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Title: Heterokaryotic state of a point mutation (H249Y) in SDHB protein drives the evolution of thifluzamide resistance in *Rhizoctonia solani*

Running title*: Evolution of thifluzamide resistance in *R. solani

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ps.6155

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Abstract

BACKGROUND: The sheath blight, caused by *Rhizoctonia solani*, can be effectively controlled by the application of the succinate dehydrogenase inhibitor (SDHI) thifluzamide. Although the resistant risk of thifluzamide in *R. solani* had been reported, but the thifluzamide-resistance mechanism and the evolution of thifluzamide-resistance in *R. solani* have not been investigated in detail.

RESULTS: No differences were found between the sequences of the SDHA, SDHC and SDHD protein among the thifluzamide-sensitive isolates and the thifluzamide-resistant mutants, but a single point mutation H249Y was found in SDHB protein. Two different types of thifluzamide-resistant *R. solani* mutants were characterized: homokaryotic type, carrying only the resistance allele, and heterokaryotic type, retaining the wild-type allele in addition to the resistance allele. The resistance level differed according to nuclear composition at position of codon 249 in *sdhB* gene. Molecular docking results suggested that the point mutation (H249Y) might significantly altered the affinity of thifluzamide and SDHB protein. Heterokaryotic mutants were able to evolve into a homokaryon when repeatedly

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cultured on agar media or rice plants in the presence of thifluzamide, but thifluzamide treatment had no effect on the genotypes of the homokaryotic mutants or the sensitive isolates.

CONCLUSION: This study showed that H249Y in SDHB protein could cause thifluzamide-resistance in *R. solani*. Fungicide application could promote heterokaryotic mutants to evolve into a homokaryon.

1 INTRODUCTION

Rhizoctonia solani Kühn is a soil-borne pathogen that can cause economically important diseases in a broad range of plants including vegetables, field crops, and fruit and forest trees, as well as turf grasses and ornamental species grown throughout the world.[1-2] Disease symptoms can vary depending on the crop, from stalk rot in cereals and corn, and damping-off in cotton and soybean, to black scurf in potatoes, and root rot in sugar beet[3] In rice, *R. solani* causes sheath blight and can infect all stages of growth from seedling to heading, penetrating sheaths, leaves, and even panicles and stems when the temperature and humidity are high enough. Diseased plants are susceptible to lodging and produce reduced numbers of tillers and poorly filled grains.[4-5]

R. solani can be categorized into 14 anastomosis groups (AGs) based on their pattern of hyphal fusion (anastomosis), the dominant group globally being AG 1-IA.[5-9]

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Although *R. solani* occasionally produces haploid basidiospores in the field, it primarily exists as vegetative mycelia and asexual sclerotia.[10] The hyphae of *R. solani* lack septa and contain three or more nuclei per cell.[6] Genetic exchange and the formation of heterokaryons occur when the hyphae of two different isolates belonging to the same AG.[6,10,11] However, precise genetic studies regarding the reproductive characteristics of *R. solani* are difficult to conduct, because the movement of nuclei during anastomosis is difficult to control. Consequently, most investigations have focused on haploid protoplasts rather than single basidiospores. Such studies have confirmed that AG 1-IA can form both homokaryotic and heterokaryotic progeny.[12] In addition, changes to the protoplast-releasing procedure have been found to result in protoplasts that contain different numbers of nuclei, making them genetically different from their parental isolates.[6]

Although the antibiotic validamycin has been widely used to control soil-borne pathogens such as *R. solani*, the control efficiency is somewhat reduced due to resistance after years of use.[13-15] Furthermore, the use of validamycin in Europe has been prohibited in response to health and environmental concerns. Fortunately, several other groups of fungicides can also provide effective control of the disease caused by *R. solani*, including the benzimidazoles, triazoles, strobilurins, and succinate dehydrogenase inhibitors (SDHIs).[4, 16-18]

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SDHIs, which comprise 23 separate compounds, have been classified into eleven groups based on their chemical structures (FRAC, https://www.frac.info/). Several of the most recently discovered members having broad-spectrum activity against a wide range of pathogens.[19-22] The target site of SDHI fungicides is the succinate dehydrogenase (SDH) complex, which plays an important role in the mitochondrial tricarboxylic acid cycle, as well as being an important component of the respiratory chain.[23] The SDH complex is composed of four nuclear-encoded subunits, with two subunits (flavoprotein subunit A, SDHA and iron-sulfur subunit, SDHB) forming the membrane-peripheral domain, and two (integral membrane protein, SDHC and SDHD) forming the membrane-anchor domain.[24-25]

Since SDHI fungicides have a single mode of action, they are vulnerable to the development of resistance, and SDHI resistance has already been reported in several basidiomycete and ascomycete fungi in both laboratory and field studies.[21, 26-30] Several point mutations in the SDHB, SDHC, and SDHD subunits are now known to cause SDHI resistance, the most common being associated with the conserved histidine in the [3Fe-4S] center of SDHB (Table S1). However, reports had indicated that an additional serine at position 83–84 of the SDHC subunit can also lead to isopyrazam resistance in *Fusarium graminearum*,[28] and a replacement of phenylalanine by leucine at position 48 in the SDHC subunit was observed in

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flutolanil-resistant *R. solani* isolates.[31] Furthermore, non-target site SDHI resistance has been confirmed in *Zymoseptoria tritici*, indicating that different mechanisms of resistance associated with different SDHI fungicides can occur.[32]

In China, two SDHI fungicides, thifluzamide and flutolanil are firstly registered to control rice sheath blight in 1999 and 1989 (www.chinapesticide.org.cn), respectively. Thifluzamide, developed by Dow Agrosciences, belongs to the thiazole-carboxamide group of SDHIs, and has excellent protective and curative activity against rice sheath blight.[5, 33, 34] Interestingly, although thifluzamide had been used for almost 21 years, no field SDHIs-resistant *R. solani* isolates are detected in China according to some recent studies.[5, 35-38] According to our previous study, the risk of *R. solani* developing resistance to thifluzamide is low to moderate,[38] and a point mutation H249Y in SDHB protein was found in thifluzamide-resistant *R. solani* isolates.[39] Although *R. solani* does not produce haploid asexual spores and sexual sporulation is very difficult to induce, recombination through parasexuality or heterokaryon formation is possible.[40] Under natural conditions, *R. solani* produces haploid sexual spores (basidiospores), which can fuse to form heterokaryotic hyphae. The resulting multinucleate hyphae can contain three or more nuclei per cell, which may have the potential for greater genetic diversity and recombination than homokaryotic fungi. The resulting genetic diversity could therefore facilitate the evolution of thifluzamide resistance in *R. solani*,

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especially under strong selection pressures,[41-42] although the evolution of fungicide resistance in heterokaryotic fungi has not been studied in detail.

Based on our previous research, the aims of the current study were to (i) further investigate and verify the molecular mechanism of thifluzamide resistance in *R. solani* using molecular docking method, (ii) validate the probability of thifluzamide resistance evolving from heterokaryotic isolates of *R. solani in vitro* and *in planta*.

2 MATERIALS AND METHODS

2.1 Isolates and culture conditions

Ten sensitive isolates collected from infected rice plants in the Jilin, Guangxi, Guangdong, Fujian, and Shanghai provinces of China that had never been exposed to thifluzamide and nine resistant mutants that were obtained in a previous study by fungicide adaption or UV exposure were used.[38] All the experimental isolates were maintained by dark incubation on potato dextrose agar (PDA) at 25°C.

2.2 Fungicide sensitivity assays

The pure active ingredient thifluzamide (96%; Dow AgroSciences Company, China) was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution (1×10^5 μg/mL), which was stored at 4°C in the dark. Fungicide sensitivity assays were conducted according to our previous study.[38] Series of concentrations of thifluzamide (0, 0.01, 0.025, 0.05, 01, and 0.5 μg/mL for sensitive isolates; 0, 0.2, 0.5, 1.5, 5, and

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20 μg/mL for mutants with low to intermediate resistance; and 0, 0.5, 1.5, 5, 20, and 200 μg/mL for highly resistant mutants) were used.

2.3 Sequence and expression analysis of the *sdh* genes from *R. solani*

Genomic DNA was extracted using the FastDNA Plant Kit (Biomed Co. Ltd, Beijing, China), and total RNA extracted using the SV Total RNA Isolation System (Promega Corp., Beijing, China) according to the protocol of the manufacturers. The resulting RNA was then reverse transcribed to cDNA using the EasyScript Reverse Transcriptase Kit (TransGen Biotech, Beijing, China).

Primer sets (Table S2) were designed to amplify full-length sequences of the *sdh* genes using BioEdit v7.0.9 (Ibis Biosciences, USA) and DNAMAN software 6.0 (Lynnon BioSoft, Quebec, Canada) in conjunction with data from the draft genome of *R. solani* AG-3 (http://www.rsolani.org) and the *sdh* sequences from other species including *Coprinus cinereus*, *Ustilago maydis*, *Magnaporthe grisea*, *Botrytis cinerea*, *Escherichia coli*, *Homo sapiens*, and *Gallus gallus* (Table S3). The PCR was performed using Taq PCR MasterMix (TransGen Biotech, Beijing, China), and processed in a MyCycler™ thermocycler (Bio-Rad, California, USA). All the PCR products generated in the study were sequenced by Invitrogen Life Technologies (Beijing, China) and amino acid sequences deduced from the cDNA sequences using the DNAMAN software package. Introns were then removed from the *R. solani*

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sequences using data from the *C. cinereus sdh* genes and the software hosted on the Softberry website (http://linux1.softberry.com/berry.phtml).

The real-time qPCR was performed using the ABI7500 sequence detection system (Applied Biosystems, Warrington, UK) and the SYBR Premix Dimer Eraser kit (Takara Biotechnology Co.,Ltd., Dalian, China) with the primers listed in TableS3 and the protocol of the manufacturer. The relative quantities of the PCR products were calculated using the $2^{-\Delta\Delta Ct}$ method and the *actin* gene as a reference to normalize the quantification of *sdhB* expression. The entire experiment was conducted three times.

2.4 Molecular docking of thifluzamide in the SDH complex of *R. solani*

For docking studies of thifluzamide in the SDH complex, the crystal structure of SDH complex II from *Escherichia coli* (PDB code 2WDQ), which exhibits 45% sequence identity with the sequence from *R. solani* with all of the key amino acids being highly conserved, was used as the template.[43] The binding conformation of carboxin in the *E. coli* complex II produced docking results with a good overlap to that of the crystal structure of 2WDQ, and a root mean square deviation of 0.51, which indicated that it was a suitable model for the current study. The molecular docking of thifluzamide in the quinone-binding pocket of the *R. solani* SDH complex was investigated using Sybyl 7.3 software. To prepare the protein structure for docking, the crystal of SDH complex II from *E. coli* (PDB code 2WDQ) was firstly downloaded from PDB

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database (http://www.rcsb.org/). Then, addition of hydrogen atoms, side-chain protonation states, and adjustment of the rotational states of histidine, glutamine, asparagine residues were conducted to produce the docking model. In addition, the water near the binding pocket (between SDHB_S161 and SDHB_H207) was kept in the model as several hydrogen bonds were mediated by this water molecule. The binding-pocket receptor and thifluzamide were energy minimized using the MMFF94 electric charge and the Tripos force field; the energy value was less than 0.005 kcal/mol/Å, and the cycle index 1000. Having established that these parameters were suitable, both thifluzamide and carboxin were docked into the binding-pocket using the Surflex-dock module of the Sybyl 7.3 software. The histidine at position 207 of the *sdhB* gene from *E. coli* (the analogous position 249 in *sdhB* gene from *R. solani*) was then site-directed mutated to tyrosine with the Biopolymer-Replace Sequence module to investigate how the corresponding point mutation of the histidine residue at position 249 in the SDH of *R. solani* affects thifluzamide binding. The amino acids within approximately 10 Å of the point mutation were optimized using the MMFF94 electric charge and Tripos force field with the Dynamics module of the Sybyl 7.3 software package.

2.5 Production and regeneration of protoplasts

The thifluzamide-sensitive isolate JHT158-3 (homokaryotic for C at 975 of the *sdhB*

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gene) and the highly resistant mutant T2 (homokaryotic for T at 975 of the *sdhB* gene), were paired on PDA plates and dark-incubated at 25°C for two days. Ten mycelial plugs (5 mm in diameter) were then cut from the intersection of the two colonies and used to inoculate potato dextrose broth (PDB). After 24 hours of dark incubation at 25°C with shaking (120 rpm), the mycelium was removed with forceps, washed in an osmotic medium (0.98 M $MgSO_4$, 8.4 mM Na_2HPO_4, 1.6 mM NaH_2PO_4),[44] and ground with a sterile pestle and mortar before being transferred to a 250-mL flask containing 100 mL CM medium (5 g/L glucose, 5 g/L malt extract, 5 g/L yeast extract),[12] and dark-incubated at 25°C with shaking (120 rpm) for 24 hours. The mycelium was then collected by centrifugation at 1500 rpm for 10 minutes. After the supernatant had been removed, the mycelium was washed in 10 mL osmotic medium and centrifuged as before. The supernatant was again removed and the fungal cell wall digested using 15 mL of an enzyme mixture (20 mg/mL snailase, Biodee, China; 20 mg/mL cellulase and 20 mg/mL lysing enzymes, Sigma Chemical, China), which had been dissolved in STC (1.0 M sorbitol, 10 mM Tris-HCl, and 50 mM $CaCl_2$)[44] using a magnetic stirrer, centrifuged at 8,000 rpm and passed through a 0.22-µm filter. After four hours of incubation at 28°C with shaking (60 rpm), the undigested mycelium was removed by passing the preparation through two layers of Miracloth and washing with STC. The lysates containing the protoplasts were then centrifuged

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at 4,000 rpm for 10 minutes and resuspended in STC to a final concentration of 10^4 protoplasts/mL. The resulting suspension was used to inoculate a solid RM medium (200 g/L potato, 18 g/L dextrose, 182.17 g/L mannitol, 14 g/L agar) in 200-μL aliquots and dark incubated at 25°C for two to three days, before the hyphal tips derived from individual protoplasts were subcultured onto fresh PDA plates using a sterile knife.

2.6 Pyrosequencing assays

The pyrosequencing assays in the current study were performed using the PSQ 96MA system (Gene Limited Company, Shanghai, China). Biotin-labeled samples were prepared by PCR using 40-μL reaction mixtures containing 2 μL of template DNA or cDNA, 4 μL 10×PCR buffer, 0.8 μL dNTP (10 mM), 0.3 μL of each primer (10 μM), and 0.4 μL Takara Hot Start Taq. The PB-F1/PB-R1-Bio primer set was used for the amplification of genomic DNA (Table S2). The PCR was processed in a MyCycler™ thermocycler (Bio-Rad, California, USA). The biotinylated PCR products were then immobilized on streptavidin-coated Sepharose beads and washed with 70% ethanol before being sequenced using a PyroMark ID pyrosequencer with PSQ 96MA software and 0.5 μM sequencing primer (Table S2).

2.7 The potential for thifluzamide resistance to develop in *R. solani*: *in vitro* and *in planta* experiments

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The *in vitro* tests were conducted by repeated subculture (20 in total) of the parental isolates JHT158-3 and T2, and the nine protoplast-regenerated isolates on PDA containing either 0 or 0.05 μg/mL thifluzamide (the approximate EC_{50} value for thifluzamide in field isolates of *R. solani*). Each subculture was made after three days of dark incubation at 25°C by scraping the mycelia from the original plates onto fresh media to produce the next generation. The DNA from the first, fifth, 10th, 15th, and 20th generation were extracted for the pyrosequencing analysis described above. The experiment was performed twice.

A similar experiment was conducted by subculturing the same isolates on rice plants under greenhouse conditions. The initial inoculations were made using mycelial plugs (7 mm), which were cut from four-day PDA cultures and placed beneath the leaf sheath of rice plants (cultivar Jasmine 85) at the six to seven leaf stage. Thirty rice plants were inoculated with each isolate. The inoculated leaf sheaths were then wrapped in aluminum foil. After seven days under greenhouse conditions (28°C maintained at >80% humidity), when typical lesions had formed on the inoculated plants, the aluminum foil was removed and the plants were sprayed with the commercial thifluzamide formulation Pulsor (24% thifluzamide suspension concentrate) at a rate of 100.67 μg/mL (the minimum recommended field dose), using a larynx sprayer to ensure that the fungicide was dispersed uniformly on each plant.

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Negative control treatments were prepared by spraying inoculated plants with water. After two days, one small lesion was cut randomly from the plants in each treatment and surface sterilized in NaClO for fine minutes before being placed on PDA containing 50 μg/mL of both penicillin and streptomycin. Each sample was then dark-incubated at 25°C for three days to produce the inoculum for the successive subculture and the DNA samples for the pyrosequencing analysis described above. The experiment was performed twice.

2.8 Fitness of heterokaryotic thifluzamide-resistant isolates of *R. solani*

The fitness of 14 *R. solani* was assessed using several criteria including *in vitro* mycelial growth and biomass, *in vitro* sclerotia production and germination, and *in vivo* virulence according to our previous study.[38]

2.9 Statistical analysis

The data collected in the study were analyzed using Statistical Analysis System software (version 9; SAS Inc., Cary, NC). The EC_{50} values for each isolate were calculated by linear regression using PROC REG, and Fischer's least significant difference test to assess significant among the different treatments of the fitness tests.

3 RESULTS

3.1 Mutations in SDH proteins associated with thifluzamide resistance

The full-length and complete coding sequence of *sdhA*, *sdhB*, *sdhC*, *sdhD* were

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identified and analyzed (Fig. S1) in ten thifluzamide-sensitive wild-type isolates, and nine thifluzamide-resistant mutants obtained in our previous study.[38] No differences were found between the sequences of the SDHA, SDHC and SDHD protein among the thifluzamide-sensitive isolates and the thifluzamide-resistant mutants, but a single point mutation was found in the *sdhB* gene of all the resistant mutants, which resulted in the histidine residue (CAT) at codon 249 being replaced by a tyrosine residue (TAT) in SDHB protein. Furthermore, the results of overlapping peaks produced during the Sanger sequencing indicated that the mutants with low to intermediate resistance were heterokaryotic (containing both CAT and TAT codons dislike just the CAT codon in the sensitive isolates), while the highly resistant mutants were homokaryotic, containing only the TAT codon (Table 1, Fig. S2). Real-time qPCR results indicated no overexpression of the *sdhB* gene when mutants were treated with thifluzamide at concentrations corresponding to the EC_{50} and EC_{90} of thifluzamide (Fig. 1).

3.2 Thifluzamide docking in the SDH complex of *R. solani*

The docking score of 6.03 indicated that the binding energy of carboxin was similar to that of thifluzamide. The key interactions suggested that four direct hydrogen bonds were formed between thifluzamide and residues SDHB_S161, SDHB_W164, SDHC_R31, and SDHD_Y83 of *E. coli*, while two hydrogen bonds (less than classical hydrogen bond distance) mediated by water molecules were formed,

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including one between hydrogen (No. 1) and one of thifluzamide's fluorines, and another one between hydrogen (No. 2) and the oxygen of SDHB_S161 (Fig. 2A). The results also suggested that a salt bridge was formed between SDHB_H207 and the heme prosthetic group (Fig. 2A).

The effect of changes to SDHB_H207 (the analogous SDHB_H249 in *R. solani*) on the affinity of the SDH binding pocket for thifluzamide was investigated by the introduction of Y207 instead of H207 in the *E. coli* SDH complex II. The resulting docking score of 3.83 was almost two orders of magnitude lower than that of the wild-type protein, suggesting that reduced affinity was therefore responsible for the reduced thifluzamide sensitivity of the mutants. The corresponding docking image indicated that the mutation SDHB_H207Y (the analogous SDHB_H249Y in *R. solani*) reduced the number of hydrogen bonds between H_2O and thifluzamide (Fig. 2B). This point mutation (SDHB_H207Y) also changed the position and orientation of the water, and caused the ligand's center-of-mass moved and the distance is greater than the classical hydrogen bond distance. This change caused the loss of the hydrogen bond between thifluzamide and SDHD_Y83 (Fig. 2B). The mutation also broke the salt bridge between the heme prosthetic group and thifluzamide. These changes to the key interactions of SDHB and thifluzamide indicated that the mutation significantly altered the docking of thifluzamide (Fig. 2B).

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3.3 Pyrosequencing for the rapid detection of thifluzamide-resistant *R. solani* isolates with a point mutation H249Y in the *sdhB* gene

The accuracy of the Pyrosequencing used in current study was confirmed using regression analysis of standardized DNA mixtures (pEASY®-T1 cloning vectors containing the full-length of *sdhB* gene from thifluzamide-sensitive isolate JHT158-3 or thifluzamide-resistant isolate T2). The measured mutation allele frequency (T in 975 of *sdhB*) using Pyrosequencing and the expected frequency showed good linear relationship (Fig. 3). Then, a total 207 *R. solani* isolates with different thifluzamide sensitivity were determined using Pyrosequencing, and the frequency of T in 975 of *sdhB* gene showed a positive correlation with the thifluzamide sensitivity (Fig. S3).

3.4 Evolution of thifluzamide resistance in heterokaryotic mutants of *R. solani in vitro* and *in vivo*

The genetic background of each isolate was investigated using protoplasts derived from hyphal fusions between the sensitive isolate JHT158-3 and the resistant isolate T2. Eight single protoplast isolates were selected for evolution experiment (Table 1). Sanger-sequencing and Pyrosequencing analysis results showed that P97 had only C975 which was similar to JHT158-3, while P48 contained only T975. The other six mutants (P37, P45, P196, P216, P229, P232), which showed moderate resistance and contained two alleles that encoded either histidine or tyrosine at position 249 (Table

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1).

Then, the eight mutants were subjected to repeated subculture in either the absence or presence of thifluzamide *in vitro*, and the T/C ratio of the isolates were measured after the first, fifth, 10th, 15th, and 20th subcultures. All the highly resistant and sensitive isolates remained homokaryotic. However, the heterokaryotic mutants P229 changed to be homokaryotic for the mutated allele after just five subcultures on PDA containing 0.05 μg/mL thifluzamide (Fig. 4A, 4B).

Seven resistant mutants (P48, P37, P45, P196, P216, P229, P232) were also subjected to repeated subculture in either the absence or presence of thifluzamide *in vivo*, and the T/C ratio of the isolates were measured after the first, third, fifth, seventh, and 10th subcultures. P216 becoming homokaryotic after the fifth generation when sprayed with the recommended dose (100.67 μg/mL) of the commercial thifluzamide formulation Pulsor (Fig. 4C, D).

3.5 Fitness of the parasexual progeny of thifluzamide-resistant mutants

In general, the mycelial growth, mycelial biomass, sclerotium weight and sclerotium germination of the isolates containing the thifluzamide-resistance allele were similar to or lower than that of the sensitive isolates (Table 2). Consequently, the compound fitness index (CFI), which collated all the fitness parameters into a single value, was lower for most of the isolates bearing the thifluzamide-resistance allele than that of

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the sensitive isolates, except for P229 and P196, the CFI value of which was significantly higher and comparable to that of parental isolate JHT158-3 (Table 2).

4 DISCUSSION

Many studies had shown that point mutations in the SDHB, SDHC, and SDHD subunits of various fungi can lead to resistance to SDHI fungicides (Table S1). The current study was initiated to discover whether the thifluzamide-resistant *R. solani* mutants identified in our preliminary study[34] utilized a similar mechanism of resistance. The deduced amino acid sequences of the cloned *sdhA*, *sdhB*, *sdhC*, and *sdhD* genes, which encode the flavoprotein (Fp), the iron-sulfur protein (Ip), and the large and small membrane-anchor proteins SDHC and SDHD, respectively, were found to have a high degree of similarity—97%, 90%, 96%, and 94%—with the SDHA, SDHB, SDHC, and SDHD sequences of *R. solani* AGI-IB listed on the NCBI website. Comparison of the novel *R. solani* sequences with those of other basidiomycetes, ascomycetes, bacteria, and animals revealed that the SDHA subunit exhibited the highest degree of conservation (74%), followed by SDHB (64%), SDHC (43%), and SDHD (43%). These results were consistent with other studies that have found the Fp and Ip to be more highly conserved than the two membrane-anchor subunits.[45-46]

No nonsynonymous point mutations were found in SDHA, SDHC, and SDHD

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subunits from the resistant mutants, and only H249Y was identified in their SDHB subunit. Considering that real-time PCR analysis displayed no overexpression of the *sdhB* gene in any kinds of the mutants, and H249Y in SDHB protein has also been reported in other fungi with SDHI fungicdes resistance (Table S1), it is highly likely that this point mutation is responsible for the thifluzamide resistance observed in the *R. solani* mutants.

The results of the docking analysis for thifluzamide in the quinone-binding pocket of the SDH complex supported the resistance mechanism. Similar to the docking of carboxin in the crystal of the *E. coli* SDH complex,[43] several key residues from the SDHB, SDHC, and SDHD subunits, including SDHB_H207Y (the corresponding SDHB_H249Y in *R. solani*), were directly involved in thifluzamide binding. Furthermore, the results showed that the SDHB_H207Y mutation weakened the interaction with thifluzamide due to conformational changes associated with the loss of the water-mediated hydrogen bond between the now-absent histidine residue and S161, and the loss of the salt bridge with the heme prosthetic group. These results were consistent with other studies that have also found similar mutations can change the docking of other SDHI fungicides to the quinone-binding pocket of the SDH complex.[21, 27, 47] The negative impact of the SDHB_H249Y point mutation on the interaction between thifluzamide and the binding pocket of the SDH complex in *R.*

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solani support that this mutation is responsible for the observed thifluzamide resistance of the mutant isolates. A mutation of SDHB_H267Y (the corresponding SDHB_H249Y in *R. solani*) could lead to high *in vitro* resistance factors for boscalid (RF= 473.8), isopyrazam (RF =142.6), and carboxin (RF = 65.9) in *Mycosphaerella graminicola*, which was also verified by molecular docking.[27] SDHB_H267Y lead to aromatic sidechain makes edge to face stacking to the SDHIs and the tyrosine hydroxyl group a hydrogen bond to SDHD_D129. A direct hydrogen bond of the tyrosine to the hydrogen bond accepting groups of boscalid, isopyrazam, and carboxin is unlikely potentially impairing the binding of these molecules and thereby explaining the higher resistance factors observed for these molecules in *M. graminicola*.[27]

Further examination revealed that the degree of thifluzamide resistance was related to the nuclear composition of the mutants, with homokaryotic mutants exhibiting much higher resistance than their heterokaryotic counterparts, which still possessed wild-type versions of the *sdhB* allele. The relationship between nuclear composition and fungicide sensitivity is important because *R. solani* readily undergoes anastomosis, and therefore nuclei bearing resistance mutations can readily be transmitted to sensitive strains. These characteristics result in a high potential for the recombination and selection of different alleles, including those associated with

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fungicide resistance. In the context of the current study, it seems likely that the low to intermediate level of thifluzamide resistance observed in the heterokaryotic mutants should be caused by the different proportions of the resistant allele in the nuclei, while the homokaryotic mutants only harbored resistant allele thus showed the high level of thifluzamide resistance.

Genetic studies of *R. solani* are difficult to conduct because the fungus rarely produces sexual or asexual spores, in either the field or the laboratory. Consequently, the transformation and fusion of protoplasts has become an important tool for the study of fungal strains with commercial importance.[6, 48] The current study used this approach to generate heterokaryotic isolates by the fusion of hyphae from a homokaryotic wild-type sensitive isolate and a thifluzamide-resistant mutant. The resulting progeny confirmed not only that hyphal fusion could produce progeny with the recombinant genotype but also that the mutated *sdhB* gene could be used as a selective marker for further study.

Little is yet known regarding the evolution of resistance to SDHIs in heterokaryotic fungi such as *R. solani*, although like all organisms, fungi evolve in response to selection pressures.[42, 49] In current study, it was found that heterokaryotic isolates could become highly resistant homokaryotic ones both *in vitro* and *in planta*, under the fungicide pressure. These results indicate that heterokaryotic isolates of *R. solani*

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carrying the resistant allele pose a significant risk to the development and evolution of thifluzamide resistance, which is of great concern given that the thifluzamide resistant phenotype also exhibits cross-resistance with other SDHIs including mepronil, fenfuram, carboxin, penflufen, and boscalid.[38]

A previous study of the SDHI fungicide mepronil found that resistant isolates of *R. solani* always exhibit impaired sclerotium production and pathogenicity.[4] The current study found that the CFI of the thifluzamide-resistant mutants showed different fitness and some resistant strains showed higher CFI than the sensitive wild-types, and heterokaryons lost their allele under the fungicide pressure with no fitness penalty. Combined with the evolution risk of heterokaryotic isolates of *R. solani*, good fitness increases the resistant risk of thifluzamide in *R. solani*.

ACKNOWLEDGMENTS

The authors would like to thank Dow AgroSciences for providing the fungicides used in this study, Prof. Brett Tyler (Oregon State University of USA) for useful advice. This work was funded by the National High-Technology Research and Development Programme of China (Grant number: 2012AA101502) and partially supported by the Special Fund for Agro-scientific Research in the Public Interest of China (Grant number: 201303023) and the graduate student scientific research innovation projects of China Agriculture University.

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Table 1. Genotype and thifluzamide sensitivity of *R. solani* isolates produced by hyphal fusion and protoplast regeneration.

Strain [a]	Genotype of *sdhB* at 975	Pyrosequencing		EC_{50} (μg/mL)
		C (%)	T (%)	
JHT158-3	C	100	0	0.057 (0.044-0.069) [b]
T2	T	1.6	98.4	15.631 (11.015-22.084)
P97	C	94.2	5.8	0.098 (0.065-0.136)
P37	C and T	49.1	50.9	1.601 (1.430-1.846)
P45	C and T	32.9	67.1	1.935 (1.689-2.212)
P196	C and T	34.6	65.4	2.696 (2.210-3.272)
P216	C and T	34.8	65.2	2.731 (2.296-3.246)
P229	C and T	35.7	64.3	2.641 (2.186-3.179)
P232	C and T	34.0	66.0	2.093 (1.683-2.570)
P48	T	2.1	97.9	11.597 (7.866-15.507)

[a]JHT158-3 and T2 are the parental isolates of the protoplast-regenerated isolates (P97,

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P37, P45, P196, P216, P229, P232, P48).

[b]Values in parentheses are 95% confidence limits.

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Table 2 Fitness of sensitive isolates and thifluzamide-resistant mutants of *R. solani* in the absence of thifluzamide.

Isolate	Genotype [a]	Sensitivity [b]	Mycelial growth (cm) [c]	Mycelial biomass (g) [d]	Sclerotium weight (g) [e]	Sclerotium germination (%)	Lesion length (mm) [f]	CFI [g]
JHT158-3	HM	S	8.92a	0.16 abc	0.11 ab	97.92 a	21.47 ab	325.76 c
B310	HM	S	7.83 c	0.17 ab	0.11 ab	97.00 a	22.30 ab	305.16 e
FBS-3	HM	S	8.46 b	0.16 bc	0.11 ab	98.33 a	21.80 ab	313.52 d
P97	HM	S	6.23 f	0.13 cd	0.09 b	92.45 a	20.03 bcd	134.75 j
T2	HM	HR	6.80 e	0.12 d	0.11 ab	98.21 a	16.63 cd	143.09 i
P48	HM	HR	6.28 f	0.08 e	0.11 ab	97.22 a	15.82 d	83.77 l
P232	HT	R	7.65 cd	0.12 cd	0.11ab	100.00 a	21.86 ab	245.19 f
P216	HT	R	7.75 cd	0.13 bcd	0.09 b	90.48 b	22.81 ab	195.35 h
P37	HT	R	5.82 g	0.07 e	0.10 b	100.00 a	25.76 a	114.08 k
P229	HT	R	8.96 a	0.20 a	0.14 a	100.00 a	20.43 bcd	502.18 a
P45	HT	R	7.35 d	0.13 cd	0.12 ab	97.22 a	21.14 abc	211.17 g

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P196	HT	R	7.58 cd	0.14 bcd	0.12 ab	100.00 a	25.37 a	326.02 b

[a] Genotype: (HM) homokaryotic, (HT) heterokaryotic.

[b] Thifluzamide sensitivity: (S) sensitive, (R) resistant, (HR) highly resistant.

[c] Colony diameters measured after 44 h growth on PDA.

[d] Mycelial dry biomass determined after 54 h growth in PDB.

[e] Dry weight of sclerotia per PDA plate.

[f] Lesion lengths on rice sheaths measured 7 days after inoculation under greenhouse conditions.

[g] CFI (compound fitness index) = mycelial growth × mycelial biomass ×sclerotium formation × sclerotium germination × lesion length.

Means followed by the same letter were not significantly different according to Fisher's least significant difference test at P = 0.05.

f

g

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Figure legends:

Fig. 1. Relative expression levels of the *sdhB* gene in *R. solani* isolates with different degrees of thifluzamide resistance in response to different thifluzamide concentrations. Columns and bars indicate means ± standard deviation. Columns marked with the same letter are not significantly different by Fisher's protected least significant difference test ($P < 0.05$).

Fig. 2. Docking interactions between thifluzamide and the quinone-binding site of the wild-type SDH and the mutated thifluzamide-resistant SDH. (A) 3D representation of thifluzamide in the putative binding pocket of the wild-type SDH. (B) 3D representation of thifluzamide in the putative binding pocket of the mutated SDH. Key amino acids are represented by cyan sticks, and thifluzamide carbon atoms by magenta sticks, while hydrogen bonds are represented by blue dotted lines.

Fig. 3. Regression analysis showing the near-linear relationship ($R^2 = 0.996$) between measured SNP obtained from pyrosequencing and the expected T ratio obtained when the DNA from plasmids carrying the sensitive (C) and mutant (T) allele were mixed in different proportions (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10). T frequencies at position 975 of the *R. solani sdhB* gene were calculated from the peak areas as follows: % = peak T/ (peak T + peak C) × 100. Peak areas represent the averages of two measurements.

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Fig. 4. Evolutionary changes in thifluzamide sensitivity of *R. solani* isolates after repeated *in vitro* and *in planta* subculture in the absence or presence of thifluzamide. T contents at position 975 of *sdhB* gene were determined by pyrosequencing. (A & B) The T contents (%) of *R. solani* isolates after the first, fifth, 10th, 15th, and 20th subcultures on PDA containing (A) 0 or (B) 0.05 µg/mL thifluzamide. (C & D) The T contents (%) *R. solani* isolates after first, third, fifth, seventh, and 10th subcultures on rice plants that were sprayed weekly with (C) water or (D) 100.67 µg/mL thifluzamide.

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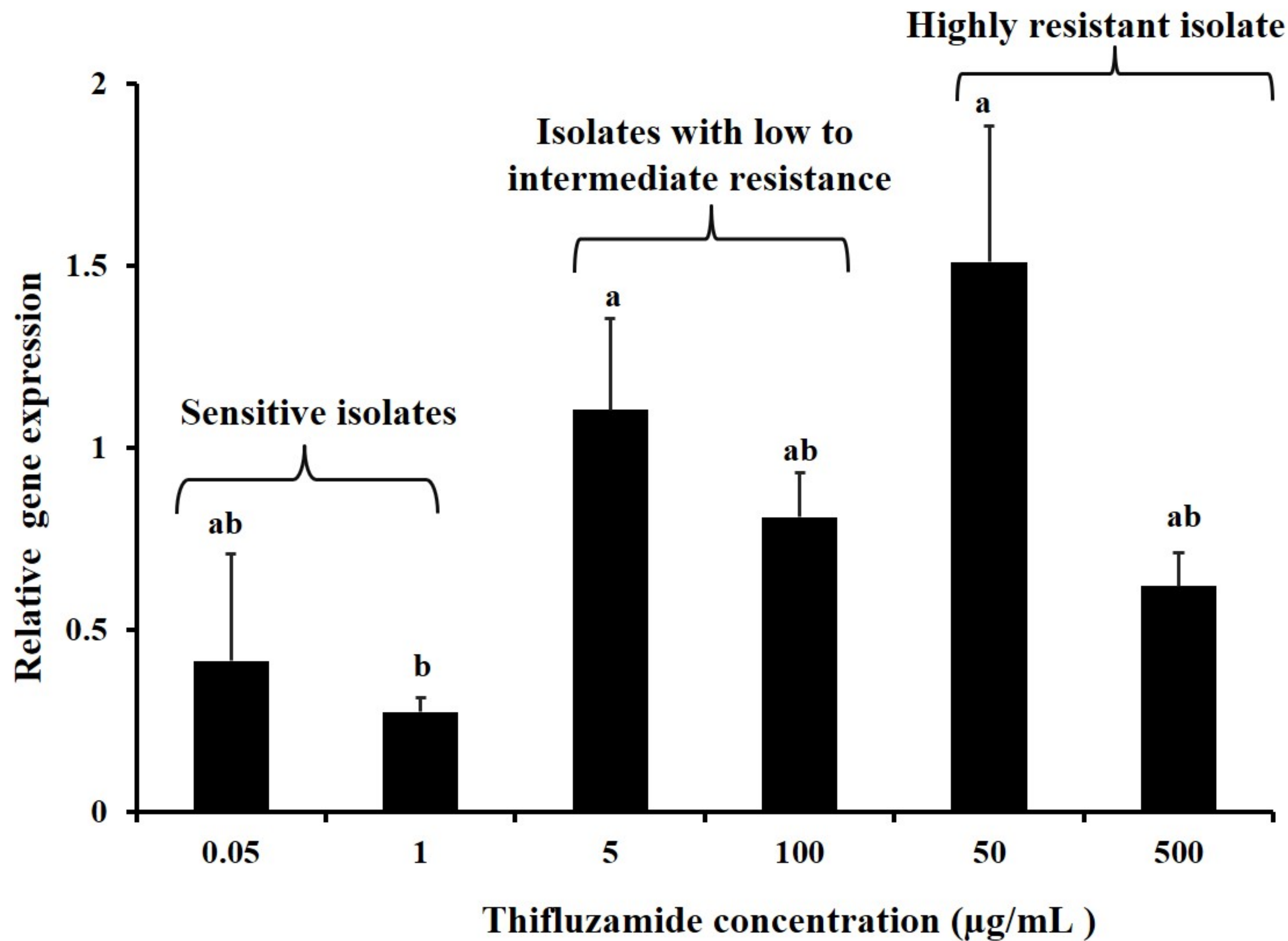

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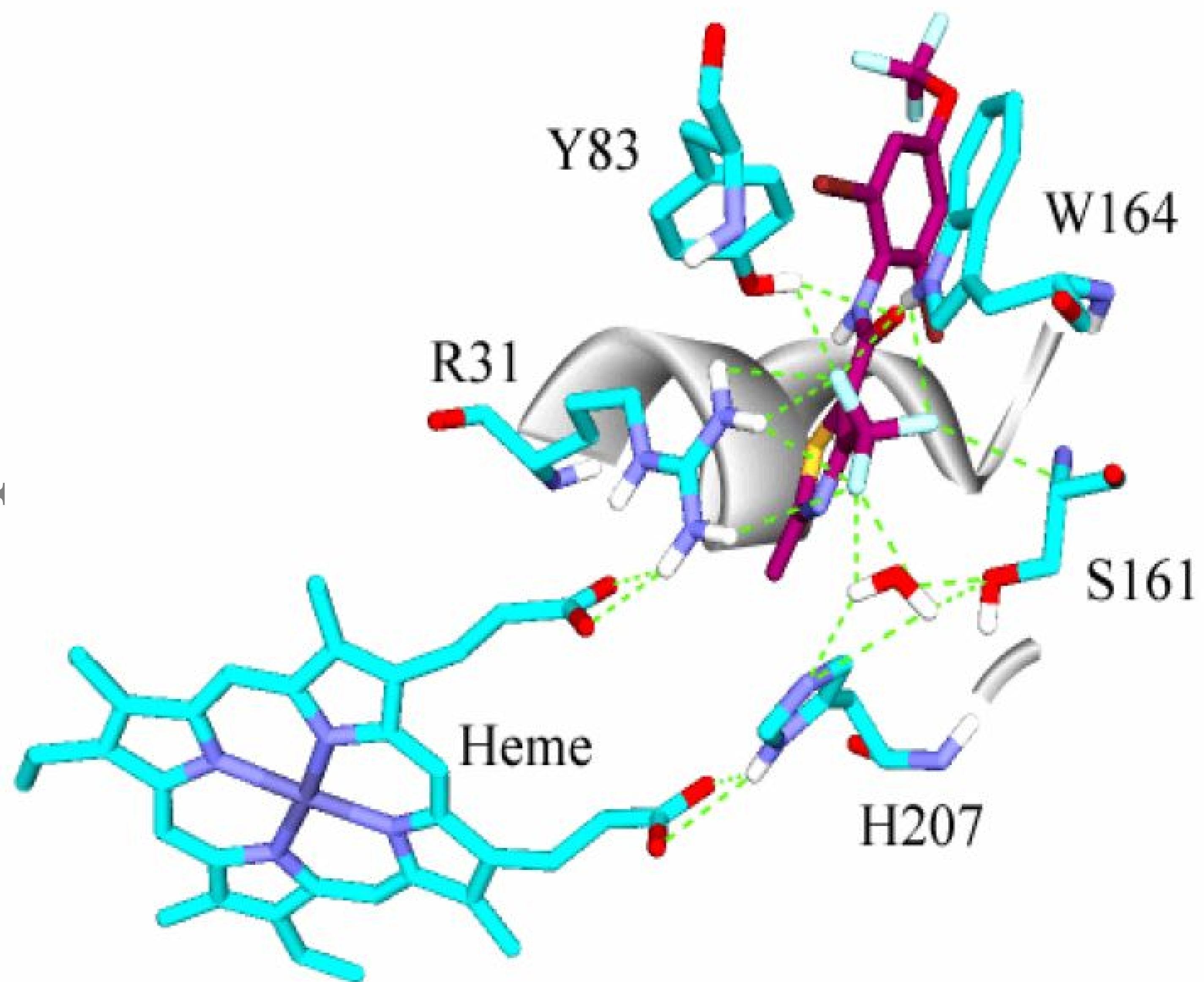

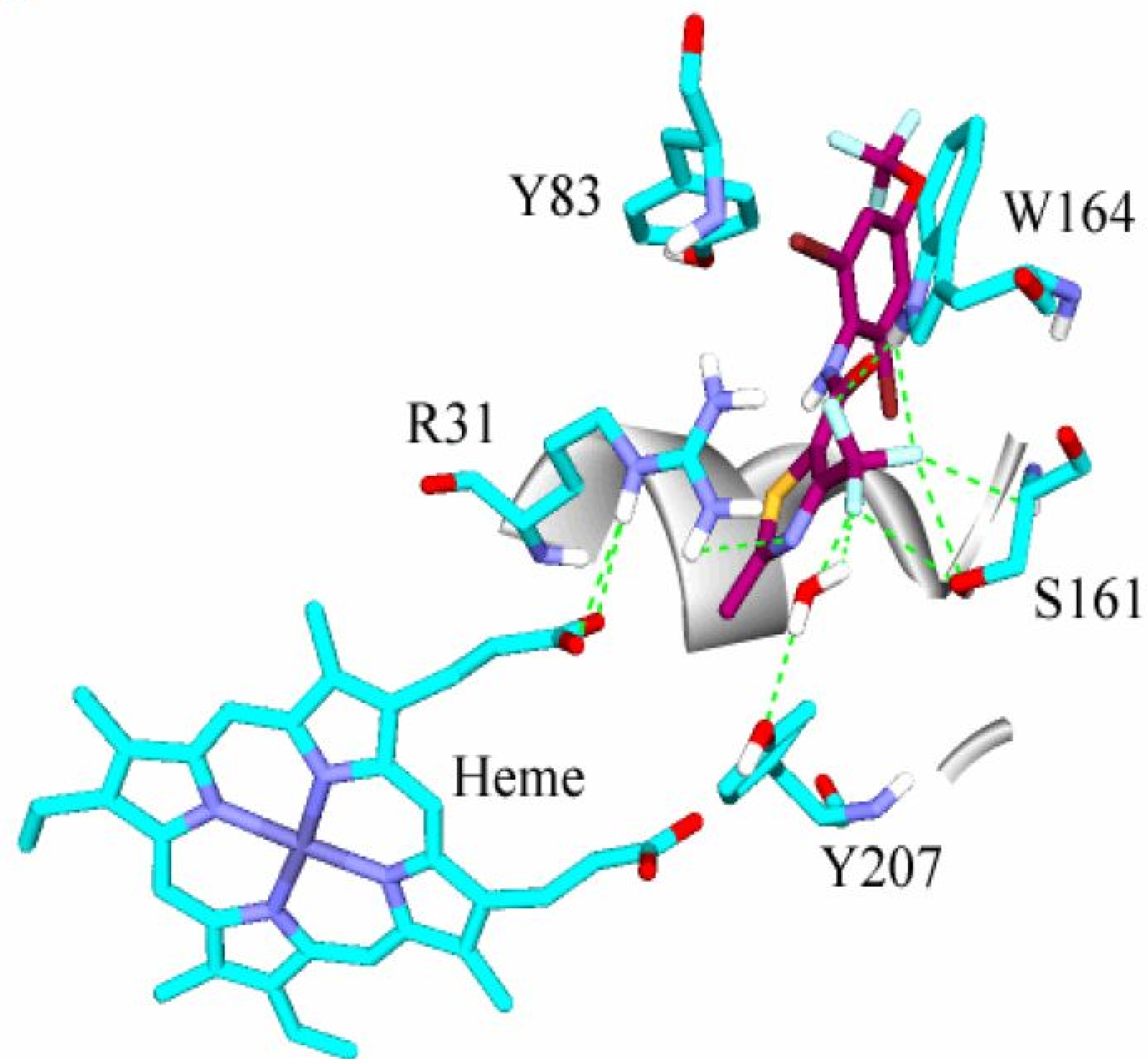

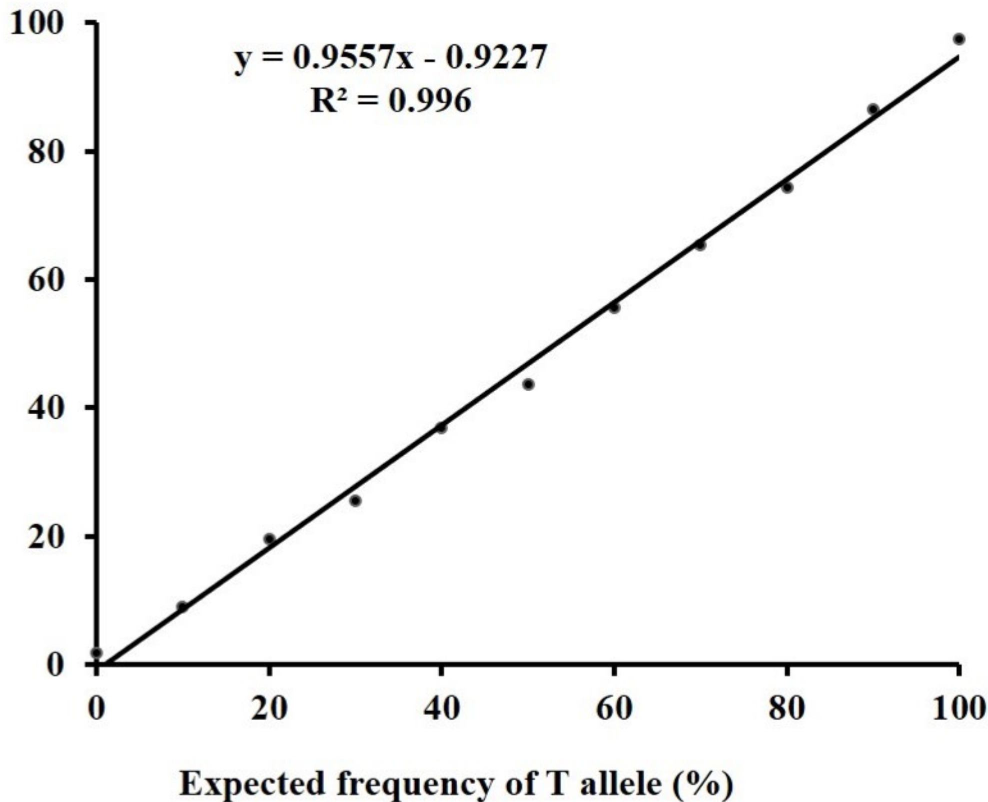

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