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Vegetative Compatibility Groups in *Fusarium graminearum* Isolates from Corn and Barley in Korea

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Fifty-three isolates of *Fusarium graminearum* were obtained from corn and barley samples in several provinces of Korea. Gas chromatography-mass spectrometric analysis of trichothecenes produced by these isolates revealed that 37 and 16 isolates were nivalenol (NIV)- and deoxynivalenol (DON)-chemotypes, respectively. Two hundred and seventy-five nitrate-nonutilizing (*nit*) mutants were obtained from the isolates. Of these mutants, 187 were identified as *nit1*, *nit3*, or NitM, but 88 could not be identified as one of these classes. The highest frequency of *nit* mutant was *nit1* (65%), followed by *nit3* (20%) and NitM (15%). Higher frequency of NitM was observed in DON-chemotypes than in NIV-chemotypes. The mutants were used for vegetative compatibility group (VCG) analysis by examining heterokaryosis using complementary mutant pairs. No heterokaryon formation was observed among all 1,248 pairwise combinations, suggesting that all isolates tested belong to different VCGs. Higher frequency of self-incompatibility was observed in NIV-chemotypes than in DON-chemotypes. These results suggest that the likelihood of asexual genetic recombination may be very low in *F. graminearum* under the field condition.

Keywords : chemotype, *Fusarium graminearum*, *nit* mutants, trichothecenes.

Fusarium graminearum Schwabe, the imperfect stage of *Gibberella zeae* (Schw.) Petch, is a fungal pathogen of cereals such as corn, wheat, and barley. It causes root rot and seedling disease (Manka et al., 1985), a head blight of wheat and barley called scab, and stalk and ear rot of corn. This fungus produces toxic trichothecenes such as deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), 3,15-diacetyldeoxynivalenol (3,15-DADON), nivalenol (NIV), 4-acetylnivalenol (4-ANIV), and 4,15-diacetylnivalenol (4,15-DANIV) (Seo et al., 1996). Trichothecenes are sesquiterpene epoxides that inhibit eukaryotic protein synthesis and thereby impair human and animal health (Marasas et al., 1984; Wei and McLaughlin, 1974). Experiments with chemically pure trichothecenes at low dosage levels have reproduced many of the features observed in moldy-grain toxicoses in animals, including anemia, immunosuppression, hemorrhage, emesis, and feed refusal (Marasas et al., 1984).

Based on the production of trichothecenes, Ichinoe et al. (1983) proposed that *F. graminearum* is chemotaxonomically divided into two groups; one is the NIV chemotype which produces NIV with 4-ANIV, and the other is the DON chemotype which produces DON and 3-ADON. There are regional differences in the natural distribution of the two chemotypes. The presence of the NIV chemotype was reported in Korea, Japan, Taiwan, and Italy (Abbas and Mirocha, 1988; Ichinoe et al., 1983; Lee et al., 1994; Logriego et al., 1988). However, the NIV chemotype was not reported in North American countries such as Canada and the United States, although grains contaminated with NIV was reported in Canada (Tanaka et al., 1988). In Korea, there is a remarkable difference in trichothecene production by *F. graminearum* isolates from corn compared to those from barley (Kim et al., 1993; Seo et al., 1996). The major chemotypes of corn and barley isolates are the DON-chemotype and the NIV-chemotype, respectively. Most of the isolates from corn produced 15-ADON in quantities larger than 3-ADON, which is characteristic of isolates from North America (Mirocha et al., 1989). The host factor on the production of different trichothecenes by the two different chemotypes was excluded (Seo et al., 1998). There appears to be an uneven geographic distribution of the two chemotypes between Kangwon province and the southern provinces of Korea.

Heterokaryon formation between different fungal individuals is an important component of many fungal life cycles, and may serve as the first step in the parasexual cycle (Leslie, 1993). In plant pathogenic fungi, the entity that emerges following heterokaryosis may differ from its constitutes in aggressiveness or host range; some of these aspects have been previously reviewed (Glass and Kuldau, 1992; Nuss and Koltin, 1990). Sexual and vegetative heter-

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okaryons are quite distinct from one another in many fungi. Strains capable of forming a successful sexual heterokaryon may be unable to form a successful vegetative heterokaryon and *vice versa* (Leslie, 1993). Heterokaryosis, and perhaps complementation or other types of mycelial interactions have been recognized in the genus *Fusarium* for a long time (Puhalla et al., 1983). Heterokaryosis using nitrate-nonutilizing (*nit*) mutants has been developed (Puhalla, 1985) and widely used in the areas of genetic diversity (Bowden and Leslie, 1992), pathogenicity (Jacobson and Gordon, 1988), and other aspects of phenotypes in plant pathogenic fungi (Correll et al., 1986; 1987)

The objective of this study was to determine genetic diversity of trichothecene-producing isolates of *F. graminearum* by means of vegetative compatibility grouping.

Materials and Methods

Fungal isolates. Fifty-three isolates of *F. graminearum* were obtained from corn and barley samples from 7 provinces in Korea during 1991-1994. These isolates were confirmed to produce 8-ketotrichothecenes by gas chromatography-mass spectrometry (Seo et al., 1996). Among 53 isolates, 37 were the NIV-chemotypes and 16 were the DON-chemotypes. These isolates were stored in sterile water at 4°C. Each culture was used as the starting inoculum for all subsequent tests.

Generation of *nit* mutants. All the media, except the chlorate medium, used in this study were the same as those of Correll et al. (1987). Minimal medium (MM) containing nitrate as the sole source of nitrogen was used for routine culturing and complementation test. Nitrate-nonutilizing (*nit*) mutants were generated on MM with chlorate (MMC) with concentrations of 2.0, 2.5, 3.0, 3.5 or 4.0%, but L-asparagine was removed from the standard recipe for this medium. For the generation of *nit* mutants, mycelial plugs of each isolate from actively growing colonies on potato dextrose agar (PDA) were placed on MMC (three blocks per plate, three plates per isolate). Each plate was incubated at 25°C and examined periodically for the appearance of fast-growing sectors from the initially restricted colonies. Fast-growing sectors appeared during 4~15 days after the mycelial plugs were transferred to MM. The growth of wild-type isolates is presumably restricted by reduction of chlorate to chlorite by nitrate reductase. All chlorate resistant mutants showed wild-type growth on complete medium.

Identification of *nit* mutant phenotypes. The *nit* mutants were assigned to one of three phenotypic classes on the basis of their growth on media containing one of different nitrogen sources. Four phenotyping media were used as the basal media supplemented with nitrite (0.5 g/L), ammonium tartrate (1.0 g/L), hypoxanthine (0.2 g/L), and uric acid (0.2 g/L), respectively. A small mycelial plug of each mutant was placed on each of four phenotyping media. The plates were incubated at 25°C and colony morphology (thin and transparent growth or dense and fuzzy growth) was scored.

Complementation test. Pairings were made by placing small mycelial blocks from each complementary *nit* mutants at 1.0~1.5 cm apart on MM plates. The plates were incubated for 7~15 days under the conditions described above and then scored for complementation. The major pairing was *nit1* and NitM, and the mutants which did not produce NitM were paired with *nit1* and *nit3*. All possible pairwise combinations (1,248) were made and tested.

Results

Isolation of *nit* mutants. Fifty-one of 53 *F. graminearum* isolates produced chlorate-resistant sectors on MM complemented with chlorate. The most number of sectors was observed at 3.0% (w/v) of chlorate concentration, followed by at 2.5%, 3.5%, and 4.0%. There were also great differences in sectoring frequency of each isolate. The majority of the chlorate-resistant sectors recovered was unable to utilize nitrate as a sole nitrogen source and consequently grew as thin expansive colonies without aerial mycelium on MM. However, the color of colonies turned to carmine-red when they were exposed to light (white fluorescence, 5,000 lux) for 12 hr, and they produced wild-type like mycelia. A few chlorate-resistant sectors were able to utilize nitrate. No chlorate-resistant sector was observed in PDA complemented with chlorate.

Identification of *nit* mutant phenotypes. The phenotypes of the *nit* mutants from *F. graminerum* were determined by their colony morphology on media containing nitrate, nitrite, hypoxanthine, uric acid, or ammonium tartrate as a sole nitrogen source. The *nit* mutants could be divided into three classes; *nit1* (a mutation of nitrate reductase structural locus), *nit3* (a mutation of nitrate-assimilation pathway specific locus), and NitM (mutations that affect the assembly of a molybdenum-containing cofactor necessary for nitrate reductase activity). Phenotypes of some mutants of *F. graminearum* could not be determined on nitrite medium; they did not grow or reversed to aerial mycelia. The majority of *nit* mutants was *nit1* (65%), and followed by *nit3* (20%) and NitM (15%) (Table 1). The frequencies of *nit1* and *nit3* were similar between DON-chemotypes and NIV-chemotypes. However, the frequency of NitM was higher in DON-chemotypes (7 of 16 isolates) than NIV-chemo-

Table 1. Frequency and phenotypes of *nit* mutants recovered from *F. graminearum*

Chemotype	Source	Number of isolates	Phenotypes of *nit* mutants		
			nit1	*nit3*	NitM
DON	Corn	15	13 (40)[a]	6 (14)	7 (11)
	Barley	1	1 (1)	–	
NIV	Corn	22	18 (45)	11 (12)	3 (13)
	Barley	15	14 (36)	6 (11)	3 (4)
Total		53	46 (122)	23 (37)	13 (28)

[a] Numbers indicate the number of isolates which produced each class of *nit* mutants from all isolates used. Numbers in parentheses indicate the total number of *nit* mutants recovered.

types (6 of 37 isolates).

Complementation test. Physiological complementation among *nit* mutants with different mutations was indicated by the development of dense aerial mycelia where the mycelia of the *nit* mutants came in contact and anastomosed to form a heterokaryon. Forty-nine isolates of *F. graminearum* produced at least one type of *nit* mutants. When all pairwise combinations (1,248 combinations), *nit1*/NitM or *nit1*/*nit3*, from 49 isolates were made on MM, no heterokaryon formation was observed except self-compatible interactions. Among 28 isolates that produced at least two different types of *nit* mutants, 12 and 16 isolates were identified as self-compatible and self-incompatible, respectively. Based on these complementation tests, each of 12 self-compatible isolates was assigned to genetically distinct vegetative compatibility group (VCG). The other isolates could not be grouped into VCG.

Self-incompatibility. High level of self-incompatibility was observed in isolates of *F. graminearum*. The frequency of self-incompatibility was higher in NIV-chemotypes (12 of 18 isolates) than DON-chemotypes (4 of 10 isolates). Within the NIV-chemotypes, much higher frequency of self-incompatibility was observed from corn isolates (10 of 11) than barley isolates (2 of 7).

Discussion

The *nit* mutants from *F. graminearum* were recovered on MM supplemented with chlorate. The *nit* mutants have also been generated from a number of *Fusarium* species. (Bowden and Leslie, 1992; Correll et al., 1987; Jacobson and Gordon, 1988). The frequency of resistant sectoring on MMC was different when different concentrations of chlorate was supplemented. The 3.0% of chlorate produced the highest frequency of sectoring in this study, while 1.5% of chlorate was effective in *F. graminearum* and other *Fusarium* spp. (Bowden and Leslie, 1992; Correll et al., 1987). Sectoring frequency of *F. moniliforme* has been shown to be heritable and to vary among isolates (Klittich and Leslie, 1988). The wide range of sectoring frequency in plant pathogenic fungi on different concentrations of chlorate has also been suggested as a selective advantage for rapid adaptation to environmental stresses such as fungicides and host resistance (Klittich and Leslie, 1988). No chlorate-resistant sector was observed in PDA complemented with chlorate. However, similar frequency of sectoring was observed in both PDA and MM complemented with chlorate in other *Fusarium* spp. (Correll, et al., 1987).

The *nit* mutants of *F. graminearum* have unique characteristics compared to other *Fusarium* spp. These include reversion of *nit* mutants to wild-type like aerial mycelia when exposed to light for 12 hr, difficulty in phenotyping on nitrite medium, and reddish brown color in some isolates. Among the 275 *nit* mutants, *nit1* was recovered at the highest frequency (65%), followed by *nit3* (20%) and NitM (15%). Similar frequencies were observed in several *Fusarium* species including *F. moniliforme* (Klittich and Leslie, 1988) and *F. oxysporum* (Jacobson and Gordon, 1988). However, *nit3* was recovered at the highest frequency in the previous study of *F. graminearum* (Bowden and Leslie, 1992).

Fusarium graminearum isolates used in this experiment showed high frequency of self-incompatibility. The lack of complementation observed may be due to the inability of the isolates to anastomose. In *F. moniliforme*, heterokaryon self-incompatibility is heritable trait and may be controlled by a single nuclear gene (Correll et al., 1987). Isolates carrying mutations that prevent them from fusing to form heterokaryons, even with themselves, have been identified in field population of *Fusarium oxysporum* (Bosland and Williams, 1987), *F. moniliforme* (Klittich and Leslie, 1988), and *F. subglutinans* (Correll et al., 1992). *Fusarium graminearum* isolates can lead to an incorrect diagnosis of vegetative incompatibility since they will usually not form heterokaryons with any other isolates. Thus, it is important to identify heterokaryon self-incompatible isolates to prevent the overestimation of the number of VCGs within a population. However, no heterokaryon formation was observed among all isolates tested in this experiment. These results indicate that each isolate belongs to a different VCG and the likelihood of asexual genetic recombination appears to be very low among isolates of *F. graminearum* under the field condition. Bowden and Leslie (1992) reported that all strains of *F. graminearum* from 23 locations in Kansas represented genetically distinct VCGs.

Since *F. graminearum* is homothallic, vegetative heterokaryon formation between two chemotypes would be the choice to become co-producer of NIV and DON trichothecenes. However, VCG analysis in this study ruled out heterokaryon formation among isolates producing different types of trichothecenes. Furthermore, recent report also ruled out the host-related factor on the production of different types of trichothecenes by *F. graminearum* (Seo et al., 1998). Taken together, the production of different types of trichothecenes by *F. graminearum* appears to be dependent upon genetic make-up of isolates rather than environmental factors. Isolates belonging to NIV-chemotypes showed more complex DNA amplification band pattern than DON-chemotypes in random amplified polymorphic DNA analysis; NIV-chemotypes were scattered, and DON-chemotypes were clustered into three groups. This implies that DON-chemotypes are more genetically uniform than NIV-chemotypes (data not shown). Although, trichothecene biosynthesis genes have been cloned and characterized

(McCormick et al., 1996), further characterization of the genes would clarify the mechanisms involved in production of the different chemotypes.

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