A New Mutation Genotype of K218T in Myosin-5 Confers Resistance to Phenamacril in Rice Bakanae Disease in the Field

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Abstract

In 2017 and 2018, a total of 294 *Fusarium fujikuroi* isolates were collected from bakanae-diseased rice plants in Jinhua, Shaoxing, and Jiaxing in Zhejiang Province, China. Phenamacril sensitivity of these isolates was determined by the 50% effective concentration value or minimum inhibitory concentration methods. Our results indicated that the phenamacril resistance frequency of *F. fujikuroi* increased from 18% in 2017 to 47% in 2018, and rice plants infected with *F. fujikuroi*-resistant isolates could not be protected effectively with 50 mg/liter of phenamacril. Phenamacril-resistant *F. fujikuroi* isolates obtained from rice fields showed stable resistance, because their fitness levels (i.e., mycelial growth, sporulation, and pathogenicity) were similar to the phenamacril-sensitive isolates. In addition to the point mutation at

codon 219 in the *myosin-5* gene that conferred resistance to phenamacril, our results also showed another point mutation at codon 218 (AAG→ACG) in *myosin-5* that also conferred resistance to phenamacril. In this study, we found rapid development and persistence of diversified genotypes of phenamacril resistance, highlighting the importance of proper use of phenamacril in rice fields. Our results may also help researchers develop new fungicides or new control strategies using combinations of different fungicides in the control of phenamacril-resistant *F. fujikuroi* isolates.

Keywords: fitness, K218T mutant, rice bakanae disease, phenamacril resistance

Rice bakanae disease is caused by four *Fusarium* species (*F. andiyazi*, *F. fujikuroi*, *F. proliferatum*, and *F. verticillioides*). Among these four pathogens, *F. fujikuroi* is the most common species and is only found in seed samples from Asia (Amatulli et al. 2012; Wulff et al. 2010). Infected rice plants present with the classic characteristics of seedling elongation, slender leaves, leaf chlorosis, grain sterility, and discoloration (Li et al. 2018; Wulff et al. 2010). Rice bakanae disease is a destructive seedborne disease and can cause significant yield losses and mycotoxin contamination in infested grains. Mycotoxins produced by rice bakanae disease in rice are mainly fumonisins and gibberellin A3, which can threaten human and animal health (McMullen et al. 2012; Pestka and Smolinski 2005; Zhang et al. 2015). Various fungicides with different modes of action have been used to control rice bakanae disease. These fungicides include carbendazim (benzimidazole fungicides [MBCs] that interact with tubulin), tebuconazole (demethylation inhibitors [DMIs] that interact with CYP51), and prochloraz (DMI). After fungicides have been used to control *Fusarium* spp. infection in rice for consecutive years, the pathogenic fungi tend to develop resistance to MBCs through point mutations (i.e., GAG→GTG mutation at codon 198, TTC→TAC mutation at codon 200, and GGC→GGT at mutation codon 235) in the β_2 tub gene (Chen et al. 2014; Zhou and Wang 2001). Mutations in the *CYP51* gene can also result in amino acid changes and the overexpression of CYP51 in *Fusarium* spp. can lead to resistance to DMIs (Fan et al. 2014; Qian et al. 2017; Sewell et al. 2017; Sun et al. 2011).

Phenamacril is a novel fungicide with a *Fusarium*-specific mode of action that has been used to control rice bakanae disease and *Fusarium* head blight caused by several *Fusarium* isolates. Phenamacril is known to inhibit *Fusarium* conidia germination and mycelial growth and to interfere with the function of Myosin-5, which plays a key role in mycotoxin biosynthesis in *F. graminearum* (Li et al. 2018; Tang et al. 2018). Myosin-5 of *Fusarium* spp. is the target protein of phenamacril, and mutations in myosin-5 were reported to be responsible for the development of phenamacril resistance. These findings are supported by the fact that *Fusarium* spp. mutants produced through ultraviolet irradiation and point mutations (e.g., K216R/E, S217P/L, and E420K/G/D) in Myosin-5 are highly resistant to phenamacril (Chen et al. 2008; Li et al. 2016; Zheng et al. 2015). Fortunately, the phenamacril-resistant *Fusarium* spp. isolates are sensitive to other commercial fungicides with different modes of action, including MBCs and DMIs (Chen et al. 2011; Li et al. 2008, 2016, 2018).

In 2016, a S219P/L mutation in Myosin-5 was reported in *F. fujikuroi* that is highly resistant to phenamacril application in the field (Hou et al. 2018). Phenamacril has been used for rice seed dressing in different regions of Zhejiang Province, China. Considering the high risk of phenamacril resistance, we decided to revisit the strategy of rice bakanae disease control using phenamacril, to investigate the current distribution of phenamacril-resistant *F. fujikuroi* isolates, and to delay the occurrence of new resistant isolates in rice fields in Zhejiang Province. This study aimed to (i) evaluate the development of phenamacril resistance in rice plant populations in Zhejiang Province, (ii) assess the resistance stability of resistant mutants collected from bakanae-diseased rice plants, and (iii) elucidate the molecular mechanism of phenamacril resistance in *F. fujikuroi*.

Materials and Methods

Fungicide and culture media. Phenamacril (95.5% active ingredient) was provided by the Jiangsu Branch of the National Pesticide Research and Development South Center of China, and its experimental code is JS399-19. Phenamacril was dissolved in methanol to 100 mg/ml and stored at 4°C. Pentachloronitrobenzene (94% active ingredient) was purchased from Aladdin (Shanghai, China), dissolved in acetone to 30 mg/ml, and stored at 4°C.

Mung bean broth medium was prepared and used to induce conidia production as described (Li et al. 2018). Potato dextrose agar (PDA)

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medium was prepared and used for regular growth tests (Yun et al. 2015).

Sample collection and fungal isolation. Infected rice plants with characteristic elongation and aerial adventitious roots were collected from three geographical regions of Zhejiang Province in July and August of 2017 and 2018, respectively. In 2017, we randomly sampled two rice fields in Jinhua and Jiaxing, respectively, and one rice field in Shaoxing. The sampled rice fields in the same geographical region were >1 km apart. In 2018, four rice fields were sampled in Jinhua or Shaoxing, and six rice fields were sampled in Jiaxing.

Isolates were obtained from diseased rice plants showing typical *Fusarium* spp. infection symptoms. For plants with aerial adventitious roots, small pieces of tissue were cut from tillers at approximately 1 cm away from the aerial adventitious roots. For plants without aerial adventitious roots, the samples were cut from the tillers at approximately 2 cm away from the roots. All isolates were isolated from the collected samples using standard procedures. Briefly, the collected tissue was surface disinfected in 70% ethanol for 30 s and then in 5% NaClO for 3 min. After three rinses with sterile water, the tissue was placed on PDA plates containing 50 mg/liter of streptomycin sulfate (Sangon Biotech, Shanghai, China) and 30 mg/liter of pentachloronitrobenzene (Aladdin). The plates were incubated at 25°C for 6 days in the dark. Fungal isolates showing characteristics of *Fusarium* infection were transferred individually to fresh PDA medium and cultivated for another 6 days in the dark. The resulting isolates were further purified as previously described (Wulff et al. 2010) and then maintained on PDA slants at 4°C.

Identification of *Fusarium* isolates through PCR. All *Fusarium* isolates were used for DNA extraction. After 8 days of cultivation, mycelia were harvested from individual *Fusarium* isolates for genomic DNA extraction using a fungal genomic DNA kit (Sangon Biotech). PCR was used to amplify a 700-bp fragment representing a partial sequence of the *translation elongation factor* (TEF) gene from each fungal isolate with a primer pair described previously (O'Donnell et al. 1998). After electrophoresis, the correct-sized PCR products were cut from 1% agarose gels, purified and cloned into the pMD19-T vector (simple) (TaKaRa, Dalian, China), and then sequenced by Sangon. The resulting DNA sequences were used to construct a phylogenetic tree using RAXMLGui ML version 8 software (provided by Zhejiang University).

Measurement of the 50% effective concentration value representing mycelial growth inhibition. Seventy-two isolates of *F.*

fujikuroi, obtained in 2017, were used for analyses of the 50% effective concentration (EC₅₀; the fungicide concentration that results in 50% radial mycelial growth inhibition). One fresh mycelium plug (size 5 × 5 mm) of each isolate was cut from the growing edge of active mycelia on PDA and transferred onto the center of new PDA plates amended with 0, 0.1, 0.2, 0.4, 0.8, and 1.6 mg/liter of phenamacril. If the EC₅₀ value was outside of this dose range, mycelia plugs were placed on PDA plates amended with 0, 6.25, 12.5, 25, 50, and 100 mg/liter of phenamacril as described previously (Hou et al. 2018). Each treatment was repeated four times, and the experiment was conducted three times.

Assessment of *F. fujikuroi* resistance to phenamacril. A total of 222 *F. fujikuroi* isolates obtained in 2018 were tested with the minimum inhibitory concentration (MIC) method to determine their resistance to phenamacril. Based on the EC₅₀ values obtained using the 2017 isolates, a fresh mycelium plug (size 5 × 5 mm) from each isolate was transferred onto a PDA medium plate amended with 5, 30, and 100 mg/liter of phenamacril. Isolates that showed normal colony growth were considered to be resistant to phenamacril; otherwise, they were considered sensitive isolates. This experiment was conducted three times, and similar results were obtained with both replicates for each isolate.

Fitness components of resistant and sensitive isolates. To determine the fitness of isolates showing different phenamacril sensitivity phenotypes, two sensitive isolates (FFSX18-02^S and FFJX18-54^S) and four resistant isolates (FFJX17-08^R, FFJX17-09^R, FFJX18-14^R, and FFJX18-76^R) were chosen for inspection of some of their biological characteristics, including hyphal growth, sporulation, and spore morphology, as described previously (Chen et al. 2014; Hou et al. 2018). Four biological replicates were analyzed for each isolate.

Seeds of rice cultivar Rice 39 were used for pathogenicity tests. A conidia suspension containing 1 × 10⁶ conidia/ml was prepared for each assayed isolate. Rice seeds were subjected to disinfection by 70% ethanol and 1% NaClO to remove any surface contaminants (Wulff et al. 2010) and germinated for 48 h at room temperature. Thirty uniformly germinated rice seeds were selected and inoculated with 10 ml of conidial suspensions (1 × 10⁶ conidia) representing an assayed isolate or with 10 ml of sterile distilled H₂O in a 50-ml flask. Rice seeds in different fluids were shaken at 75 rpm for 12 h at 25°C. After three rinses with sterile water, the rice seeds were transferred to sterilized, perforated tubes and cultivated in nutrient solution for 15 days inside a humid chamber at 25°C. The height of each rice seedling was measured and then used to determine the average height of the plants with the same treatment. The excessive growth rate caused by *F. fujikuroi* infection was calculated as follows:

$$\begin{aligned} \text{excessive growth rate(\%)} &= (\text{average height of the infected rice seedlings} \\ &\quad - \text{average height of the healthy rice seedlings}) \\ &\quad / \text{average height of the healthy rice seedlings} \times 100 \end{aligned}$$

The experiment was performed twice with 30 seedlings per treatment.

Control efficacy of phenamacril against *F. fujikuroi* in rice seedlings. Elongation of rice primary leaves is a typical symptom caused by *F. fujikuroi* infection. In this study, the effectiveness of phenamacril against *F. fujikuroi* infection was determined by measuring the height of rice seedlings inoculated with isolates FFJX18-54^S, FFJX17-09^R, and FFJX18-76^R. Phenamacril (50 mg/liter) was applied with conidia suspensions either 6 h before the germinated seeds were inoculated (protective treatment) or 20 h after the germinated seeds were inoculated (curative treatment). Germinated rice seeds inoculated with *F. fujikuroi* and methyl alcohol or with methyl alcohol only were used as controls in this study. After inoculation, the rice seeds were cultivated for 15 days inside the chamber at 25°C and then measured for plant height. The experiment was performed twice with 24 seeds per treatment.

Fig. 1. Phenamacril sensitivity of *Fusarium fujikuroi* isolates collected from three different rice fields in Zhejiang Province in 2017. Frequency distribution of the observed 50% effective concentration (EC₅₀) values for phenamacril based on the inhibition of mycelial growth of 31, 10, and 31 isolates collected from **A**, Jinhua, **B**, Shaoxing, and **C**, Jiaxing, respectively.

Analysis of *myosin-5* gene sequences from the phenamacril-resistant and phenamacril-sensitive isolates. The full-length *myosin-5* gene was PCR amplified from the genomic DNA of all *F. fujikuroi* collected in 2017 and 2018 using primer pair JY29_F

(AGAGCATCCGCTAAACTCCCAC) and JY31_R (TCTCAGG GAATATCAAGACGC), designed according to a known full-length *F. fujikuroi myosin-5* gene sequence (GenBank HF679023.1). The PCR products were purified and sequenced as described above.

Fig. 2. Phenamacril resistance of *Fusarium fujikuroi* isolates collected in Zhejiang Province. **A**, Growth condition of eight *F. fujikuroi* isolates on potato dextrose agar (PDA) plates amended with 5, 30, and 100 mg/liter. **B**, Minimum inhibitory concentration method for the detection of phenamacril resistance of field samples. An asterisk indicates the number of isolates that grew on PDA plates with or without phenamacril. **C**, Number of phenamacril-resistant (R) and phenamacril-sensitive *F. fujikuroi* isolates sampled from each field in Jinhua, Shaoxing, and Jiaxing. R = resistant isolates grew on PDA plates amended with 100 mg/liter of phenamacril or the 50% effective concentration (EC_{50}) values were >50 mg/liter and S = sensitive isolates did not grow on PDA plates containing 5 mg/liter of phenamacril or the EC_{50} values were <1 mg/liter. **D**, Development of the frequency of phenamacril resistance in rice fields in Jinhua, Shaoxing, and Jiaxing. The upper and lower graphs are the resistance frequency and sensitivity frequency of *F. fujikuroi* isolates sampled from each field in Jinhua, Shaoxing, and Jiaxing in 2017 and 2018, respectively.

The *myosin-5* sequences in different *F. fujikuroi* isolates were used to identify the cause of phenamacril resistance in field *F. fujikuroi* strains.

Results

Development of phenamacril resistance in *F. fujikuroi* isolates in the field. *Fusarium* isolates collected from bakanae-diseased rice plants were identified by phylogenetic analysis using TEF gene sequences, and the results showed that the *Fusarium* isolates, including isolates FFSX18-02, FFJX18-54, FFJX17-08, FFJX17-09, FFJX18-14, and FFJX18-76 used in this study, were *F. fujikuroi* (Supplementary Fig. S1).

Using the 72 isolates obtained from three different regions in Zhejiang Province in 2017, we determined that the EC₅₀ values of the

isolates collected from Jinhua and Shaoxing were <1 mg/liter (Fig. 1A and B), but the EC₅₀ values of the isolates obtained from Jiaxing ranged from 0.538 to 80.33 mg/liter (Fig. 1C). This result showed that phenamacril-resistant *F. fujikuroi* isolates were found in Jiaxing, and the resistance ratio of phenamacril was 42% (13 of 31 isolates).

When the MIC method was used to determine the sensitivity level of the 222 isolates obtained from the same cities in 2018, a total of 104 isolates collected from Shaoxing and Jiaxing grew on PDA plates amended with 100 mg/liter of phenamacril. None of the isolates obtained from Jinhua grew on the PDA plates amended with phenamacril (Fig. 2B). The ratio of phenamacril-resistant *F. fujikuroi*

Fig. 3. Biological characteristics of phenamacril-resistant (R) and phenamacril-sensitive (S) *Fusarium fujikuroi* isolates. **A**, Mycelial growth. **B**, Spore morphology. **C**, Sporulation. **D**, Pathogenicity. FFSX18-02^S and FFJX18-54^S are sensitive isolates, whereas FFJX17-08^R, FFJX17-09^R, FFJX18-14^R, and FFJX18-76^R are resistant isolates.

Table 1. Characterization of *Fusarium fujikuroi* isolates

Strain ^y	Minimum inhibitory concentration (mg/liter)	Mycelia growth (mm/8 days) ^z	Conidia production capacity (× 10 ⁶ conidia/ml)	Conidia type	Excessive growth rate (%) ^z
FFSX18-02 ^S	<5	79.4 ± 0.07 bc	12.5 ± 0.13 b	Microconidia	62.54
FFJX18-54 ^S	<5	82.8 ± 0.06 a	14.0 ± 0.33 b	Microconidia	61.67
FFJX17-08 ^R	>100	81.5 ± 1.00 ab	14.7 ± 0.09 b	Microconidia	75.47
FFJX17-09 ^R	>100	80.0 ± 0.44 ab	6.1 ± 0.54 c	Microconidia	72.39
FFJX18-14 ^R	>100	75.9 ± 0.32 d	20.8 ± 0.62 a	Microconidia	61.77
FFJX18-76 ^R	>100	76.7 ± 0.66 dc	19.7 ± 0.78 a	Microconidia	61.81

^y S = phenamacril-sensitive isolate and R = phenamacril-resistant isolate.

^z Values are the means ± SE of 16 seedlings from three independent experiments. Values in each column followed by different letters are significantly different (*P* < 0.05) according to Fisher's least significant difference test.

obtained from Shaoxing was 12% and that obtained from Jiaxing was 96% (Fig. 2C). The ratio of phenamacril-resistant *F. fujikuroi* in rice field 1 in Jiaxing increased from 53% (8 of 15) in 2017 to 94% (15 of 16) in 2018 (Fig. 2C and D). This finding indicates that the

development of phenamacril resistance occurs gradually and is variable in different regions.

Fitness of phenamacril-resistant and phenamacril-sensitive *F. fujikuroi* isolates. Mycelial growth, spore production, spore

Fig. 4. Two types of phenamacril-resistant field isolates of *Fusarium fujikuroi*. **A**, Control efficacy of 50 mg/liter of phenamacril against *F. fujikuroi* on rice seedlings. FFJX18-54^S is a sensitive (S) isolate, whereas FFJX17-09^R and FFJX18-14^R are resistant (R) isolates. I = Germinating seeds were incubated with 50 mg/liter of phenamacril for 6 h, followed by incubation with the spore suspension for another 20 h; and II = Germinating seeds were incubated with the spore suspension for 20 h, followed by incubation with 50 mg/liter of phenamacril for another 6 h. **B**, Alignment of partial gene sequences of the myosin-5 gene from sensitive and resistant isolates. A schematic representation of the site of codons 218 and 219 in the motor domain of the myosin-5 gene is shown. FfMyo1 = partial gene sequences (631 to 672 nt) of the myosin-5 gene in *F. fujikuroi* (GenBank HF679023.1). FFSX18-02, FFJX18-54, FFJX17-08, FFJX17-09, FFJX18-14, and FFJX18-76 are the partial gene sequences (631 of 672 nt) of the myosin-5 gene of tested isolates. **C**, Number of each mutation type of resistant *F. fujikuroi* isolates sampled from each field. S219P and K218T are the resistant isolates that contained a change at amino acids 219 and 218, respectively. An asterisk indicates the number of each mutation type.

morphology, and pathogenicity were investigated using the six selected isolates to determine the fitness of phenamacril-resistant and phenamacril-sensitive isolates collected from the field. Isolates FFSX18-02^S and FFJX18-54^S could not grow on PDA plates containing 5 mg/liter of phenamacril, isolates FFJX18-14^R and FFJX18-76^R grew on PDA plates amended with 100 mg/liter of phenamacril, and the EC₅₀ values of isolates FFJX17-08^R and FFJX17-09^R were >50 mg/liter. After 8 days of cultivation on PDA medium, the mycelial growth of isolates FFJX17-08^R, FFJX17-09^R, and FFJX18-54^S was similar (i.e., 81.5, 80.0, and 82.8 mm, respectively). Meanwhile, similar mycelial growth was found for isolates FFSX18-02^S and FFJX18-76^R. Slower mycelia growth was observed for isolate FFJX18-14^R (Fig. 3A; Table 1). Based on the conidia production results, significant differences were observed among the six tested isolates. Isolates FFJX18-14^R and FFJX18-76^R produced significantly ($P < 0.05$) more spores than the other four isolates (Fig. 3C; Table 1). All six isolates produced a substantial number of microconidia and very few falcate macroconidia (Fig. 3B).

The pathogenicity of each isolate was determined by measuring the excessive plant growth after inoculation. Compared with the control rice seedlings inoculated with sterile water, the rice seedlings inoculated with isolates FFSX18-02^S, FFJX18-54^S, FFJX17-08^R, FFJX17-09^R, FFJX18-14^R, or FFJX18-76^R developed longer and slender leaves (Fig. 3D). The excessive plant growth rates for infected rice seedlings were 62.54, 61.67, 75.47, 72.39, 61.77, and 61.81% for FFSX18-02^S, FFJX18-54^S, FFJX17-08^R, FFJX17-09^R, FFJX18-14^R, and FFJX18-76^R, respectively (Table 1). Hence, our biological characteristics and pathogenicity assays showed that there were no remarkable differences in the fitness of the phenamacril-resistant and phenamacril-sensitive isolates.

Protective and curative activity of phenamacril on *F. fujikuroi* infection in rice seedlings. Whether applied 6 h before or 20 h after inoculation, we found that phenamacril could successfully control the excessive growth of plants inoculated with isolate FFJX18-54^S but not the plants inoculated with isolates FFJX17-08^R and FFJX18-76^R. The results in Figure 4A showed that after the treatment with phenamacril, the average height of rice seedlings inoculated with FFJX18-54^S was similar to the control rice seedlings inoculated with sterile water, suggesting that the protective and curative activity reached 100%. In contrast, phenamacril had little effect on rice seedlings inoculated with isolate FFJX17-08^R or FFJX18-76^R. For example, the average heights of the FFJX17-08^R-inoculated rice seedlings treated with sterile water or phenamacril were 182.0 and 181.6 mm, respectively (Fig. 4A; Table 2). These results showed that isolates FFJX17-08^R and FFJX18-76^R could not have been controlled by phenamacril because their mycelial growth could not be inhibited by phenamacril.

A single point mutation in Myosin-5 results in *F. fujikuroi* resistance to phenamacril. To investigate the cause of phenamacril resistance in *F. fujikuroi* isolates, the *myosin-5* gene fragment was amplified for sequencing. We found that FFJX17-08^R and FFJX17-09^R both contained a point mutation from TCA to CCA at codon 219, resulting a change of serine to proline (S→P) in Myosin-5 (Fig. 4B); this amino acid change was reported previously (Hou et al. 2018). In addition, we compared the *myosin-5* gene sequences from FFSX18-02^S and FFSX18-54^S with that from *F. fujikuroi*

(GenBank HF679023.1) and a point mutation (AAG→ACG) at codon 218, resulting in a lysine to threonine (K→T) substitution, was observed in *myosin-5* from FFJX18-14^R and FFJX18-76^R (Fig. 4B). The sequencing results also showed that all of the resistant isolates collected from Jiaying in 2017 contained the S219P mutation at position 219, and all of the resistant isolates collected from Shaoxing in 2018 contained the K218T mutation at position 218 (Fig. 4C). In 2018, 12 resistant *F. fujikuroi* isolates collected from rice fields 1 and 2 in Jiaying contained the substitution at position 219 and the remaining 84 resistant isolates collected from Jiaying had the K218T mutation at position 218 (Fig. 4C). These findings showed that most phenamacril-resistant *F. fujikuroi* isolates (92 of 104 isolates) collected in 2018 had the K218T mutation. Therefore, we considered K218T to confer high resistance to phenamacril during *F. fujikuroi* infection in rice plants in the field.

Discussion

Phenamacril is a novel cyanoacrylate fungicide and targets myosin-5 protein in *Fusarium* spp. (Tang et al. 2018). *Fusarium* spp. mutant isolates resistant to phenamacril can be generated through ultraviolet irradiation and selection, and the fitness levels of the mutant isolates are similar to their parental isolates during laboratory tests (Chen et al. 2008; Zheng et al. 2015). The phenamacril resistance of *Fusarium* isolates cannot be altered by sexual or asexual reproductions (Chen et al. 2009). In our study, we found that phenamacril-resistant *F. fujikuroi* isolates are a severe problem in rice production in Zhejiang Province and these mutant isolates have high resistance frequency and speed of spread. In this study, we also found a new point mutation at codon 218 in the *myosin-5* gene in *F. fujikuroi* responsible for conferring resistance to phenamacril.

High levels of resistance to phenamacril are common in the rice population in Zhejiang Province, especially in Jiaying. A total of 72 *F. fujikuroi* isolates were collected from phenamacril-treated rice fields in 2017 and 222 isolates were collected in 2018. Analysis of the sensitivity level of the 294 *F. fujikuroi* isolates to phenamacril showed that the total phenamacril resistance frequency increased from 18% in 2017 to 47% in 2018. A previous report mentioned that once phenamacril resistance in *F. fujikuroi* has appeared in a rice field, resistance can develop quickly, leading to poor control of rice bakanae disease in the field (Hou et al. 2018). In our study, detection of resistance levels in two consecutive years showed that the resistance ratio of *F. fujikuroi* isolates obtained from Jiaying was the highest, whereas the resistance ratio of *F. fujikuroi* isolates collected from Jinhua was zero. This finding suggests that the population of *F. fujikuroi* isolates in rice fields is geographically different. The resistance frequency of *F. fujikuroi* isolates collected from Shaoxing and Jiaying increased from 0 to 12% and 42 to 96%, respectively. In addition, the resistance ratio of the *F. fujikuroi* isolates obtained from the same rice field in Shaoxing and Jiaying also increased from 2017 to 2018. These results cautioned us not to ignore the development of resistance when phenamacril is used to prevent the spread of *F. fujikuroi* isolates in the field.

Phenamacril was formally registered in 2012 for controlling rice bakanae disease and *Fusarium* head blight. The first phenamacril-resistant

Table 2. Activity of phenamacril against *Fusarium fujikuroi* in rice seedlings

Treatment ^y	Seedling height (mm) ^z							
	CK		FFJX18-54 ^S		FFJX17-09 ^R		FFJX18-76 ^R	
	–	+	–	+	–	+	–	+
I	117.8 ± 11.5 b	115.1 ± 10.8 b	184.4 ± 20.4 a	120.8 ± 16.7 b	182.0 ± 35.4 a	181.6 ± 13.7 a	179.2 ± 21.0 a	183.0 ± 15.8 a
II	107.8 ± 6.3 c	110.6 ± 5.1 c	204.1 ± 21.6 ab	121.4 ± 10.5 c	215.7 ± 13.2 a	220.0 ± 20.4 a	189.6 ± 26.2 b	190.0 ± 22.3 b

^y I = germinating seeds were incubated with 50 mg/liter of phenamacril for 6 h, followed by incubation with the spore suspension for another 20 h; and II = germinating seeds were incubated with the spore suspension for 20 h, followed by incubation with 50 mg/liter of phenamacril for another 6 h.

^z Height in millimeters is the unit of the average rice height of each treatment. Values are means ± SE of 24 seedlings from three independent experiments. Values in each column followed by different letters are significantly different ($P < 0.05$) according to Fisher's least significant difference test. Minus signs indicate germinating seeds that were incubated with methyl alcohol, whereas plus signs indicate germinating seeds that were incubated with phenamacril. S = phenamacril-sensitive isolate and R = phenamacril-resistant isolate.

F. fujikuroi isolate was documented in 2016 and has since developed rapidly in the rice plant field. It is noteworthy that phenamacril is still a good fungicide for *Fusarium* head blight disease management. We speculate that the difference in phenamacril resistance levels between rice bakanae disease and wheat head blight is caused by the patterns of fungicide application. Rice bakanae disease is commonly controlled by seed dressing, which may add a selective pressure of fungicides and cause a higher risk for and development of phenamacril resistance. Similar resistance development occurred when MBCs and DMIs against *F. fujikuroi* were used to control *F. graminearum*. Through field studies, carbendazim-resistant *F. fujikuroi* isolates were found in 1980 and carbendazim-resistant *F. graminearum* isolates were found in 1992 (Zhou et al. 1994). We previously reported prochloraz-resistant *F. fujikuroi* isolates in rice fields (Zhang 2018); however, prochloraz-resistant *F. graminearum* isolates have not been found in wheat fields (Yin et al. 2009). Prior studies also proved that the fitness of the tebuconazole-resistant and phenamacril-resistant *F. graminearum* isolates is reduced (Gao 2017; Zheng et al. 2015).

According to previous studies, laboratory-induced phenamacril-resistant mutants have the same fitness as the parents (Chen et al. 2008, 2011). In this study, both phenamacril-sensitive and phenamacril-resistant isolates produced abundant microconidia and induced similar elongated primary leaves in rice seedlings with similar excessive growth. Except for isolate FFJX18-14^R, other phenamacril-resistant isolates showed mycelial growth similar to the phenamacril-sensitive isolates. In addition, except for isolate FFJX17-09^R, the other phenamacril-resistant isolates produced either similar or more spores compared with isolates FFSX18-02^S and FFJX18-54^S. Therefore, the “excellent” fitness of the phenamacril-resistant isolates allowed them to maintain their phenamacril resistance continuously in rice fields.

In this study, we found phenamacril-resistant *F. fujikuroi* isolates with the S219P mutation in the field, similar to a previous report (Hou et al. 2018). In a different report, Li et al. (2016) reported that their laboratory-induced phenamacril-resistant mutants contained a point mutation at codon 216 (AAG→AGG or AAG→GAG) in *myosin-5* of *F. asiaticum*, which is a pathogen of *Fusarium* head blight (Li et al. 2016). Here, we reported (to our knowledge, for the first time) a lysine to threonine substitution at codon 218 in the field-collected phenamacril-resistant *F. fujikuroi* isolates. We also found that all of the phenamacril-resistant *F. fujikuroi* isolates collected from Shaoxing in 2018 contained an amino acid substitution at codon 218. It was suggested that phenamacril can dock into the Myosin-5 binding pocket (Hou et al. 2018; Zhang et al. 2015). *F. fujikuroi* isolates containing the Myosin-5 protein with the S219P mutation or the K218T mutation may have lower affinity for the fungicide, leading to the loss of phenamacril efficacy in controlling rice bakanae disease. In this study, phenamacril-resistant isolates carrying the K218 mutation were the dominant isolates in rice fields. A total of 104 phenamacril-resistant isolates were found in the 2018 collected samples, and 92 of the 104 isolates carried the K218T mutation. The reason this caused the occurrence of K218T mutation in *F. fujikuroi* remains unknown and requires further research.

Fungicide applications are economical and effective tools for rice bakanae disease management. However, the emergence of various mutant isolates makes DMIs, MBCs, and phenamacril less effective in rice fields (Chen et al. 2014; Hou et al. 2018; Zhang et al. 2013). Fortunately, we found that isolates FFJX18-76^R and FFJX18-14^R with the K218T mutation were sensitive to tebuconazole (DMI), prochloraz (DMI), or carbendazim (MBC), and isolates FFJX17-09^R and FFJX17-08^R with the S219P mutation were sensitive to DMIs (*data not shown*). Collectively, in this study, we revealed the rapid development of phenamacril resistance in *F. fujikuroi* and the “excellent” fitness of mutant isolates, and these results will help farmers use phenamacril properly in controlling rice bakanae disease. Furthermore, the sensitivity of K218T mutants to DMIs and MBCs may help farmers use a combination of two or more fungicides to control *Fusarium* diseases in the field or help researchers develop more comprehensive control strategies for phenamacril-resistant *F. fujikuroi* isolates.

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