Genotypes and Characteristics of Phenamacril-Resistant Mutants in *Fusarium asiaticum*

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Abstract

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Fusarium asiaticum is a critical pathogen of Fusarium head blight (FHB) in the southern part of China. The fungicide phenamacril has been extensively used for controlling FHB in recent years, which reduced both FHB severity and mycotoxin production. Our previous report indicated that resistance of *F. asiaticum* to phenamacril was related to mutations in *myosin5*. A recent article revealed that the resistance level of phenamacril-resistant mutants was associated with the genotypes of *myosin5* in these mutants. In total, we obtained 239 resistant isolates by fungicide domestication, and 82 resistant mutants were randomly selected for further study. Of these mutants, 25.6, 7.3, and 67.1% showed low resistance (LR), moderate resistance (MR), and high resistance (HR), respectively, to phenamacril determined

by 50% effective concentration values. Point mutations A135T, V151M, P204S, I434M, A577T, R580G/H, or I581F led to LR. Point mutations S418R, I424R, and A577G were responsible for MR and point mutations K216R/E, S217P/L, or E420K/G/D conferred HR. Interestingly, all of the mutations concentrated in the myosin5 motor domain and mutations conferring HR occurred at codon 217 and 420, which we called the core region. Homology modeling revealed that mutations far from the core region led to a lower resistance degree. Phenotype assays revealed that the most highly resistant mutants did not significantly change pathogenicity but decreased conidia production compared with the wild type, which may slow down the formation of the resistant pathogen population in the fields.

Fusarium head blight (FHB) of wheat and other small cereal grains is a devastating disease worldwide, which can produce harmful mycotoxins in infected grain and threaten animal and human health (Bai and Shaner 2004; Bottalico 1998; Goswami and Kistler 2004). Thus far, 11 phylogenetic species are causal agents of FHB and these species belong to the *Fusarium graminearum* Schwabe species complex of β -trichothecene toxin producers (O'Donnell et al. 2004; Starkey et al. 2007; Suga et al. 2008; Tóth et al. 2005). FHB disease has become increasingly severe in the middle and lower reaches of the Yangtze River, the southern winter wheat region, and the northeastern spring wheat region in China since FHB was first reported in 1936 (Chen et al. 2000). Zhang et al. (2007) reported that 77.3% of the 299 isolates collected from wheat of various regions that experienced FHB epidemics in China were identified as *F. asiaticum*, and another 68 isolates were confirmed to be *F. graminearum*.

Because few wheat cultivars are available with natural resistance to *Fusarium* spp., the control of FHB has depended on application of fungicides during wheat anthesis in the past few decades (Chen et al. 2011; Parry et al. 1995; Windels 2000). A novel cyanoacrylate fungicide, phenamacril (JS399-19), has been extensively used to control FHB and reduce mycotoxin contamination, such as 25% SC (certificate number LS20072657). (Chen and Zhou 2009; Diao et al. 2012; Yu et al. 2011; Zhang et al. 2010b). However, mutants with levels of resistance to phenamacril have been easily obtained by fungicide domestication and UV irradiation in *F. asiaticum* (Chen et al. 2008). We also found that point mutations of *myosin5* were responsible for phenamacril resistance in *F. graminearum* through comparison of the *myosin5* gene sequences between wild-type and highly resistant mutants (Zheng et al. 2015).

In addition, Zhang et al. (2015) revealed that JS399-19 (phenamacril) inhibited ATPase activity of the FgMyo1 (myosin5) motor domain and had a serious effect on localization of FgMyo1 at the tips of germlings.

Myosin5 is actin-dependent ATPase motor and contains two motor domains: tail homology 1 and src homology 3. In *F. graminearum*, myosin5 encodes an essential gene and the myosin5 deletion mutant had a lethal effect on this fungus (Zhang et al. 2015).

It is well known that mutations at different drug target sites generate variable degrees of drug resistance. For example, a change at codon 198 in β -tubulin of *F. asiaticum* will confer high resistance to benzimidazoles, and a change at codon 167 will confer moderate resistance (Qiu et al. 2011). In this article, we studied the frequency of low, moderate, and high resistance to phenamacril in *F. asiaticum* mutants, and evaluated their fitness with the biological characteristics of growth rate, colony morphology, and asexual reproduction. Genotypes associated with resistance degree were also analyzed by sequencing the whole *myosin5* gene. These results will help to understand the risk of phenamacril resistance and develop molecular techniques to diagnose phenamacril resistance in *F. asiaticum*.

Materials and Methods

Fungicide and media. Technical-grade phenamacril (95%; experimental code JS399-19; active ingredient 2-cyano-3-amino-3-phenylacrylic acetate) was provided by Jiangsu Branch of the National Pesticide Research & Development South Center and dissolved in methanol to 1 and 4 mg/ml for stock solution, which was added into autoclaved media for testing inhibition of mycelia growth. Potato dextrose agar (PDA; 200 g of potato, 20 g of glucose, 15 g of agar, and 1 liter of water) was used for routine cultures and for testing sensitivity of phenamacril. Mung bean liquid (MBL) medium (30 g of mung bean boiled in 1 liter of water for 20 min; the mixture was then filtered through cheesecloth) was prepared for conidia production. Water agar (2% agar) medium was used to recover single-spore isolates.

Recovery of phenamacril-resistant mutants. In all, 1,000 fresh mycelial plugs from the colony margins of sensitive strain 2021 were transferred to the 250 PDA plates amended with phenamacril at 10 μ g/ml and incubated in a growth chamber at 25°C. Any spontaneous and fast-growing sectors from the restricted colonies on PDA plates were selected for resistance identification on PDA plates amended with phenamacril at 1.5, 15, or 75 μ g/ml. For the stable phenamacril-resistant mutants, single-spore isolates were established. The number of resistant mutants was counted (Chen et al. 2008).

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Stability of the resistance to phenamacril. All the resistant mutants and their parental *F. asiaticum* strain 2021 were transferred eight times onto new PDA plates with no fungicide, by transferring single mycelial plugs to the new PDA plates every 5 days for a period of 40 days, or stored at 4°C in the refrigerator for 40 days. Resistance to phenamacril was determined again for resistance stability.

Characterization of *F. asiaticum* mutants resistant to phenamacril. For all of these *F. asiaticum* mutants, the levels of resistance to phenamacril were determined by transferring 5-mm plugs from the edge of an actively growing colony (3 days old) to PDA media amended with phenamacril at 0, 1.5, 15, and 75 µg/ml simultaneously; plugs were incubated at 25°C in the growth chamber for 3 to 5 days. Then, we randomly selected 82 mutants from among the 239 mutants to narrow the sample. Based on the colony growth of the 82 mutants at various concentrations of phenamacril (0, 1.5, 15, and 75 µg/ml), we placed the mycelial plugs (5 mm in diameter) which grew at 1.5 to 15, 15 to 75, and >75 µg/ml on the center of a PDA plate amended with JS399-19 at 0, 1, 2, 4, or 8 µg/ml; 0, 10, 20, 40, or 80 µg/ml; or 0, 50, 100, 200, or 400 µg/ml, respectively. There were three replicates for each concentration. After cultures were kept at 25°C for 3 to 14 days, the 50% effective concentration (EC₅₀) values of resistant mutants were calculated by regressing percent growth inhibition against the log of fungicide concentration with DPS software, as described previously (Li et al. 2008). According to mean EC₅₀ values, these 82 resistant mutants were divided into groups showing low resistance (LR, 1.5 to 15 µg/ml), moderate resistance (MR, 15.1 to 75 µg/ml), and high resistance (HR, >75 µg/ml), determined by EC₅₀ values as in the previous classified method (Chen et al. 2008).

Hyphal growth and conidia production of *F. asiaticum* mutants resistant to phenamacril. After culturing these mutants for 3 days on PDA, mycelial plugs (5 mm in diameter) of the mutants were transferred to PDA plates at 25°C for 3 days. Three replicates were performed for each of the selected mutants and the tests were repeated twice. Colony diameter (in centimeters) was measured to compare mycelial linear growth, and means were compared with Fisher's least significant difference test with DPS software. Photographs of colony morphology were taken on the third day.

For conidium production assays, 10 mycelial plugs (5 mm in diameter), taken from the periphery of a 3-day-old colony of randomly selected resistant mutants, were added to a 200-ml flask containing 100 ml of MBL medium. Each strain was represented by three flasks. The flasks were incubated at 25°C for 7 days with shaking (185 rpm). Production of conidia was assessed after 7 days of incubation using a hemocytometer and a microscope. The experiment was conducted three times.

DNA extraction. The selected mutants were cultured on PDA for 5 to 14 days and aerial hyphal were obtained for DNA extraction. The aerial hyphal were suspended in 700 ml of cetyltrimethylammonium bromide (100 mM Tris-HCl [pH 8.0], 20 mM EDTA, and 1.5 M NaCl) and 700 ml of phenol + chloroform + isoamyl alcohol (25:24:1). After the suspensions had been incubated at 37°C for 2 h, the aqueous layers were extracted once with chloroform, and isopropyl alcohol was added to precipitate DNA. The DNA pellet was collected by centrifugation, washed with 70% ethanol, and resuspended after drying in 50 µl of Millipore water (Brandfass and Karlovsky 2008).

Cloning and sequencing of *myosin5* gene in phenamacril-resistant isolates of *F. asiaticum*. Complete DNA sequences of a *myosin5* locus (ranging from 313 bp upstream to 202 bp downstream of the *myosin5* coding region) in *F. asiaticum* were obtained using a BLAST search of the *Fusarium* genome (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html). These sequences were used to design the polymerase chain reaction (PCR) primer pairs 01410F 5'-TCCCGTCAGGCACCACTA-3' and 01410R 5'-CGGTGCCCCAGTTTCAGTC-3', that were used for amplification of the whole fragment of the *myosin5* gene (FGSG 01410). PCR mixtures contained total DNA (25 ng/µl), 1.5 mM MgCl₂, 200 µM dNTP, and 2.5 U of LA Taq DNA polymerase. PCR was performed using a TaKaRa PCR Thermal Cycler Dice. The PCR program included an initial denaturation step at 94°C for 5 min; followed by 35 cycles at 94°C for 30 s, 53°C for 1 min, and 72°C for 4 min; a final extension for 10 min at 72°C; and holding at 12°C. The amplified

myosin5 gene, of approximately 3.8 kb in size (eliminated upstream 313 bp and downstream 202 bp of the *myosin5* coding region), was obtained from genomic DNA of *F. asiaticum*. Individual amplified fragments were purified, cloned, and then sequenced and used to compare the *myosin5* sequence between wild-type strain 2021 and resistant mutants using V7.0.9.0 Bioedit (Isis Pharmaceuticals) (Zheng et al. 2015).

The virulence assays of mutants with different genotypes. Seed of 'Zhenmai' wheat were incubated on moist filter paper at 20°C for 3 days and used to assay mutant virulence (Wu et al. 2005). The top 2 to 3 mm of the coleoptiles were cut from the 3-day-old seed, and the tip was injected with 10 µl of conidial suspension. The coleoptiles were then incubated at 25°C, 95% relative humidity, and 12 h of fluorescent light per 24 h. There were 10 coleoptiles per isolate and three repeat experiments to ensure the accuracy the assays. The pathogenicity of the strains and mutants were assessed by measuring the length of brown lesions on diseased stems at 14 days postinoculation (dpi) (Zhang et al. 2010a).

Homology modeling of the *F. asiaticum* myosin5 motor domain. The sequence alignment of the myosin5 motor domain displayed 46% identity with the crystal structure of the actin-tropomyosin-myosin complex in *Dictyostelium discoideum* (PDBID:4A7F), which was used to perform the homology modeling on the Discovery Studio 3.5. A three-dimensional model of the *F. graminearum* myosin5 motor domain was built. The resulting structure was viewed with the Cn3D 4.3.1 three-dimensional structure viewer.

Results

Induction of phenamacril-resistant mutants from phenamacril-sensitive isolate 2021. All 1,000 fresh mycelial plugs from phenamacril-sensitive strain 2021 grew poorly on PDA plates amended with phenamacril at 10 µg/ml during the first 10 days. Only a few spontaneous sectors of these plugs grew fast after 15 days, and 239 resistant mutants from the growing sectors were obtained by selection for resistance to the fungicide after 20 days. However, their parental strain 2021 cannot grow any mycelia. The 239 resistant mutants, which were recovered from the phenamacril-sensitive strain 2021, grew well on the PDA plates amended with phenamacril at 1.5, 15, or 75 µg/ml.

Resistance stability of the resistant mutants. All of the 239 single-spore progenies of each resistant mutant grew normally on PDA plates amended with phenamacril at 1.5, 15, or 75 µg/ml, indicating stability of resistance to this fungicide. Based on colony growth on PDA plates amended with phenamacril at 1.5, 15, or 75 µg/ml, all of the resistant mutants maintained their resistance to phenamacril through eight transfers on PDA plates for 40 days, and when stored on PDA slants at 4°C for 40 days (Fig. 1).

Frequency of different resistance levels of *F. asiaticum* mutants to phenamacril. After testing the EC₅₀ of all 82 resistant mutants, we found that 21 mutants belonged to category LR, 6 mutants belonged to MR, and 55 mutants belonged to HR. Therefore, the frequency of LR, MR, and HR was 25.6, 7.3, and 67.1%, respectively (Table 1). This small sample could indirectly reflect the frequency of the big sample (239 mutants) or the actual laboratory and fields sample.

Genotypes of *myosin5* gene in the laboratory induced phenamacril-resistant *F. asiaticum* mutants. Comparison of the nucleotide sequence of the *myosin5* gene in *F. asiaticum* strain 2021 revealed that 12 codon positions of point mutations (codon 135, 151, 204, 216, 217, 418, 420, 424, 434, 577, 580, and 581) were present in the sequences of all the resistant mutants. Among these mutants, changes at codons 135 (A→T), 151 (V→M), 204 (P→S), 434 (I→M), 577 (A→T), 580 (R→G, R→H), and 581 (I→F) were found in 21 LR mutants out of the 82 phenamacril-resistant mutants. Six MR mutants expressed three kinds of point mutations in the *myosin5* gene, including codons 418 (S→R), 424 (I→R), and 577 (A→G). However, 55 resistant mutants belonged to HR mutants and point mutations occurred at codons 216 (K→R, K→E), 217 (S→P, S→L), and 420 (E→K, E→G, E→D) (Table 2). Among all of the point mutation types, mutation type E→K showed extremely high resistance (Table 2). It was noticeable that the resistant mutants with differential resistance levels (LR, MR, and HR) exhibited different point mutations in the *myosin5* gene at codons 135, 151, 204, 216, 217, 418, 420, 424, 434, 577, 580, and 581. In addition, all of these mutations

concentrated in the myosin5 motor domain and, as is common, two kinds of mutation positions occurred at codon 217 and 420.

Hyphal growth rate, colony morphology, and conidia production of *F. asiaticum* mutants resistant to phenamacril. Compared with phenamacril-sensitive *F. asiaticum* strain 2021, many resistant

mutants did not show any changes in mycelial growth rate on fungicide-free PDA plates, and only a few mutants exhibited growth defects, indicating no significant reduction of this fitness parameter due to resistance. However, many resistant mutants had a distinctive colony morphology on PDA plates. Mutants with mutations at codon

Fig. 1. Resistance stability of *Fusarium asiaticum* sensitive strain 2021 and several mutants with low resistance (LR), moderate resistance (MR), and high resistance (HR) on potato dextrose agar amended with phenamacril at 1.5, 15, or 75 µg/ml.

Table 1. Frequency and 50% effective concentration (EC₅₀) of phenamacril-resistant mutants from *Fusarium asiaticum*-sensitive strain 2021

Level, codon ^a	Type ^b	N ^c	Freq (%) ^d	Mutants	EC ₅₀
LR					
135	A→T	14	17.1	T13, T26, T64, T66, T111, T2-23, T2-25, T2-26, T2-35, T2-38, T2-39, T2-51, T2-75, T2-94	1.88–8.24
151	V→M	1	1.2	T93	2.17
204	P→S	1	1.2	T4	1.48
434	I→M	1	1.2	T2-60	2.95
577	A→T	1	1.2	T76	3.22
580	R→G	1	1.2	T54	12.41
	R→H	1	1.2	T86	2.53
581	I→F	1	1.2	T2-102	3.45
MR					
418	S→R	1	1.2	T40	33.96
424	I→R	2	2.4	T2-89, T2-56	18.54–20.11
577	A→G	3	3.7	T21, T2-14, T2-76	19.21–28.38
HR					
216	K→R	1	1.2	T79	192.05
	K→E	1	1.2	T122	186.16
217	S→P	2	2.4	T105, T2-5	149.31–206.54
	S→L	27	32.9	T1, T3, T10, T12, T18, T25, T51, T91, T99, T106, T2-1, T2-7, T2-16, T2-18, T2-29, T2-32, T2-37, T2-41, T2-48, T2-67, T2-69, T2-71, T2-85, T2-112, T2-117, T2-125, T2-127	91.17–167.40
420	E→K	22	26.8	T11, T14, T32, T48, T69, T73, T89, T95, T101, T102, T2-3, T2-9, T2-21, T2-61, T2-78, T2-81, T2-82, T2-86, T2-90, T2-93, T2-98, T2-107	259.41–448.88
	E→G	1	1.2	T50	209.50
	E→D	1	1.2	T112	232.18

^a Resistance level is determined by EC₅₀ values: low resistance (LR, 1.5 to 15 µg/ml), moderate resistance (MR, 15.1 to 75 µg/ml), and high resistance (HR, >75 µg/ml). Codon = mutation position.

^b Mutation types.

^c Number of mutants; 82 mutants were randomly selected from the 239 resistant mutants.

^d Resistance frequency.

Table 2. Mutation types, resistance level, and growth rate (GR) of the selected 82 resistant mutants^a

Strains	Codon 135	Codon 151	Codon 204	Codon 216	Codon 217	Codon 418	Codon 420	Codon 424	Codon 434	Codon 577	Codon 580	Codon 581	EC ₅₀ (µg/ml)	GR (cm/day)
2021	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	0.21	2.44 ± 0.12
T13	ACC (T)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	2.38	2.34 ± 0.09
T26	ACC (T)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	3.40	1.82 ± 0.10
T64	ACC (T)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	2.52	2.17 ± 0.04
T66	ACC (T)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	2.86	1.07 ± 0.06
T111	ACC (T)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	5.23	1.08 ± 0.13
T2-23	ACC (T)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	4.06	2.06 ± 0.03
T2-25	ACC (T)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	2.55	2.32 ± 0.05
T2-26	ACC (T)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	3.05	1.41 ± 0.09
T2-35	ACC (T)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	1.88	2.47 ± 0.10
T2-38	ACC (T)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	8.24	1.29 ± 0.05
T2-39	ACC (T)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	5.99	1.72 ± 0.09
T2-51	ACC (T)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	4.06	2.66 ± 0.02
T2-75	ACC (T)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	7.26	1.72 ± 0.02
T2-94	ACC (T)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	2.57	1.69 ± 0.07
T93	GCC (A)	ATG (M)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	2.17	2.66 ± 0.05
T4	GCC (A)	GTG (V)	TCT (S)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	1.48	1.91 ± 0.06
T79	GCC (A)	GTG (V)	CCT (P)	AGG (R)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	192.05	1.74 ± 0.02
T122	GCC (A)	GTG (V)	CCT (P)	GAG (E)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	186.16	1.41 ± 0.03
T105	GCC (A)	GTG (V)	CCT (P)	AAG (K)	CCA (P)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	206.54	2.46 ± 0.03
T2-5	GCC (A)	GTG (V)	CCT (P)	AAG (K)	CCA (P)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	149.31	1.93 ± 0.04
T1	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	109.75	2.07 ± 0.12
T3	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	150.96	1.99 ± 0.06
T10	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	126.71	2.51 ± 0.02
T12	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	156.29	1.62 ± 0.07
T18	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	119.07	2.47 ± 0.04
T25	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	120.36	1.87 ± 0.01
T51	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	155.10	2.43 ± 0.03
T91	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	104.35	2.57 ± 0.05
T99	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	119.73	1.95 ± 0.03
T106	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	167.40	1.28 ± 0.04
T2-1	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	105.85	2.31 ± 0.07
T2-7	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	98.95	2.38 ± 0.08
T2-16	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	149.77	2.39 ± 0.02
T2-18	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	125.65	1.32 ± 0.02
T2-29	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	124.69	2.45 ± 0.03
T2-32	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	106.36	0.57 ± 0.05
T2-37	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	92.41	2.52 ± 0.02
T2-41	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	91.17	2.07 ± 0.01
T2-48	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	95.34	2.44 ± 0.05
T2-67	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	98.95	2.22 ± 0.02
T2-69	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	124.68	2.62 ± 0.02
T2-71	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	140.49	2.20 ± 0.10
T2-85	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	102.91	1.91 ± 0.05
T2-112	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	118.91	2.45 ± 0.03
T2-117	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	140.65	1.52 ± 0.03
T2-125	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	125.86	1.92 ± 0.02
T2-127	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TTA (L)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	131.45	0.65 ± 0.03
T40	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGG (R)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	33.96	2.21 ± 0.07
T11	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	431.03	1.33 ± 0.11
T14	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	308.31	1.71 ± 0.12
T32	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	448.88	1.32 ± 0.12
T48	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	320.16	0.61 ± 0.01
T69	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	426.62	1.85 ± 0.04
T73	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	330.09	1.26 ± 0.12
T89	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	293.84	1.79 ± 0.02
T95	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	259.41	1.44 ± 0.05
T101	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	320.38	1.43 ± 0.07
T102	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	301.36	1.54 ± 0.05
T2-3	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	338.38	1.62 ± 0.10
T2-9	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	319.86	1.06 ± 0.09
T2-21	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	239.43	1.64 ± 0.06
T2-61	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	332.09	1.15 ± 0.03
T2-78	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	287.37	1.32 ± 0.07
T2-81	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	309.46	1.55 ± 0.03
T2-82	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	289.42	1.82 ± 0.07
T2-86	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	301.25	1.34 ± 0.05
T2-90	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	286.37	1.44 ± 0.05
T2-93	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	278.31	1.22 ± 0.02
T2-98	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	269.94	0.82 ± 0.02
T2-107	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	AAA (K)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	269.36	1.52 ± 0.02
T50	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GGA (G)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	209.50	2.08 ± 0.05
T112	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAT (D)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	232.18	2.00 ± 0.07
T2-89	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ACT (T)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	20.11	2.03 ± 0.03
T2-56	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ACT (T)	ATC (I)	GCA (A)	CGT (R)	ATC (I)	18.54	0.67 ± 0.05
T2-60	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATG (M)	GCA (A)	CGT (R)	ATC (I)	2.95	1.80 ± 0.04
T76	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	ACA (T)	CGT (R)	ATC (I)	3.22	2.37 ± 0.05
T21	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GGA (G)	CGT (R)	ATC (I)	28.38	2.26 ± 0.03
T2-14	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GGA (G)	CGT (R)	ATC (I)	26.21	2.52 ± 0.07
T2-76	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GGA (G)	CGT (R)	ATC (I)	19.21	2.37 ± 0.05
T54	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	GGT (G)	ATC (I)	12.41	2.35 ± 0.04
T86	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CAT (H)	ATC (I)	2.53	2.57 ± 0.05
T2-102	GCC (A)	GTG (V)	CCT (P)	AAG (K)	TCA (S)	AGT (S)	GAA (E)	ATT (I)	ATC (I)	GCA (A)	CGT (R)	TTC (F)	3.45	1.89 ± 0.02

^a Strains = strains and mutants and EC₅₀ = 50% effective concentration.

217 produced slightly different colonies but mutants with mutations at codon 420 had distinctive colony morphologies and significantly reduced hyphal growth rates (Fig. 2; Table 2). Most of the highly resistant mutants produced significant fewer conidia than the parental strain 2021 (Fig. 3).

Virulence assays of induced mutants at codons 135, 217, and 420. Because the frequency of mutations at codons 135, 217, and 420 was much higher than at others, we choose the mutants

T13 (with mutation at codon 135); T25 (with mutation at codon 217); and T32, T50, and T112 (with mutation at codon 420) to assay virulence. T32, T50, and T112 represent the change of the amino acid E→K, E→G, and E→D, respectively. In addition, T25 represents the change of amino acid S→L; we did not choose the change of amino acid S→P because the conidia production of T105 and T2-5 was too little to assay the virulence. Assays with sensitive strain 2021 and dH₂O as the blank control were conducted. Stems started to turn

Fig. 2. Colony morphology of the *Fusarium asiaticum*-sensitive strain 2021 and the resistant mutants which have mutations at codon positions 217 and 420. Mycelial plugs (5 mm in diameter) of *F. asiaticum*-sensitive strain 2021 and mutants were transferred to potato dextrose agar plates at 25°C for 3 days.

Fig. 3. Conidia production of randomly selected resistant mutants and the *Fusarium asiaticum*-sensitive strain 2021. All strains were represented by three flasks, and the flasks were incubated at 25°C for 7 days with shaking (185 rpm). LR = low resistance, MR = moderate resistance, and HR = high resistance.

brown within 3 dpi, and dark-brown lesions were evident on the coleoptiles of the inoculated seedlings by 7 dpi (Fig. 4). The brown lesions of induced phenamacril-resistant mutants did not differ from the phenamacril-sensitive *F. asiaticum* strain 2021.

Homology modeling of *F. asiaticum* myosin5 motor domain. Because all of the mutations concentrated in the myosin5 motor domain, a three-dimensional model of the *F. asiaticum* myosin5 motor domain was constructed (Fig. 5). Twelve codon positions of point mutations (codon 135, 151, 204, 216, 217, 418, 420, 424, 434, 577, 580, and 581) in the myosin5 motor domain conferring resistance to phenamacril were highlighted in the model (Fig. 5A). Mutations at amino acids 216, 217, and 420, which conferred high resistance to phenamacril, formed a similar “pocket” structure on the inside of the myosin5 motor domain. Because most mutations conferring high resistance occurred at codon positions 217 and 420, we called that the core region. Mutations located far from the core region were associated with lower resistance to phenamacril (Fig. 5B).

Discussion

Phenamacril is a novel cyanoacrylate fungicide that can strongly inhibit the mycelial growth of *F. graminearum* and exhibit an excellent efficacy in controlling FHB in the field (Li et al. 2008). In recent years, application of this fungicide for managing FHB has been extensively relied on. Although we have not found large numbers of isolates resistant to phenamacril in the fields, the selection pressure in the existence of this fungicide could not be ignored. To assess the potential risk in the development of resistance to phenamacril in *F. asiaticum* in the fields, we produced many mutants resistant to phenamacril in the laboratory. In this study, 1,000 mycelial plugs of the *F. asiaticum*-sensitive strain 2021 were used to produce resistant mutants on PDA amended with phenamacril at 10 µg/ml. Of these sensitive mycelial plugs, 239 mutants resistant to phenamacril were obtained, with an overall phenamacril resistance frequency of 23.9%. These mutants maintained their resistance through eight transfers on PDA plates for 40 days and when stored on PDA slants at 4°C for 40 days. This stability indicated that the resistance to phenamacril was not a temporary adaptation of the pathogen to this fungicide.

Previous studies revealed that most phenamacril-resistant mutants exhibited similar mycelial growth rate when compared with sensitive strain 2021. However, some highly resistant mutants had distinctive colony morphologies. Several researchers found that gene deletion mutants would change colony morphology because the deletion strategy disrupted some gene functions (Farhat et al. 2013; Gu et al. 2015; Yu et al. 2014; Yun et al. 2015). However, distinctive colony morphologies of laboratory-induced *F. asiaticum*-resistant mutants might result from point mutations. Selection of fungicide led to the quick evolution of this fungus and changed some regions of the genome. Mutations in some regions directly conferred resistance whereas mutations in other regions directly or indirectly changed colony morphology. Conidia are responsible for the spread and infection of *F. asiaticum* during wheat flowering. However, most laboratory-induced HR mutants did not influence pathogenicity

but sometimes reduced conidial production. This may reduce the fitness of the resistant mutants in the field and may slow down the formation of the resistant pathogen population for a few years.

Previous studies have reported that mutations at codons K216E, S217P, L, S418R, and E420G of *myosin5* were responsible for resistance to the fungicide phenamacril (Zhang et al. 2015; Zheng et al. 2015). However, recent studies have confirmed that mutations in *myosin5* conferred resistance to phenamacril in the *F. graminearum* species complex. In our study, we found that mutations not only at codons 216, 217, 418 and 420 of *myosin5* but also at codons 135, 151, 204, 424, 434, 577, 580, and 581 of *myosin5* conferred resistance to phenamacril in *F. asiaticum*, and all of the mutations concentrated in the myosin5 motor domain. In addition, we also found new point mutation types at codon positions K216R, E420K, and E420D. Among the selected 82 resistant mutants, the frequencies of HR, MR, and LR mutants were 67.1, 7.3, and 25.6%, respectively. Interestingly, The quantity of mutants which had point mutations at codons 217 and 420 took up a large proportion (64.6%) in resistant mutants; we named that the core region. According to our homology modeling of the *F. asiaticum* myosin5 motor domain, we can see that mutations located far from the core region led to lower resistance to phenamacril. To our knowledge, this is the first report of an experiment studying phenamacril resistance by inducing *F. asiaticum* resistance in the laboratory and the point mutation in *myosin5* gene conferring resistance to phenamacril of laboratory-induced *F. asiaticum* populations.

Among resistant mutants in this study, different genotypes in *F. asiaticum* showed various resistance levels. We divided these mutations into two classes: one was resistance caused by target effect (HR) and the other was off-target effect (MR and LR). For the resistance mechanism of HR, we can assume that mutations occurred in different amino acid residues which influence binding to phenamacril to different degrees. For example, site-directed mutagenesis of the P225, N230, and H272 residues of succinate dehydrogenase subunit B from *Botrytis cinerea* could influence the binding to carboxamide fungicides (Lalève et al. 2014). For MR and LR, we hypothesized that the different changes in the amino acid residue affected inordinately the expression level of the proteins FgFim and FgApsB, which can regulate the resistance level of phenamacril (Zheng et al. 2013, 2014). The exact mechanism of resistance to phenamacril needs to be explored further.

Resistance is encoded in the *F. graminearum* species complex genome and is thought to arise through the serial acquisition of point mutations in the *myosin5* gene. Resistance-associated mutations can serve as biomarkers that can be rapidly identified in the fields by PCR. Current molecular diagnostics amplify and detect known fungicide resistance-associated mutations, and their performance depends on the inclusion of a comprehensive catalog of these mutations. Although known mutations indicated larger resistance risk to phenamacril, phenamacril-resistant field isolates would not exhibit the same level of resistance and the same point mutation types. Large-scale monitoring for resistance to this fungicide should be performed, because field phenamacril resistance would help to clarify

Fig. 4. Representative photographs of infected wheat coleoptiles inoculated with conidia of the *Fusarium asiaticum*-sensitive strain 2021 and mutants T13, T25, T32, T50, and T112. Brown lesions on the coleoptiles are indicated with arrows. CK = coleoptiles inoculated with distilled water.

this point. Also, in order to delay the development of phenamacril resistance, tank-mixing the fungicide phenamacril with other fungicides will be effective in controlling FHB.

Our study showed the diversity within resistant mutations in *myosin5* and the genotype frequency of different drug-resistant mutants, as well as the resistance level of these mutations. The study provided a theoretical basis for assessing the JS399-19 risk of resistance, and the molecular technology for detecting and managing the resistance.

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Fig. 5. Schematic representation of homology modeling of the *Fusarium asiaticum* myosin5 motor domain. **A**, Sites of Ala135, Val151, Pro204, Lys216, Ser217, Ser418, Glu420, Ile424, Ile434, Ala577, Arg580, and Ile581 mutations are indicated with black arrowheads. The conserved motor domain, myosin tail homology 1 (TH1), and src homology domain 3 (SH3) are highlighted. Bar indicates 100 amino acids (aa). **B**, Amino acids Ala135, Val151, Pro204, Lys216, Ser217, Ser418, Glu420, Ile424, Ile434, Ala577, Arg580, and Ile581 of the myosin5 motor domain are shown in yellow. Amino acids at these sites have been identified as mutations which conferred resistance in *F. asiaticum*.

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