows the horizontal and vertical cross-sections of the leaves
499 against the vein. Apparent internal air spaces were observed within leaves grown under
500 control conditions. In contrast, cells were highly condensed in leaves grown under Mn-toxic
501 conditions, and the internal air space was hardly observed (Fig. 7A). These results showed
502 that in addition to stomatal complex, leaf anatomical development was also altered under
503 Mn-toxic conditions.

Fig. 7

504 The vertical cross-section revealed that the bundle-sheath cells were brownish

under Mn-toxic conditions (Fig. 7A). To investigate whether the brown spots were artifacts caused by the chemical fixing treatment, intact leaf sections were also studied. Subsequently, we found that the brownish spots natively existed in the bundle-sheath cells, both small and large bundles grown under Mn-toxic conditions (Fig. 7B, C). These results indicated that under Mn-toxic conditions, rice leaves caused apoplastic Mn toxicity-like symptoms in addition to symplastic Mn toxicity, and the symptoms are more evident in the bundle-sheath cells in rice leaves.

512

513 **Auxin content in the leaves and mRNA expression of auxin-responsive genes**

Our findings demonstrated that excess Mn accumulation affects the developmental process of leaves. IAA is an important hormone that determines cell fate and differentiation in plants (Perrot-Rechenmann, 2010). Morgan et al. (1966) reported that Mn toxicity stimulates IAA oxidation activity in cotton leaves, indicating that the auxin concentration could be lowered by accelerated auxin degradation in leaves under excess Mn conditions. To examine this possibility, the IAA concentration in leaves was quantified by GC-MS. Figure 8A shows the total ion chromatograms (TIC) with fragment ion chromatograms of the 202 (*m/z*) and 319 (*m/z*) of the IAA standard and leaf extract from the control and Mn-toxic conditions (Fig 8A) (see Materials and Methods). Subsequently, this analysis revealed that the IAA concentration was significantly decreased by 76% in the leaves under Mn-toxic conditions (Fig. 8B).

Fig. 8

To confirm that the decrease in IAA concentration affects physiological reactions within the leaves, the IAA-responsive gene expression was investigated. *OsARF1*, *OsARF15*, *OsARF16*, *OsAUX/IAA1*, and *OsAUX/IAA24* showed increases in their expression level with an increase in IAA concentration in the leaves (Waller et al., 2002; Jain et al., 2006; Wang et al., 2007). Among these genes, the expression levels of *OsARF1*, *OsARF16*, and *OsAUX/IAA1* were significantly decreased under Mn-toxic conditions (Fig. 8C). *OsARF15* showed similar trends to other *ARF* genes, but the difference was not significant between the growth conditions. In contrast, *OsAUX/IAA24*, and *OsACT1-1*

533 showed comparable expression levels between the control and Mn-toxic conditions (Fig.
534 8C). From these results, the auxin-responsive gene expression indicated a lower IAA
535 concentration in leaves under Mn-toxic conditions in concert with the results of GC-MS
536 although all genes were not responded to equally.

537 To regulate auxin concentration in plant cells, DIOXYGENASE FOR AUXIN
538 OXIDATION (DAO) undertakes IAA catabolism to maintain IAA homeostasis by oxidising
539 IAA to an inactive form, oxindole-3-acetic acid, in plant cells (Zhao *et al.*, 2013). Because
540 Mn-toxicity stimulates oxidative IAA degradation *in vitro* (Morgan *et al.*, 1966), we
541 hypothesised that *OsDAO* is upregulated following an increase in Mn concentration;
542 however, the transcriptional activation of *OsDAO* was suppressed under Mn-toxic conditions
543 (Fig. 8). The expression of *OsDAO* is activated under high IAA concentrations to maintain
544 IAA concentrations in plant tissues (Zhao *et al.*, 2013). Based on this homeostatic response
545 of *OsDAO*, the decrease in *OsDAO* expression under Mn-toxic conditions would be a
546 consequence of the lower IAA concentration in leaves to avoid further inactivation of IAA.

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561 DISCUSSION

562 In acidic or waterlogged agricultural fields, Mn is easily released from the soil and causes
563 Mn toxicity in crop plants (Fernando *et al.*, 2016). In addition to the current agricultural
564 situations, the effects of climate change, such as the rises in the atmospheric temperature
565 and the frequency of flooding or acidic rainfall, can further accelerate Mn dissolution in soils
566 (Fernando and Lynch, 2015). Therefore, the mechanisms of Mn toxicity should be
567 elucidated to manipulate the future threat of increasing Mn availability on agricultural lands.
568 To address the mechanisms of symplastic Mn toxicity, we investigated the physiological
569 effects of high Mn application in rice. In this study, we propose that the disturbance of IAA
570 homeostasis is one of the critical causes of symplastic Mn-toxicity in rice leaves.

571

572 Mn toxicity disturbs IAA homeostasis and attenuates auxin signalling in the leaves

573 Our results showed that the IAA concentration was decreased in the leaves under Mn-toxic
574 conditions (Fig. 8). A previous study demonstrated that IAA oxidation activity increased in
575 leaf extracts grown under high Mn concentration conditions (Morgan *et al.*, 1966). Our
576 observations suggest that the increase in IAA oxidation activity functions *in vivo*. In addition
577 to the decrease in *ARF* and *AUX/IAA* gene expression levels in rice, the microRNA
578 expression profiles revealed that miRNA160 which targets *ARF* genes was downregulated,
579 and miRNA164 which targets the auxin-signal related F-box protein (TRANSPORT
580 INHIBITOR RESPONSE 1, TIR1) was upregulated in the leaves of common beans under
581 Mn toxicity (Valdés-López *et al.*, 2010). From these observations, we suggest that Mn
582 toxicity affects the auxin signalling cascade at the transcriptional level by decreasing the IAA
583 concentration in the leaves.

584 We suggest that the decrease in IAA concentration, together with the change in
585 auxin signalling activities, causes a change in leaf anatomy and stomatal
586 patterning/development under Mn-toxic conditions. When IAA deficiency is caused by the
587 overexpression of GRETCHEN HAGEN3 which conjugates IAA to amino acids or the anion
588 peroxidase which oxidatively inactivates IAA *in planta*, the leaf cell size becomes smaller

589 and the leaf anatomy shows more condensed cell structures (Lagrimini *et al.*, 1997*a, b*; Du *et*
590 *al.*, 2012). Alternatively, the transgenic plants that inhibit auxin signalling or auxin polar
591 transport activities represent the IAA-deficient phenotype and show the abnormal leaf
592 anatomical development (Pérez-Pérez *et al.*, 2010; Guo *et al.*, 2013; Qi *et al.*, 2014;
593 Muñoz-Nores *et al.*, 2017). The aforementioned relationships among the IAA-deficient
594 phenotypes and the change in leaf structure caused by attenuating auxin signal transduction
595 are similar to those among the leaf phenotypes under Mn-toxic conditions (Figs. 3B, 6).
596 Moreover, stomatal development is regulated by auxin signalling (Balcerowicz and Hoecker,
597 2014). Exogenous IAA application decreased stomatal density in a concentration-depending
598 manner; whereas the attenuation of ARF-dependent auxin signal transduction increased it
599 (Le *et al.*, 2014; Zhang *et al.*, 2014). Based on these results, a decrease in IAA
600 concentrations could affect the change in leaf development and stomatal
601 patterning/development.

602 Generally, the terrestrial plants with smaller stomata and higher stomata density
603 show higher transpiration ability than those with larger stomata and lower density (Franks
604 and Beerling, 2009). This relationship does not apply to g_s and stomatal patterning (Figs. 3B,
605 6C); therefore, additional factors to suppress stomatal opening should exist under Mn
606 toxicity. IAA stimulates stomatal opening by activating plasma membrane H^+ -ATPase (Jezek
607 and Blatt, 2017), and the exogenous application of IAA stimulates stomatal opening in the
608 leaves (Klein *et al.*, 2003). Therefore, the decrease in g_s could be caused by reducing the
609 IAA concentration in the leaves. However, the regulation of stomatal function is quite
610 complex, and the ROS and organic acid composition are also determinants of stomatal
611 movement (Jezek and Blatt, 2017). Considering the stimulation of oxidative stress under Mn
612 toxicity (see the following discussion), the decrease in IAA would not be the only cause of
613 stomatal dysfunction. Interestingly, fumarase activity increased under Mn toxicity (Fig. 5I).
614 Because the increase in malate/fumarate ratio is important for stomatal opening (Araújo *et*
615 *al.*, 2011), the increased fumarase activity might be a counteraction toward stomatal
616 dysfunction under Mn toxicity.

617 Currently, we cannot determine how the IAA concentration is lowered under Mn
618 toxicity at the molecular level. Further research is required on a molecular genetic basis to
619 elucidate the key factors that cause IAA deficiency. We now consider the possibility that IAA
620 oxidation can be undertaken by unknown proteins such as class III PODs as reported
621 previously (Ray, 1958; Lagrimini *et al.*, 1997a, b; Tognetti *et al.*, 2012). Because IAA is
622 transported through the apoplast, the increase in POD activity in the apoplast might
623 influence IAA catabolism under Mn toxicity (Robert and Friml, 2009).

624

Suppression of photosynthesis is caused by stomatal dysfunction in rice

626 We suggest that the simultaneous prolonged limitation of the CO₂ assimilation reaction and
627 stimulation of photorespiration caused oxidative stress by ROS under Mn toxicity. Under Mn
628 toxicity, ROS-scavenging enzyme activities are upregulated in various terrestrial plants
629 (González *et al.*, 1988; Lei *et al.*, 2007; Santos *et al.*, 2017). This response indicates that
630 oxidative stress is stimulated by ROS in cells under Mn toxicity. Furthermore, Houtz *et al.*
631 (1988) showed an increase in the RuBP concentration in leaves under Mn toxicity. This
632 result indicated that the turnover of photosynthates is limited by Rubisco activity (Suzuki *et al.*
633 *et al.*, 2012). Here, *g_s* was lowered and the CO₂ assimilation rate was limited under Mn-toxic
634 conditions (Fig. 2B). In contrast to the CO₂ assimilation rate, which showed a 39% decrease
635 at the highest light irradiance, Y(II) only decreased by 9% at the same light intensity (Fig. 3).
636 This difference would have been caused by the difference in the electron distribution
637 between carboxylation and oxygenase reaction by Rubisco (Takagi *et al.*, 2016b; Wada *et al.*
638 *et al.*, 2019). Even when the CO₂ availability within chloroplasts is limited by stomatal closure,
639 O₂ can diffuse within the leaves; therefore, Rubisco drives photorespiration (Zivcak *et al.*,
640 2013). Under such conditions, and the *proton motive force* is built up to stimulate luminal
641 acidification of the thylakoid membranes owing to the lower ATP consumption rate in the
642 photorespiration reaction compared with that in the carboxylation reactions in the
643 Calvin-Benson cycle (Takagi *et al.*, 2016b, 2017). Luminal acidification is represented by an
644 increase in Y(ND) (Fig. 3), which is increased by limiting the electron flow from

645 plastoquinone to cytochrome *b₆f* depending on the luminal acidification (Kramer *et al.*, 1999),
 646 under Mn-toxic conditions. These results indicated that Mn toxicity stimulates
 647 photorespiration via stomatal closure. The increase in photorespiration activity can also be
 648 supported by the change in the Gly/Ser ratio under Mn-toxic conditions (Fig. 5B). Wada *et al.*
 649 (2019) reported that the limitation of the CO₂ assimilation rate by drought stress with a
 650 decrease in *g_s* stimulates PSI photoinhibition rather than PSII photoinhibition by ROS. A
 651 similar situation would occur under Mn toxicity, which is why the decrease in PSII activity is
 652 modest, although PSI inhibition is accentuated under Mn-toxic conditions (Kitao *et al.*, 1997;
 653 Lidon *et al.*, 2004; Millaleo *et al.*, 2013). Previous studies have shown that Mn toxicity
 654 targets PSI by attenuating Fe absorption (Andressen *et al.*, 2018). However, the
 655 concentration was maintained within a sufficient range for Fe nutrition in rice [from 70 to 300
 656 µg g⁻¹ (D.W.)] (Fageria and Stone, 2008). Furthermore, a decrease in the PSI content
 657 caused an increase in Y(NA), instead of Y(ND) during steady-state photosynthesis (Brestic
 658 *et al.*, 2015). Hence, we suggest that the decrease in PSI content due to Mn toxicity would
 659 be caused not only by the Fe status but also by PSI photoinhibition, as a consequence of
 660 limited CO₂ assimilation. Other nutrients such as Ca, Mg and Zn were also reported to be
 661 deficient under Mn toxicity (Broadley *et al.*, 2012). We did not observe a decrease in these
 662 minerals in the leaves, but rather increased Mg, Cu, and Zn concentrations under Mn
 663 toxicity (Fig. 2). Because Cu/Zn-type SOD activities are upregulated under Mn toxicity
 664 (González *et al.*, 1988; Lei *et al.*, 2007; Santos *et al.*, 2017) and Mg supply rescues Mn
 665 toxicity symptoms (Broadley *et al.*, 2012), the requirement of these minerals might be
 666 upregulated.

667 Our results suggest that chloroplasts have homeostatic systems to evade
 668 excessive Mn accumulation. *In vitro* studies have shown that excess free Mn stimulates
 669 ROS production by PSII and causes severe oxidative damage to thylakoid lipids and PSII
 670 (Panda *et al.*, 1987). Although the Mn concentration was high (Fig. 2), clear evidence of
 671 severe damage to PSII was not detected here (Fig. 3J). Furthermore, Mn is to bind Rubisco,
 672 and an *in vitro* study indicated that the substitution of Mg²⁺ with Mn²⁺ decreases the

specificity of Rubisco for CO₂ (Jordan and Ogren, 1983; Bloom and Kameritsch, 2017). Previous studies have suggested that Mn binding to Rubisco causes a decrease in photosynthesis under Mn toxicity (Houtz *et al.*, 1988; Kitao *et al.*, 1997). However, the contribution of Mn-binding Rubisco to decrease CO₂ assimilation reactions remains unknown under Mn-toxic conditions *in vivo* (Houtz *et al.*, 1988; Chatterjee *et al.*, 1994). To address this question, we simulated the change in CO₂ fixation rate between Mg²⁺- and Mn²⁺-binding Rubisco using photosynthetic biochemical model (von Caemmerer and Farquhar, 1981). Calculated CO₂ assimilation rate using Mn²⁺-binding Rubisco kinetics was negative value below C_c = 50 Pa and greatly decreased compared to calculated CO₂ assimilation rate using Mg²⁺-binding Rubisco kinetics (Supplementary Fig. S3). However, such a drastic decrease in CO₂ assimilation rate was not observed (Fig. 3). Although Mn²⁺-binding Rubisco showed a higher photorespiration rate than Mg²⁺-binding Rubisco, this difference cannot compensate the difference in CO₂ assimilation rate. Moreover, investigation of the relationship between *g_s* and the CO₂ assimilation rate did not show a decrease in the CO₂ assimilation rate independent of the change in *g_s* (Supplementary Fig. S4). These considerations reconciled the cause of the decrease in CO₂ assimilation as decrease in *g_s*, and Rubisco is protected from excess Mn in chloroplasts. To protect chloroplasts from excess free Mn accumulation, the homeostatic function of Mn²⁺ in the chloroplasts should function. At this point, two mechanisms can be considered. The first mechanism is binding Mn to the thylakoid membranes. Although this modulates membrane structure of the thylakoid membranes, no inhibitory effect on photosynthetic activity was observed (Lidon and Teixeira, 2000a, b). The binding of Mn to the thylakoid membrane could be a storage system for Mn homeostasis. The second mechanism is the sequestration or limiting Mn transport within chloroplasts. Indeed, Mn distributed to the chloroplasts was decreased under Mn-toxic conditions in rice (Führs *et al.*, 2010). These homeostatic systems would prevent the deleterious effects of Mn²⁺ within the chloroplasts.

Fig. S4

Carbon catabolism due to reduced carbohydrates suppresses growth

701 A decrease in respiration rate under Mn toxicity has been reported in wheat and cotton
 702 plants (Sirkar and Amin, 1974; Macfie and Taylor, 1992). We also observed a decrease in
 703 leaf respiration, but not in sucrose feeding (Fig. 5). Moreover, no decreases in mitochondrial
 704 enzyme activities, including TCA cycle and respiratory electron transport chain enzymes,
 705 were observed, except for the NAD-malic enzyme (Fig. 5). The physiological importance of
 706 the NAD-malic enzyme is highly limited in C3 plants, and a pronounced phenotype has not
 707 been observed in NAD-malic enzyme mutants in *Arabidopsis* (Tronconi *et al.*, 2008). This
 708 could be because of the lower contribution to the supply of pyruvate into the TCA cycle by
 709 NAD-malic enzyme activity (Williams *et al.*, 2008). These observations suggested that the
 710 significant decrease in NAD-malic enzyme activity could be independent of the growth
 711 defect and decreased respiration rate under Mn-toxic conditions (Figs. 1, 5). However, the
 712 change in amino acid composition could be influenced by a decrease in the NAD-malic
 713 enzyme (Tronconi *et al.*, 2008). From these observations, the decreased respiration rate was
 714 caused by a decrease in carbohydrate concentrations due to the suppression of
 715 photosynthesis (Sulpice *et al.*, 2014). Carbohydrate catabolism during the night is a
 716 determinant of plant growth (Takagi *et al.*, 2016a). We suggest that a decrease in the
 717 capacity of carbon catabolism causes growth defects in rice under Mn toxicity. Interestingly,
 718 Hibberd and Quick (2002) reported that similar to C4 plants, the NAD-malic enzyme activity
 719 is high in the bundle-sheath cells in C3 plants. Here, we found that the bundle-sheath cells
 720 turned brown under Mn-toxic conditions (Fig. 7B, C). Based on a previous study on
 721 apoplastic Mn toxicity, brown spots contained oxidised phenol, oxidised Mn, and callose.
 722 Fernando *et al.* (2016) showed that wheat leaves concentrate Mn in the bundle-sheath cell.
 723 However, this specific localisation was not observed in soybean and canola, suggesting that
 724 the bundle-sheath cell could function as storage organs for attenuating Mn toxicity in
 725 monocots. The Mn accumulation in the bundle sheath cell could cause the suppression of
 726 the NAD-malic enzyme activity in leaves. These results provide insight for elucidating the
 727 function of bundle-sheath cells in C3 monocots.

728

729 **CONCLUDING REMARKS**

730 We proposed that the disturbance of IAA homeostasis is an initial cause of the inhibition of
 731 CO₂ assimilation and photoinhibition caused chlorosis in the leaves (Fig. 9). As a short-term
 732 effect, the decrease in IAA suppresses stomatal opening. Subsequently, the decrease in IAA
 733 concentration modifies leaf anatomy and stomatal development in newly emerged leaves as
 734 a long-term effect. Because leaf anatomy determines the CO₂ pathway for efficient CO₂
 735 absorption by the chloroplasts, the lower internal air space could further lower the CO₂
 736 fixation efficiency under Mn toxicity (Earles *et al.*, 2018). The decreased CO₂ assimilation
 737 rate and increased photorespiration rate decelerate carbon acquisition for catabolism; thus,
 738 vegetative growth is suppressed. To our knowledge, there are limited studies focusing on
 739 the relationship between auxins and Mn toxicity (Morgan *et al.*, 1966). Tsunemitsu *et al.*
 740 (2018) reported that the *osmtp11* mutant decreased grain fertility and yield in rice. *OsMTP11*
 741 undertakes Mn sequestration to the endoplasmic reticulum and is highly expressed in
 742 inflorescences, including the anthers, pistil, lemmas, paleas, and ovaries (Tsunemitsu *et al.*,
 743 2018). Together with our conclusion, Mn toxicity may also affect grain fertilisation by
 744 disturbing IAA homeostasis. Not only the photosynthetic measurements but also the
 745 developmental analyses from leaf emergence to the reproductive phase would provide more
 746 insights into the mechanisms of the symplastic Mn-toxicity by focusing on IAA.

Fig. 9

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758 **SUPPLEMENTARY DATA**

759 **Figure S1;** The sucrose-feeding respiration activities and mitochondrial respiratory electron
760 transport activities depending on ATP synthase (uncoupled), COX, and AOX based on leaf
761 fresh weight

762

763 **Figure S2;** The enzyme activities involved in the TCA cycle calculated on the fresh weight
764 basis.

765

766 **Figure S3;** The simulated CO₂ assimilation in leaves containing Mg-binding Rubisco and
767 Mn-binding Rubisco.

768

769 **Figure S4;** The relationship between g_s and CO₂ fixation rate under the control and the
770 Mn-toxic conditions.

771

772 **Table S1;** The primer list for the gene expression analysis in rice leaf blade

773

774 **Table S2;** Mg²⁺-binding Rubisco and Mn²⁺-binding Rubisco kinetics.

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792

793 **Conflicts of interest:**

794 The authors declare no conflicts of interest.

795

796 **Author contributions:**

797 Conceptualisation: DT; investigation: DT, KI, MS, TU, TF, YT, MK, and KO; original draft: DT;
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799 and AM.

800

801 **Data availability statements**

802 All data supporting the findings of this study are available within the article and its
803 supplementary figures and tables published online.

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1205 **FIGURE LEGENDS**

1206 **Figure 1**

1207 Phenotypes of rice plants grown under control and Mn-toxic conditions. The pictures of fully
1208 expanded leaf blades grown under control and Mn-toxic conditions are shown in (A), and (B)
1209 shows an enlarged picture of these. The white bars indicate lengths of 1 cm (A) and 1 mm
1210 (B), respectively. (C) The dried weights of the leaf blade, leaf sheath, and root, respectively.
1211 (D) The sum of the dried weights shown in (C). Data are shown as box plots ($n = 9$), black
1212 squares indicate the mean value, and bars indicate the range of the maximum or minimum
1213 data within a $1.5 \times$ interquartile range (IQR). Green boxes indicate the results of the control
1214 conditions, and red boxes indicate those of the Mn-toxic conditions. Asterisks show
1215 significant differences between the control and Mn-toxic conditions (*: $p < 0.05$,
1216 Kruskal–Wallis test).

1218 **Figure 2**

1219 Mineral concentration in the rice leaf blade. Data are shown as box plots (control conditions
1220 $n = 8$, Mn-toxic conditions $n = 7$), black squares indicate the mean value, and bars indicate
1221 the range of the maximum or minimum data within a $1.5 \times$ interquartile range (IQR). Green
1222 boxes indicate the results of the control conditions, and red boxes indicate those of the
1223 Mn-toxic conditions. Asterisks show significant differences between the control and Mn-toxic
1224 conditions (*: $p < 0.05$, **: $p < 0.01$, Kruskal–Wallis test).

1226 **Figure 3**

1227 Photosynthetic activities in leaves grown under control and the Mn-toxic conditions. The
1228 CO_2 assimilation rate (A), stomatal conductance (g_s) (B), and intercellular CO_2 concentration
1229 (C) are shown. For the PSII photosynthetic parameters, the quantum yields of PSII [Y(II)] (D),
1230 non-photochemical quenching [Y(NPQ)] (E), and non-radiative decay [Y(NO)] (F) are shown.
1231 The maximum quantum yield of PSII (F_v/F_m) is shown (J). For PSI photosynthetic
1232 parameters, the quantum yields of PSI [Y(I)] (G), non-photochemical quenching at the donor

side [Y(ND)] (H), and non-photochemical quenching at the acceptor side [Y(NA)] (I) are shown. (K) and (L) show the total Chl and N concentrations in the leaves, respectively. Data of photosynthetic parameters are shown as means with standard deviation (SD) (n = 7). The results for Fv/Fm (n = 7) and total Chl (n = 7) and N (n = 9) concentrations are shown as box plots. Black squares indicate the mean value, and bars indicate the range of the maximum or minimum data within a 1.5 × interquartile range (IQR). Asterisks show significant differences between the control and Mn-toxic conditions (*: $p < 0.05$, **: $p < 0.01$, Kruskal–Wallis test).

1241

1242 **Figure 4**

Carbohydrate content in leaf blades at the end of the day and end of the night. Data are shown as means with standard deviation (SD) (n = 3–4), and squares and diamonds show the distribution of the raw data. Asterisks show significant differences between the control and Mn-toxic conditions (*: $p < 0.05$, Kruskal–Wallis test).

1247

1248 **Figure 5**

Mitochondrial respiration activities and amino acid contents in leaf blades grown under control and Mn-toxic conditions. (A) Leaf respiration rate (n = 12–13) in the dark and (B) sucrose feeding respiration activities and mitochondrial respiratory electron transport activities depending on ATP synthase (uncoupled), COX, and AOX (n = 7). (C) Amino acid content in leaves sampled in the middle of the night (n = 6). (D–I) Enzyme activities involved in the TCA cycle (n = 13–14). Data are shown as box plots, black squares indicate the mean value, and bars indicate the range of the maximum or minimum data within a 1.5 × interquartile range (IQR). Green boxes indicate the results under control conditions, and red boxes indicate those under Mn-toxic conditions. Asterisks show significant differences between the control and Mn-toxic conditions (*: $p < 0.05$, **: $p < 0.01$, Kruskal–Wallis test).

1259

1260 **Figure 6**

1261 Stomatal structures and characteristics in the rice leaf blade. Pictures of the stomata on the
1262 adaxial side of the leaf blade grown under control (A) and Mn-toxic (B) conditions. The upper
1263 pictures focus on the stomatal distributions, and the lower pictures focus on the single
1264 stomata complex. (C) Showed the stomatal density ($n = 10-12$). (D) and (E) showed the
1265 major and the minor axis of the stomatal complex, respectively ($n = 53-63$). Data are shown
1266 as box plots, black squares indicate the mean value, and bars indicate the range of the
1267 maximum or minimum data within a $1.5 \times$ interquartile range (IQR). Green boxes indicate
1268 the results under control conditions, and red boxes indicate those under Mn-toxic conditions.
1269 Asterisks show significant differences between the control and Mn-toxic conditions (*: $p <$
1270 0.05 , ***: $p < 0.001$, Kruskal–Wallis test).

1271

1272 **Figure 7**

1273 Characteristics of the leaf section grown under control and Mn-toxic conditions. (A) Fixed
1274 leaf sections sectioned from the horizontal and vertical side against the leaf vein. Fresh leaf
1275 sections of the small (B) and large (C) bundles. Black bars show the scales.

1276

1277 **Figure 8**

1278 Leaf indole acetic acid (IAA) concentration and gene expression analysis relating to IAA and
1279 stomata development. (A) The result of GC-MS analysis targeting IAA, together with the
1280 chemical structures of IAA and its derivatives. The total ion chromatography (TIC) and
1281 chromatographs of $m/z = 202$ and $m/z = 319$ in the standard IAA solution and the leaf extract
1282 from plants under control and Mn-toxic conditions are shown. The retention time of the
1283 silylated IAA was 15.2 min. (B) The IAA concentrations in the leaf blade ($n = 8$). (D) The
1284 results of gene expression analysis involving IAA-responsive gene and stomatal patterning
1285 ($n = 8$). The quantified results are expressed on an *OsATC1-2* expression basis. Asterisks
1286 show significant differences between the control and Mn-toxic conditions (*: $p < 0.05$, **: $p <$
1287 0.01 , Kruskal–Wallis test).

1288

1289 **Figure 9**

1290 Visual scheme of symplastic Mn toxicity to suppress CO₂ assimilation. When Mn is
 1291 excessively accumulated in leaves, the leaf IAA concentration is lowered, which might cause
 1292 the stimulation of IAA degradation activities under Mn toxicity (Morgan et al., 1996). As a
 1293 short-term effect, the decrease in IAA concentration affects stomatal opening (Blatt & Thiel,
 1294 1994; Klein et al., 2003). Subsequently, as a long-term effect, the auxin-dependent signal
 1295 transduction involving ARF transcriptional factor is perturbed in the leaves to cause an
 1296 IAA-deficient phenotype, changing both stomatal and leaf anatomical structures (Le et al.,
 1297 2014; Zhang et al., 2014). The change in stomatal function and leaf structure severely limits
 1298 CO₂ diffusion to the chloroplasts, and CO₂ assimilation by Rubisco is inhibited. In contrast,
 1299 the photorespiration reaction limits the photosynthetic electron activities to cause ROS
 1300 production (Wada et al., 2019). The decreased sugar production efficiency in
 1301 photosynthesis suppresses sugar catabolism. Therefore, growth is inhibited under Mn-toxic
 1302 conditions.

1303

Figure 1

Phenotypes of rice plants grown under control and Mn-toxic conditions. The pictures of fully expanded leaf blades grown under control and Mn-toxic conditions are shown in (A), and (B) shows an enlarged picture of these. The white bars indicate lengths of 1 cm (A) and 1 mm (B), respectively. (C) The dried weights of the leaf blade, leaf sheath, and root, respectively. (D) The sum of the dried weights shown in (C). Data are shown as box plots ($n = 9$), black squares indicate the mean value, and bars indicate the range of the maximum or minimum data within a 1.5 $\times$ interquartile range (IQR). Green boxes indicate the results of the control conditions, and red boxes indicate those of the Mn-toxic conditions. Asterisks show significant differences between the control and Mn-toxic conditions (*: $p < 0.05$, Kruskal–Wallis test).

Figure 2

Mineral concentration in the rice leaf blade. Data are shown as box plots (control conditions $n = 8$, Mn-toxic conditions $n = 7$), black squares indicate the mean value, and bars indicate the range of the maximum or minimum data within a 1.5× interquartile range (IQR). Green boxes indicate the results of the control conditions, and red boxes indicate those of the Mn-toxic conditions. Asterisks show significant differences between the control and Mn-toxic conditions (*: $p < 0.05$, **: $p < 0.01$, Kruskal–Wallis test).

Figure 3

Photosynthetic activities in leaves grown under control and the Mn-toxic conditions. The CO_2 assimilation rate (A), stomatal conductance (g_s) (B), and intercellular CO_2 concentration (C) are shown. For the PSII photosynthetic parameters, the quantum yields of PSII [$Y(\text{II})$] (D), non-photochemical quenching [$Y(\text{NPQ})$] (E), and non-radiative decay [$Y(\text{NO})$] (F) are shown. The maximum quantum yield of PSII (F_v/F_m) is shown (J). For PSI photosynthetic parameters, the quantum yields of PSI [$Y(\text{I})$] (G), non-photochemical quenching at the donor side [$Y(\text{ND})$] (H), and non-photochemical quenching at the acceptor side [$Y(\text{NA})$] (I) are shown. (K) and (L) show the total Chl and N concentrations in the leaves, respectively. Data of photosynthetic parameters are shown as means with standard deviation (SD) ($n = 7$). The results for F_v/F_m ($n = 7$) and total Chl ($n = 7$) and N ($n = 9$) concentrations are shown as box plots. Black squares indicate the mean value, and bars indicate the range of the maximum or minimum data within a $1.5\times$ interquartile range (IQR). Asterisks show significant differences between the control and Mn-toxic conditions (*: $p < 0.05$, **: $p < 0.01$, Kruskal–Wallis test).

Figure 4

Carbohydrate content in leaf blades at the end of the day and end of the night. Data are shown as means with standard deviation (SD) ($n = 3-4$), and squares and diamonds show the distribution of the raw data. Asterisks show significant differences between the control and Mn-toxic conditions (*: $p < 0.05$, Kruskal–Wallis test).

Figure 5

Mitochondrial respiration activities and amino acid contents in leaf blades grown under control and Mn-toxic conditions. (A) Leaf respiration rate ($n = 12-13$) in the dark and (B) sucrose feeding respiration activities and mitochondrial respiratory electron transport activities depending on ATP synthase (uncoupled), COX, and AOX ($n = 7$). (C) Amino acid content in leaves sampled in the middle of the night ($n = 6$). (D-I) Enzyme activities involved in the TCA cycle ($n = 13-14$). Data are shown as box plots, black squares indicate the mean value, and bars indicate the range of the maximum or minimum data within a $1.5\times$ interquartile range (IQR). Green boxes indicate the results under control conditions, and red boxes indicate those under Mn-toxic conditions. Asterisks show significant differences between the control and Mn-toxic conditions (*: $p < 0.05$, **: $p < 0.01$, Kruskal-Wallis test).

Figure 6

Stomatal structures and characteristics in the rice leaf blade. Pictures of the stomata on the adaxial side of the leaf blade grown under control (A) and Mn-toxic (B) conditions. The upper pictures focus on the stomatal distributions, and the lower pictures focus on the single stomata complex. (C) Showed the stomatal density ($n = 10-12$). (D) and (E) showed the major and the minor axis of the stomatal complex, respectively ($n = 53-63$). Data are shown as box plots, black squares indicate the mean value, and bars indicate the range of the maximum or minimum data within a 1.5 $\times$ interquartile range (IQR). Green boxes indicate the results under control conditions, and red boxes indicate those under Mn-toxic conditions. Asterisks show significant differences between the control and Mn-toxic conditions (*: $p < 0.05$, ***: $p < 0.001$, Kruskal–Wallis test).

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