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Unraveling the Toxicity Mechanisms of the Herbicide Diclofop-Methyl in Rice: Modulation of the Activity of Key Enzymes Involved in Citrate Metabolism and Induction of Cell Membrane Anion Channels

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Supporting Information

ABSTRACT: Residual soil concentrations of the herbicide diclofop-methyl (DM) can be toxic to other nontarget plant species, but the toxicity mechanisms at play are not fully understood. In the present study, we analyzed the toxic effect of DM on root growth and metabolism in the rice species *Oryza sativa*. The results show that a 48-h exposure to a trace level (5 μg/L) of DM inhibits rice root growth by almost 70%. A 48-h exposure to 5 μg/L DM also leads to an ≈2.5-fold increase in citrate synthase (CS) activity (and CS gene transcription) and an ≈2-fold decrease in the citrate lyase gene transcripts, which lead to an increase in the intracellular concentration of citrate and in citrate exudation rate. Addition of a specific inhibitor of cell membrane anion channel, anthracene-9-carboxylic acid, decreased citrate release in the culture, suggesting that DM-induced citrate loss from the cells is mediated by a specific membrane-bound channel protein. This study brings new insights into the key biochemical mechanisms leading to DM toxicity in rice.

KEYWORDS: *herbicide, diclofop-methyl, rice, citrate, organic acid, cell wall*

INTRODUCTION

Crop production around the world is dependent on herbicide application for weed control. Diclofop-methyl or 2-[4-(2,4-dichlorophenoxy) phenoxy] propanoate (DM) is a phenoxypropanoic acid herbicide that selectively controls wild oat and other graminaceous weeds in wheat, barley, and soybean crops. DM may inhibit fatty acid synthesis in graminaceous weeds by inhibiting the activity of acetyl-CoA carboxylase (ACCase).[1−3] DM is a widely used herbicide; its annual application has reached approximately 750 000 pounds in the United States, according to the United States Environmental Protection Agency (2000),[4] and 1.5-fold more in China.[5] Upon DM application, approximately 99.9% of the herbicide may move into the environment without reaching their target weeds,[6] and may thus negatively affect several nontarget plants.[7] It is now known that DM residues in soils are absorbed in plants and affect several other metabolic pathways, not only fatty acid synthesis.[8,9] For instance, Ye et al.[8] showed that DM inhibited the Hill reaction activities in rice chloroplasts both in vivo and in vitro, which may affect photosynthesis. Moreover, microarray results from Qian et al.[9] demonstrated that low DM concentrations inhibited rice growth, affected starch and sucrose metabolism, and decreased oxidative phosphorylation, biosynthesis, and metabolism of amino acids.

A recent study from our laboratory conducted in rice has also shown that the production and exudation of citrate, a key metabolite involved in fatty acid synthesis, could be induced after exposure to 100 μg/L DM.[10] This enhanced production and exudation of citrate and malate in plant cells is not only observed under pesticide stress, but is also commonly observed at high metal concentrations, in anoxia, or under phosphorus limitation.[11−13] Evidence suggests that specific anion channel proteins (OsFRDL4) in the plasma membrane of rice cells may transport citrate and may be up-regulated in the presence of Al.[14] These anion channel proteins belong to the multidrug and toxic compound extrusion family (MATE), but their involvement in herbicide-induced organic acid exudation in rice is currently unknown. The toxicity mechanisms of DM in nontarget plants at the biochemical and genetic level and their interplay with organic acid production remain also poorly known. In the present study, we examined the toxicity mechanisms of DM in one plant inadvertently affected by herbicides, the japonica rice Xiushui 63 (*Oryza sativa* L.). To do so, we analyzed the toxic effect of DM on root growth of rice seedlings of *Oryza sativa*, on the activity of citrate synthase and ROS-detoxifying enzymes, on the production of sugar in cell wall, on intracellular or exuded citrate, and on the transcription level of several genes coding for key proteins and enzymes involved in rice metabolism. We further

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investigated the mechanism of citrate exudation by following the effect of DM on the transcription of the gene OSFRDL4 coding for an anion channel protein believed to mediate citrate efflux in plants[15] in the presence or absence of anthracene-9-carboxylase (a specific anion channel inhibitor).

MATERIALS AND METHODS

Culture Conditions and Herbicide Treatment. The sterilized Japonica rice seeds (*Oryza sativa* L. *japonica* cv. Xiushui 63) were germinated on filter papers at 30 °C in the dark for 2 d. The germinated seeds were then transferred onto a net floating on a culture medium containing 0.5 mM $CaCl_2$ (initial pH ≈ 5.7) at 25 °C in the dark for 4 d. For the DM exposure experiments, the seedlings were uniformly placed in an environmentally controlled growth room under a 12 h/12 h light/dark cycle. The rice seedlings were exposed for 24, 48, and 72 h to DM concentrations of 0, 5, and 50 μg/L (or 0, 14.7, and 147 nM), which were added to the $CaCl_2$ culture medium. Note that DM may rapidly hydrolyze in diclofop acid (DH) on a time scale of hours to days.[4] Because DH has a pK_a of 3.57,[16] the unprotonated or negatively charged species (D^{-1}) of DH is then expected to predominate over the protonated species at the pH of the culture medium. The rice seedlings were thus exposed to a mixture of DM, D^{-1}, and a low concentration of DH. The toxicity of DM on root growth and various biochemical targets in root explants of *O. sativa* L. was then investigated after 48 h of exposure. The transcription of genes related to citrate production and incorporation in fatty acids, as well as the activity of citrate synthase, were, however, measured after 24, 48, and 72 h of exposure to DM.

Root Growth Measurement, Microscopic Observation of Leaf Protoplasts and Root Tissue Section. The degree of inhibition in root elongation due to DM was measured using a vernier caliper. In parallel, we also isolated protoplasts from the leaves of rice seedlings using cellulase and pectinase incubation according to the protocol described in our previous study, and tissue sections were prepared from the roots of rice seedlings by paraffin block embedding.[17] The leaf protoplasts and root tissue sections were then observed with a light microscope at 1000×.

Quantitative Real-Time PCR (qRT-PCR). Root tissues were ground with a mortar and pestle in liquid nitrogen, and total RNA was extracted in 1 mL of RNAiso reagent according to the manufacturer's instructions (TaKaRa Company, Dalian, China). Total RNA was treated with DNase. RNA (500 ng) was then reverse transcripted in cDNA using a M-MLV reverse transcript kit (TaKaRa Company, Dalian, China). cDNA was diluted 10 times and used for real-time quantitative RT-PCR (qRT-PCR) which was carried out on an Eppendorf MasterCycler ep RealPlex[4] (Wesseling Berzdorf, Germany) according to the manufacturer's instructions using primers specific to the DNA sequence of several genes (Supporting Information (SI) Table SI.1). The housekeeping gene *25S* rDNA was used as an internal standard. qRT-PCR was performed in 10-μL aliquots, containing 1 μL of cDNA, 0.2 μL of each primer (10 μM), 5 μL of 2× mix buffer (Master mix, TOYOBO), and supplemented with sterile distilled water, with the following program: 95 °C 1 min; 40 cycles of 95 °C 15 s, and 60 °C 1 min. Gene expression normalized to 25S rDNA was analyzed by the $2^{-\Delta\Delta Ct}$ method, where C_t is the cycle number at which the fluorescent signal rises statistically above the background.[18] qRT-PCR was repeated with three independent replicates.

Enzyme Activity and Lipid Peroxidation Analyses. To extract the enzymes, root tissues were ground and homogenized with a mortar and pestle in 1.5 mL of 20 mM phosphate buffer (pH 7.4) in an ice bath. The homogenate was centrifuged at 10 000*g* for 10 min at 4 °C and the supernatant was used for the enzyme, protein, and malondialdehyde assays. The activity of citrate synthase (CS) was measured according to the method of Srere.[19] The activity of superoxide dismutase (SOD) was determined with a SOD kit (SOD kit, Sangon Company, China). Peroxidase (POD) activity was measured according to Andrews et al.[20] Lipid peroxidation level was determined by following the root malondialdehyde (MDA) content according to Zhang and Kirkham.[21] The activity of each enzyme was normalized to protein concentration, which was determined using the bicinchoninic acid (BCA) method (BCA protein kit, Sangon Company, China).

Intracellular and Extracellular Citrate Analyses by HPLC. After the DM exposures, the root tissues of rice seedlings were rinsed with distilled water and homogenized with a mortar and pestle in 1.2 mL of ice-cold 0.6 M $HClO_4$. The homogenates were transferred into Eppendorf tubes, which were centrifuged at 14 000*g* at 4 °C for 5 min. Eighty microliters of 5 M K_2CO_3 was added to the homogenates to neutralize the $HClO_4$. The intracellular concentration of citrate was measured by HPLC using an ion-exclusion column (Shodex Rspak Kc811, 300 mm × 8 mm) and a spectrophotometry detector at the wavelength of 210 nm. The mobile phase was perchloric acid solution (pH 2.1) with a flow rate of 0.8 mL/min.

The dissolved concentration of citrate in the culture medium was also analyzed according to the following procedure. The culture medium was collected to purify the organic acids exuded in solution by *O. sativa* L. The medium was then passed through a cation-exchange column (16 mm × 20 cm) filled with 5 g of Amberlite IR-120B resin (H^+ form) and an anion-exchange column (16 mm × 20 cm) filled with 2 g of Dowex 1 × 8 (100−200 mesh, formate form). The organic acid anions retained on the anion-exchange resin were eluted with 2 M HCl. The eluate was then dried in a 40 °C water bath, redissolved in 1 mL of Milli-Q water, and filtered through a 0.45-μm filter membrane. Determination of dissolved citrate exuded in the culture medium was done by HPLC according to the same method as that used for the measurement of intracellular citrate.

Measurements of Polysaccharides and Uronic Acid in Root Cell Wall. Root tissues were disrupted with a mortar and pestle in liquid nitrogen, and put in 75% (v/v) ethanol for 20 min in a cool water bath. The sample was then centrifuged at 12 000*g* for 10 min. After the supernatant was removed, the pellet was washed with acetone, methanol/chloroform (1:1), and methanol, for 20 min each. The remaining pellet, composed of cell wall materials, was freeze-dried for further use. Two mg of cell wall was dissolved in 1 mL of water, and the pectin fraction was extracted with a 1-h heating treatment (100 °C). The samples were again centrifuged at 14 400*g* for 10 min, and the supernatants were collected into the tube. The above two steps were repeated three times. The pellet was further extracted with 1 mL of 24% (w/v) KOH and 0.1% (w/v) KBH_4 for 12 h and centrifuged at 12 000*g* for 10 min. The hemicellulose (HCl) fraction was recovered in the supernatant. Pectin extracts (200 μL) were then incubated with 1 mL of 98% (v/v) H_2SO_4 containing 0.0125 M $Na_2B_4O_7{\cdot}10H_2O$ at 100 °C for 5 min. After cooling, 20 μL of M-hydro-diphenyl (0.15%, w/v) was added to the solution and let to react with the samples for 20 min. The uronic acid concentration in the pectin fraction was then measured spectrophotometrically by following the absorbance at 520 nm. Extraction of total polysaccharides in the hemicellulose fraction of the cell wall was performed using the phenol sulfuric acid method in which 200 μL of hemicellulose was incubated in 1 mL of 98% H_2SO_4 and 10 μL of 80% (v/v) phenol for 15 min, followed by incubation in boiling water for 15 min. The total polysaccharide content in the HCl fraction was finally measured spectrophotometrically using an absorbance wavelength set to 490 nm.

Statistical Analyses. Significant differences among the means of different treatments were evaluated by using analyses of variance (ANOVA). The conditions of ANOVAs (the normality and homogeneity of variance) were evaluated with the Kolmogrov−Smirnov test and the Levene test, respectively. If the test conditions were not validated, the data were transformed prior to the ANOVAs. Statistical analyses were performed using the program StatView. Differences among means were considered to be significant when the probability (p) was less than 0.05. All means and standard errors were calculated from three independent replicates.

RESULTS

Effect of DM on Root Growth. After a 48-h exposure to 5 and 50 μg/L DM, the mean root growth was only about 31.2%

($p < 0.01$) and 15.6% ($p < 0.01$) of the control value, respectively (Figure 1). DM also affected the shape and color of

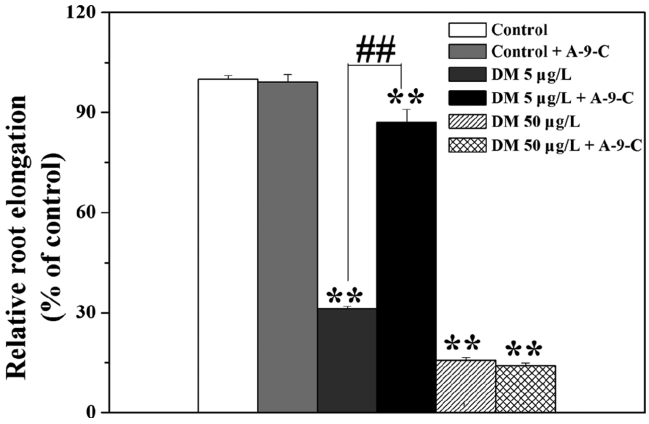

Figure 1. Relative root growth inhibition after a 48-h exposure to 5 and 50 μg/L DM in the presence or absence of the anion channel inhibitor anthracene-9-carboxylic acid (A-9-C). (*), (**) represent statistically significant differences relative to controls at $p < 0.05$ and at $p < 0.01$, respectively. (#), (##) show statistically significant differences due to the presence of A-9-C at $p < 0.05$ and at $p < 0.01$, respectively. Error bars represent the standard errors of three replicates.

cell walls and chloroplasts based on microscopic observations (SI Figure SI.1). Indeed, the root cell wall appeared darker and thicker after DM exposures (SI Figure SI.1). Moreover, at 50 μg/L DM, the chloroplasts could not be distinguished in the leaf protoplast and the green color of chlorophyll also weakened relative to that of the control (SI Figure SI.1).

DM Effect on Cell Wall Composition. Uronic acid and polysaccharides accounted for a relatively high proportion (>50%) of the pectin and hemicellulose fraction of the cell wall, respectively. The mean proportion of uronic acid significantly increased to a level 1.54-fold higher than that of the control after a 48-h exposure to 50 μg/L DM ($p < 0.01$) (SI Figure SI.2A). The proportion of polysaccharides in the cell wall also significantly increased by around 1.60-fold relative to that of the control after the 48 h exposure to 5 and 50 μg/L DM ($p < 0.01$) (SI Figure SI.2B). Furthermore, the transcription of five *pectin methylesterase* homologous genes was strongly induced by DM (SI Figure SI.2C). After the 48-h exposure to 50 μg/L DM, the transcripts of PME7, PME11, PME12, and PME22 significantly increased to levels 2.03-, 2.69-, 4.16-, and 3.10-fold higher than those of the control, respectively ($p < 0.01$ for PME11, PME12, PME22, but $p < 0.05$ for PME7) (SI Figure SI.2C). Gene transcription of PME29 was only significantly induced in response to the 5 μg/L DM treatment ($p < 0.05$), but did not change significantly at the highest DM concentration tested (50 μg/L) (SI Figure SI.2C). The microarray results also showed that the transcripts of many genes involved in the biosynthesis of cell wall can be induced in response to DM exposure (SI Table SI.2). The transcripts of phenylalanine ammonia-lyase (PAL) and peroxidases (PODs) were all induced by more than 2-fold in response to DM exposure.

Effect of DM on Citrate Metabolism and Exudation. Figure 2A shows that the transcript of ATP-cirate lyase (ACLY) already significantly decreased by 19.4% and 60.8% after 24 h of exposure to 5 and 50 μg/L DM, respectively ($p < 0.01$). Between 24 and 72 h, the transcript of ACLY continued to

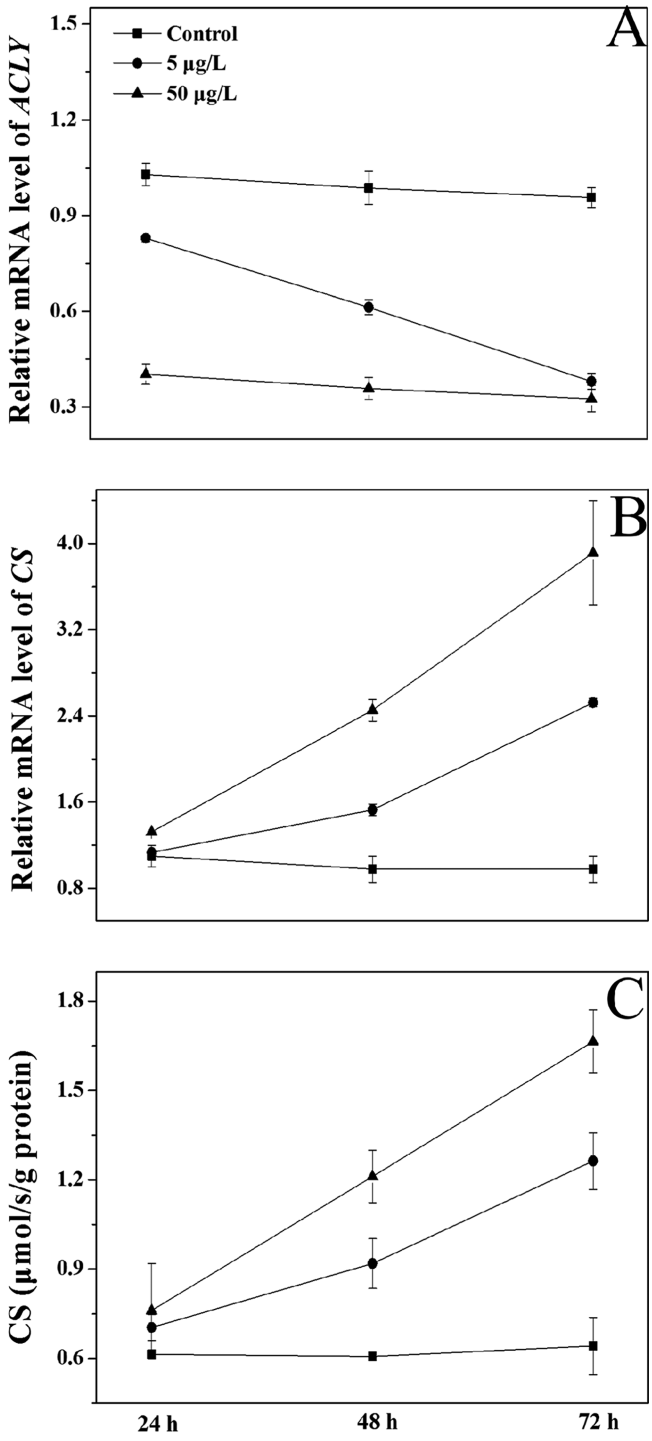

Figure 2. Effect of a 5 and 50 μg/L DM exposure for 24, 48, and 72 h on the transcription level of the citrate lyase (ACLY) gene (A) and the citrate synthase gene (B). Expression levels were normalized to that of the 25S rRNA gene. The effect of DM on CS activity (C). Error bars are the standard errors of three replicates.

decrease in the presence of 5 μg/L DM; at 72 h, the transcription of ACLY was inhibited by 63.0%. In contrast, at 50 μg/L DM, ACLY transcripts were inhibited by around 68% throughout the 72 h exposure ($p < 0.01$). The CS transcript level and CS enzyme activity both significantly increased in the presence of the two tested DM concentrations after 48 and 72 h exposure. After 72 h of exposure to 5 and 50 μg/L DM, CS

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enzyme activities reached levels 1.97- and 2.62-fold higher than those of the control, respectively (Figure 2B,C).

The citrate concentration in rice roots was not affected significantly after 24 h of exposure to 5 or 50 μg/L DM relative to control value, but significantly increased by approximately 15% after 48 h of exposure to 50 μg/L DM or 72 h of exposure to 5 or 50 μg/L DM. (Figure 3A). The amount of exuded

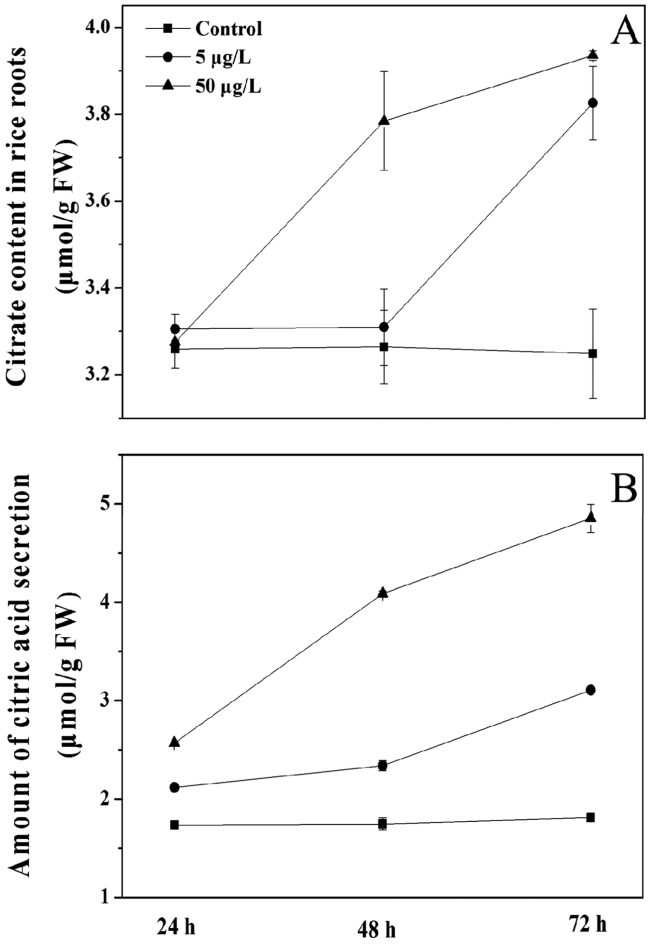

Figure 3. Citrate concentration in rice roots (A) and citrate concentration exuded in the medium normalized to root biomass (B) as a function of DM concentrations and time. Error bars are the standard errors of three replicates.

citrate in the culture medium normalized to root biomass showed a 1.79-fold increase after 72 h of exposure to 5 μg/L DM ($p < 0.01$) relative to that measured in control. The mean exudation of citrate increased by a factor of 1.48, 2.34, and 2.68 relative to control after 24, 48, and 72 h, respectively, of exposure to 50 μg/L DM (Figure 3B).

Anion-Channel Inhibitor and Citrate Exudation. Preliminary experiments demonstrated that 1 mg/L anthracene-9-carboxylic acid (A-9-C) did not affect the root growth of *O. sativa* L. over 48 h. In Figure 1A, we showed that addition of 1 mg/L A-9-C can even significantly decrease the toxicity of DM on root growth after 48 h of exposure to 5 μg/L DM, although 1 mg/L A-9-C did not affect significantly the toxicity of 50 μg/L DM. The results in Figure 4A shows that the transcription of *OsFRDL4* significantly increased in the presence of DM relative to control cultures without DM. Exposure of rice seedlings to 5 μg/L DM and 1 mg/L A-9-C

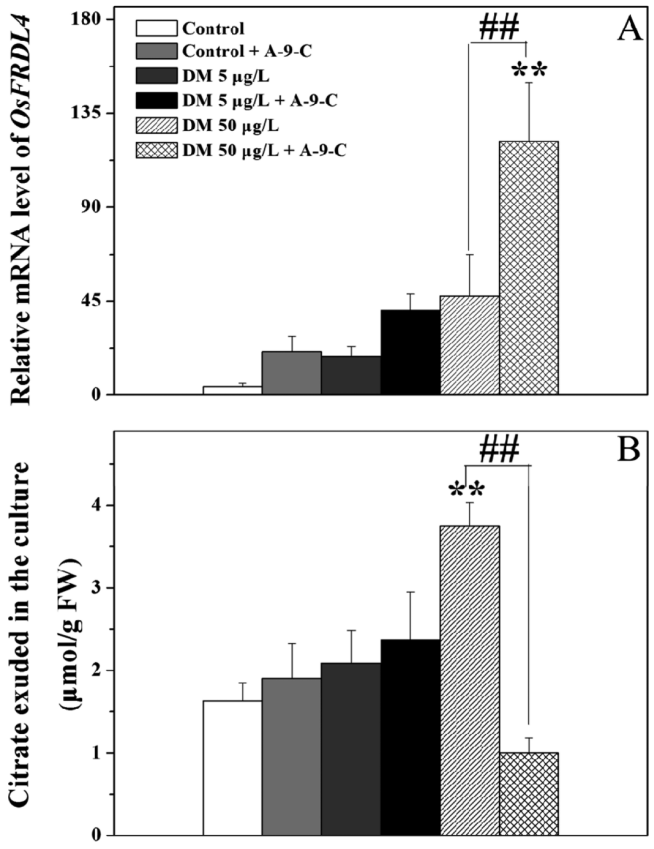

Figure 4. (A) Effect of a 5 and 50 μg/L DM exposure for 24, 48, and 72 h on the transcription level of the anion channel protein gene (OsFRDL4) normalized against 25S rRNA. The experiments in panel A were done in the presence or absence of the anion channel inhibitor Anthracene-9-carboxylic acid (A-9-C). B: Amount of citrate exuded in the culture medium normalized to root biomass. Error bars are the standard errors of three replicates. (*), (**) represent statistically significant differences relative to controls at $p < 0.05$ and at $p < 0.01$, respectively. (#), (##) show statistically significant differences due to the presence of A-9-C at $p < 0.05$ and at $p < 0.01$, respectively.

significantly increased the transcription of *OsFRDL4* relative to that measured after the exposure to 5 μg/L without A-9-C. In the presence of 50 μg/L DM and 1 mg/L A-9-C, the transcription of *OsFRDL4* was markedly induced by almost one order of magnitude with respect to its transcription at 50 μg/L DM without A-9-C (Figure 4A). A-9-C alone or combined with 5 μg/L DM did not affect significantly citrate exudation relative to control cultures, but addition of 1 mg/L A-9-C and 50 μg/L DM decreased citrate excretion by approximately 4-fold relative to that measured at 50 μg/L DM without A-9-C ($p < 0.01$)(Figure 4B).

Antioxidant Enzymes and Oxidative Stress in Roots. A 48-h exposure to 5 and 50 μg/L DM induced a significant increase in SOD activities relative to control values ($p < 0.05$ at 5 μg/L DM; $p < 0.01$ at 50 μg/L DM). In contrast, when 1 mg/L A-9-C was added in combination with DM, the SOD activity remained unaffected by the presence of DM (SI Figure SI.3A). The activity of total peroxidase (POD) also significantly increased in response to 5 or 50 μg/L DM ($p < 0.01$) and this stimulation of POD activity was less pronounced when A-9-C was added in combination with DM (SI Figure SI.3B). The malondialdehyde content in root tissues nearly doubled at 50 μg/L DM relative to control value, and the addition of A-9-C in

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combination with DM tended to decrease this effect, although this decrease was not statistically significant (SI Figure SI.3C).

DISCUSSION

DM Toxicity on Root Growth. Residual low concentrations of the widely used herbicide diclofop-methyl have been shown to inhibit the growth of nontarget monocots and aquatic algae.[8,9,22] Qian et al.[9] have previously shown that exposures to 50 μg/L DM for 2 weeks significantly inhibited by around 50% the root growth of rice seedlings. Our results here show that DM toxicity on root growth of rice seedlings for a 48-h exposure period may be more important that that measured after a much longer exposure time of 2 weeks, indicating that DM toxicity may be particularly important in the first hours or days of exposure to DM (Figure 1A). This is consistent with a relatively rapid uptake of the hydrophobic DM species followed by a relatively rapid hydrolysis of DM in mostly deprotonated diclofop acid (D^-) (at pH of our culture medium).[4] Indeed, hydrophobic molecules such as DM (and perhaps DH) are typically taken up by living cells at a much higher rate compared to that of charged species (such as D^-).[12,23]

Interaction between DM and Cell Wall Biosynthesis. To exert its toxicity in plant cells, DM first needs to pass through the cell wall. Several studies have shown that both biotic stresses (e.g., bacteria, fungi, and virus) and abiotic stresses (metals, pesticide, UV−B, and temperature) may alter the composition of cell wall by inducing a thickening of cuticles or trichomes.[24,25] The present study confirms that the herbicide DM may strongly affect the cell wall of *O. sativa*. Our results show that a 48-h exposure to trace DM concentrations may increase the proportion of uronic acid and polysaccharides in cell wall fractions (SI Figure SI.2A, B) and induce the transcription of various genes involved in the biosynthesis of cell wall (*pectin methylesterase, pectinesterase, cellulose synthase, phenylalanine ammonia-lyase* genes) (SI Figure SI.2C; Table SI.1 and SI.2). Different hypotheses may explain this energy investment in cell wall biosynthesis: (1) The increased biosynthesis of cell wall components might be a toxic effect of DM with no cellular function per se. (2) If not accompanied by a net increase in the absolute amount of cell wall components, the DM-induced up-regulation of cell wall biosynthesis rate could be triggered to repair the cell wall damages caused by DM. (3) If accompanied by a net increase in the absolute amount of cell wall components, DM might trigger a cellular defense mechanism associated with the thickening of the cell wall, which could reduce DM or DH diffusive rate across the cell wall (depending on the cell wall porosity), as well as DM or DH uptake rate and its toxicity. Additionally, if the unprotonated diclofop acid species (D^-) could be internalized in cells of rice roots via a specific cell membrane transporter, then the increase in the activity of pectin methylesterase, which converts the methoxyl groups on the polygalacturonic acid chain of the cell wall into negatively charged carboxyl groups,[26] would decrease (to more negative values) the cell surface charge of plant cells, which could decrease the effective concentration of D^- close to the cell surface and help decrease D^- uptake rate. Further experiments are, however, needed to confirm or refute the above hypotheses stipulating that DM-induced cell wall thickening might decrease DM, DH, or D^- uptake in rice roots.

DM Toxicity on Fatty Acid Precursors. The high toxicity of DM in monocots was previously shown to be due to the ability of DM to inhibit the activity of the enzyme acetyl-CoA carboxylase, which catalyzes the conversion of two acetyl-CoA molecules in one molecule of malonyl-CoA, the primary precursor for fatty acid biosynthesis in the chloroplast.[27] Another key enzyme upstream of the acetyl-CoA carboxylase that might also be affected by DM is the ATP-dependent citrate lyase (ACLY), which catalyzes the conversion of citrate and CoA in acetyl-CoA and oxaloacetate in the chloroplast.[28] This citrate comes from the mitochondrion matrix, where another important enzyme, citrate synthase (CS) produced citrate and initiates the tricarboxylic acid cycle. The present study shows that the activity and gene transcription of the enzyme CS increased in response to DM exposure, while DM decreased the gene transcription of the enzyme ACLY (Figure 2). This regulation pattern of CS and ACLY gene expression and enzyme activity will lead to an increase in citrate concentration in root cells as observed in Figure 3A.

DM-Induced Citrate Exudation from Root Cells. Our results show that the herbicide DM may increase citrate exudation from root cells since dissolved citrate concentrations increased over time in plant cultures exposed to DM (Figure 3B) as observed in the study of Ding et al.[10] who performed longer (2−3 weeks) DM exposure of rice seedlings.

Our data also show that the transcription level of *OsFRDL4* increased under DM exposure (Figure 4A) and that addition of the anion channel inhibitor A-9-C (at nontoxic concentration, Figure 1A) further increased the transcription of *OsFRDL4* (Figure 4A). Moreover, the amount of citrate exuded in the culture medium contaminated with 50 μg/L DM (but not with 5 μg/L DM) strongly decreased in response to the addition of the anion channel inhibitor A-9-C (Figure 4B). Together, the results strongly suggest that DM may up-regulate the synthesis of anion channel proteins in the plasma membrane of rice roots and may mediate the transport of excess intracellular citrate out of the cells (as shown in Figure 3B). However, this up-regulation of citrate channels and its associated increase in citrate exudation (only at 50 μg/L DM) caused by DM exposure did not decrease DM toxicity. Our results agree with those of previous reports showing that citrate exudation from plant roots probably involved a specific transport system in the plasma membrane.[14,29,30] The latter reports dealt with the effect of metals or phosphorus limitation, but, to our knowledge, the present study is the first showing that citrate exudation in the presence of an herbicide, DM, is mediated by an anion channel protein. Contrary to the protective effect of citrate exudation on Al toxicity,[14] our study shows that DM-induced citrate exudation did not protect rice roots against DM toxicity.

DM Toxicity and Oxidative Stress. Besides the toxicity of DM at the level of fatty acid biosynthesis, other studies of Shimabukuro et al.[31,32] have proposed that DM toxicity might be due to the DM-induced production of free radicals in plant cells. In the present study, we show that DM increased oxidative stress at the level of cell membranes (MDA production SI Figure SI.3C) in the roots of rice seedlings, and that the activity of two antioxidant enzymes may increase in response to this stress (SOD and POD) (SI Figure SI.3A,B). Furthermore, the addition of 1 mg/L A-9-C in cultures contaminated with 5 μg/L DM significantly decreased the level of oxidative stress in cell membrane (SI Figure SI.3C) as well as DM toxicity on root growth (Figure 1A). Then, our results are also consistent with a putative DM toxicity cellular mechanism based on the production of free radicals.

Conclusions. This study brings new insights into the key biochemical mechanisms leading to DM toxicity in rice. Here

we show that DM may increase the activity of CS and decrease the activity of ACLY, which increase the intracellular pool of citrate in rice roots. This cellular excess in citrate may be exuded by specific anion channels located in the cell membrane of root cells, but this DM-induced citrate exudation did not protect against DM toxicity on the root growth of rice seedlings. The exudation of citrate induced under DM stress is noteworthy since an increase in organic acid release in soils contaminated by residual herbicide concentrations may affect the chemistry of soil pore water (e.g., pH, organic carbon respiration) and the microbial community structure in the rhizosphere, which could in turn affect plant nutrition.

■ ASSOCIATED CONTENT

Supporting Information

Table SI.1 Sequences of primer pairs used to measure the transcription of several genes by real-time PCR. Table SI.2 Changes in cell wall-related gene transcription relative to control after treatment with DM enantiomer (*S*-DM and *R*-DM), as analyzed by microarray. Figure SI.1 Microscopic observations of root tissue sections and leaf protoplasts of rice seedlings exposed to 0, 5, and 50 μg/L DM for 48 h. Figure SI.2 Effect of a 48-h exposure to 5 and 50 μg/L DM on the proportion of uronic acid in the pectin fraction of cell wall (A) and on the proportion of polysaccharides in the hemicellulose (HCl) fraction of cell wall (B). (C) Transcripts of five pectin methylesterase homologous genes normalized against 25s rRNA. Figure SI.3 Activity of superoxide dismutase (SOD) (A), and peroxidase (POD) (B) in rice roots after a 48-h exposure to 5 or 50 μg/L DM with and without the anion channel inhibitor, anthracene-9-carboxylic acid (A-9-C). Concentrations of malondialdehyde in rice roots (C) exposed for 48 h to 5 or 50 μg/L DM with or without A-9-C. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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