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Analysis of Enantioselective Biochemical, Physiological, and Transcriptional Effects of the Chiral Herbicide Diclofop Methyl on Rice Seedlings

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Supporting Information

ABSTRACT: Diclofop methyl (DM) is a chiral herbicide that is widely used as a racemic mixture. This study analyzed the enantioselective effects of *R*- and *S*-DM on rice at the physiological and molecular levels. DM exerts an enantioselective effect on rice growth, reactive oxygen substance (ROS) formation, and antioxidant gene expression, with *R*-DM acting as a more potent stressor than *S*-DM. An analysis of chlorophyll fluorescence demonstrated that photosynthesis process was more strongly inhibited by *R*-DM than by *S*-DM. Microarray results showed that many metabolic pathways, including starch and sucrose metabolism, oxidative phosphorylation, and amino acid biosynthesis and metabolism, were affected by DM in an enantioselective manner. These results suggest that *R*-DM is more active to plant growth than *S*-DM and that this activity is induced not only by repression of fatty acid synthesis but also by *R*-DM affecting the transcription of genes in other metabolic pathways in an enantioselective manner.

KEYWORDS: *diclofop methyl, enantioselectivity, fatty acid, microarray, real-time PCR, rice*

INTRODUCTION

With the rapid expansion of the chemical manufacturing industry, a large number of pesticides have been introduced into the environment to improve agricultural production. Approximately 25% of the most widely used pesticides are chiral compounds with one or more pairs of enantiomers.[1] Enantiomers have identical physical and chemical properties in achiral environments, but they display dramatic differences in biochemical processes because of selective interactions with enzymes and other chiral molecules.[2−5] However, a large majority of chiral pesticides are still released into the environment as racemic mixtures; one reason is lack of investigation to the mechanisms of enantioselectivity.

Recent studies on the enantioselective mechanisms involved in the activity of herbicides, such as metolachlor and imazethapyr (IM),[6−10] have mainly focused on plants. Metolachlor belongs to the family of acylanilide chiral herbicides. Although racemic metolachlor is used to control the growth of grass in some countries, *S*-metolachlor has been demonstrated to be more active than the racemic form.[11,12] Imazethapyr is an imidazolinone herbicide[10] and also showed enantioselectivity that *R*-IM is more active than *S*-IM.[7−9] Diclofop methyl (DM) is a phenoxypropanoic acid herbicide that is widely used on wheat, barley, and golf courses to control or suppress wild oats and annual grasses. The usage of DM in the United States reached approximately 750 000 pounds in 2000, according to a report of United States Environmental Protection Agency,[13] and (1−5) × 10^6 kg in China.[14] One proposed mechanism of DM is to bind acetyl-CoA carboxylase (ACCase) to inhibit the synthesis of fatty acids, causing cellular destruction, particularly in rapidly growing meristematic regions.[15,16] ACCase mutants showed great resistance to phenoxypropanoic acid herbicide,[17] demonstrating again that ACCase is one of the important targets in plant. The other proposed mechanism is a catabolic mechanism where membrane disassembly and the production of free radicals results in plant death.[18,19] DM has one pair of enantiomers, *R*- and *S*-DM, which derive from a chiral carbon in the molecule. Early studies showed that DM has enantioselective effects on fatty acid biosynthesis and that *R*-DM inhibited fatty acid biosynthesis at a lower concentration than *S*-DM in maize chloroplasts in vitro.[20] Cai et al.[21] found that exposure of three freshwater algae species to *R*- or *S*-DM induced different rates of aquatic ecotoxicity and degradation, with *S*-DM having a more active effect. Ye et al.[22] reported that DM enantiomers show enantioselective phytotoxicity on rice growth. This information is not sufficient to completely infer the mechanism of enantioselectivity; therefore, the specific enantioselective mechanism of chiral diclofop and related herbicides will require further in-depth study. In the present study, we selected japonica rice Xiushui 63 (*Oryza sativa*) as the target organism for an analysis of DM enantioselectivity from plant morphology, physiology, and metabolic pathways at the transcriptional level.

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Table 1. Root Elongation (cm) of Rice Exposed to DM Enantiomers or Racemate at 25, 50, or 100 μg L^{-1} for 2 or 3 weeks[a]

	2 weeks exposure			3 weeks exposure		
	25 (μg L^{-1})	50 (μg L^{-1})	100 (μg L^{-1})	25 (μg L^{-1})	50 (μg L^{-1})	100 (μg L^{-1})
control	6.00 ± 0.29			6.90 ± 0.22		
R-DM	4.28 ± 0.16**	3.26 ± 0.13**##	2.02 ± 0.14**##	5.16 ± 0.19**##	4.10 ± 0.14**##	2.62 ± 0.10**##
S-DM	4.92 ± 0.56*	4.38 ± 0.13**	3.20 ± 0.23**	6.12 ± 0.27*	5.02 ± 0.21**	4.06 ± 0.10**
Rac	4.98 ± 0.22 *	4.62 ± 0.25**	3.44 ± 0.17**	5.78 ± 0.23 *	5.40 ± 0.24**	4.02 ± 0.18**

[a]"*" and "**" indicate that the values are significantly different as compared to the control ($p < 0.05$ and 0.01, respectively). "##" indicates that the values are significantly different as compared to those of the *S*-DM-treated plants ($p < 0.01$).

MATERIALS AND METHODS

Plant Materials. Rice (*Oryza sativa* L. cv. Xiushui 63) seeds were sterilized and germinated in Petri dishes filled with 10 mL of ddH_2O at 30 °C in the dark for 2 days. Uniformly germinated seedlings were placed in a growth chamber at 25 °C with a 300 μmol/m^2/s light intensity and a 12 h:12 h light/dark cycle. The concentrations of 25, 50, and 100 μg L^{-1} DM enantiomers were added to 1/2 Murashirrg and Skoog (MS) liquid medium and applied to the rice seedlings. Triplicate cultures were prepared for each treatment, and every replicate contained at least five plantlets. The relative inhibition rate of root elongation caused by the DM enantiomers was calculated via the following formula: relative inhibition rate (RI) (%) = $((X_0 - X_n)/(X_0)) \times 100\%$. X_0 presents root length of the control, and X_n presents root length of treatments.

Preparation of Rice Protoplasts. To obtain protoplasts, calli were first induced from rice seeds in MS (Murashige and Skoog) basal medium supplemented with 2.0 mg L^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D), and then were suspended in MS liquid medium with 2.0 mg L^{-1} of 2,4-D to transform into approximately 2 mm callus particles. Callus particles were enzymolyzed into protoplasts according to our previously reported method.[9] Protoplasts were exposed to 100 μg L^{-1} DM enantiomers from 6 to 24 h, and the number of protoplasts was then counted under an optical microscope. Triplicate cultures were prepared for each treatment.

Determination of Chlorophyll Fluorescence. The rice seedlings treated with DM enantiomers for 2 and 3 weeks were selected to measure chlorophyll fluorescence. Chlorophyll fluorescence was measured with a Dual-PAM-100 Chlorophyll Fluorometer (Heinz Walz, Effeltrich, Germany) using previously described methods.[23] Before the chlorophyll fluorescence was determined, the rice seedlings were adapted to the dark for more than 30 min. The following parameters were calculated: maximum quantum yield of PSII (F_v/F_m), photochemical quenching (qP), electron transport rate (ETR), and quantum yield of photosystem II (ΦPSII).

Antioxidant Genes Transcript Analysis. The total RNA of treated rice seedlings was extracted using RNAiso Reagent (TaKaRa, Dalian, China). RNA was reverse-transcribed into cDNA, and then real-time PCR analysis was performed in an Eppendorf MasterCycler ep RealPlex[4] (Wesseling-Berzdorf, Germany). Three Cu/Zn-SOD genes (CSD1, CSD2, and CSD3), two Fe-SOD genes (FSD1 and FSD2), three Mn-SOD genes (MSD1, MSD2, and MSD3), three CAT genes (CAT1, CAT2, and CAT3), two GPX genes (GPX1 and GPX2), and eight APX genes (APX1−APX8) were selected for analysis. The primer pairs used for the APX genes have been described previously,[24] and the others are listed in Table S1. The PCR reaction mixture was prepared according to the instruction of the SYBR Green real-time PCR Master Mix (Toyobo, Tokyo, Japan). The PCR protocol was similar to that in a previous study: one denaturation step at 95 °C for 1 min and 40 cycles of 95 °C for 15 s, followed by 60 °C for 1 min.[9] Actin was used as a housekeeping gene to normalize the expression profiles.

Fatty Acid Measurements. The rice seedlings were treated with DM enantiomers for 1 week. Harvested plant material was then dried and ground into powders. Fatty acids were isolated by Soxhlet extraction with petroleum ether for 12 h. Diatomite and activated carbon were added in a 3:1 ratio, respectively, to remove pigment. Fatty acid methyl esters were generated according to Zhang et al.[25] and analyzed by GC−MS.

Microarray Hybridization and Real-Time PCR Verification. The microarray analysis was performed using a GeneChip rice genome array (Affymetrix) containing approximately 48 564 japonica and 1269 indica transcripts. Microarray hybridization was performed and analyzed by the CapitalBio Co (Beijing, China). To increase the likelihood of detecting differentially expressed genes, transcripts with a ratio of change >2.0 or <0.5 were classified as up- or down-regulated, respectively. The functional annotation of differentially expressed genes was performed with MAS 3.0 (http://bioinfo.capitalbio.com/mas3/). Real-time PCR was performed to verify the results of the microarray.

Data Analysis. The data are presented as the mean ± standard error of the mean (SEM) and were tested for statistical significance by one-way ANOVA. Values were considered significantly different when the probability (p) was less than 0.05 or 0.01.

RESULTS AND DISCUSSION

The Enantioselective Effects of DM on Plant Growth. Rice seedlings were exposed to the DM enantiomers or racemate at concentrations of 25, 50, and 100 μg L^{-1}. The DM treatments significantly inhibited root growth relative to untreated controls, but *R*-DM had a more pronounced effect than does *S*-DM or racemate (see Figure S1, Supporting Information). The degree of root elongation inhibition was positively related to the dosage applied (Table 1). At a concentration of 100 μg L^{-1}, the *R*-DM treatment showed stronger inhibitory effect on root elongation than *S*-DM treatment ($p < 0.01$). The average root lengths were only 33.7% and 38.0% of the control after 2 and 3 weeks of *R*-DM exposure, respectively, but were approximately 53.3% and 58.8% of the control after 2 and 3 weeks of *S*-DM exposure, respectively. The effect of racemate was between that of *R*-DM and *S*-DM.

The number of adventitious roots per plantlet is shown in Figure 1. Adventitious root formation was significantly inhibited by DM treatment (100 μg L^{-1}), with *R*-DM having the strongest effect ($p < 0.01$). After 3 weeks of exposure, the number of adventitious roots was only 2.5 in the 100 μg L^{-1} *R*-DM treatment, which was lower than that of the control. The number of adventitious roots in the *S*-DM treatment was also lower than that of the control after 2 weeks of exposure but recovered by the third week. These results are similar to those of Ye et al.[22] in that *R*-DM was more active to rice roots than *S*-DM. However, our results differ from those of Cai et al.,[21] who found that the active effect of *S*-DM on algae was similar to or higher than that of *R*-DM. The discrepancy might be attributed to the different physiological and metabolic pathways of plants and algae. Figure S1 also showed evidently that *R*-DM was more active to rice leaves than *S*-DM (data not shown); this is contrary to the results of Ye et al.,[22] who reported that *S*-DM at

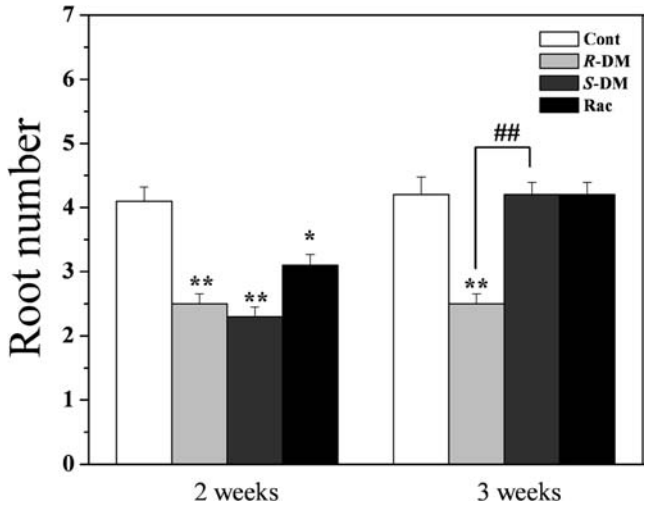

Figure 1. The number of adventitious roots counted in seedlings subjected to 100 μg L^{-1} DM enantiomer treatment for 2 or 3 weeks. "*" and "**" indicate that the values are significantly different as compared to the control ($p < 0.05$ and 0.01, respectively). "##" indicates that the values are significantly different as compared to the *S*-DM-treated plants ($p < 0.01$).

high concentrations (mg L^{-1}) was more active to rice leaves than *R*-DM.

The Effects of DM on Protoplast Survival. The isolated plant protoplast is a single cell bounded by the plasma membrane that contains all of the normal cell components except for the cell wall, and it is very sensitive to environmental factors. Therefore, protoplasts provide an important biochemical tool for short-term environmental activity research.[26,27] In this study, we utilized an enzymatic method to obtain protoplasts and subsequently exposed them to DM enantiomers. We observed that the protoplast survival rate decreased significantly following DM treatment as compared to the control (see Figure S2, Supporting Information). The protoplast survival rate significantly decreased after 6 to 12 h of *R*-DM and racemate exposure but did not decrease significantly under *S*-DM treatment until the 18 h time point. The minimal survival rates of the protoplasts were 27.2%, 66.0%, and 40.1% of the control after *R*-, *S*-DM, and racemate exposure, respectively (Figure 2). This result demonstrates that DM

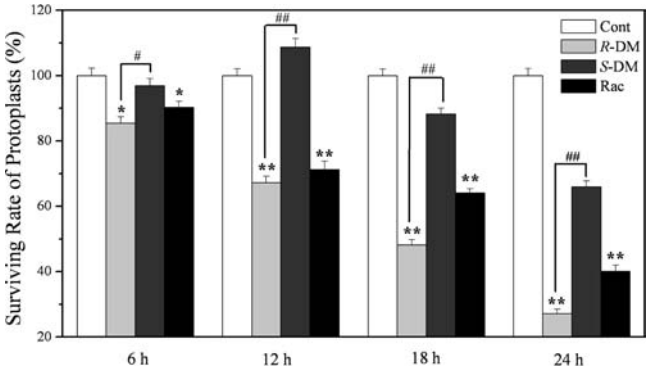

Figure 2. The effect of DM enantiomers on protoplast survival. "*" and "**" indicate that the values are significantly different as compared to the control ($p < 0.05$ and 0.01, respectively). "#" and "##" indicate that the values are significantly different as compared to the *S*-DM-treated plants ($p < 0.05$ and $p < 0.01$, respectively).

toxicity is enantioselective in rice cells and that *R*-DM exhibited stronger cytocidal effects than the *S*-DM, which is in accordance with the morphological research on plantlets.

The Effects of DM on Chlorophyll Fluorescence. Chlorophyll fluorescence reflects thylakoid membrane organization and photosynthetic function.[28] The inhibitory effect of DM enantiomers on rice photosynthesis was evaluated by analyzing several fluorescence parameters. As shown in Figure 3, F_v/Fm was decreased after 2 and 3 weeks of DM enantiomer exposure. The F_v/F_m values were approximately 88.9% and 89.6% of the control after 2 and 3 weeks of *R*-DM exposure, respectively, and were significantly lower than that of the control and *S*-DM treatments. Several previous reports have observed a similar phenomenon. In a study of four tropical evergreen tree species, Huang et al.[20] observed that cold temperatures decreased the F_v/F_m. UV light also decreased the F_v/F_m of *Arabidopsis*, possibly through PSII photodamage.[29] These reports suggest that the decrease in F_v/F_m is closely accompanied by a decrease in D1 protein content or damage to the thylakoid membranes.[30] Therefore, we speculated that *R*-DM might damage the thylakoid membranes in PSII and inhibit the transfer of energy from the antenna molecules to the reaction centers more potently than *S*-DM.

In the present study, the qP value did not change after 2 weeks of DM enantiomer treatment; however, the qP was reduced to approximately 75.6% of the control value as the exposure period increased to 3 weeks, which was significantly lower than that observed after *S*-DM exposure. qP occurs in response to the reoxidation of QA^-. This process helps to protect the cell against photodamage by photochemical quenching energy dissipation. qP also reduces the relative quantum yield of PSII to maintain an adequate balance between photosynthetic electron transport and carbon metabolism.[31] The decrease of qP upon *R*-DM exposure revealed the existence of photoinhibition in seedling leaves and is reminiscent of the effects of cold treatment on trees,[23] cinnamic acid treatment on *Lactuca sativa* L.,[32] and salt treatment on maize.[33] Also, decreases in the qP can significantly reduce the PSII electron transport rate and affect photosynthesis.[33] To determine whether these parameters were affected by the DM enantiomers, we measured the electron transport rate (ETR) at the PSII as well as the ΦPSII (Figure 4). The ETR decreased to approximately 89.6% and 75.8% of control levels after *R*-DM treatment for 2 and 3 weeks, respectively. These values were significantly lower than those observed after treatment with *S*-DM. This result demonstrated that *R*-DM can strongly inhibit the PSII electron transport rate. Similar patterns were observed with ΦPSII; ΦPSII decreased to 88.1% and 68.4% of control levels after 2 and 3 weeks of *R*-DM exposure, respectively. *S*-DM treatment did not affect ΦPSII after 2 weeks of exposure, but a significant decrease was noted after 3 weeks of exposure. The effect on ΦPSII was therefore enantioselective, with *R*-DM having a stronger inhibitory effect than *S*-DM.

Photosynthesis is commonly affected by physiological stress, such as salt, high or low temperatures,[29] and herbicides, as reported in this study. DM also exhibited an enantioselective effect on chlorophyll fluorescence, as *R*-DM decreased chlorophyll fluorescence more than *S*-DM. Overall, *R*-DM treatment strongly inhibited photosynthesis and caused plant growth retard.

The Effects of DM on ROS Accumulation and Antioxidant Gene Transcription. The chloroplast is a

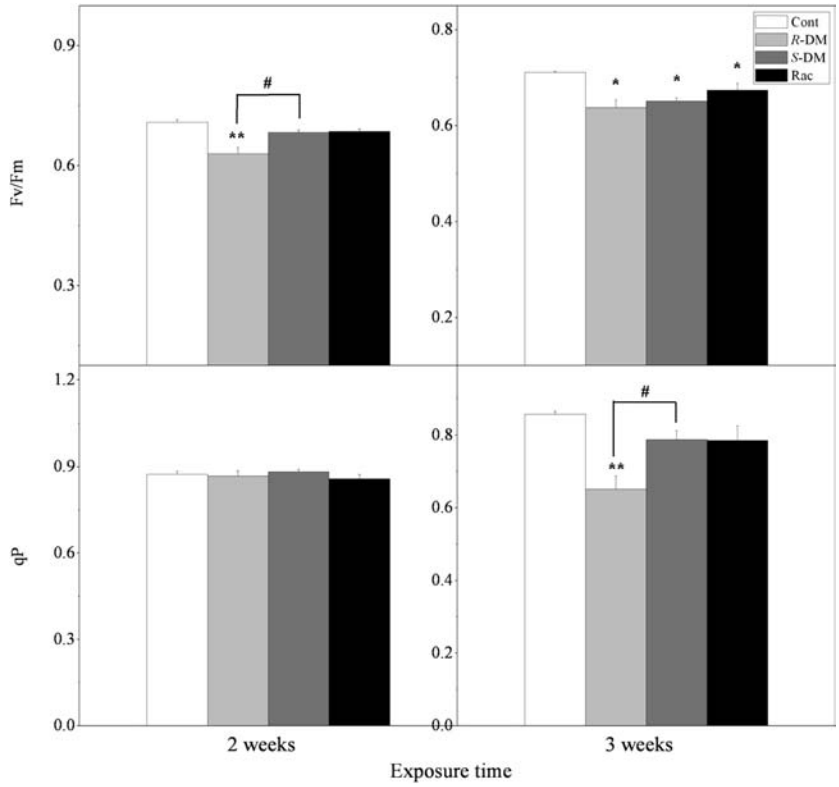

Figure 3. The effect of DM enantiomers on the chlorophyll fluorescence parameters maximum quantum yield of PSII (F_v/F_m) and photochemical quenching (qP). "*" and "**" indicate that the values are significantly different as compared to the control ($p < 0.05$ and 0.01, respectively). "#" indicates that the values are significantly different as compared to those of the *S*-DM-treated plants ($p < 0.05$).

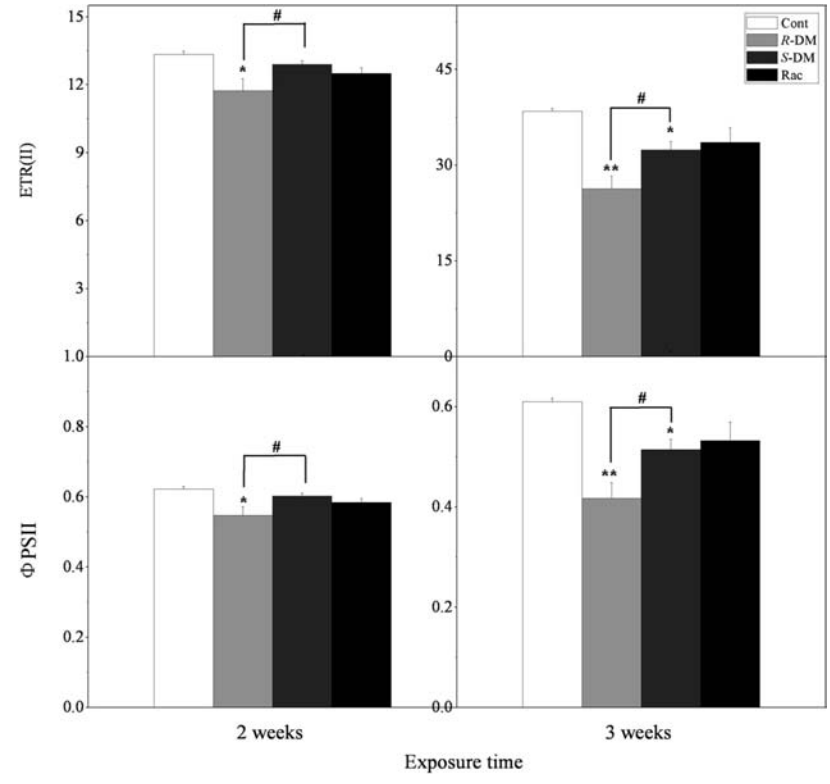

Figure 4. The effect of DM enantiomers on the chlorophyll fluorescence parameters electron transport rate (ETR) and quantum yield of photosystem II (ΦPSII). "*" and "**" indicate that the values are significantly different as compared to the control ($p < 0.05$ and 0.01, respectively). "#" indicates that the values are significantly different as compared to the *S*-DM-treated plants ($p < 0.05$).

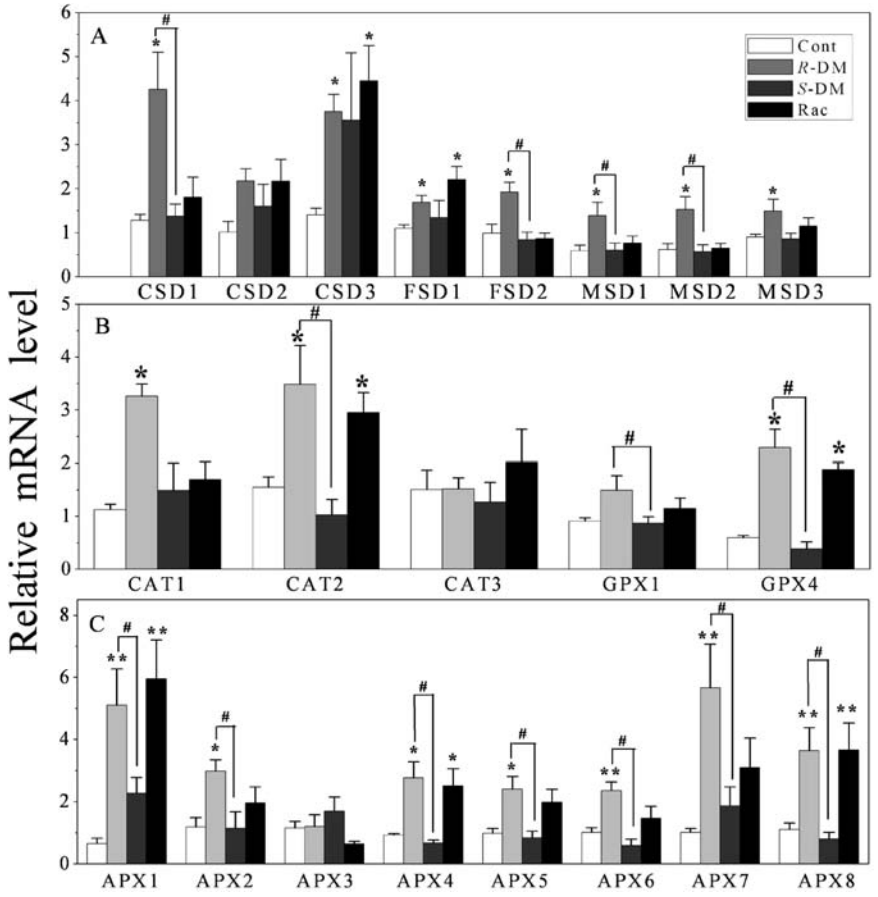

Figure 5. The effect of DM enantiomers on antioxidant gene expression in rice after 2 weeks of exposure. "*" and "**" indicate that the values are significantly different as compared to the control ($p < 0.05$ and 0.01, respectively). "#" indicates that the values are significantly different as compared to the *S*-DM-treated plants ($p < 0.05$).

primary source of ROS, through surplus electrons combining with molecular oxygen.[34] Our results on chlorophyll fluorescence show that *R*-DM disturbs the electron transport chain. To evaluate changes in ROS levels, we measured the accumulation of superoxide radical (O_2^-) and H_2O_2 using nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) staining. The intensities of blue coloration and the red-brown stain indicate the concentrations of O_2^- and H_2O_2, respectively. The results revealed an accumulation of O_2^- and H_2O_2 after *R*-DM treatment (see Figure S3, Supporting Information). We also analyzed the transcript levels of several antioxidant genes to gauge the balance between the oxidant and antioxidant systems. After 2 weeks of *R*-DM exposure, the transcript levels of seven SOD genes were up-regulated 1.5−3.3-fold over the control, whereas three SOD genes were up-regulated after 2 weeks of racemate exposure. *R*-DM induced the up-regulation of two CAT genes and one GPX gene, whereas one CAT gene and one GPX gene were up-regulated by racemate. *R*-DM treatment also caused seven APX genes to be up-regulated 2.3−7.9-fold over the control. Racemate treatment, in turn, resulted in the up-regulation of three APX genes (Figure 5). After 3 weeks of exposure, the transcript levels of all eight SOD genes were up-regulated in the plants exposed to *R*-DM; all but one was also induced by the racemate. Three weeks of *R*-DM treatment resulted in the up-regulation of two CAT genes and two GPX genes, but only CAT1 and GPX1 were up-regulated in the racemate-treated plants (Figure 6). No antioxidant gene was affected by *S*-DM treatment after 2 or 3 weeks of exposure. DM therefore exerts an enantioselective effect on the transcription of antioxidant genes, with *R*-DM showing a more potent effect than *S*-DM.

Malondialdehyde (MDA) content serves as an indicator of lipid peroxidation. The MDA content increased by 8.4- and 9.6-fold over the control after 2 and 3 weeks of *R*-DM exposure, respectively, but was not affected by *S*-DM treatment (see Figure S4, Supporting Information). It appears that DM induces oxidative stress and then stimulates the antioxidant system to maintain the balance between ROS and scavenging factors. However, the concomitant increase in MDA content suggests that the antioxidant enzymes induced by DM may be insufficient to completely eliminate the ROS, as suggested by previous reports.[35−37] DM therefore exerts an enantioselective effect on ROS formation and antioxidant gene expression, with *R*-DM acting as a more potent stressor than *S*-DM.

The Effects of DM on Fatty Acid Content. Previous reports noted that DM had an inhibitory effect on fatty acid synthesis, which subsequently blocked the production of membrane lipids and induced cell death.[15,16] We therefore measured the effects of DM enantiomers on fatty acid content. Figure 7 shows that total fatty acids were highest in the control and lowest in the *R*-DM treatment. *R*-DM treatment reduced the amount of total fatty acids to approximately 56% of the control. *S*-DM treatment, in comparison, reduced fatty acid synthesis to 91.3% of the control. These results confirmed previous reports showing that DM can inhibit fatty acid

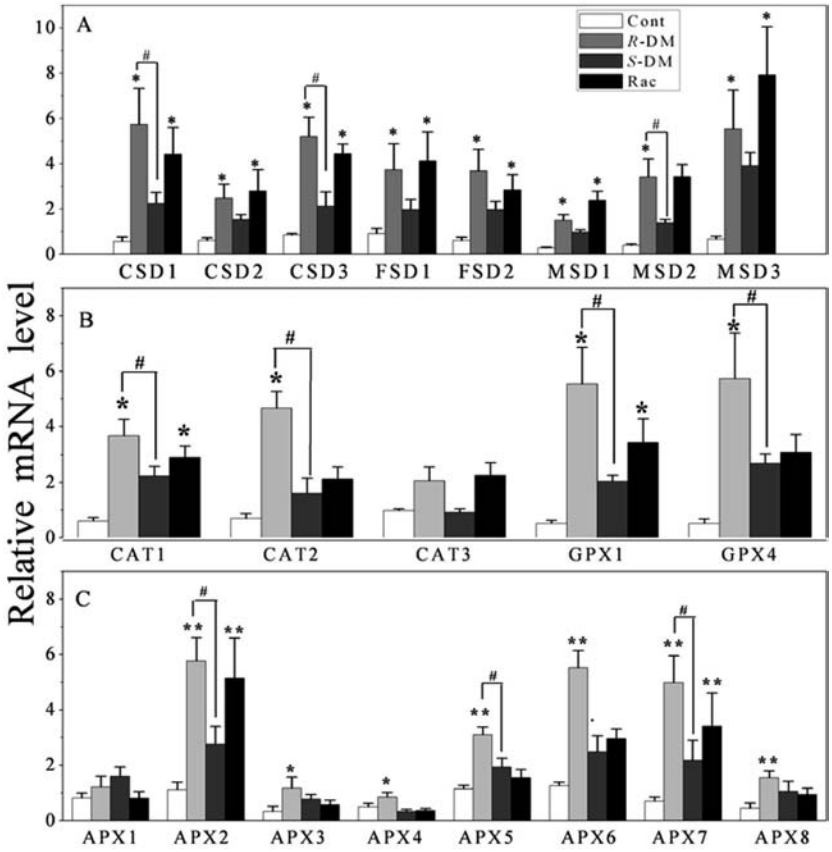

Figure 6. The effect of DM enantiomers on antioxidant gene expression in rice after 3 weeks of exposure. "*" and "**" indicate that the values are significantly different as compared to the control ($p < 0.05$ and 0.01, respectively). "#" indicates that the values are significantly different as compared to the *S*-DM-treated plants ($p < 0.05$).

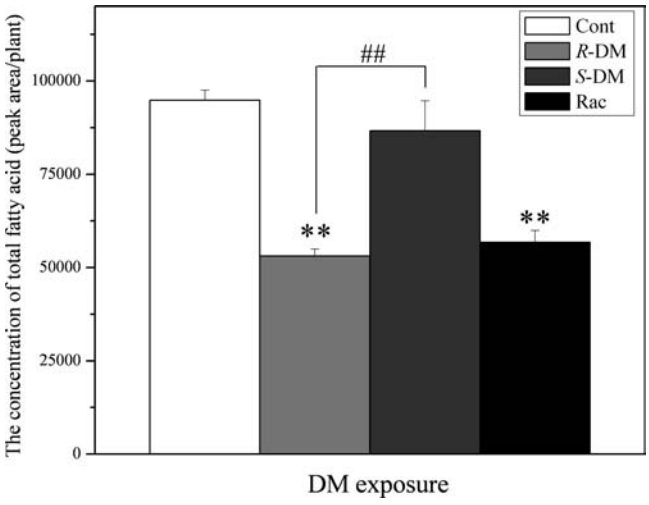

Figure 7. The effect of DM enantiomers on the fatty acid content of rice plants after 1 week of exposure. "**" indicates that the values are significantly different as compared to the control ($p < 0.01$). "##" indicates that the values are significantly different as compared to the *S*-DM-treated plants ($p < 0.01$).

synthesis and revealed that it acts in an enantioselective manner.

Microarray Analysis of the Effects of DM on Transcription. To better understand the mechanism of enantioselectivity, we investigated the effects of DM on global gene transcription. We selected root tissue for this analysis because DM was absorbed by the roots in this research. Given that transcription is more sensitive than physiology or biochemistry, rice seedlings were treated with DM for only 1 week before the total RNA was extracted and subjected to microarray analysis. As shown in Figure S5 (Supporting Information), 1482 and 769 root genes met the criteria for up- or down-regulation after *R*- and *S*-DM exposure, respectively, as compared to the control. In plants treated with *R*-DM, 38.46% of the 1482 affected genes were up-regulated, and 61.54% were down-regulated, whereas in the plants treated with *S*-DM, 51.24% of the 769 affected genes were up-regulated, and 48.76% were down-regulated. The observation that *R*-DM affected more genes than *S*-DM demonstrates that there was enantioselective regulation of transcription.

We classified these differentially expressed genes according to their function by performing a homology search. Most are involved in starch or sucrose metabolism, oxidative phosphorylation, amino acid biosynthesis and metabolism, photosynthesis and carbon fixation, or other physiological processes. In a functional analysis, we observed that three genes (Os12g0274700, Os11g0171300, and Os12g0291100) in the carbon fixation pathway were up-regulated by *R*-DM, but not by *S*-DM. Two ribosomal-related proteins (Os05g0169100 and Os09g0258600) were up-regulated only by *R*-DM. One gene (Os01g0803800) belonging to the cytochrome P450 family was up-regulated 2.33-fold over the control after *R*-DM treatment, higher than the level induced by *S*-DM treatment. Another cytochrome P450 family protein (Os10g0515200) was up-regulated more than 2-fold over the control by *S*-DM

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Table 2. Changes in Gene Transcription after DM Enantiomer Treatment, As Analyzed by Microarray and Real-Time PCR

		R-DM control		*S*-DM control	
gene bank	annotation	fold change by microarray	fold change by real-time PCR	fold change by microarray	fold change by real-time PCR
Os01g0185200	glutamyl-tRNA synthetase	0.45	0.36	0.69	0.47
Os01g0681900	NADH-dependent glutamate synthase precursor	0.31	0.62	0.45	0.46
Os03g0836800	IAA-amino acid hydrolase	0.43	0.22	0.46	0.89
Os01g0854800	P450-dependent fatty acid omega-hydroxylase	0.55	0.81	0.48	0.30
Os03g0318500	precursor of glucose-6-phosphate 1-dehydrogenase	0.36	0.46	0.89	0.65
Os03g0643300	arginine and proline metabolism urea cycle and metabolism of amino groups	0.40	0.17	0.67	0.25
Os10g0515200	cytochrome P450 family protein	1.65	1.77	2.04	2.37
Os01g0803800		2.33	3.2	1.94	1.88

treatment. Many genes were down-regulated only by *R*-DM, including Os06g0131300, Os01g0185200, and Os06g0131400 (glutamate metabolism), Os03g0318500 (pentose phosphate pathway), Os01g0791500 (photosynthesis), and Os09g0255400 (phenylalanine, tyrosine, and tryptophan biosynthesis) (Supporting Information, Tables S2 and S3).

Validation of the Microarray Results by Real-Time PCR. Eight genes were used to validate the microarray results by real-time PCR. We found that the results obtained from the real-time PCR analysis approximated the results of the microarray (Table 2). Two glutamate metabolism-related genes were selected for this analysis: glutamyl-tRNA synthetase (Os01g0185200) and NADH-dependent glutamate synthase precursor (Os01g0681900), which is the major enzyme involved in the assimilation of NH_4^+.[38] Both were down-regulated by *R*- and *S*-DM, as determined by microarray and real-time PCR analysis. A decrease in the transcript levels of these two genes after *R*- or *S*-DM treatment is expected to inhibit glutamate synthesis and block NH_4^+ assimilation.[39] Os03g0836800 encodes IAA-amino acid hydrolase, which hydrolyzes IAA-amino acid conjugates, producing free IAA and promoting plant development.[40] In the microarray analysis, the Os03g0836800 transcript levels were down-regulated to approximately 50% of the control after both *S*- and *R*-DM treatments, but the real-time PCR analysis showed that it was down-regulated to 22% of the control after *R*-DM treatment, lower than that observed after *S*-DM treatment. We therefore speculated that the active IAA content might be lower in the *R*-DM-treated group than in the control or *S*-DM-treated groups, causing *R*-DM-treated seedlings to grow slowly. This is consistent with the results on plant growth, where the *R*-DM treatment had the strongest inhibitory effect on seedling root length (Table 1). Os01g0854800 encodes P450-dependent fatty acid omega-hydroxylase, which can catalyze fatty acids into hydroxyl-fatty acids.

Hydroxyl-fatty acids are considered passive constituents of cutin, which is the first barrier that protects plants against chemical or biological stress.[41,42] The transcription of this gene (Os01g0854800) was down-regulated by DM enantiomer treatment, as determined by both the microarray and the real-time PCR analyses. Down-regulation of the Os01g0854800 gene is expected to result in a decrease in hydroxylated fatty acids, which can induce the accumulation of defensive substances[41,43] by an unknown mechanism.[39]

Os03g0318500 encodes the precursor of glucose-6-phosphate 1-dehydrogenase (G6PDH) in the chloroplast. The transcription of Os03g0318500 was down-regulated to less than 50% of the control after *R*-DM treatment, which was lower than observed after *S*-DM treatment. This enzyme catalyzes the oxidation of Glc-6-phosphate (G6P) to 6-phosphogluconolactone and is regarded as the key enzyme controlling the oxidative pentose-phosphate pathway.[44] The main function of the pentose-phosphate pathway is to produce NADPH and other intermediates, such as pentose and erythrose-4-phosphate.[45] NADPH is a limiting factor in the incorporation of acetyl-CoA into fatty acids,[46] and pentose and erythrose-4-phosphate are the precursors for the biosynthesis of lignin, aromatic amino acids, nucleic acids, and coenzymes, which are potentially involved in the plant response to stress.[44] Therefore, we speculate that DM inhibited G6PDH transcription and decreased the NADPH content, which in turn limited the rate of fatty acid synthesis. Similarly, several types of xenobiotics can modulate the activity of G6PDH, such as metal compounds, salt stress, and pesticides.[46−48]

Os03g0643300 transcript levels were also down-regulated by DM treatment in an enantioselective manner: *R*-DM displayed a stronger inhibitory effect than *S*-DM. By sequence clustering, we found that Os03g0643300 has aminotransferase and pyridoxal phosphate binding motifs. The DM-induced down-regulation of this gene likely affects amino acid metabolism and the urea cycle. Os10g0515200 and Os01g0803800 are part of the cytochrome P450 protein family, which is a large and diverse group of enzymes that are present in all domains of life, including plants, animals, fungi, bacteria, and even viruses. Cytochrome P450s play a primary role in detoxifying herbicides (reviewed by Siminszky[49]). Transcript levels of Os10g0515200 and Os01g0803800 were up-regulated by approximately 2−3-fold after DM treatment, as determined by both microarray and real-time PCR analyses. This *R*- and *S*-DM-induced up-regulation of genes involved in detoxification is reminiscent of a report by Forthoffer et al.,[50] who demonstrated that cytochrome P450 is involved in the detoxification of diclofop. Interestingly, they determined that diclofop has strong effects on the total P450 levels.

This study analyzed the enantioselective and phytotoxic effects of DM on rice at the physiological and metabolic levels. DM clearly inhibited seedling growth, especially root development, in an enantioselective manner. DM also decreased carbon assimilation and disturbed electron transport, increased ROS accumulation, and affected various metabolic pathways in the enantioselective manner. All of these results demonstrated that *R*-DM had a stronger herbicidal effect than *S*-DM. Therefore,

using the active enantiomer (such as *R*-DM) alone would decrease the amount of total pesticides' usage, and reduce the release of thousands of tons of inactive enantiomer into environment, which may have adverse impacts on nontarget species.

■ ASSOCIATED CONTENT

Supporting Information

Table S1: The sequences of the primer pairs used for real-time PCR. Table S2: Genes up-regulated in rice root tissue after DM enantiomer exposure. Table S3: Genes down-regulated in rice root tissue after DM enantiomer exposure. Figure S1: The phenotype of rice seedlings subjected to DM enantiomer treatment for 1 (A) or 2 weeks (B). Figure S2: The number of protoplasts observed under an optical microscope after DM enantiomer treatment: (A) control; (B) *R*-DM; (C) *S*-DM; (D) racemate. Figure S3: Superoxide radical (A) and hydrogen peroxide (B) accumulation after DM enantiomer treatment, detected by NBT and DAB staining, respectively. Figure S4: The effect of DM enantiomers on MDA content. "**" indicates that the values are significantly different as compared to the control ($p < 0.01$). "##" indicates that the values are significantly different as compared to the *S*-DM-treated plants ($p < 0.01$). Figure S5: The number of differentially expressed genes in rice root tissue: (A) The number of up-regulated genes; (B) the number of down-regulated genes. 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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■ ABBREVIATIONS USED

APX, ascorbate peroxidase; CAT, catalase; CSD, Cu/Zn-SOD gene; DM, diclofop methyl; DAB, 3,3′-diaminobenzidine; ETR, electron transport rate; FSD, Fe-SOD gene; F_v/F_m, maximum quantum yield of PSII; G6P, Glc-6-phosphate; G6PDH, glucose-6-phosphate 1-dehydrogenase; GPX, glutathione peroxidase; IM, imazethapyr; MDA, malondialdehyde; MS, Murashirrg and Skoog; MSD, Mn-SOD gene; NADPH, nicotinamide adenine dinucleotide phosphate; NBT, nitroblue tetrazolium; QA^-, reduced electron acceptor quinone molecule; qP, photochemical quenching; ROS, reactive oxygen species; SEM, standard error of the mean; SOD, superoxide dismutase; ΦPSII, quantum yield of photosystem II

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