The velvet family proteins mediate low resistance to isoprothiolane in

*Magnaporthe oryzae*

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Abstract

Isoprothiolane (IPT) resistance has emerged in Magnaporthe oryzae, due to the long-term usage of IPT to control rice blast in China, yet the mechanisms of the resistance remain largely unknown. Through IPT adaptation on PDA medium, we obtained a variety of IPT-resistant mutants. Based on their EC$_{50}$ values to IPT, the resistant mutants were mainly divided into three distinct categories i.e., low resistance (LR, 6.5 ≤ EC$_{50}$ < 13.0 μg/mL), moderate resistance 1 (MR-1, 13.0 ≤ EC$_{50}$ < 25.0 μg/mL), and moderate resistance 2 (MR-2, 25.0 ≤ EC$_{50}$ < 35.0 μg/mL). Molecular analysis of MoIRR (Magnaporthe oryzae isoprothiolane resistance related) gene demonstrated that it was associated only with the moderate resistance in MR-2 mutants, indicating that other mechanisms were associated with resistance in LR and MR-1 mutants. In this study, we mainly focused on the characterization of low resistance to IPT in M. oryzae. Mycelial growth and conidial germination were significantly reduced, indicating fitness penalties in LR mutants. Based on the differences of whole genome sequences between parental isolate and LR mutants, we identified a conserved MoVelB gene, encoding the velvet family transcription factor, and genetic transformation of wild type isolate verified that MoVelB gene was associated with the low resistance. Based on molecular analysis, we further demonstrated that the velvet family proteins VelB and VeA were indispensable for IPT toxicity and the deformation of the LaeA-VeA-VelB heterotrimer played a vital role for the low IPT-resistance in M. oryzae, most likely through the down-regulation of the secondary metabolism-related genes or CYP450 genes to reduce the toxicity of IPT.

Keywords: Magnaporthe oryzae, Isoprothiolane, low resistance, velvet family transcription factor, secondary metabolism
Author summary

Isoprothiolane (IPT) resistance has emerged in *Magnaporthe oryzae*, due to the long-term usage of IPT to control rice blast in China, yet the mechanisms of the resistance remain largely unknown. Here, we explored the mechanisms of low IPT resistance in *M. oryzae*. Combining the whole genome sequencing and genetic transformation, we identified a conserved *MoVelB* gene, encoding the velvet family transcription factor to be a determinant for IPT toxicity. We further demonstrated that the deformation of the LaeA-VeA-VelB heterotrimer conferred the low IPT-resistance in *M. oryzae*, most likely through down-regulating the secondary metabolism-related genes or CYP450 genes to reduce the toxicity of IPT. This study improved our understanding of the resistance mechanism as well as the mode of action of IPT which will be helpful for making suitable strategies to manage the emerging resistance of IPT in *M. oryzae*.

Introduction

*Magnaporthe oryzae* is the causal agent of rice blast, the most destructive disease of rice worldwide. Each year it causes significant yield losses to rice production, enough to feed 60 million people [1]. *M. oryzae*, previously known as *Magnaporthe grisea*, is a filamentous ascomycete fungus, which attacks rice at all stages of growth and infects seedlings, leaves, nodes, panicles and grains [2]. Routine use of fungicides to control crop diseases, including rice blast has become an important part of modern agriculture, helping to increase crop yields, improve quality and ensure stable production [3]. However, intensive monocropping and long-term usage of single-site fungicides provide an favorable environment for the development of fungicide resistance [4].

Isoprothiolane (IPT), an agricultural fungicide developed by Nihon Nohyaku Co., Ltd. in 1974, is a systemic fungicide in the dithiolanes class with both protective and curative effects for controlling rice blast disease [5]. In China, IPT has been routinely used to control leaf blast and panicle blast for 1-3 times a season. It is not
only effective in controlling rice blast, but is also used as insecticide to control rice plant hopper [6], and as plant-growth regulator to promote the growth of plant roots [7]. IPT inhibits the methylation of phosphatidylethanolamine to phosphatidylcholine, and is thus considered as a choline biosynthesis inhibitor with cross resistance with organophosphorus fungicides such as iprobenfos [8, 9]. Resistance to IPT (also known as Fuji One) in M. oryzae has already been detected in rice fields in recent years [10], but little is known about the molecular basis of resistance. Lab IPT-resistant mutants did not possess changes in the DNA sequence and expression level of genes related to the synthesis of phosphatidylcholine [11], indicating the existence of resistance mechanisms unrelated to the putative target enzyme. Interestingly, mutations in a Zn$_2$Cys$_6$ transcription factor MoIRR, resulted in a moderate level of resistance to IPT in M. oryzae [12]. Our research group found that most of the IPT resistance in field isolates were unstable, and only showed low resistance (LR). Thus, we adapted the wild type isolate H08-1a on PDA amended with IPT in a series of concentrations and obtained a variety of resistant mutants with different levels of resistance. The objective of this study was to explore the resistance mechanisms of low resistance to IPT in M. oryzae.

**Results**

**Induction of IPT-resistant mutants in M. oryzae**

To explore the mechanism of IPT resistance, the wild type isolate H08-1a was adapted on PDA amended with IPT at 3, 5, 10, 30 μg/mL. Mycelium fanning out from inoculation plugs situated on IPT-amended medium was transferred in form of mycelial plugs (3 mm in diameter) onto PDA for 6 consecutive generations (one generation: six days on fungicide free PDA followed by another six days on IPT-amended PDA). We found that the proportion of stable resistant mutants increased with increasing concentrations of IPT (Fig 1A). A total of 77 IPT-resistant mutants were obtained with EC$_{50}$ values between 7.5-45 μg/mL. Except for the mutant 30-50 which was considered as highly resistant (HR; EC$_{50}$ value 43.55 μg/mL), the
resistant mutants were divided into three distinct categories, i.e., LR (6.5 ≤ EC$_{50}$ < 13 μg/mL), MR-1 (13 ≤ EC$_{50}$ < 25 μg/mL), and MR-2 (25 ≤ EC$_{50}$ < 35 μg/mL) containing 10, 35, and 31 mutants, respectively. There were significant differences of EC$_{50}$ values between different resistance categories (Fig 1B). Even at low dose IPT exposure, multiple phenotypic resistant mutants readily emerged, indicating a high risk of resistance emergence upon frequent exposure to IPT in fields.

Positive cross-resistance between IPT and iprobenfos (IBP) has been established in the past. Thus, we determined the sensitivity of the resistant mutants to IBP to clarify whether the IPT mutants showed resistance to IBP. Unexpectedly, the R-value of the linear regression of the EC$_{50}$ values of IPT and IBP was 0.4939, indicating that the IPT-resistant mutants were not significantly cross-resistant to IBP (Fig 1C). These results suggested that IPT and IBP may not have the same mode of action.

**Fitness assessment of IPT-resistant mutants**

To assess the fitness of IPT-resistant mutants, mycelial growth rate, melanin production, conidia, conidial germination and virulence were evaluated. Compared with the wild-type isolate H08-1a, the LR mutant showed significantly reduced mycelial growth, conidial germination, increased sporulation, and increased melanin production (Fig 2A-2D), while other mutants did not show significant changes for any of the investigated phenotypes (Fig 2). The virulence of the LR mutant was comparable with parental isolate and the other mutants (Fig 2). These results suggest that mutants with different IPT phenotypes possess different resistance mechanisms.

To further characterize IPT-resistant mutants, we determined the sensitivity of different mutants to seven common fungicides with different modes of action. IPT-resistant mutants were more sensitive than isolate H08-1a to fungicides associated with osmotic pathway regulation, i.e., the antibiotics rapamycin (RAP), PP fungicide fludioxonil (FLU), and DCF fungicide iprodione (IPR), although each mutant had specific sensitivity profiles (Fig 2F). The resistant mutants did not differ in their sensitivity to other fungicides when compared to parental isolate H08-1a (Fig 2F).
Fludioxonil has been shown to control IPT-resistant MoIRR mutants [13]. In this study, we further characterized the response of IPT-resistant mutants cultured on 5 μg/mL IPT and 5 μg/mL FLU. Results showed that FLU could effectively inhibit the mycelial growth of all types of resistant mutants, and the combination of IPT and FLU had better control efficacy than IPT or FLU alone (Fig 2G).

**Mutations in MoIRR were mainly associated with moderate IPT-resistance**

The MoIRR (MGG_04843) gene was sequenced from all 77 IPT resistant-mutants. It was found that 31 mutants containing MoIRR mutations showed moderate resistance to IPT and grouped into the MR-2 resistance phenotype (Fig 3A, 3B), while the LR mutants did not reveal mutations in MoIRR gene (Table S1). MR-2 mutants accounted for 40% of all resistant mutants, and the main types of mutation in MoIRR gene were frameshifts and substitutions (Fig 3C). The mutation sites were located in exons and introns, but they were mainly concentrated in the fourth exon that corresponded to the Fungal_TF_MHR domain (fungal transcription factor regulatory middle homology region) (Fig 3D, 3E). Nevertheless, resistance to IPT was similar among MoIRR mutants with different mutation types and that mutations in MoIRR caused moderate but not low IPT resistance in M. oryzae.

**The velvet family protein MoVelB was associated with the IPT low resistance**

To further explore the mechanism of low IPT resistance in M. oryzae, we performed whole-genome sequencing, then SNP and InDel analysis for the LR mutant 3-15. As shown in Table 1, we obtained 6 candidate IPT-resistance genes, including three transcription factor encoding genes MGG_01620, MGG_01870, and MGG_02962, a metalloproteinase encoding gene MGG_15370, a linker histone family protein encoding gene MGG_12797, and a helicases encoding gene MGG_16993.

MGG_01620 encodes the developmental regulation protein VelB, which usually forms a complex with members of the velvet factor family and is involved in fungal growth, development and secondary metabolism. It was found that the CAG (glutamine) at the 305th codon (Q305X) of the MoVelB gene was substituted to the terminator (TAG) in the LR mutant 3-15 (Fig 4A). Phylogenetic analysis showed that
VelB was conserved in filamentous fungi and contained a DNA binding velvet domain (Fig 4B). Further investigation of other LR mutants showed that MoVelB mutations could be detected from 60% of the LR mutants and that the main types of mutation were frameshifts (Fig S1F).

To explore the resistance mechanisms in the LR mutants, we generated knockout transformants of six candidate genes and investigated their corresponding resistance phenotypes. MoVelB (MGG_01620) knockout transformants exhibited significantly increased IPT resistance, just like the resistance level in LR mutant 3-15 (Fig 4C, Fig S1B), transformants of other candidate genes did not show significantly different sensitivity to IPT compared to parental isolate H08-1a.

Transformation of the 2k promoter region and the coding region of MoVelB into the knockout transformant ΔVelB-2 restored the IPT sensitivity to wild type level (Fig 4C), in addition, OEVelB transformants increased sensitivity to IPT (Fig S1D), indicating that MoVelB negatively regulates the low IPT resistance in M. oryzae.

To further clarify the molecular mechanism by which MoVelB negatively regulates the resistance to IPT in M. oryzae, we examined the expression of MoVelB when subjected different concentrations of IPT together with impact on MoIRR expression, a gene related to moderate IPT resistance. Results showed that the expression of MoVelB was significantly suppressed at high concentrations of IPT (Fig 4D), and that the knockout of MoVelB did not affect the expression of MoIRR (Fig 4E).

Neither the knockout nor the overexpression of MoIRR affected the expression of MoVelB (Fig 4F), indicating that the down regulation of MoVelB upon IPT exposure is a novel mechanism, different from the MoIRR regulatory pathway.

Biological functions of MoVelB in M. oryzae

To investigate whether MoVelB is involved in the growth and development of M. oryzae, the colony morphology of the different strains were investigated, and the mycelial growth rates were measured. Compared to parental isolate H08-1a, knockout transformants showed fluffy colonies (Fig S2A), produced fewer conidia, and the mycelial growth rate decreased on the CM, MM, PDA or OTA media. The
complemented transformants restored the colony growth rate and sporulation (Fig 5A, 5B). These results indicated that VelB played important roles in regulating mycelial growth and sporulation. MoVelB knockout transformants produced significantly more melanin when cultured in PDB for 6 days compared to the parental isolate or the complemented transformant (Fig 5C, Fig S2B), indicating VelB plays a negative regulation role in the synthesis of melanin.

To determine whether VelB is involved in response to environmental stresses, H08-1a, ΔVelB, ΔVelB-C were inoculated onto PDA plates amended with different stress agents, including osmotic stresses NaCl, KCl and SOR, cell wall/cell membrane stresses SDS, CFW and CR, and oxidative stress H₂O₂. After incubation at 27°C for 5 days, ΔVelB showed a decreased tolerance to H₂O₂ and increased tolerance to SOR, but no difference was observed upon other stresses in comparison to the wild-type H08-1a and ΔVelB-C transformant (Fig 5D, Fig S2C). Catalase gene Cat3 plays an important role in oxidative stress response. As expected, the expression of Cat3 was significantly reduced in ΔVelB transformants (Fig 5E), indicating that MoVelB plays an important role in response to oxidative stress by regulating the expression of Cat3 in M. oryzae.

To further characterize whether VelB-related IPT-resistant mutants are also resistant to other fungicides, we tested the sensitivity of ΔVelB transformants to iprobenfos (IBP), fludioxonil (FLU), iprodione (IPR), tebuconazole (TEB), azoxystrobin (AZO), boscalid (BOS), carbendazim (CAR). Compared to parental isolate H08-1a and complemented transformant ΔVelB-C, ΔVelB showed resistance to the organophosphorus fungicide IBP (Fig 5F, Fig S2D). In addition, ΔVelB is remarkably sensitive to PP fungicide FLU and DCF fungicide IPR (Fig 5F, Fig S2D). FLU and IPR are thought to stimulate MAP kinases in osmotic signaling, leading to excessive glycerol accumulation and consequent cell death. As shown in the Fig 5G, 5H, we found that MoVelB was involved in FLU and IPR tolerance by regulating the expression of Gpd1 and Gpp1 which are participated in glycerol synthesis in M. oryzae.
Velvet family proteins were indispensable for toxicity of IPT

The velvet family proteins include MoVeA, MoVelB, MoVelC and MoVosA in *M. oryzae*, all of which have a conserved velvet domain (Fig 6A). The velvet complex is a class of global transcription factors involved in secondary metabolism, growth and development in filamentous fungi. To characterize the functional roles of velvet genes in IPT-resistance of *M. oryzae*, corresponding knockout transformants ΔVeA, ΔVelC, and ΔVosA were generated through PEG mediated protoplast transformation. Results showed that the IPT-resistance in ΔVeA was comparable to that of ΔVelB and that ΔVosA and ΔVelC also showed resistance to IPT, but at lower levels than ΔVelB (Fig 6B). It was rather intriguing to note that ΔVeA also showed reduced tolerance to H₂O₂, FLU and IPR and increased resistance to IBP, similar with that appeared in ΔVelB (Fig S3). These results suggest that MoVeA and MoVelB may jointly be involved in the regulation of IPT toxicity and oxidative stress in *M. oryzae*.

The expression pattern of *MoVeA* under IPT stress was determined by using RT-qPCR. In contrast to *MoVelB*, the *MoVeA* expression was induced by IPT (Fig 6C). The interaction between MoVelB and MoVeA for IPT toxicity was further demonstrated by using the yeast two-hybrid method. The results showed that MoVelB interacted with MoVeA (Fig 6D), suggesting that MoVelB determines IPT toxicity by a direct interaction with MoVeA.

The cytosolic protein LaeA can form heterotrimers with the VelB and VeA complexes in the dark to co-regulate secondary metabolism, growth and development processes in *Aspergillus nidulans* and *MoLaeA* (*MGG_01233*) has been reported to be involved in the regulation of tenuazonic acid synthesis in *M. oryzae*. In the current study, yeast two-hybrid assay showed that MoLaeA can directly interact with MoVeA, demonstrated that MoLaeA formed a heterotrimer complexes with the MoVeA and MoVelB through direct interactions (Fig 6E). Knockout of *MoLaeA* caused defects in vegetative growth and colony pigmentation on PDA compared with the parental isolate, and ΔLaeA exhibited low resistance to IPT, comparable to resistance levels found for ΔVeA and ΔVelB (Fig 6F). These results suggested that the deformation of the heterotrimer LaeA/VeA/VelB was involved in the low IPT-resistance in *M. oryzae*. 
Identification of a regulatory network of VelB-VeA-LaeA heterotrimers

To further verify the involvement of VelB-VeA-LaeA heterotrimers for the IPT toxicity in *M. oryzae* through the regulation of secondary metabolism, genome-wide expression of wild-type H08-1a and *ΔVelB* responding to IPT was analyzed by RNA-seq. There were 1717 differentially expressed genes (DEG) in *ΔVelB*, of which 657 were up-regulated and 1060 were down-regulated, 4 h after IPT treatment (Fig 7A). Analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway showed that the DEGs were mainly enriched in the metabolic pathway, with the most significant enrichment in the synthesis of secondary metabolites (Fig 7B). Heat map with the DEGs in biosynthesis of secondary metabolites was shown in Figure 7C. Meanwhile, RT-qPCR confirmed that the DEGs in *ΔVelB* showed similar expression patterns in *ΔVeA* and *ΔLaeA*, indicating that the VelB-VeA-LaeA heterotrimer significantly regulated secondary metabolism-related genes under IPT pressure. Particularly, five DEGs were down regulated in *ΔVelB*, similar results were observed in *ΔVeA* and *ΔLaeA* (Fig 7D). These data suggested that the deformation of VelB-VeA-LaeA heterotrimer determined the low IPT resistance, most likely through down-regulating secondary metabolism-related genes to reduce the toxicity of IPT.

**Discussion**

IPT, tricyclazole and azoxystrobin are the most important chemicals for controlling rice blast in China. As an effective and low-toxic fungicide, IPT is used for the prevention and treatment of rice blast. However, it can easily bring about the emergence of resistance under long-term usage, and the frequency of resistance to IPT resistant isolates has been more than 50% in Liaoning Provinces in China [14]. In this study, we investigated the mechanism of resistance and assessed the risk of resistance to IPT in *M. oryzae*.

Very little is known about the molecular basis of IPT resistance in *M. oryzae* or any other plant pathogenic fungus. The Zn₂Cys₆ transcription factor MoIRR was shown to be associated with isoprothiolane resistance in *M. oryzae* due to the frameshifts and SNPs in the *MoIRR* gene [12]. In this study, the IPT resistance
mechanisms were further investigated. It was found that the \textit{MoIRR} point mutants including Q48Stop, A72V, H213Y, L346F, R413H, F544S, W562Stop, Stop743Y confer moderate resistance. In mutants with low resistance, no \textit{MoIRR} mutations were observed, indicating that other mechanisms determine low IPT resistance in \textit{M. oryzae}.

In recent years, isolates with low IPT resistance and lacking variations in the \textit{MoIRR} gene, have been reported in Liaoning and Jiangsu provinces in China [10, 14]. Meanwhile we identified a large number of low resistance mutants without mutations in \textit{MoIRR}. Therefore, it is important to explore the molecular mechanisms of low resistance in \textit{M. oryzae}. Based on whole-genome sequencing, the velvet transcription factor MoVelB was identified to be associated with the low resistance in the LR mutants. Genetic transformation proved that knockout of \textit{MoVelB} gene resulted in the development of resistance. It has been reported that VelB plays crucial roles in the fungal development, production of conidia and pigments in \textit{A. nidulans} [15], \textit{B. cinerea} [16], \textit{C. lunata} [17], \textit{M. oryzae} [18], \textit{V. dahlia} [19], \textit{V. mali} [20]. In addition, VelB is indispensable for virulence in several filamentous phytopathogenic fungi [21]. For the first time, we found that MoVelB negatively regulated low IPT resistance, and the regulatory mechanism of MoVelB on IPT resistance is different from the regulatory pathway of MoIRR which confers moderate resistance in \textit{M. oryzae} [12].

Velvet family proteins usually function as homodimers or heterodimers. Nuclear entry of VelB requires the formation of a heterodimer with VeA and the assistance of \(\alpha\)-importin KapA protein. In the dark, VeA-VelB heterodimers enter the nucleus, promote sexual fruiting body formation [22]. VelB also forms homodimers and participates in asexual reproduction [23]. LaeA can bind VeA and VelB to form heterodimers that participate in secondary metabolism [24, 25]. We also found that \(\Delta VeA\) and \(\Delta LaeA\) showed low resistance to IPT, with similar levels of resistance to \(\Delta VelB\). Furthermore, we verified by yeast two-hybrid assay that MoVeA could interact with MoVelB and MoLaeA, respectively, but MoVelB could not interact with MoLaeA. Thus, it was proposed that the heterotrimer LaeA-VeA-VelB was generated through MoVeA interacted with MoVelB and MoLaeA, respectively. Through the
deformation of the heterotrimer, the downstream genes involving secondary metabolisms were down regulated to reduce the toxicity of IPT and caused the corresponding low IPT resistance in *M. oryzae* (Fig 8).

NF-κB is a protein heterodimer consisting of p50 and RelA, which could be activated by many types of cellular stresses, leading to the promotion of cell survival [26]. The velvet proteins with a DNA binding domain structurally similar to that of NF-κB p50, VelB and VeA heterodimer can activate p450 BapA expression in response to stimulation of the five-ring PAH BaP (benzo[a]pyrene) in *A. nidulans* [27]. Interestingly, DEGs with monooxygenase activity were highly enriched by Gene Ontology analysis, with 18 p450 proteins being expressed by MoVelB activation under IPT stress (Fig S3), suggesting that the regulation of low IPT resistance may also be realized through similar mechanism (Fig 8).

In conclusion, we explored the mechanisms involved in the low IPT resistance in *M. oryzae*. Genomic sequencing combined with genetic transformation demonstrated that the low resistance was negatively regulated by the *MoVelB* gene. Further molecular analysis showed that another velvet protein MoVeA interacted with MoVelB and MoLaeA to form the heterotrimer LaeA-VeA-VelB, which regulated the genes involving secondary metabolisms to achieve the toxicity of IPT, deformation of the heterotrimer LaeA-VeA-VelB conferred the low IPT resistance, most likely through down-regulating the secondary metabolism related genes to reduce the toxicity of IPT in *M. oryzae*.

**Materials and Methods**

**Mutants, media and fungicides**

The *M. oryzae* strains including wild type isolate H08-1a and resistant mutants are listed in Table S1. All mutants were cultured on potato dextrose agar (PDA) medium for 5 days at 27 °C in the dark. For vegetative growth, 3 mm × 3 mm mycelial plugs from the periphery of freshly cultured mutants were inoculated onto complete medium (CM), minimal medium (MM), PDA or Tomato Oat Agar (OTA). The fungicide IPT was dissolved in acetone to make stock solution at the concentration of 8000 µg a.i.
Sensitivity to IPT was assessed on PDA amended with IPT at 0, 1, 2, 5, 10, 30 and 50 µg/mL. Growth inhibition was calculated, and regression against the logarithm of fungicide concentrations was analyzed to obtain EC_{50} values. *Escherichia coli* JM109 was grown in LB medium with ampicillin (100 µg/mL) for plasmid amplification. Sensitivity to rapamycin (Rap), fludioxonil (FLU), iprodione (IPR), tebuconazole (TEB), azoxystrobin (AZO), boscalid (BOS), carbendazim (CAR) was assessed on PDA amended with corresponding fungicides at concentrations 0.15, 5, 20, 0.4, 10, 20, 0.35 µg/mL, respectively.

**Sequence analysis of MoIRR gene in resistant mutants**

*MoIRR* gene was identified previously to be associated with IPT resistance [12]. Therefore, the primer pair MoIRR-Check-F/R was designed based on the MG8 genome to amplify the full-length of *MoIRR* gene in resistant mutants to detect the presence of mutations in *MoIRR* (Table S2).

**Whole genome sequencing**

Cetyltrimethylammonium bromide (CTAB) method was used to extract genomic DNA from the LR mutant 3-15. Genome sequencing was conducted on the Illumina HiSeq 4000 PE150 platform using 150 bp paired-end libraries with 500 bp inserts at Wuhan SeqHealth Technology Company. We used Lofreq (version 2.1.5) software to perform SNP and InDel assays. In order to reduce unnecessary mutation sites, we specified strict screening conditions: 1. The mutation appears in the open reading frame region; 2. The mutation can only appear once in a gene; 3. The candidate gene is expressed in the hyphal growth stage; 4. The candidate gene is not mutated in the 1a_mut genome because the colony morphology of 3-15 is completely different from that of 1a_mutant, a mutant with moderate resistance to IPT.

**Phylogenetic analysis**

The amino acid sequences of the VelB and BLAST Servers at
NCBI from *Magnaporthe* genome (https://www.ncbi.nlm.nih.gov/genome/51706) were used. Phylogenetic trees were constructed by comparing the identified amino acid sequences using MEGA7.0 and the neighbor-joining method (number of bootstrap replications were 1000). Protein domain architecture analysis was performed by a Conserved Domains Database search (https://www.ncbi.nlm.nih.gov/cdd).

**Genetic manipulations including knockout, complementation and overexpression**

As mutations were identified in *VelB* (*MGG_01620*), genetic transformation was carried out to validate the roles of *VelB* in IPT resistance. Double-joint PCR was used to generate the knockout constructs of *M. oryzae* *VelB* [28]. Three knockout transformants for each gene were obtained, confirmed, and one transformant was randomly selected from each group for further analysis. To generate complemented transformants of the Δ*VelB* knockout transformant, a full *VelB* genomic region, including its upstream 2-kb region, was inserted into the plasmid pGTN for transformation. Overexpression transformants were obtained through the construct including the *VelB* -coding region and the 1.5 kb promoter region of histone 3 in the plasmid pTNHG. Genetic transformation was conducted by using PEG-mediated protoplast transformation. At the same time, other members of velvet family, i.e., *VeA* (*MGG_08559*), *VelC* (*MGG_14719*) and *VosA* (*MGG_00617*), as well as the VeA-interaction protein encoding gene *LaeA* (*MGG_01233*) were also knocked out to demonstrate the contribution of these genes in IPT resistance.

**Stress measurement**

To test sensitivity against different stresses, mycelial growth was assayed after incubation at 27 °C for 5 days on PDA plates and PDA amended with 0.7 M NaCl, 0.7 M KCl, 1.2 M sorbitol (SOR), 0.015% SDS (w/v), 1200 μg/mL Congo red (CR) or 1200 μg/mL calcofluor white (CFW), 6 mM H₂O₂.

**RNA preparation and qRT-PCR**
Mycelia from the relevant strains were collected at indicated conditions and times, frozen rapidly in liquid nitrogen and stored at -80°C until further use. Total RNA isolation was conducted by using TRIzol. cDNA was prepared using a HifairII 1st Strand cDNA Synthesis kit (YEASEN Biotech Co., Ltd) with oligo (dT). Reverse transcription quantitative PCR (RT-qPCR) was performed with ChamQTM SYBR® qPCR Master Mix (Vazyme biotech co., Lth) on a Bio-Rad CFX96 real-time PCR detection system. The comparative cycle threshold (CT) method was used for data analysis and relative fold difference was expressed as \( 2^{-\Delta\Delta CT} \) [29]. As an internal reference, primers for MoActin were used for each quantitative real-time PCR analysis. Primer sequences are shown in Table S2.

**Yeast two-hybrid analysis**

The full-length cDNA sequences of VelB, VeA, and LaeA were amplified to verify the potential interactions among VelB, VeA, and LaeA using Y2H assay. The cDNAs of VelB and VeA were respectively inserted into the EcoRI site of the pGBK7 vector containing the GAL4 binding domain, and the VeA and LaeA cDNA was inserted into the EcoRI site of the pGADT7 vector containing the yeast GAL4 activation domain. The plasmid pairs of pGBK7-VelB/pGADT7-VeA and pGBK7-VelB/pGADT7-LaeA, pGBK7-VeA/pGADT7-LaeA were co-transformed into the AH109 using the LiAc/Carry-DNA/PEG3350 transformation method [30]. The plasmid pairs of pGADT7/pGBK7-53 and pGADT7/pGBK7-Lam served as the positive and negative controls, respectively.

**RNA sequencing**

RNA sequencing was conducted on the Illumina HiSeq 4000 PE150 platform using 150 bp paired-end libraries with 500 bp inserts at Wuhan SeqHealth Technology Company. Transcriptome data quality was controlled using fastp (version 0.23.0) and over 35 million high-quality reads per sample were achieved. The RPKM (Reads per Kilobase per Million Reads) value used as a measure of gene expression, the gene was considered as a differentially expressed gene, when \( \log_2(\text{FoldChange}) \) \( \Delta \)
VelB_RPKM/H08-1a_RPKM)) > 1 or < -1 and p-value < 0.05 [31]. KEGG and GO analyses were performed using the DAVID Bioinformatics Resources online website (https://david.ncifcrf.gov/home.jsp) [32].

Statistical analysis
Statistical differences were evaluated by one-way ANOVA with Duncan’s Multiple Range tests in SPSS for Windows Version 19.0. Graphs were produced using GraphPad Prism 8. Statistical tests used for each reported experiment are detailed within figure legends.

Supporting information
S1 Table. Characteristics of Magnaporthe oryzae strains used in this study.
S2 Table. Primers used in this study.
S3 Table. Origin of mutants associated with Magnaporthe oryzae.
S1 Fig. Generation and identification of MoVelB knockout, complementation, overexpression transformants and determination of sensitivity to IPT. (A) Gene replacement strategy of MoVelB and identification of MoVelB knockout transformants by PCR. (B) Sensitivity analysis of MoVelB knockout transformants to IPT. Data presented are the mean ± SD (n=3). Bars followed by the same letter are not significantly different according to a LSD test at P = 0.01. (C) Gene complementation strategy of MoVelB and identification of MoVelB complementation transformants by RT-PCR. (D) Sensitivity analysis of MoVelB overexpression transformants to IPT. Data presented are the mean ± SD (n=3). Bars followed by the same letter are not significantly different according to a LSD test at P = 0.01. (E) Detection of VelB expression in MoVelB overexpression transformants by western blot. (F) Detection of variations of MoVelB in low resistant mutants.
S2 Fig. Biological functions of velvet family gene VelB. (A) Colony morphology of VelB knockout and complemented transformants. (B) Melanin production in VelB knockout and complemented transformants. (C) Tolerance of VelB knockout and complemented transformants to different stresses. (D) Sensitivity of VelB knockout and complemented transformants to different fungicides. Strains were inoculated on different media with different stresses or fungicides at 27°C for 5 days. IBP, FLU, IPR, TEB, AZO, BOS, and CAR indicate the fungicides iprobenfos, fludioxonil, iprodione, tebuconazole, azoxystrobin, boscalid, and carbendazim, respectively.

S3 Fig. VelB involved in IPT resistance through the regulation of monoxygenases. (A) GO analysis of DEGs in VelB compared to H08-1a. (B) Expression heat map of differentially expressed monoxygenases in ΔVelB compared to H08-1a.

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Competing interests
The authors have declared that no competing interests exist. All authors contributed to the article and approved the submitted version.

References


Table 1. Identification of candidate genes for low IPT resistance in *M. oryzae*

<table>
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<tr>
<th>Gene ID</th>
<th>Type of mutation</th>
<th>cDNA changes</th>
<th>Protein changes</th>
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Figure legends

**Fig 1.** Isoprothiolane taming of *M. oryzae*. (A) Proportion of stable IPT resistant mutants at different taming fungicide concentrations. (B) The IPT resistant mutants exhibited significant differences in their resistance to IPT. Data presented are the mean ± SD (n=3). Bars followed by the same letter are not significantly different according to a LSD test at P = 0.05. (C) Detection of cross-resistance to IBP in IPT resistant mutants. Linear regression analysis was performed with EC\textsubscript{50} values of IPT and IBP for IPT-resistant mutants.

**Fig 2.** Assessment of fitness of different IPT resistant mutants. (A) Growth rate of IPT resistant mutants on PDA and OTA. (B) Sporulation of different IPT resistant mutants. (C) Conidial germination of different IPT resistant mutants. (D) Detection of melanin accumulation in IPT resistant mutants. (E) Pathogenicity test of different IPT resistant mutants. (F) Mycelial inhibition rate heat map of IPT resistant mutants to different fungicides. H08-1a and IPT resistant mutants were inoculated on PDA or PDA amended with 0.15 μg/mL rapamycin (RAP), 5 μg/mL fludioxonil (FLU), 20 μg/mL iprodione (IPR), 10 μg/mL azoxystrobin (AZO), 20 μg/mL boscalid (BOS), 0.35 μg/mL carbendazim (CAR), 0.4 μg/mL tebuconazole (TEB). (G) Control efficacy of IPT resistant mutants. H08-1a and IPT resistant mutants were inoculated on PDA or PDA amended with 5 μg/mL IPT, 5 μg/mL FLU, combination of 5 μg/mL IPT and 5 μg/mL FLU. Data presented are the mean ± SD (n=3). Bars followed by the same letter are not significantly different according to a LSD test at P = 0.01.
Fig 3. Mutation of MoIRR is one of the main causes of IPT resistance in *M. oryzae*. (A) Mutations of MoIRR gene in IPT resistant mutants. (B) Percentage of mutants with MoIRR mutations in all resistant mutants. (C) Mutation types in MR-2 mutants. (D) Mutation sites of MoIRR gene in MR-2 mutants. (E) Distribution of mutations in different domains in MR-2 mutants. Bars followed by the same letter are not significantly different according to a LSD test at P = 0.01.
Fig 4. MoVelB negatively regulates the low resistance to IPT in *M. oryzae*. (A) Identification of IPT resistance loci in the LR mutant 3-15. (B) Phylogenetic tree of MoVelB homologous proteins. The amino acid sequences of proteins in phylogenetic tree were retrieved from the NCBI database. Domains were aligned with ClustalW, and the tree was constructed with the neighbor-joining method. The VelB velvet domain was indicated as green boxes. (C) Knockout transformant ΔVelB (ΔVelB-2) displayed low resistance to IPT. A 3-mm mycelial plug of each strain was inoculated on PDA or PDA amended with 4, 6, 8 μg/mL IPT and then incubated at 27°C for 5 days (top panel), and the mycelial growth inhibition was calculated for each strain (below panel). (D) Expression of MoVelB in wild type isolate H08-1a at different concentrations of IPT. (E) Expression of MoIRR in different strains with or without IPT. (F) Expression of MoVelB in wild type isolate H08-1a, MoIRR knockout (ΔIRR-1) and overexpression (OEIRR-1) transformants with or without IPT. Ace: acetone, the solvent of IPT; IPT: isoprothiolane. The MoActin gene was used as the internal reference for normalization. Data presented are the mean ± SD (n = 3). Bars followed by the same letter are not significantly different according to a LSD test at P = 0.01.
**Fig 5.** MoVelB regulates mycelial growth, sporulation, melanin synthesis, and oxidative stress. (A) Comparisons in colony morphology among the parental isolate H08-1a, ΔVelB and the complemented transformant ΔVelB-C grown on CM, MM, PDA, or OTA, mycelial growth rate was calculated accordingly. (B) Sporulation of different types of strains. (C) Production of melanin of different strains cultured in PDB for 7 days. (D) The H08-1a, ΔVelB and ΔVelB-C strains were incubated on PDA amended with different stress agents at 27 °C for 5 days and statistical analysis of the growth inhibition rate. (E) RT-qPCR analyses of the expression of Cat3 in ΔVelB, compared to H08-1a. The MoActin gene was used as the internal reference for normalization. (F) The H08-1a, ΔVelB and ΔVelB-C strains were incubated on PDA amended with different fungicides at 27 °C for 5 days and statistical analysis of the growth inhibition rate. (G, H) RT-qPCR analyses of the expression of Gpd1, Gpp1 in ΔVelB, compared to H08-1a. The MoActin gene was used as the internal reference for normalization. Data presented are the mean ± SD (n=3). Bars followed by the same letter are not significantly different according to a LSD test, lower case letters indicate the p-value <0.05 and capital letters indicate the p-value <0.01.
Fig. 6. Involvement of velvet family proteins in resistance to IPT in *M. oryzae*. (A) Velvet family proteins contain a velvet structural domain identified using the NCBI protein database (https://www.ncbi.nlm.nih.gov/cdd). (B) Knockout of Velvet family proteins encoding genes leads to reduced sensitivity to IPT. (C) Expression of *MoVeA* at different concentrations of IPT. The *MoActin* gene was used as the internal reference for normalization. (D) The yeast two-hybrid (Y2H) assay revealed that *MoVeA* interacted directly with *MoVelB*. The plasmid pairs of pGADT7/pGBK7T7-53 and pGADT7/pGBK7T7-Lam served as the positive and negative controls, respectively. Coordinated involvement of the heterotrimer LaeA-VeA-VelB in regulating the low resistance to IPT in *M. oryzae*. (E) The yeast two-hybrid (Y2H) assay revealed that *MoLaeA* interacted directly with *MoVeA*, but not with *MoVelB*. The plasmid pairs of pGADT7/pGBK7T7-53 and pGADT7/pGBK7T7-Lam served as the positive and negative controls, respectively. (F) Knockout of LaeA reduced sensitivity to IPT. Data presented are the mean ± SD (n = 3). Bars followed by the same letter are not significantly different according to a LSD test at P = 0.01.
Fig 7. Involvement of heterotrimers in IPT resistance is possibly through regulation of secondary metabolism in *M. oryzae*. (A) Volcano plot analysis of DEGs (log$_2$(fold change $\geq 1$ or $\leq -1$ and P value of $\leq 0.05$) in $\Delta$VelB, compared to H08-1a. (B) KEGG enrichment analysis of DEGs in $\Delta$VelB under IPT pressure. (C) Head map analysis of DEGs (log$_2$(fold change $\geq 1.5$ or $\leq -1.5$)) in biosynthesis of secondary metabolites. (D) Regulation of DEGs in biosynthesis of secondary metabolites by MoVelB, MoVeA, MoLaeA determined by RT-qPCR. Data presented are the mean ± SD ($n = 3$). Statistical significance was determined using Student’s t test with a two-tailed distribution and two-sample unequal variance, *, P $\leq 0.05$; **, P $\leq 0.01$. 
**Fig 8.** Model of velvet family proteins in regulating low IPT resistance in *M. oryzae*. VelB, VeA, LaeA, and KapA indicate the MoVelB, MoVeA, MoLaeA, and MoKapA, respectively.