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Original Article

Physiological effects of mandestrobin

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Mandestrobin is a novel and potent fungicide with a methoxyacetamide structure, and inhibits complex III on the mitochondrial respiratory chain of fungi. It is widely accepted that some fungicides, including Q_OIs and SDHIs, have additional physiological effects on treated plants. In this study, we evaluated the physiological effects of mandestrobin both in the field and the laboratory. Mandestrobin treatment increased the yield of *Brassica napus* by an average of 6.3% in the field under disease-free conditions. Mandestrobin treatment delayed chlorophyll degradation and the senescence of *B. napus* leaf discs, and excised *Arabidopsis thaliana* leaves in darkness. Analyses of transcriptome and gene ontology enrichment of mandestrobin-upregulated genes showed that chlorophyll degradation genes and jasmonate-related genes were downregulated while salicylate-related genes were upregulated by mandestrobin treatment. A possible mechanism by which mandestrobin triggered the physiological effects observed in the field and the laboratory was discussed.

Keywords: mandestrobin, fungicide, physiological effect.

Electronic supplementary materials: The online version of this article contains supplementary materials (Supplemental Fig. S1, Table S1), which are available at http://www.jstage.jst.go.jp/browse/jpestics/.

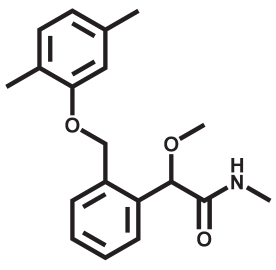
Introduction

Mandestrobin is a novel and potent fungicide with a methoxyacetamide structure (Fig. 1). It shows good fungicidal efficacy against a broad spectrum of agriculturally important plant pathogens, including the Sclerotiniaceae and Venturiaceae families. Its fungicidal efficacy is based on the fungal respiration suppression through the inhibition of cytochrome bc_1 complex (Complex–III) at the quinone outside (Q_O) site on the respiratory chain of plant pathogens. Furthermore, mandestrobin shows both preventive and curative (post infection) efficacy against plant diseases.[1)]

Fungicides are invaluable tools for securing crop yield in modern agriculture. In addition to their fungicidal effects, some fungicides—including Q_O inhibitors (Q_OI) and succinate dehydrogenase inhibitors (SDHI)—have been reported to have additional effects on the physiology of treated plants both in field and greenhouse conditions.[2–8)] For example, it has been reported that nitrate uptake was increased in pyraclostrobin-treated wheat (*Triticum aestivum*) compared to untreated plants.[2)] Also, azoxystrobin treatment of wheat reportedly delayed plant senescence and reduced oxidative stress.[3)] Foliar application of benzovindiflupyr, an SDHI, suppressed transpiration of treated plants and increased grain yield as compared to untreated plants.[4)]

Although it is widely accepted that Q_OI fungicides probably have beneficial physiological effects, it is still not clear whether all Q_OI fungicides have positive physiological effects on treated plants and how they interact with plants to cause such effects. Understanding the physiological effects of Q_OI fungicides is important because it will contribute to maximizing crop yields and achieving higher productivity. In addition, the physiological effects will give growers benefits of using preventive fungicides even in the absence of disease pressure. This can happen because outbreaks of some plant diseases are observed in a patchy fashion, and some areas are eventually found to be disease free.

The objective of this work is to reveal the beneficial effects of mandestrobin on plant physiology. To this aim, we first evalu-



Mandestrobin

Fig. 1. The chemical structure of mandestrobin.

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ated the effect of mandestrobin on the yield of *Brassica napus* in the field under disease-free conditions. We then evaluated the effect of mandestrobin on dark-induced leaf senescence as well as chlorophyll (Chl)-degradation gene expression in the laboratory. We also analyzed its effect on the expression of salicylate (SA)- and jasmonate (JA)-responsive genes, using a model plant, *Arabidopsis thaliana*. A possible mechanism by which mandestrobin triggered the physiological effects observed in the field and the laboratory will be discussed.

Materials and Methods

1. Chemicals and plant materials

Mandestrobin was synthesized at Health & Crop Sciences Research Laboratory, Sumitomo Chemical Co., Ltd. (Hyogo, Japan). *Brassica napus* plants were grown at 22°C in long-day conditions (100 μmol photons $m^{-2} s^{-1}$, 16 hr light/8 hr dark). For dark-induced senescence experiments, leaf discs were prepared from the third and fourth leaves. The leaf discs were floated on mandestrobin solution containing 0.1% DMSO in petri dishes, and incubated in the dark for six days at 18°C.

Arabidopsis thaliana plants were grown at 22°C in long-day conditions (100 μmol photons $m^{-2} s^{-1}$, 16 hr light/8 hr dark). For dark-induced senescence experiments, excised leaves were floated on mandestrobin solution containing 0.1% DMSO in petri dishes and incubated in the dark for four days at 22°C. For mandestrobin spray treatment, *A. thaliana* plants just after bolting were sprayed with 1000 ppm mandestrobin solution containing 10% *N*,*N*-dimethylformamide (DMF) and 0.1% Tween 20. After two and seven days, leaves were collected and used for RNA extraction. More than five leaves were collected and pooled as one sample and used for RNA extraction.

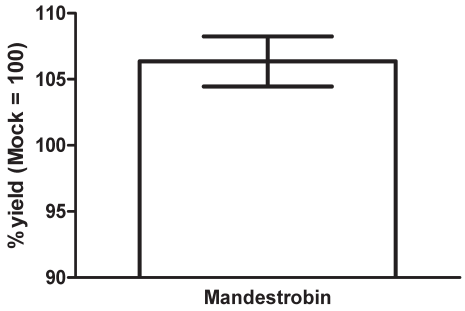

Fig. 2. The effect of mandestrobin treatment on *B. napus* yield under disease-free conditions. The yield of the mock treatment in each field trial was set to 100, and the relative yield of the mandestrobin treatment was calculated. Data are the average±standard error (n=21).

2. B. napus *field trials*

Field trials of *B. napus* were conducted between 2010 and 2017 in European countries, namely France, the United Kingdom, Austria, Sweden, and Germany. Mandestrobin was sprayed at a rate of 200 g active ingredient/ha. Twenty-one trials in disease-

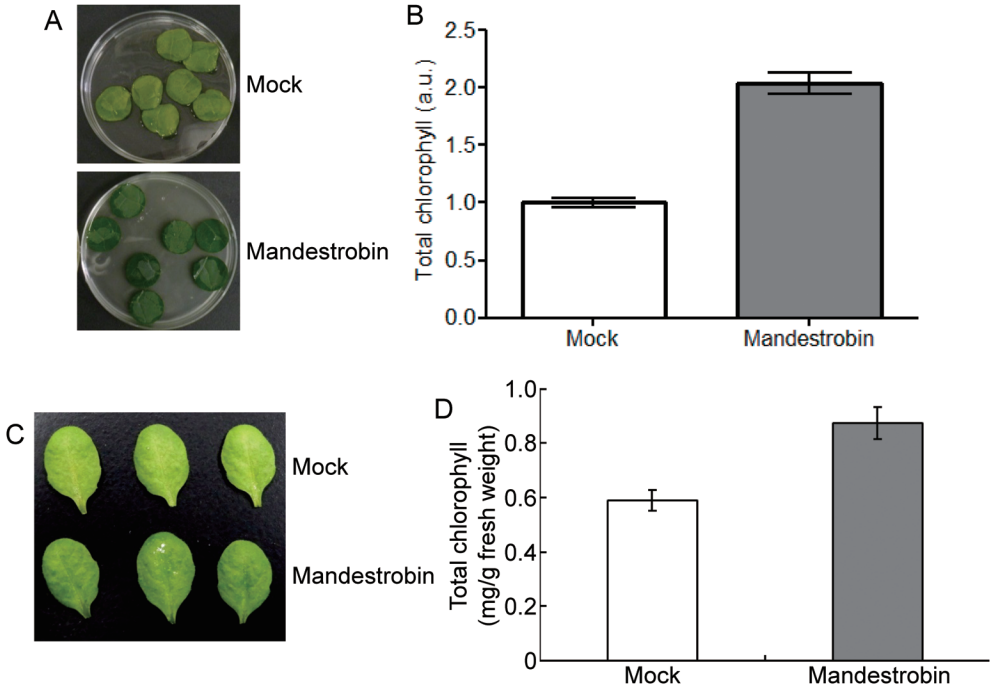

Fig. 3. The effect of mandestrobin on dark-induced senescence and Chl degradation. (A) The leaf discs of *B. napus* were dark incubated for six days in the absence (upper) or presence (lower) of 100 μM mandestrobin. (B) Chl was extracted from leaf discs shown in (A), and the relative total Chl content was quantified. Error bars represent standard errors (n=4). The difference was statistically significant (Student's t-test, $p<0.01$). (C) The excised leaves of *A. thaliana* were dark incubated for four days in the absence (upper) or presence (lower) of 100 μM mandestrobin. (D) Chl was extracted from the excised leaves shown in (C), and the total Chl content was quantified. Error bars represent standard errors (n=6). The difference was statistically significant (Student's t-test, $p<0.01$).

free conditions—*i.e.*, plants without fungicide treatment were not visibly affected by plant disease—were selected, and the yield data were recorded.

3. Chl content measurements

Chl was extracted from the *B. napus* leaf discs and *A. thaliana* detached leaves after dark incubation by submerging them in DMF at 4°C overnight in darkness. The absorbances at 646.8 and 663.8 nm of the DMF solution were recorded, and the Chl concentration was calculated with the equation described by Arnon.[9)]

4. Gene expression analysis

Total RNA extraction from *A. thaliana* excised leaves was conducted using RNeasy Plant Mini Kit (QIAGEN) in accordance with the manufacturer's protocol. More than five leaves from the same treatment were pooled and used for the RNA extraction. *A. thaliana* Oligo DNA Microarray Ver.4.0 (Agilent Technologies) was used to analyze the global gene expression of the samples. Fluorescent probe labeling, hybridization, and scanning were performed in accordance with the manufacturer's instructions. Genes with a change greater than twofold and a *p*-value of less than 0.05 in the mandestrobin-treated samples as compared to mock samples were considered to be significantly upregulated genes, and the upregulated gene list was used for gene ontology enrichment analysis. Gene ontology enrichment analysis was conducted using PANTHER[10)] (http://www.pantherdb.org/).

Reverse transcription of RNA and quantitative PCR were conducted with ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan) and Luna Universal qPCR Master Mix (New England BioLabs, Beverly, MA, USA), respectively, in accordance with the manufacturers' protocols. The sequences of the primers used in qRT-PCR analysis are listed in Supplementary Table 1. *ACTIN2* was used as a reference gene.

Results

1. Mandestrobin treatment increased B. napus *yield in disease-free conditions in the field*

In order to evaluate the physiological effects of mandestrobin on *B. napus*, the yield of mandestrobin-treated plants was compared to that of nontreated plants in field trials. In this analysis, results of the field trials in nondisease conditions—*i.e.*, untreated plants were visibly free from disease pressure—were used because mandestrobin has fungicidal effects and it is difficult to distinguish its physiological effects from fungicidal effects in the presence of disease pressure. In the 21 field trials with no disease pressure conducted in France, the United Kingdom, Austria, Sweden, and Germany between 2010 and 2017, mandestrobin treatment increased *B. napus* yields by an average of 6.3% as compared to untreated plants (Fig. 2); this increase was statistically significant (Wilcoxon matched-pairs signed rank test, $p<0.01$).

It has been reported that some Q_OIs delayed the leaf senescence of treated plants, leading to extended green leaf area duration and increased yield.[2,3,5,7)] Therefore, the effect of mandestrobin in combination with tebuconazole on SPAD values of *B. napus* top leaves was evaluated. Treatment significantly increased SPAD values by 27%, indicating that a larger amount of Chl was retained in the top leaves of treated plants than in those of mock-treated plants (Fig. S1). The yield increase in field trials and the higher SPAD values suggested that mandestrobin treatment triggered beneficial physiological effects on treated plants.

2. Mandestrobin treatment delayed Chl degradation of B. napus *and* A. thaliana *in darkness*

In order to evaluate the physiological effects of mandestrobin in the laboratory, we evaluated the effect of mandestrobin treatment on dark-induced senescence using *B. napus* leaf discs. Mandestrobin treatment strongly suppressed the Chl degradation of *B. napus* leaf discs during dark incubation after six days (Figs. 3A, B). We also evaluated the effect of mandestrobin on detached leaves of *A. thaliana*, because *A. thaliana* is a widely used model plant and, like *B. napus*, it belongs to the Brassicaceae family. The suppression of Chl degradation by mandestrobin treatment after dark-induced senescence was also observed in detached leaves of *A. thaliana* (Figs. 3C, D). These data suggested that the physiological effects of mandestrobin could also be evaluated as to its ability to suppress Chl degradation in dark-induced senescence in the laboratory.

3. The expression of Chl degradation genes was downregulated by mandestrobin treatment

As described above, mandestrobin treatment suppressed dark-

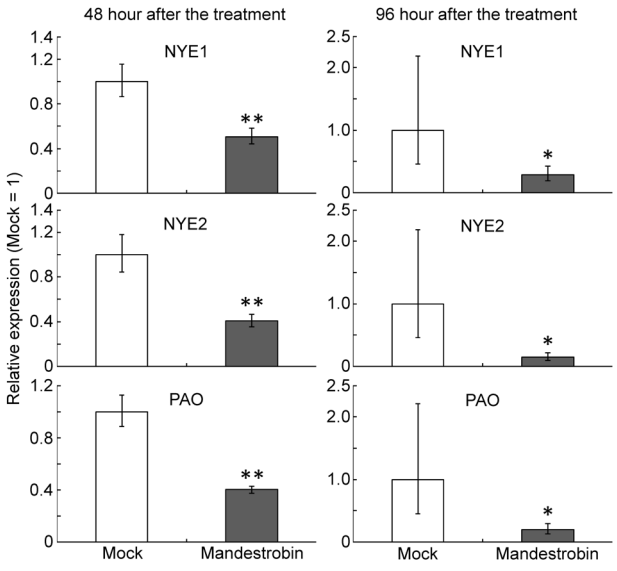

Fig. 4. The effect of mandestrobin on Chl-degradation gene expression. Total RNA was extracted from excised leaves of *A. thaliana* after 48 or 96 hr of dark incubation in the absence or presence of 100 μM mandestrobin, and relative gene expression was quantified by qRT-PCR. *Actin2* was used as a reference gene. Error bars represent 95% confidence intervals ($n=3$). Asterisks indicate statistical difference between mock- and mandestrobin-treated plants (Student's *t*-test, * $p<0.05$; ** $p<0.01$).

Table 1. GO terms that were significantly enriched in the mandestrobin-upregulated genes

No.	GO term	Count	FDR
1	Defense response to bacterium	37	3.4E-15
2	Response to bacterium	21	5.8E-11
3	Response to chitin	22	1.2E-09
4	Response to salicylic acid	23	3.0E-09
5	Protein phosphorylation	51	2.6E-07
6	Defense response to bacterium, incompatible interaction	12	2.2E-06
7	Defense response	40	1.3E-05
8	Response to oxidative stress	22	1.9E-03
9	Systemic acquired resistance	9	3.0E-03
10	Plant-type hypersensitive response	11	5.4E-03

induced Chl degradation in *B. napus* and *A. thaliana*. It is conceivable that the suppressed Chl degradation was related to the downregulation of Chl degradation gene expression. To test this hypothesis, the gene expression level of *NONYELLOWING 1* and *2* (*NYE1* At4g22920 and *NYE2* At4g11910) and *PHEOPHORBIDE A OXYGENASE* (*PAO* At3g44880) 48 and 96 hr after treatment were analyzed by quantitative RT-PCR (qRT-PCR).[11–13] After 48 hr of mandestrobin treatment, the expression of *NYE1*, *NYE2*, and *PAO* were significantly downregulated as compared to that after mock treatment (Fig. 4). The downregulation of the three genes by mandestrobin was more prominent after 96 hr than after 48 hr (Fig. 4). These data suggest that the physiological effects—including Chl degradation suppression by mandestrobin in darkness—were possibly related to the transcriptional level control.

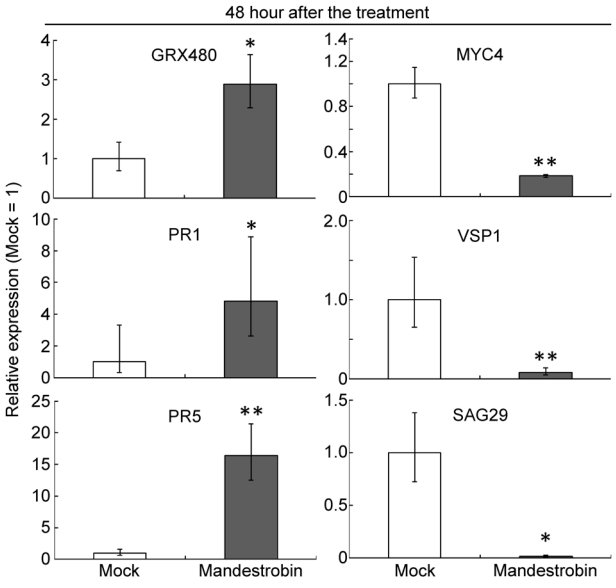

Fig. 5. The effect of mandestrobin on SA- and JA-responsive gene expression. Total RNA was extracted from excised leaves of *A. thaliana* after 48 hr of dark incubation in the absence or presence of 100 μM mandestrobin, and relative gene expression was quantified by qRT-PCR. *Actin2* was used as a reference gene. Error bars represent 95% confidence intervals (n=3). Asterisks indicate statistical difference between mock- and mandestrobin-treated plants (Student's t-test, * $p<0.05$; ** $p<0.01$).

4. Gene ontology enrichment analysis of upregulated genes suggests that mandestrobin treatment induced SA-related gene expression

To gain further insights into how mandestrobin treatment induced physiological effects, we analyzed the effects of mandestrobin on the global gene expression profile in detached leaves of *A. thaliana*. For the microarray analysis, samples collected after 48 hr of treatment were used because we were interested in gene expression modulation before a senescence event was visibly recognizable. Upregulated genes in the mandestrobin-treated samples as compared to mock samples were subjected to gene ontology (GO) enrichment analysis.[10] The GO term *Response to Salicylic Acid* was present in the top 10 GO term list with 23 counts and a false discovery rate (FDR) of 3.04×10^{-9} (Table 1). In addition, most of the other GO terms in the list were considered to be closely related to the SA response, suggesting that mandestrobin treatment induced SA-responsive gene expression.

5. SA-related genes were upregulated, while JA-related genes were downregulated by mandestrobin treatment

In order to confirm that the mandestrobin treatment upregulated SA-responsive gene expression, these gene expressions were quantified by qRT-PCR. Glutaredoxin 480 (GRX480) and pathogenesis-related proteins 1 and 5 (PR1 and PR5) were selected as SA-responsive genes that were upregulated in the microarray analysis by mandestrobin treatment. In accordance with the microarray analysis, these three genes were significantly upregulated after 48 hr of mandestrobin treatment (Fig. 5). Because it has been reported that GRX480 suppressed JA-related gene expression,[14] and it is widely accepted that SA signaling antagonizes JA signaling, we then focused on JA-responsive gene expression. As JA-responsive genes, MYC4, Vegetative Storage Protein 1 (VSP1), and Senescence-Associated Gene 29 (SAG29) were selected. These three genes were downregulated after 48 hr of mandestrobin treatment (Fig. 5).

In addition to the effect on detached leaves, the effect of mandestrobin on gene expression was also analyzed using

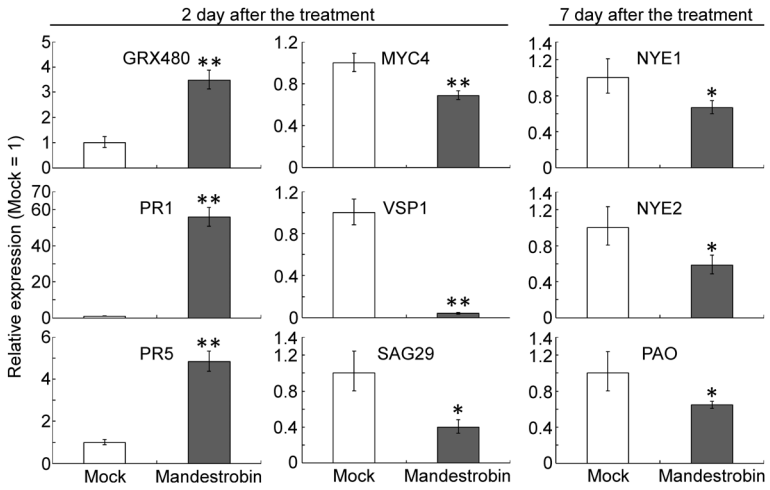

Fig. 6. The effect of mandestrobin on SA-responsive, JA-responsive, and Chl-degradation gene expression in intact *A. thaliana* plants. Total RNA was extracted from leaves of *A. thaliana* two or seven days after treatment with mandestrobin spray, and relative gene expression was quantified by qRT-PCR. *Actin2* was used as a reference gene. Error bars represent 95% confidence intervals ($n=3$). Asterisks indicate statistical difference between mock- and mandestrobin-treated plants (Student's *t*-test, * $p<0.05$; ** $p<0.01$).

mandestrobin-sprayed *A. thaliana* intact plants. SA-responsive genes GRX480, PR1, and PR5 were significantly upregulated, while JA-responsive genes MYC4, VSP1, and SAG29 were significantly downregulated in mandestrobin-treated *A. thaliana* plants, as compared to mock-treated plants (Fig. 6). Seven days after treatment, the expression of Chl degradation genes (NYE1, NYE2, and PAO) were downregulated in mandestrobin-treated plants (Fig. 6). Therefore, gene expression responses in the mandestrobin-treated plants were similar to those observed in the detached leaves.

Discussion

Mandestrobin is a potent strobilurin fungicide that inhibits complex III on the mitochondrial respiratory chain.[1] In this study, the physiological effects of mandestrobin were evaluated in the field in disease-free conditions. A significant increase of *B. napus* yield (6.3% on average) with mandestrobin treatment as compared to mock treatment was observed (Fig. 2). Furthermore, treatment with mandestrobin in combination with tebuconazole significantly increased the Chl content of *B. napus* top leaves (Fig. S1). These results indicate that using mandestrobin is beneficial even in the absence of apparent disease pressure. Several studies have reported that treatment with Q_OI fungicides increased Chl content, delayed leaf senescence, and enhanced crop yield both in the greenhouse and the field, suggesting that prolonged green leaf area duration and, possibly, concomitant increased photosynthesis activity contributed to the increased yield.[2,5,15] The results presented in this study suggest that mandestrobin also affected treated plants in a manner similar to other Q_OI fungicides.

In order to clarify how mandestrobin triggered physiological effects in plants, we analyzed the effect of mandestrobin treatment on the gene expression of *A. thaliana*. Gene expression analysis showed that mandestrobin treatment downregulated the expression of Chl-degradation genes, both in excised leaves after dark incubation and in intact plants (Figs. 4 and 6). Gene ontology enrichment analysis of genes upregulated by mandestrobin treatment revealed that SA-related genes were significantly overrepresented (Table 1). Quantitative RT-PCR analysis confirmed the upregulation of SA-related genes (GRX480, PR1, and PR5) and also showed that JA-related genes (MYC4, VSP1, and SAG29) were downregulated by mandestrobin treatment (Figs. 4, 5). Köhle *et al.* reported that the pretreatment of tobacco leaves with pyraclostrobin solution by infiltration accelerated the induction of PR1-gene expression after tobacco mosaic virus infection.[2] The authors proposed that pyraclostrobin treatment stimulate SA signaling possibly *via* nitric oxide formation, leading to suppression of JA signaling and leaf senescence. In this study, we showed that mandestrobin treatment alone upregulated SA-related genes and downregulated JA-related genes. Although SA is known to promote leaf senescence, the low concentration of SA repressed methyl JA-induced leaf senescence.[16] Among the upregulated SA-related genes, GRX480 is a

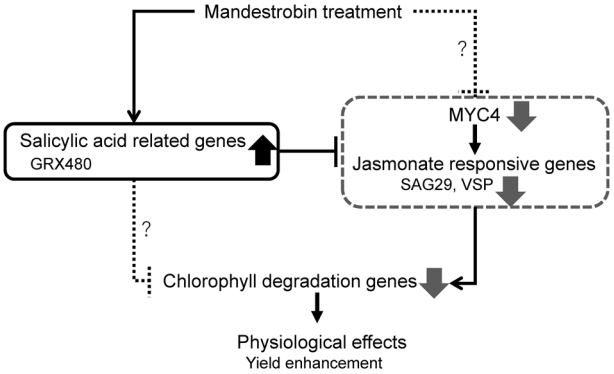

Fig. 7. The possible mechanism of mandestrobin physiological effects. For details, please refer to the discussion section.

glutaredoxin known to suppress JA signaling.[14] Therefore, the upregulation of GRX480 by mandestrobin treatment seems to be involved in the downregulation of JA-related gene expression. MYC2/3/4 transcription factors bind to the G-box in the *PAO* promoter and induce its expression,[17] so MYC4 downregulation by mandestrobin may lead to Chl-degradation gene downregulation.

The possible mechanism of mandestrobin's physiological effects is proposed in Fig. 7. Mandestrobin induces SA-related gene expression and downregulates JA-related gene expression. JA-related genes are possibly downregulated through SA-JA antagonistic control, and SA-induced GRX480 may be involved in this process. The downregulation of JA-related genes, including MYC4, seems to lead to the downregulation of Chl-degradation genes. Consequently, mandestrobin treatment leads to beneficial physiological effects, such as increased Chl content and yield enhancement. Presently, we cannot exclude the possibility that the SA-independent pathway contributes to the physiological effects. For example, mandestrobin might directly repress JA-related genes. Another possibility is that SA-related genes might be directly involved in Chl-degradation gene expression. Further studies are required to fully elucidate the contribution of each pathway to the physiological effects of mandestrobin.

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