Vegetative Compatibility of *Fusarium graminearum* Isolates and Genetic Study on Their Carbendazim-Resistance Recombination in China

Yu Chen, Jian-Xin Wang, Ming-Guo Zhou, Chang-Jun Chen, and Shan-Kui Yuan

College of Plant Protection, Nanjing Agricultural University, Nanjing, 210095, China.

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ABSTRACT

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Monoconidial isolates of 33 carbendazim-sensitive isolates and 31 carbendazim-resistant isolates of *Fusarium graminearum* were selected from three regions of China for vegetative compatibility group (VCG) analysis. A total of 213 and 224 *nit* mutants were recovered from the 33 sensitive and the 31 resistant isolates, respectively. Of all the *nit* mutants, the frequency of the different phenotypes was 44.6, 46.5, 5.7, and 3.2% for *nit1*, *nit3*, *nitM*, and *nitA*, respectively. VCG analysis identified 30 different VCGs among the 33 sensitive- and the 31 carbendazim-resistant isolates, with VCG diversity 0.91 and 0.97, respectively. Both, a carbendazim-sensitive and a -resistant isolate from the same field belonged

to the same VCG. In all then, a total of 59 VCGs were identified among the 64 isolates with an overall VCG diversity 0.92. Direct hyphal fusion was observed in six pairs of vegetatively compatible complements, which is evidence of heterokaryon formation. It was hypothesized that carbendazim resistance could not be transferred by hyphal fusion or there is a small chance to be transferred between two compatible isolates. Three stable sexual recombinants of *F. graminearum* were randomly chosen from each of the three genetic crosses to study their biological properties. There were no significant differences in mycelial linear growth and pathogenicity between recombinants and their parents, but they differ in sporulation ability and capacity to produce perithecia. We concluded that sexual recombination presumably played a role in the development of carbendazim resistance under field conditions.

Additional keywords: fungicide resistance, Fusarium head blight.

Fusarium graminearum (teleomorph *Gibberella zeae*) is the main pathogen of Fusarium head blight (FHB) on wheat and barley in China. FHB was the most economically important disease worldwide especially in the United States (20) and China (8).

In China, FHB generally occurs in the middle and lower reaches of the Yangtze River, Huaihe River Valley, and the eastern coastal region. The incidence and severity of FHB have increased in recent years (32). FHB not only resulted in the loss of grain yields by 5 to 15% in moderate epidemic years and up to 40% in severe epidemic years, but also caused decrease in grain quality because of containing mycotoxins (27), such as the deoxynivalenol (DON, vomitoxin) (24,26). The mycotoxins could inhibit amino acid incorporation and protein production in plant tissues (7), and also, caused emesis and feed refusal in animals (11,30).

To control FHB, fungicide sprays have been used during the last few decades because only few cultivars with effective resistance to this disease are available. The benzimidazole fungicides, particularly carbendazim (MBC), have been consistently used during the period of wheat heading and flowering in areas with warm and moist weather to control FHB in China for over 30 years (41). MBC resistance develops in many fungi after the fungicide has been used for 2 to 3 years (1,25,34). However, MBC resistance had not been detected in *F. graminearum* or reported worldwide until 1992, when the first MBC-resistant isolate of *F. graminearum* from the field was detected in the Zhejiang Province of China (41). A previous study determined that 100 sensitive isolates of *F. graminearum* could not grow on

potato sucrose agar (PSA) amended with MBC at 1.4 µg/ml, whereas resistant isolates grew normally at this concentration (40). Therefore, 1.4 µg/ml had been used as the critical concentration for detecting the resistance of field isolates in vitro. Continuous MBC-resistance monitoring showed that the frequency of resistant isolates in some regions of China increased gradually, and the efficacy of MBC against *F. graminearum* decreased dramatically after 1998 (31). Essentially, the effectiveness of carbendazim decreased along with the increase of the frequency of resistant pathogen populations in the field (40).

In many filamentous fungi, physiologically distinct individuals can fuse asexually to form a stable heterokaryon. Heterokaryosis provides an opportunity for genetic recombination via the parasexual cycle in sterile, homothallic, and imperfect fungi. The individuals that anastomose and form a stable heterokaryon are said to be vegetatively compatible and therefore belong to the same vegetative compatibility group (VCG). In *F. graminearum*, VCGs can be determined by using two basic techniques: (i) direct assessment of heterokaryon formation using auxotrophic mutants such as nitrate nonutilizing (*nit*) mutants (4), and (ii) direct assessment of the inability to form heterokaryons through barrage formation (18). *F. graminearum* is primarily homothallic, and the mode of inheritance of many traits has not been studied. Bowden and Leslie stated that the population structure of *F. graminearum* is poorly understood, and a better understanding of spatial and temporal patterns of genotypic diversity, potential sexual and asexual reproduction, and selection pressures, could lead to better control strategies for FHB (2). The objectives of this study were to (i) determine the vegetative compatibility between MBC-sensitive and -resistant *F. graminearum* from several regions of China, (ii) investigate whether the MBC resistance could be transferred by hyphal fusion in *F. graminearum*, and (iii) explore MBC

Corresponding author: M. G. Zhou; E-mail address: mgzhou@njau.edu.cn

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resistance in sexual recombination and characterization of sexual recombinants in *F. graminearum*. Presumably, this information might be important for the assessment of resistance risk of MBC, so that strategies to manage the resistance could be developed and implemented to avoid unexpected control failures and to sustain the usefulness of MBC.

MATERIALS AND METHODS

Fungicide and media. Technical grade carbendazim (MBC) was provided by Shenyang Academy of Chemistry and Industry (Shengyang, China). The material was dissolved in 0.1 mol/liter of hydrochloric acid (HCl) at 10 mg/ml as stock solution. MBC stock solution was added to autoclaved media that had cooled to 40 to 50°C and the pH was adjusted to 6.8 in all the media using 0.1 mol/liter of HCl.

PSA was used for routine culture and in tests for MBC sensitivity. Media, such as MMC (potassium chlorate medium), MM (minimal medium), MO₂ (nitrite medium), MH (hypoxanthine medium), and MA (ammonium medium), were used for isolating *nit* mutants and determining the types of mutation, respectively. The MM was made by adding 2.0 g of NaNO₃ to 1.0 liter of the basal medium (19). Mutants of *nit* were generated on the MM and MMC media. The MMC was prepared by adding 1.6 g of L-asparagine and 15.0 g of KClO₃ to 1.0 liter of the MM. The defined MM was amended with 0.2% of NaNO₂ (MO₂), 0.2% hypoxanthine (MH), and 1.0% ammonium tartrate (MA) (10). Carrot agar medium was used for producing perithecia (15). Mung bean broth (MBB, 50.0 g of mung bean per liter of distilled water) was prepared for conidia production. Water agar (WA) medium (1.8% agar) was used to recover single-spore isolates.

Fungal isolates and phenotypic characterization of carbendazim resistance. Wheat samples were collected from three commercial wheat fields, Tongzhou in Jiangsu Province, Jiaying in Zhejiang Province, and Shanghai of China during the harvest season of 2004. The wheat fields were separated from each other by more than 100 km. In each field several spikes with symptoms of FHB were randomly collected from a designated 5.0 × 5.0 m² area, air-dried, placed in paper envelopes, and stored at -20°C. Thirty-three monoconidial isolates sensitive to MBC (S01-S33) and 31 resistant (MBC at 1.4 µg/ml) isolates (R01-R31; previously characterized [31]), were used in this study. Each isolate originated from different diseased heads of wheat. All isolates were grown on PSA at 25°C for 3 to 5 days and stored on PSA slants at 4°C.

A wild-type isolate (ZF43) that was stored in our laboratory and two other isolates, ZF43-06 and ZF43-02-05, which were recovered from ZF43 by fungicide training, showed high resistance (HR) to MBC. These three isolates were used to determine whether the MBC mutation could change the vegetative compatibility and whether MBC resistance could be transferred by hyphal fusion in these studies.

The fungal isolates were re-assessed for MBC sensitivity by transferring 5-mm-diameter plugs from the leading edge of an actively growing colony to a series of PSA dishes amended with MBC at 0, 0.5, 1.4, 50, and 100 µg/ml and incubating at 25°C for 3 days. The isolates were classified into three different sensitivity phenotypes, such as sensitive (S), moderately resistant (MR), and highly resistant (HR), based on their growth at the various concentrations of MBC as described previously (36).

Seeds/cultivars. Yangmai56 is a local planted wheat cultivar sensitive to FHB. The seeds were used for perithecia production test and the spikes of the cultivars were used for pathogenicity test in *F. graminearum*, respectively.

Recovery and identification of *nit* mutants. After 3 days cultured on PSA, mycelial plugs of parental isolates (5 mm in diameter) were taken from colony margins and placed equidistantly

apart on a plate of MMC. Any spontaneous and fast-growing sectors from the restricted colonies on MMC plates were picked up for identification (4). Genetic terminology for these mutants followed the guidelines for plant pathogenic fungi (35). The phenotypes of the spontaneous and fast-growing sectors which could be resistant to chlorate were identified as previously described (9,10,39).

Vegetative compatibility test and genetic study on MBC resistance in hyphal fusion. At least two different *nit* mutants were obtained from each isolate and complementary pairings of *nit1*, *nit3*, and *nitM* were initially made to establish self-compatibility. Vegetative compatibility was tested by placing a mycelial plug of *nit1* mutant paired with another mycelial plug of *nit3* or *nitM* mutant approximately 2 cm apart on MM, with six replicate plates for each pairing. Plates were observed daily for up to 14 days and two isolates were judged to be compatible if wild-type growth (heterokaryotic growth) occurred at the interaction zone between their different *nit* (4). Lack of complementation might have meant that the mutants were not complementary and that the isolates were in different VCGs. VCGs were established by pairing in all possible pairwise combinations.

An MBC-sensitive *nit* mutant and an MBC-resistant *nit* mutant were taken as one pair for the complementary test as previously described (4), and pairings between vegetative compatible isolates with different *nit* mutant phenotypes were repeated three times. A mycelial plug was picked up from the hyphal fusion zone where wild-type mycelia growth had appeared after 7 to 15 days, transferred to MBB, and shaken for 7 days. The progeny (conidia) of the mycelial plug were collected and the conidia suspension was adjusted to 10³ conidia per ml. An aliquot of 200 µl of conidia suspension was spread onto a water agar plate and incubated at 25°C for 10 h. More than 1,000 germinated spores were picked up for further determination of *nit* mutant phenotypes and sensitivity to MBC as described above.

Crosses, preparation of hybrid progeny, and determination of sexual recombinants. Five isolates, representing the three phenotypes of the MBC sensitivity levels, were randomly selected to study the inheritance of MBC resistance by three genetic crosses (Table 1). Each parent in all crosses was marked with a different class of nitrate nonutilizing (*nit*) mutation. Mycelial plugs (5 mm in diameter) from cultures of each parent were placed 4 cm apart on opposite sides of 9-cm-diameter dishes containing carrot agar. The cultures were incubated at 25°C with a 12-h photoperiod for 7 days, which allowed sufficient time for the cultures to cross and produce perithecia (5).

About 50 putative heterozygous perithecia for each cross were sampled along the interface of colonies on carrot agar of the two parents under a dissecting microscope. The perithecia were crushed with a sterile needle on a concave slide, and the ascospores were washed into a tube with sterile water. The mixed ascospore suspensions were adjusted to 10³ spores per ml. An aliquot of 200 µl of ascospore suspension was spread onto a WA plate and incubated at 25°C for 10 h. For each cross, approximately 100 single-ascospore isolates were picked up and transferred to MM plates amended with streptomycin to minimize contamination with bacteria. The plates were incubated at 25°C for 3 to 5 days when the *nit* mutant phenotypes and sensitivity

TABLE 1. Parental isolates used in genetic crosses of *Fusarium graminearum* isolates collected from wheat fields in China

Genetic crosses	Parental isolates (and <i>nit</i> physiological phenotype)	Parental MBC phenotype ^z
A	S07 (<i>nitM</i>) × R14 (<i>nit1</i>)	S × MR
B	S09 (<i>nitM</i>) × R29 (<i>nit1</i>)	S × HR
C	R03 (<i>nitM</i>) × R29 (<i>nit1</i>)	MR × HR

^z S: sensitive isolate; MR: moderately resistant isolate; HR: highly resistant isolate.

level to MBC were determined as described above. The presence of both *nit* mutants and wild-type progeny indicated that outcrossing between the two parental isolates had occurred. Once outcrossing of the parents was confirmed, single perithecia were sampled along the interface and washed individually with sterile water at least three times to remove contaminating conidia. Perithecia were then crushed on a concave slide, and the ascospores were washed with 0.2 ml of sterile water and smeared onto MM plates amended with streptomycin. About 100 germinating single ascospores from each perithecium where the *nit*/wild-type ratio was 3:1 were examined further. All of the progeny of each heterozygous perithecium were transferred to PSA plates, cultures of the F₁ hybrid progeny were established, and the *nit* mutant phenotypes and sensitivity level to MBC were determined as described above. A sexual progeny with wild type, or with *nit* mutant phenotype and MBC sensitivity level combination different from its parents, was defined as a sexual recombinant. Three nonparental sexual recombinants were picked up randomly from the *nit* mutants of the progeny in each cross to study their biological properties (Table 2).

Biological properties of sexual recombinants (culture characterization, asexual and sexual reproduction ability, and pathogenicity). After 3 days cultured on PSA, mycelial plugs of sexual recombinant and their parental strains (5 mm in diameter) were transferred to PSA plates at 25°C for 3 days. Colony diameter (cm) was measured to compare mycelial linear growth and means were compared with Fisher's least significant difference (LSD) test. The color of the isolates and the growth of the aerial mycelium were also observed simultaneously.

After 3 days cultured on PSA, five mycelial plugs of sexual recombinant and their parental isolates (5 mm in diameter) were transferred to each conical flask containing 100 ml of MBB for preparing a shake culture for 7 days (150 rpm, 25°C, 12-h photoperiod). There were three replicates for each of the recombinants and their parental isolates, and the tests were repeated twice. Sporulation capacity of the isolates was determined by counting the number of spores with a hemacytometer as described previously (40). The conidial production of both recombinants and their parental isolates were compared using Fisher's LSD test.

To evaluate production of perithecia, autoclaved seeds of Yangmai56 wheat cultivar were inoculated with 5 mm in diameter mycelial plugs of the sexual recombinant and their parental isolates, incubated at 25°C for 7 days. Subsequently, the seeds were covered with sterile wet sands in petri plates (diameter = 9 cm) and incubated in a humid room (RH = 80%) at 25°C and under light for 12 h per day. Production of perithecia was scored as “+++”, “++”, and “+”, after 3 days incubation when the perithecia

covered more than 2/3, between 1/3 and 2/3, and less than 1/3 of the grain area, respectively.

Pathogenicity of the sexual recombinant and their parental isolates in the field in both 2005 and 2006 was compared using Fisher's LSD test. Firstly, conidial suspensions of each isolate were prepared in MBB and adjusted to a concentration of 10⁵ spores per ml. Then, 10 µl of conidia suspension of each isolate was injected into a flowering grain in the middle of the spike and at least 20 spikes were inoculated for each isolate. Control wheat spikes were injected with the same volumes of MBB. All treated and control plants were grown in the same wheat field in Nanjing Agricultural University (Nanjing, China). The severity of scabbed wheat heads was calculated as described previously (29).

Data analysis. Data were analyzed with ANOVA (SAS Institute, Cary, NC) to test for significant differences. Fisher's LSD test was used to determine significant difference on the biological properties between sexual recombinants and their parental isolates.

RESULTS

Vegetative compatibility of the MBC-sensitive and -resistant *F. graminearum* isolates in China. We arbitrarily selected monoonidial isolates of 33 sensitive isolates and 31 resistant isolates from the three regions of China to perform VCG analysis. A total of 213 *nit* mutants were recovered from the 33 sensitive isolates, and 224 *nit* mutants were recovered from the 31 resistant isolates. Of all the *nit* mutants, the frequency of the different phenotypes was 44.6, 46.5, 5.7, and 3.2% for *nit1*, *nit3*, *nitM*, and *nitA*, respectively. When the *nit1* mutants were paired with either *nit3* or *nitM* mutants on MM, all 64 isolates were self-compatible. When mutants from different isolates were paired on MM with each other, 30 different VCGs were identified in the 33 sensitive isolates and the VCG diversity (number of VCGs/number of isolates) averaged over the three regions was 0.91. In addition, 30 different VCGs were identified in the 31 resistant isolates and the VCG diversity was 0.97. However, between the sensitive and the resistant isolates, only R03 could complement with S21 and the two isolates both came from the same field in Zhejiang Province. Therefore, a total of 59 VCGs were identified among the 64 isolates with the overall VCG diversity 0.92.

Genetic analysis of MBC resistance in the hyphal fusion. Direct hyphal fusion was observed in six pairs in which wild-type mycelia growth appeared over the hyphal fusion zone as evidence of heterokaryons formation. All the progeny of “the hyphal fusion zone” exhibited their parental phenotype and no asexual recombinants were observed. This indicated that MBC resistance could

TABLE 2. Comparison of biological properties between sexual recombinants and their parental strains of *Fusarium graminearum*

Isolate (<i>nit</i> and sensitivity phenotype)	Mycelial linear growth (mm)	Sporulation capacity (10 ⁶ CFU/ml)	Fusarium head blight severity (%)		Peritheciogenous capacity
			2005	2006	
S07 (<i>nitM</i> -S)	63.77 ± 1.96 aA ^y	0.34 ± 0.04 aA	33.55 ± 6.57 aA	37.86 ± 6.37 aA	+++ ^z
R14 (<i>nit1</i> -MR)	62.20 ± 3.21 aA	0.45 ± 0.18 aA	37.64 ± 7.31 aA	34.12 ± 4.69 aA	+++
A-1 (<i>nit1</i> -S)	64.20 ± 1.87 aA	0.35 ± 0.09 aA	35.78 ± 7.34 aA	39.76 ± 4.62 aA	++
A-2 (<i>nitM</i> -MR)	62.47 ± 3.43 aA	0.36 ± 0.17 aA	35.46 ± 5.35 aA	34.68 ± 5.43 aA	++
A-3 (<i>nit1</i> - <i>nitM</i> -S)	64.90 ± 2.55 aA	0.87 ± 0.13 bB	36.78 ± 3.67 aA	35.43 ± 6.53 aA	+++
S09 (<i>nitM</i> -S)	63.17 ± 2.83 aA	1.95 ± 0.24 aA	38.25 ± 5.63 aA	34.67 ± 3.68 aA	+++
R29 (<i>nit1</i> -HR)	64.43 ± 2.70 aA	0.73 ± 0.09 cB	37.84 ± 5.22 aA	34.58 ± 4.07 aA	++
B-1 (<i>nitM</i> -HR)	62.77 ± 3.82 aA	1.01 ± 0.13 bcB	36.79 ± 5.34 aA	35.38 ± 3.45 aA	++
B-2 (<i>nit1</i> - <i>nitM</i> -S)	62.50 ± 3.40 aA	1.03 ± 0.17 bcB	37.53 ± 5.65 aA	36.36 ± 3.19 aA	+
B-3 (<i>nit1</i> - <i>nitM</i> -HR)	64.93 ± 3.07 aA	1.25 ± 0.11 bB	38.32 ± 5.24 aA	37.65 ± 4.87 aA	+
R03 (<i>nitM</i> -MR)	62.60 ± 1.50 aA	1.76 ± 0.29 aA	34.55 ± 5.76 aA	36.45 ± 3.34 aA	++
R29 (<i>nit1</i> -HR)	64.97 ± 2.04 aA	0.81 ± 0.11 cB	35.74 ± 6.47 aA	35.70 ± 4.28 aA	++
C-1 (<i>nitM</i> -HR)	62.53 ± 3.12 aA	1.05 ± 0.15 bcB	35.67 ± 6.71 aA	35.74 ± 5.78 aA	+
C-2 (<i>nit1</i> -MR)	63.50 ± 2.20 aA	1.07 ± 0.17 bcB	36.32 ± 5.83 aA	33.72 ± 5.34 aA	+++
C-3 (<i>nit1</i> -MR)	63.24 ± 1.97 aA	1.28 ± 0.13 bB	35.98 ± 6.39 aA	33.65 ± 4.53 aA	++

^y Difference analysis between every parent and resistant mutants respectively (a: *P* = 0.05; A: *P* = 0.01) and figures were mean ± SD.

^z “+++”, “++”, and “+” scores for perithecia covering more than 2/3, between 1/3 and 2/3, and less than 1/3 of the grain surface, respectively.

not be transferred by hyphal fusion or could be transferred with low chance between two compatible isolates (Table 3). Thus, we assumed that hyphal fusion might take little part in the development of MBC resistance in the field.

Recovery of sexual recombinants. A total of three crosses were performed and all were found to be fertile (Table 1). Individual perithecia were sampled along the interface line for each cross, and single-ascospore progeny of each perithecium were cultured for further identification. The viability of ascospores was never lower than 97%. Generally, nearly 25% of the progeny from the perithecia produced from hybridization of the two parental isolates exhibited wild-type growth on MM. The ratio was not significantly different from the expected 3:1 segregation ratio for *nit* mutants and wild-type recombinants (Tables 4 and 5). Also, in crosses between resistant (MR and HR) and sensitive isolates (crosses A and B), segregation fit a 1:1 ratio of S:MR and S:HR, indicating that MBC resistance was controlled by a single Mendelian gene. From cross C, in which the parents had different resistant phenotypes, the progeny segregation for parental resistance phenotypes fit a 1:1 ratio. Therefore, both moderate (MR) and high resistance (HR) phenotypes were controlled by the same locus in the isolates characterized. These results were consistent with a previous study (36), in which it was concluded that a single gene controlled resistance to MBC in *F. graminearum* in China, and was generally expressed as a high degree of resistance to this fungicide.

Biological properties of sexual recombinants. Three stable sexual recombinants were randomly chosen from each cross. There were no significant differences in mycelial linear growth and pathogenicity between recombinants and their parents, but not in sporulation ability. In addition, differences in perithecial capacity were observed between recombinants and their parents. Nevertheless, most of the sexual recombinants possessed fitness levels comparable to their parents (Table 2).

DISCUSSION

The present study is the first report on VCG diversity of MBC-sensitive and -resistant *F. graminearum* populations isolated from different agroecological regions in China. We found a high degree of regional VCG diversity among the 33 sensitive and 31 resistant isolates. These results indicated there was a high degree of regional VCG diversity in MBC-sensitive and -resistant *F. graminearum* populations isolated from three regions of China. We also found that the ZF43, ZF43-06, and ZF43-02-05 isolates could complement with each other. This meant that the MBC mutation did not change the vegetative compatibility of *F. graminearum*. Noticeably, an MBC-sensitive isolate (S21) and an MBC-resistant isolate (R03) that belonged to the same VCG were found in the same field. This might be due to gene mutation under the highly selective pressure of MBC in the field. Vegetative compatibility is a good indicator of clonal ancestry and a predictor of genotypic similarity among isolates. Similar results have been obtained in *F. graminearum* populations worldwide by using vegetative compatibility tests as a marker in so many previous studies (2–4, 13,17,19,21,37). Also, similar reports showed that the *F. graminearum* clade was composed of eight worldwide lineages by using six gene genealogies (22,33). Furthermore, these lineages were biogeographically structured, indicating limited gene flow among these populations. The *F. graminearum* clade lineages have been formally described as phylogenetically distinct species (23). The high genotypic variation revealed in all these studies was somewhat surprising, as many of the ascospores were probably formed in a homothallic manner in which no recombination or genetic exchange occurred. Likewise, all the progeny of “the hyphal fusion zone” exhibited their parental phenotype and no asexual recombinants were observed in this study, which indicated that MBC resistance could not be transferred by hyphal fusion or could be transferred only with low chance between two compatible isolates. The likelihood of asexual genetic recombination seemed to be low, because the probability of vegetative compatibility between two random isolates is low, and when vegetatively compatible isolates anastomose, the resulting heterokaryon appeared to be limited in stability. The apparent lack of

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TABLE 5. χ^2 test of the progeny from three genetic crosses of isolates of *Fusarium graminearum*

Genetic cross	Number of the progeny		χ^{2y} (3:1) ^z	Number of each phenotype			χ^2 (1:1) ^z
	Nit	Wild type		S	MR	HR	
A	89	37	1.06	61	65	0	0.07
B	117	29	1.79	71	0	75	0.06
C	101	36	0.06	0	67	70	0.03

^y $\chi^2_{0.05, 1} = 3.84$.
^z Expected segregation ratio of progeny.

TABLE 3. *Nit* phenotype and sensitivity to carbendazim of the asexual progeny of fused mycelia of two vegetative compatible isolates of *Fusarium graminearum* collected from wheat fields in China

Name of the paired isolates (<i>nit</i> phenotype and sensitivity to MBC)		Total number of single spore tested	<i>Nit</i> phenotype and sensitivity to MBC (number of the progeny)
Paired mutant 1	Paired mutant 2		
S06 (<i>nit1</i> -S) ^z	S09 (<i>nitM</i> -S)	1,021	<i>nit1</i> -S (268); <i>nitM</i> -S (753)
S16 (<i>nit3</i> -S)	S22 (<i>nit1</i> -S)	1,009	<i>nit3</i> -S (697); <i>nit1</i> -S (312)
R14 (<i>nit1</i> -MR)	R17 (<i>nit3</i> -MR)	1,004	<i>nit1</i> -MR (474); <i>nit3</i> -MR (530)
R03 (<i>nit3</i> -MR)	S21 (<i>nit1</i> -S)	1,152	<i>nit3</i> -MR (383); <i>nit1</i> -S (769)
ZF43 (<i>nit1</i> -S)	ZF43-06 (<i>nit3</i> -HR)	1,036	<i>nit1</i> -S (658); <i>nit3</i> -HR (378)
ZF43-06 (<i>nit1</i> -HR)	ZF43-02-05 (<i>nit3</i> -HR)	1,024	<i>nit1</i> -HR (729); <i>nit3</i> -HR (295)

^z S06 is the number of isolate; *nit1* is the physiological type of nitrate nonutilizing mutation; S indicates sensitive to MBC.

TABLE 4. *Nit* phenotype and sensitivity to MBC of the sexual progeny in the three genetic crosses of isolates of *Fusarium graminearum*

Genetic crosses	Ascospore viability on WA (%)	Total progeny observed	Number of progeny			
			Phenotype of the <i>nit</i> mutants (S + R)/(MR + HR) ^z			Wild type (S + R)
			<i>nit1</i>	<i>nitM</i>	<i>nit1</i> - <i>nitM</i>	
A	98	126	34 (18 + 16)	24 (12 + 12)	31 (14 + 17)	37 (17 + 20)
B	99	146	38 (15 + 23)	38 (17 + 21)	41 (19 + 22)	29 (16 + 13)
C	97	137	33 (14 + 19)	34 (21 + 13)	34 (18 + 16)	36 (14 + 22)

^z S + R: the number of progeny sensitive to MBC + the number of progeny resistant (MR or HR) to MBC for crosses A and B; MR + HR: the number of progeny moderately resistant to MBC + the number of progeny highly resistant to MBC for cross C.

propagation of the heterokaryon would greatly reduce the probability of asexual recombination (4). Therefore, we concluded that MBC resistance could hardly be transferred by hyphal fusion or could be transferred with low chance, and MBC resistance might not take place and spread in the field by hyphal fusion.

Although *F. graminearum* isolates had the ability to reproduce both sexually and asexually, and both macroconidia and ascospores could infect cereal heads (28), the relative proportion of sexual and asexual reproduction was still unknown. Nevertheless, Bowden and Leslie reported an out-crossing rate of 35% under laboratory conditions by crossing strains with different nitrate nonutilizing mutants (5), although heterozygous perithecia and sexual recombination in the field had not yet been found. Out-crossing appeared to be common in *F. graminearum* populations from the United States and China because they displayed low levels of gametic disequilibrium (12,38). If sexual recombination does occur in the field, it can facilitate the assembly of selective advantageous multilocus genotypes through recombination of favorable alleles from diverse strains. This might improve the ability of *F. graminearum* populations to adapt to MBC. In our study, the monogene that controlled the resistance to MBC could be transferred by sexual recombination under laboratory conditions. However, the relative frequency of selfing and out-crossing in nature had not been rigorously investigated. The widespread MBC resistance among *F. graminearum* populations in the field in China could be explained by sexual recombination, mutation, gene flow or high selective pressure of the fungicide MBC. Somehow, the biological properties of sexual recombinants of *F. graminearum* have changed just a little as compared to those of their parents. Moreover, the large quantities of VCGs could facilitate out-crossing between *F. graminearum* isolates in different VCGs with different sensitivity levels to MBC under suitable weather conditions. Thus, sexual recombination presumably played a role in the development of MBC resistance, although further studies on the sexual recombination under field conditions should be carried on in the future.

Benzimidazole fungicides bind to β -tubulin and affect microtubule function in many fungi (6). The β -tubulin gene occurs as a single copy in *Gibberella pulicaris*, but it was not linked to thiabendazole resistance (14). Also, the β -tub gene of *G. zeae* had already been cloned and sequenced (16). This gene has a length of 1,631 bp, including three introns, encoding 447 amino acid residues, but no difference was found in the β -tubulin gene between the sensitive and resistant isolates. Work to identify the novel MBC resistance mechanism of *F. graminearum* is currently in progress in our laboratory in order to provide information about the exact gene which controls the resistance to MBC.

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