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Genetic Differentiation Associated with Fumonisin and Gibberellin Production in Japanese *Fusarium fujikuroi*

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ABSTRACT *Fusarium fujikuroi* is a pathogenic fungus that infects rice. It produces several important mycotoxins, such as fumonisins. Fumonisin production has been detected in strains of maize, strawberry, and wheat, whereas it has not been detected in strains from rice seedlings infested with bakanae disease in Japan. We investigated the genetic relationships, pathogenicity, and resistance to a fungicide, thiophanate-methyl (TM), in 51 fumonisin-producing strains and 44 nonproducing strains. Phylogenetic analyses based on amplified fragment length polymorphism (AFLP) markers and two specific genes (a combined sequence of translation elongation factor 1α [*TEF1*α] and RNA polymerase II second-largest subunit [*RPB2*]) indicated differential clustering between the fumonisin-producing and -nonproducing strains. One of the AFLP markers, EATMCAY107, was specifically present in the fumonisin-producing strains. A specific single nucleotide polymorphism (SNP) between the fumonisin-producing and nonproducing strains was also detected in *RPB2*, in addition to an SNP previously found in *TEF1*α. Gibberellin production was higher in the nonproducing than in the producing strains according to an *in vitro* assay, and the nonproducing strains had the strongest pathogenicity with regard to rice seedlings. TM resistance was closely correlated with the cluster of fumonisin-nonproducing strains. The results indicate that intraspecific evolution in Japanese *F. fujikuroi* is associated with fumonisin production and pathogenicity. Two subgroups of Japanese *F. fujikuroi*, designated G group and F group, were distinguished based on phylogenetic differences and the high production of gibberellin and fumonisin, respectively.

IMPORTANCE *Fusarium fujikuroi* is a pathogenic fungus that causes rice bakanae disease. Historically, this pathogen has been known as *Fusarium moniliforme*, along with many other species based on a broad species concept. Gibberellin, which is currently known as a plant hormone, is a virulence factor of *F. fujikuroi*. Fumonisin is a carcinogenic mycotoxin posing a serious threat to food and feed safety. Although it has been confirmed that *F. fujikuroi* produces gibberellin and fumonisin, production varies among strains, and individual production has been obscured by the traditional appellation of *F. moniliforme*, difficulties in species identification, and variation in the assays used to determine the production of these secondary metabolites. In this study, we discovered two phylogenetic subgroups associated with fumonisin and gibberellin production in Japanese *F. fujikuroi*.

KEYWORDS fumonisin, *Fusarium fujikuroi*, gibberellin

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The *Fusarium fujikuroi* species complex, which mostly corresponds to *Fusarium* section Liseola, includes more than 50 phylogenetic species (1, 2), including 13 independent mating populations (MPs) (3, 4). *Fusarium fujikuroi* Nirenberg, corresponding to MP-C in the *F. fujikuroi* complex, causes rice bakanae disease. Diseased rice plants are slender, have pale yellow leaves, and are taller than nondiseased plants (5). In Japan, the disease has been controlled with benzimidazole fungicides, although it is now also controlled using other types of fungicides, such as ipconazole, or by disinfestation by hot-water immersion. One benzimidazole fungicide, thiophanate-methyl (TM), has been used extensively for seed disinfection, but TM-resistant strains have emerged since 1984 in Japan (6). Historically, *F. fujikuroi* has been known as *Fusarium moniliforme*, along with many other species in the *F. fujikuroi* complex. The precise characteristics and diversity of *F. fujikuroi* have been obscured by the traditional appellation of *F. moniliforme*, although this broad species concept is no longer used (7). *F. fujikuroi* has been found in rice, wheat, maize, strawberry (8), and tomato (9) plants, in sugarcane, and in grape vines (10), although *F. moniliforme* has been reported in a wider variety of plant species.

F. fujikuroi is known to produce a large variety of secondary metabolites, and 45 putative secondary metabolite gene clusters have been found in its genome (11). Among such metabolites, gibberellins and fumonisins have been studied intensively. Gibberellin, which is a well-known plant hormone, was originally identified as a virulence factor in *F. fujikuroi* rice infestation. Gene disruption studies have indicated the contribution made by gibberellin to initial fungal colonization in the host roots (11). Fumonisin is a carcinogenic mycotoxin that was first discovered in *Fusarium verticillioides*, a relative of *F. fujikuroi* (12, 13). Fumonisin contamination of maize poses a serious global threat to food and feed safety.

Although it has been confirmed that *F. fujikuroi* produces gibberellin and fumonisin, production varies among strains, and individual production has been obscured by confusion over species classification, difficulties in species identification (14), and variation in the assays used to determine the production of these secondary metabolites. Furthermore, many researchers have focused only on *F. fujikuroi* strains in rice and on either gibberellin or fumonisin production. In a previous investigation of Japanese *F. fujikuroi* in a wide variety of host plants, fumonisin production was detected in 46 strains obtained from maize, strawberry, wheat, and rice seeds, whereas it was undetectable in 43 strains obtained from bakanae-diseased rice, rice seeds, and unknown sources (8). In the present study, strains unable to produce fumonisins at detectable levels are referred to as fumonisin nonproducers, although they may have been able to produce low levels of fumonisins. Although both fumonisin producers and nonproducers were isolated from rice seeds, all 21 strains from bakanae-diseased rice were fumonisin nonproducers (8). Eighteen out of 31 strains (58%) of *F. fujikuroi* from rice in the Philippines were fumonisin nonproducers (15).

Combined investigations of fumonisin and gibberellin production in *F. fujikuroi* from rice have been carried out by several researchers (16–18). In these studies, gibberellin production was detected in all 75 *F. fujikuroi* strains, as reported by Malonek et al. (19): "all so-far-analyzed rice isolates of the species *F. fujikuroi* (MP-C) produced significant amounts of biologically active GAs without any exclusion," demonstrating gibberellin production in 5 *F. fujikuroi* strains. In contrast, fumonisin production in *F. fujikuroi* from rice tends to be minimal; none of the 4 strains investigated in one study (20) and 2 of the 7 strains investigated in another study were fumonisin producers (16); 2 of the 10 strains produced 0.1 to 1 ppm fumonisin, but the remaining 8 strains produced less than 0.1 ppm in one study (17); 8 out of 58 strains were fumonisin producers in one study (18); and none of the 21 strains from bakanae-diseased rice were fumonisin nonproducers in another study (8).

Whereas most *F. fujikuroi* strains isolated from plants other than rice are fumonisin producers, all 4 strains from wheat (21), 4 out of 5 strains from maize (22), all 4 strains from strawberry plants (8), and all 50 strains from grape vines were fumonisin producers (10). However, in case of *F. fujikuroi* from plants other than rice, only fumonisin

production has been a focus and their gibberellin production has not been investigated (8, 10, 21, 22). Therefore, there has not yet been a precise assessment of the biological hazard posed by *F. fujikuroi* as a mycotoxin producer and rice pathogen. All *F. fujikuroi* strains from bakanae-diseased rice are fumonisin nonproducers, and the presence of a specific single nucleotide polymorphism (SNP) in the translation elongation factor 1 α (*TEF1*α) gene suggests possible genetic differences between fumonisin producers and nonproducers in Japanese *F. fujikuroi* (8). Phylogenetic analyses of the *F. fujikuroi* complex from rice and maize in South Korea revealed a subclade consisting mostly of rice isolates in the *F. fujikuroi* clade (23). Recently, two distinctive pathotypes, the bakanae type and the stunting type, corresponding to gibberellin and fumonisin production, respectively, have been identified in *F. fujikuroi* (24).

In the present study, we carried out a comprehensive investigation of gibberellin production, TM resistance, and genetic variability in Japanese *F. fujikuroi* strains that had been studied previously with regard to fumonisin production (8). The results indicate that fumonisin and gibberellin production is associated with genetic differentiation in Japanese *F. fujikuroi*.

RESULTS

SNP analysis. The authors of a previous study reported an SNP (TEF_T618G) that distinguishes fumonisin producers from nonproducers (8). Additional SNPs were investigated to elucidate the genetic differences between fumonisin producers and nonproducers. First, seven SNPs that distinguish a fumonisin producer (Gfc0825009) from a nonproducer (Gfc0801001) were assessed with an additional four fumonisin producers (MAFF235463, Mo78, Gfc0825007, and Gfc0009105) and four nonproducers (MAFF235949, Gfc9424702, Gfc0625008, and APF06083) using PCR-restriction fragment length polymorphism (PCR-RFLP) or PCR sequencing. The seven SNPs were TEF_C447A, FUM1_G423A, FUM78_C41T (the intergenic region between *FUM7* and *FUM8*), FUM8_G2834A, and FUM18_G51T in a fumonisin biosynthesis gene cluster; and CPR_C1152A and P4504_C842T in the genes involving gibberellin production. In the case of FUM18_G51T, the DNA was not amplified from three fumonisin nonproducers (Gfc9424702, Gfc0625008, and APF06083), and their SNPs could not be determined. The results indicated that FUM78_C41T, FUM8_G2834A, CPR_C1152A, and P4504_C842T were associated with differences in fumonisin production in the tested strains. The specificities of these SNPs with regard to fumonisin production compared with TEF_T618G were further investigated in all 95 strains using the Luminex assay. All the SNPs except P4504_C842T were associated with a difference in fumonisin production (Table 1).

We also compared the whole-genome sequences, secondary metabolite profiles, and pathogenicity with regard to rice resulting from the SNPs in nine *F. fujikuroi* strains that had already been investigated (24) (Table 1). They were isolated from geographically diverse regions. One of the nine strains (B14) is apparently a fumonisin producer, and the other strains are fumonisin nonproducers, although a small amount of fumonisin is occasionally detected in strains IMI58289 and B20 (24). Strain B14 has the same SNP combination patterns as Japanese fumonisin producers (Table 1). Four strains had the same SNP combination patterns as the Japanese fumonisin nonproducers, and the other five strains, including Japanese isolate m657, had none of the SNP combination patterns of the Japanese fumonisin producers or nonproducers (Table 1). Among the SNPs, TEF_T618G was associated with a difference in fumonisin production in the Japanese strains and also in the nine strains investigated by Niehaus et al. (24).

Phylogenetic tree. Based on the result of the SNP analyses, we conducted phylogenetic analyses on 66 AFLP markers (see Table S3 in the supplemental material) to demonstrate the genetic relationships between the individual strains. One of the markers (EATMCAY107) was specific to fumonisin producers. Fumonisin producers and nonproducers were separated by a bootstrap value of 68% in the phylogenetic tree (Fig. 1). It was reproduced in the phylogenetic tree based on the combined sequences *TEF1*α and *RPB2* of the representative strains and the nine strains investigated by

TABLE 1 *Fusarium fujikuroi* strains used in the present study and their characteristics[a]

Fumonisin production[a]	Strain	Isolation source[b]	Geographic origin[c]	Translation elongation factor 1α sequence (GenBank accession no.)	RNA polymerase II second-largest subunit		SNP data (TEF_T618G, FUM78_C41T, FUM8_G2834A, CPR_C1152A, P4504_C842T)[e]	AFLP haplotype	Thiophanate-methyl resistance	Gibberellin spot on TLC[f]		Reference or source[g]
					Sequence (GenBank accession no.)	SNP RPB2_C3250T[d]				$GA_{4/7}$	GA_3	
Nonproducer												
	MAFF235949[h]	91 rice	N	JN695731	LC133055	D (T)	G, T, A, A, T	1	S	−	±	
	Gfc0801001[h]	08 bakanae-diseased rice	P-I	JN695733	LC133056	D (T)	(G, T, A, A, T)	2	R	+	+	
	Gfc0801002	08 bakanae-diseased rice	P-I			D	G, T, A, A, T	3	R	+	+	
	Gfc0801003	08 bakanae-diseased rice	P-I			D	G, T, A, A, T	4	R	+	+	
	MYG92-10	92 bakanae-diseased rice	J			D	G, T, A, A, T	5	R	+	+	Tateishi and Chida (48)
	SMN86-2[h]	86 bakanae-diseased rice	u			D	G, T, A, A, T	6	R	+	+	Tateishi and Chida (48)
	MIE92-4	92 bakanae-diseased rice	A			D	G, T, A, A, T	7	R	+	+	Tateishi and Chida (48)
	Gfc9424702[h]	94 bakanae-diseased rice	A	JN695735	LC133057	D (T)	G, T, A, A, T	8	R	+	+	H. Suzuki
	Gfc9424703	94 bakanae-diseased rice	A			D	G, T, A, A, T	9	R	+	+	H. Suzuki
	Gfc9424707	94 bakanae-diseased rice	A			D	G, T, A, A, T	10	R	+	+	H. Suzuki
	Gfc0625001[h]	06 bakanae-diseased rice	B-I			D	G, T, A, A, T	11	R	+	+	T. Kitazawa
	Gfc0625002	06 bakanae-diseased rice	B-II			D	G, T, A, A, T	12	R	+	+	T. Kitazawa
	Gfc0625004	06 bakanae-diseased rice	B-III			D	G, T, A, A, T	12	R	+	+	T. Kitazawa
	Gfc0625005[h]	06 bakanae-diseased rice	B-IV			D	G, T, A, A, T	12	R	+	±	T. Kitazawa
	Gfc0625006	06 bakanae-diseased rice	B-V			D	G, T, A, A, T	12	R	+	+	T. Kitazawa
	Gfc0625007	06 bakanae-diseased rice	B-VI			D	G, T, A, A, T	12	R	+	+	T. Kitazawa
	Gfc0625008	06 bakanae-diseased rice	B-IV			D	G, T, A, A, T	13	R	+	+	T. Kitazawa
	Gfc0625010	06 bakanae-diseased rice	B-IV			D	G, T, A, A, T	14	R	+	+	T. Kitazawa
	Gfc0925005[h]	09 bakanae-diseased rice	B-IV			D	G, T, A, A, T	15	R	+	±	
	Gfc0925010	09 bakanae-diseased rice	B-VII			D	G, T, A, A, T	16	R	+	+	
	Gfc0925011	09 bakanae-diseased rice	B-VIII			D	G, T, A, A, T	17	R	+	+	
	Gfc0925012	09 bakanae-diseased rice	B-IX			D	G, T, A, A, T	18	R	+	+	
	APF06083[h]	Unknown	Unknown	JN695736	LC133058	D (T)	G, T, A, A, T	19	R	+	±	S. Fuji
	Gfc8707123	Unknown	Unknown			D	G, T, A, A, T	20	R	+	+	H. Hamamura
	Gfc8707182	Unknown	Unknown			D	G, T, A, A, T	20	R	+	+	H. Hamamura
	Gfc8707117	Unknown	Unknown			D	G, T, A, A, T	21	R	+	+	H. Hamamura
	Gfc8707249	Unknown	Unknown			D	G, T, A, A, T	22	S	+	+	H. Hamamura
	Gfc8707642	Unknown	Unknown			D	G, T, A, A, T	23	R	+	+	H. Hamamura
	Gfc0825001[h]	05 rice seed	B-VII	JN695734	LC133059	D (T)	G, T, A, A, T	24	R	+	+	
	Gfc0825002	05 rice seed	B-VII			D	G, T, A, A, T	24	R	+	+	
	Gfc0825003	05 rice seed	B-VII			D	G, T, A, A, T	24	R	+	+	
	Gfc0825004	05 rice seed	B-VII			D	G, T, A, A, T	24	R	+	+	
	Gfc0825005	05 rice seed	B-VII			D	G, T, A, A, T	24	R	+	+	
	Gfc0825006	05 rice seed	B-VII			D	G, T, A, A, T	24	R	+	+	
	Gfc0901002	07 rice seed	P-II			D	G, T, A, A, T	25	R	+	+	
	Gfc0901005	07 rice seed	P-II			D	G, T, A, A, T	25	R	+	+	
	Gfc0901009[h]	07 rice seed	P-II			D	G, T, A, A, T	26	R	+	+	
	Gfc1004001	09 rice seed	J			D	G, T, A, A, T	27	R	+	+	
	Gfc1004002	09 rice seed	J			D	G, T, A, A, T	28	R	+	+	
	Gfc1034001[h]	09 rice seed	y			D	G, T, A, A, T	29	R	+	+	
	GL24[h]	Rice	California, USA	LC133052	LC133060	D (T)	G, T, A, A, T	19	S	+	+	Proctor et al. (49)
	GL25	Rice	Unknown			D	G, T, A, A, T	30	S	+	+	Proctor et al. (49)
	GL27	Rice	Unknown			D	G, T, A, A, T	31	S	+	+	Proctor et al. (49)
	GL28	Rice	California, US			D	G, T, A, A, T	32	R	+	+	Proctor et al. (49)

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TABLE 1 (Continued)

Fumonisin production[a]	Strain	Isolation source[b]	Geographic origin[c]	Translation elongation factor 1α sequence (GenBank accession no.)	RNA polymerase II second-largest subunit		SNP data (TEF_T618G, FUM78_C41T, FUM8_G2834A, CPR_C1152A, P4504_C842T)[e]	AFLP haplotype	Thiophanate-methyl resistance	Gibberellin spot on TLC[f]		Reference or source[g]
					Sequence (GenBank accession no.)	SNP RPB2_C3250T[d]				$GA_{4/7}$	GA_3	
Producer												
	MAFF235463[h]	90 wheat	N	JN695737	LC133061	(C)	T, C, G, C, C	33	S	−	−	
	Gfc1004003[h]	09 rice seed	J	LC133053	LC133062	(C)	T, C, G, C, T	34	S	−	−	
	Gfc1006001	09 rice seed	m			C	T, C, G, C, C	35	S	−	−	
	Gfc1006002	09 rice seed	m			C	T, C, G, C, C	36	S	−	−	
	Gfc1006007	09 rice seed	m			C	T, C, G, C, C	37	S	−	−	
	Gfc1019001	10 rice seed	g-I			C	T, C, G, C, C	38	S	−	−	
	Gfc1019003[h]	10 rice seed	g-I			C	T, C, G, C, C	39	S	−	−	
	Gfc1019004	10 rice seed	g-I			C	T, C, G, C, C	40	S	−	−	
	Gfc1016022	09 rice seed	H			C	T, C, G, C, C	41	S	−	−	
	Gfc1016024	09 rice seed	H			C	T, C, G, C, C	42	S	−	−	
	Gfc1016025	09 rice seed	H			C	T, C, G, C, C	43	S	−	−	
	Gfc1016026	09 rice seed	H			C	T, C, G, C, C	43	S	−	−	
	Gfc0821004[h]	08 rice seed	K-I	JN695740	LC133063	(C)	T, C, G, C, T	44	S	−	−	
	Gfc0921001	09 rice seed	K-II			C	T, C, G, C, C	45	S	−	−	
	Gfc0921002	09 rice seed	K-II			C	T, C, G, C, C	46	S	−	−	
	Gfc0921009	09 rice seed	K-II			C	T, C, G, C, C	47	S	−	−	
	Gfc0921014	09 rice seed	K-II			C	T, C, G, C, C	48	S	−	−	
	Gfc0921034	09 rice seed	K-III			C	T, C, G, C, C	49	S	−	−	
	Gfc0921039[h]	09 rice seed	K-III			C	T, C, G, C, C	50	S	+	−	
	Gfc0921040	09 rice seed	K-III			C	T, C, G, C, C	51	S	−	−	
	Gfc0921041	09 rice seed	K-III			C	T, C, G, C, C	52	S	−	−	
	Gfc1025029	09 rice seed	B-VII			C	T, C, G, C, C	53	S	−	−	
	Gfc1025037	09 rice seed	B-VII			C	T, C, G, C, C	53	S	−	−	
	Gfc1025091	09 rice seed	B-VII			C	T, C, G, C, T	54	S	−	−	
	Gfc1034002	09 rice seed	y			C	T, C, G, C, C	55	S	−	−	
	Gfc1041003	10 rice seed	p-I			C	T, C, G, C, C	56	S	−	−	
	Gfc1041010	10 rice seed	p-I			C	T, C, G, C, C	57	S	−	−	
	Gfc1041011	10 rice seed	p-I			C	T, C, G, C, C	56	S	−	−	
	Gfc1043032[h]	09 rice seed	R-I			C	T, C, G, C, C	58	S	−	−	
	Gfc1043035	09 rice seed	R-I			C	T, C, G, C, C	59	S	−	−	
	Gfc1043037	09 rice seed	R-I			C	T, C, G, C, C	60	S	−	−	
	Gfc1043045	09 rice seed	R-I			C	T, C, G, C, C	60	S	−	−	
	Gfc1043046	09 rice seed	R-I			C	T, C, G, C, C	58	S	−	−	
	Gfc1043047	09 rice seed	R-I			C	T, C, G, C, C	58	S	−	−	
	Mo78	06 forage paddy rice for livestock	C-I			C	T, C, G, C, C	61	S	−	−	T. Tsukiboshi
	Mo80	06 forage paddy rice for livestock	C-I			C	T, C, G, C, C	62	S	−	−	T. Tsukiboshi
	Mo141	07 silage of forage paddy rice for livestock	f-I			C	T, C, G, C, T	63	S	−	−	T. Tsukiboshi
	Mo136	07 silage of forage paddy rice for livestock	f-I			C	T, C, G, C, C	64	S	−	−	T. Tsukiboshi
	Mo309	Maize	C			C	T, C, G, C, C	65	S	−	−	
	IBR89-1	Unknown	N			C	T, C, G, C, C	66	S	−	−	Tateishi and Chida (48)
	Gfc0825007[h]	07 maize	B-IV	JN695738	LC133064	(C)	T, C, G, C, C	67	S	−	−	
	Gfc0825009[h]	07 maize	B-IV	JN695739	LC133065	(C)	(T, C, G, C, C)	68	S	−	−	
	Gfc0825011[h]	07 maize	B-IV			C	T, C, G, C, C	69	S	+	−	
	Gfc0009063[h]	00 strawberry	C-II			C	T, C, G, C, C	70	S	−	−	M. Morishima

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TABLE 1 (Continued)

Fumonisin production[a]	Strain	Isolation source[b]	Geographic origin[c]	Translation elongation factor 1α sequence (GenBank accession no.)	RNA polymerase II second-largest subunit		SNP data (TEF_T618G, FUM78_C41T, FUM8_G2834A, CPR_C1152A, P4504_C842T)[e]	AFLP haplotype	Thiophanate-methyl resistance	Gibberellin spot on TLC[f]		Reference or source[g]
					Sequence (GenBank accession no.)	SNP RPB2 _C3250T[d]				$GA_{4/7}$	GA_3	
	Gfc0009105[h]	00 strawberry	C-III	JN695742	LC133066	(C)	T, C, G, C, C	71	S	−	−	M. Morishima
	Gfc0009110	00 strawberry	C-IV			C	T, C, G, C, C	72	S	−	−	M. Morishima
	Gfc0009117	00 strawberry	C-V			C	T, C, G, C, C	73	S	−	−	M. Morishima
	41-79[h]	Wheat	USA	LC133054	LC133067	(C)	T, C, G, C, C	74	S	−	−	Busman et al. (21)
	41-84	Wheat	USA			C	T, C, G, C, C	75	S	−	−	Busman et al. (21)
	41-116	Wheat	USA			C	T, C, G, C, C	76	S	−	−	Busman et al. (21)
	41-108	Wheat	USA			C	T, C, G, C, C	77	S	−	−	Busman et al. (21)
F. fujikuroi (whole genome, secondary metabolite profiles, and pathogenicity to rice[i]												
(Nonproducer)[j]	IMI58289	Infected rice	China			(T)	(G, C, G, C, T)			Producer		
Nonproducer	m567	Infected rice	Japan			(T)	(G, C, G, C, T)			Producer		
Nonproducer	MRC2276	Infected rice	Philippines			(T)	(G, C, A, C, T)			Producer		
Nonproducer	C1995	Infected rice	Taiwan			(T)	(G, C, G, A, T)			Producer		
Producer	B14	Infected rice	South Korea			(C)	(T, C, G, C, C)			(Nonproducer)[j]		
(Nonproducer)[i]	B20	Infected rice	South Korea			(T)	(G, C, G, A, T)			Producer		
Nonproducer	E282	Infected rice	Italy			(T)	(G, T, A, A, T)			Producer		
Nonproducer	FSU48	Maize	Germany			(T)	(G, T, A, A, T)			Producer		
Nonproducer	NCIM 1100	Infected rice	India			(T)	(G, T, A, A, T)			Producer		

[a]All strains used in this study were previously investigated in Suga et al. (8).
[b]First two digits indicate harvest year.
[c]Letters indicate differences in geographic origin rather than actual geographic names. Prefecture-city or town (if the city or town is known) was indicated by insignia.
[d]Single nucleotide polymorphisms were determined by dCAPs. Nucleotide determined by sequencing is indicated in parentheses, but the uncut type is indicated as D (i.e., not C).
[e]Single nucleotide polymorphisms were determined by Luminex.
[f]Ten microliters of culture filtrate was spotted on TLC. +, clear spot detected; ±, ambiguous spot; −, no spot detected.
[g]All strains were used in the previous fumonisin analysis (Suga et al. [8]), and the references only of strains not originally isolated in the previous analysis were indicated.
[h]Strains used to fumonisin and gibberellin quantification by UPLC-MS/MS and pathogenicity test.
[i]Conditions for fumonisin and gibberellin detection were different from those of this study, and SNP data were obtained from the whole-genome sequences in the study by Niehaus et al. (24).
[j]Results of triplicates were not stable, and a small amount was occasionally detected (Niehaus et al. [24]).

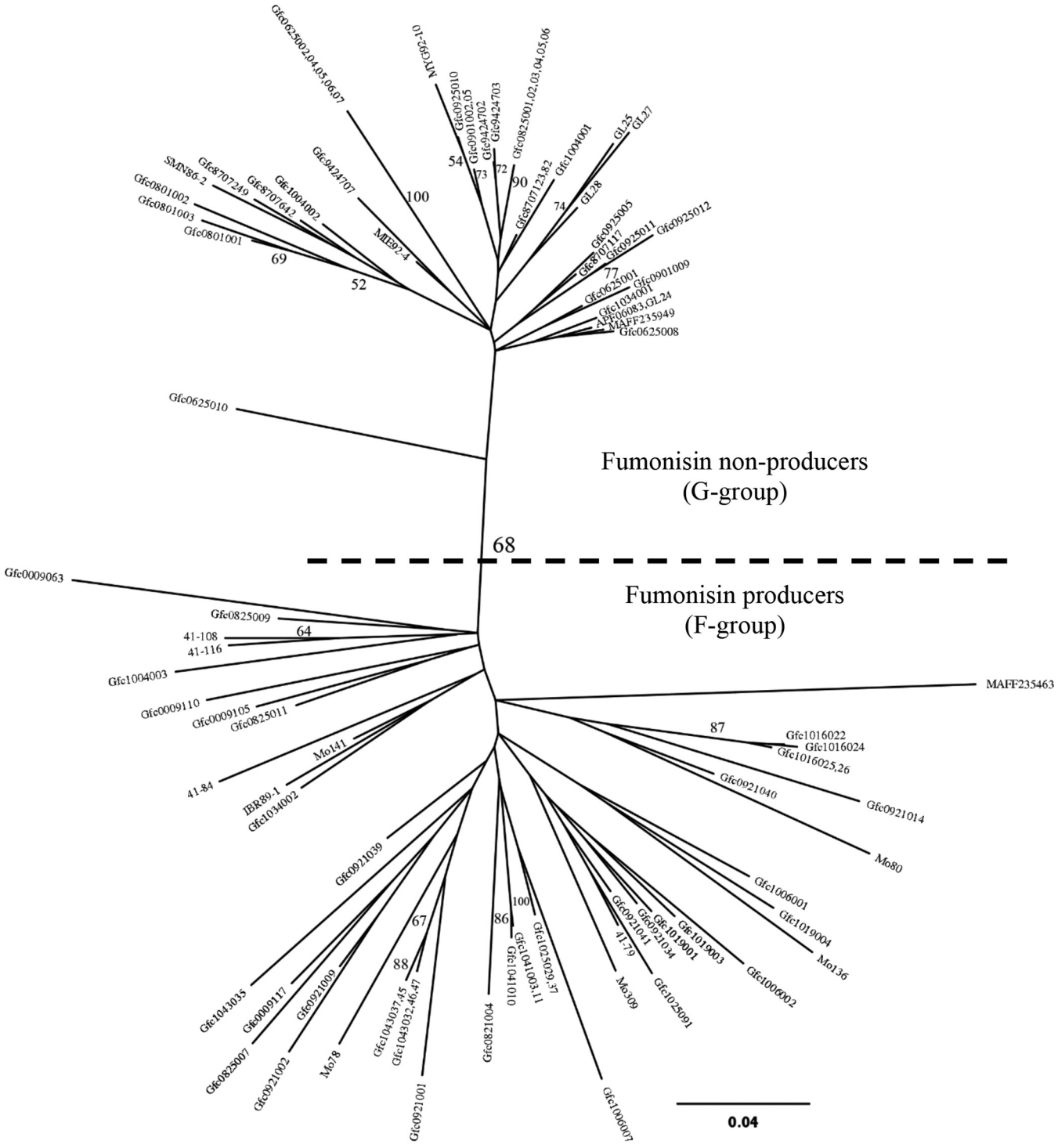

FIG 1 Phylogenetic tree of *Fusarium fujikuroi* based on amplified fragment length polymorphism (AFLP) data inferred by unrooted neighbor-joining analysis. Node support given for >50 neighbor-joining bootstrap values is shown above or below each branch. *F. fujikuroi* fumonisin producers and nonproducers are indicated as the F group and G group mentioned in the Discussion, respectively.

Niehaus et al. (24) (Fig. 2). The aligned data matrix of 1,527 sequences consisted of 1,248 characters, of which 279 characters were variable and 121 were phylogenetically informative for parsimony analysis. The MP analysis using PAUP* generated 30 equally parsimonious trees with 431 steps (consistency index [CI], 0.76; retention index [RI], 0.76; rescaled consistency index [RC], 0.58; homoplasy index [HI], 0.24). Although we

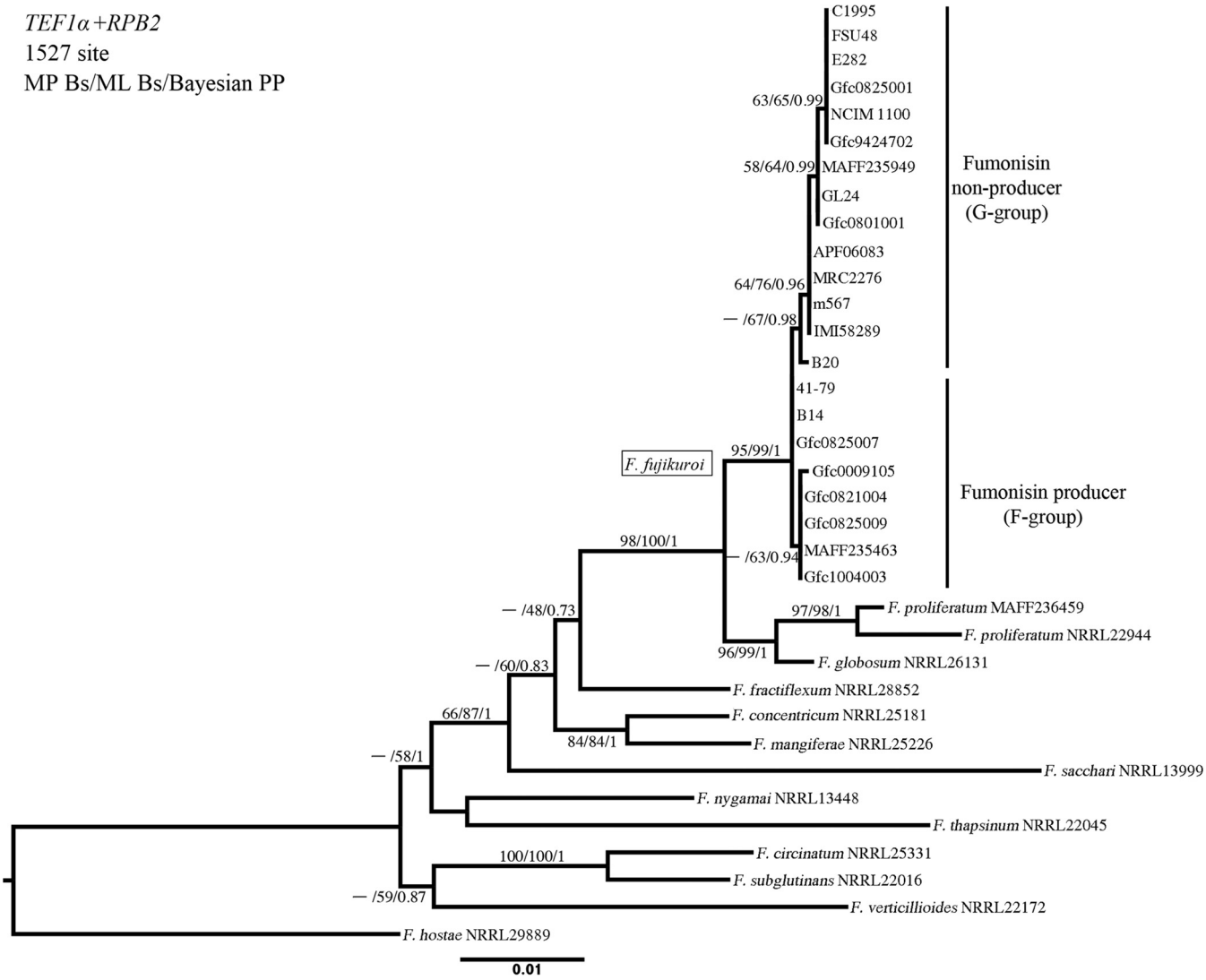

FIG 2 Phylogenetic tree of *Fusarium fujikuroi* species complex based on the sequences of the translation elongation factor 1 α (*TEF1*α) and the second largest subunits of RNA polymerase II (*RPB2*) genes inferred by maximum likelihood analysis. The sequence of *Fusarium hostae* strain NRRL29889 was used as an outgroup. Node supports given for >55 maximum-parsimony bootstrap values (MP Bs), maximum likelihood bootstrap values (ML Bs), and >0.80 Bayesian posterior probabilities (Bayesian PP) are shown above or below each branch. *F. fujikuroi* fumonisin producers and nonproducers are indicated as the F group and G group mentioned in the Discussion, respectively.

observed slight differences in the small branching orders of the terminal branches and in branch lengths, the tree topologies were generally consistent among all 30 trees (data not shown). Moreover, the topology of the tree generated by ML analysis (Fig. 2) was similar to the topology of the MP and Bayesian phylogenetic trees.

We detected an SNP (RPB2_C3250T) between the fumonisin producers and nonproducers, including the nine strains investigated by Niehaus et al. (24), in the *RPB2* sequence. We developed a diagnostic PCR-RFLP using a derived cleaved amplified polymorphic sequence (dCAPS) primer for the SNP and applied it to all 95 strains. Fifty-one fumonisin producers displayed the cut type (i.e., position 3250 was occupied by cytosine), whereas 44 nonproducers were of the uncut type (i.e., position 3250 was not occupied by cytosine) (Table 1).

Fumonisin production. The fumonisin production of all strains in Table 1 as previously investigated by enzyme-linked immunosorbent assay (ELISA) though the presence or absence of fumonisins in 10 strains was confirmed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (8). In order to compare

TABLE 2 Fumonisin and gibberellin production and pathogenicity of *Fusarium fujikuroi* strains

Fumonisin production[a]	Strain	Fumonisin production (ppm)[b]			Gibberellin production (ppm)[b]				Pathogenicity[c]
		FB_1	FB_2	FB_3	GA_3	GA_7	GA_4	GA_1	
Nonproducer									
	MAFF235949	ND	ND	ND	4.08 (±0.32)	ND	ND	ND	2.1 (±0.3)
	Gfc0801001	ND	ND	ND	31.79 (±10.92)	2.02 (±0.49)	1.20 (±0.14)	1.11 (±0.25)	2.0 (±0.4)
	SMN86-2	ND	ND	ND	12.14 (±5.06)	ND	ND	ND	1.2 (±0.1)
	Gfc9424702	ND	ND	ND	21.93 (±15.00)	3.96 (±1.69)	1.53 (±0.26)	ND	2.0 (±0.3)
	Gfc0625001	ND	ND	ND	15.93 (±5.00)	2.02 (±1.66)	ND	0.61 (±0.07)	1.7 (±0.2)
	Gfc0625005	ND	ND	ND	19.89 (±5.34)	2.24 (±0.29)	ND	ND	1.1 (±0.1)
	Gfc0925005	ND	ND	ND	6.34 (±1.70)	3.39 (±0.82)	0.77 (±0.04)	ND	2.1 (±0.4)
	APF06083	ND	ND	ND	4.15 (±1.02)	2.29 (±2.36)	1.16 (±1.11)	ND	1.5 (±0.1)
	Gfc0825001	ND	ND	ND	10.46 (±4.91)	5.01 (±0.95)	2.64 (±0.21)	0.64 (±0.15)	1.8 (±0.3)
	Gfc0901009	ND	ND	ND	12.46 (±6.91)	6.47 (±2.58)	5.96 (±0.71)	1.32 (±0.64)	2.1 (±0.1)
	Gfc1034001	ND	ND	ND	12.51 (±2.83)	5.82 (±0.57)	3.80 (±1.23)	0.76 (±0.26)	2.1 (±0.6)
	GL24	ND	ND	ND	17.75 (±1.48)	3.22 (±0.36)	1.54 (±0.13)	0.84 (±0.06)	1.6 (±0.4)
Producer									
	MAFF235463	15.02 (±3.49)	1.59 (±0.19)	1.82 (±0.43)	ND	ND	ND	ND	1.1 (±0.0)
	Gfc1004003	>50.00	>50.00	>10.00	1.34 (±0.49)	ND	ND	ND	0.9 (±0.1)
	Gfc1019001	>50.00	>50.00	>10.00	1.20 (±0.45)	ND	ND	ND	1.2 (±0.2)
	Gfc0821004	>50.00	>50.00	>10.00	0.93 (±ND)	ND	ND	ND	0.9 (±0.0)
	Gfc0921039	>50.00	>50.00	>10.00	0.83 (±0.11)	ND	ND	ND	0.9 (±0.0)
	Gfc1043032	>50.00	>50.00	>10.00	2.90 (±ND)	ND	ND	ND	1.0 (±0.2)
	Gfc0825007	>50.00	>50.00	>10.00	0.93 (±0.08)	ND	ND	ND	1.1 (±0.1)
	Gfc0825009	>50.00	>50.00	>10.00	2.21 (±1.18)	ND	ND	ND	1.1 (±0.1)
	Gfc0825011	>50.00	>50.00	>10.00	2.31 (±1.70)	1.31 (±0.54)	ND	ND	1.0 (±0.0)
	Gfc0009063	>50.00	>50.00	>10.00	ND	ND	ND	ND	1.0 (±0.1)
	Gfc0009105	>50.00	>50.00	>10.00	2.30 (±ND)	ND	ND	ND	1.0 (±0.1)
	41-79	>50.00	9.99 (±1.85)	5.28 (±0.52)	ND	ND	ND	ND	0.9 (±0.0)

[a]Fumonisin production determined in Suga et al. (8).

[b]Mean of 3 replicates. Standard deviation is indicated in parentheses. ND, below the limits of quantification (LOQs) for the fumonisin analysis, as follows: $FB_1$and FB_2, 0.1 ppm; FB_3, 0.05 ppm; and for gibberellin analysis; GA_1, GA_3, GA_4, and GA_7, 0.5 ppm.

[c]Ratio of mean height of inoculated plants to the mean height of control (uninoculated) plants. Standard deviation is indicated in parentheses.

fumonisin production levels among the strains, FB_1, FB_2, and FB_3 of 12 representative strains from each fumonisin producer and nonproducer were quantified by ultraperformance LC-MS/MS (UPLC-MS/MS). The production of FB_1 and FB_2 of higher than 50 mg/liter and FB_3 of higher than 10 mg/liter was observed in 10 out of 12 fumonisin producers (Table 2). Although the production valance of FB_1, FB_2, and FB_3 could not be determined in these fumonisin producers because their concentrations exceeded the high limit of quantification, two remaining fumonisin producers showed highest production in FB_1 (Table 2). In the case of the fumonisin nonproducers, neither FB_1, FB_2, or FB_3 was detected with LOQs, at FB_1/FB_2 of 0.1 mg/liter and FB_3 of 0.05 mg/liter.

Gibberellin production and pathogenicity in rice seedling. All strains from bakanae-diseased rice are fumonisin nonproducers, according to a previous analysis (8). This suggests that gibberellin production is different between fumonisin producers and nonproducers. We made a preliminary investigation of gibberellin production in the strains by thin-layer chromatography (TLC) (Table 1). TLC detected GA_3 in fumonisin nonproducers but not in fumonisin producers (Table 1). We used UPLC-MS/MS to determine the concentration of gibberellin in the 12 representative strains from each fumonisin producer and nonproducer and carried out a pathogenicity assay on the rice seedlings (Table 2). The concentration of GA_3 in the fumonisin nonproducers was 4.08 to 31.79 mg/liter (Table 2). We did not detect GA_3 in the fumonisin producers using TLC and detected less than 3 mg/liter in 9 out of the 12 strains using UPLC-MS/MS. The pathogenicity values of the 12 fumonisin nonproducers ranged from 1.1 to 2.1, and the pathogenicity values of the 12 fumonisin producers ranged from 0.9 to 1.2 (Fig. 3 and Table 2). The fumonisin producers did not induce typical bakanae symptoms in the rice seedlings, although they were reisolated from the stems after surface sterilization.

TM resistance. TM resistance in *F. moniliforme* isolated from bakanae-diseased rice has been well known in Japan, but preliminary experiments on several *F. fujikuroi* strains in a study by Suga et al. (8) indicate that fumonisin producers are sensitive to TM. Therefore, we investigated the sensitivity to TM in all 95 strains by measuring growth in medium containing 100 ppm of TM. None of the 51 fumonisin producers

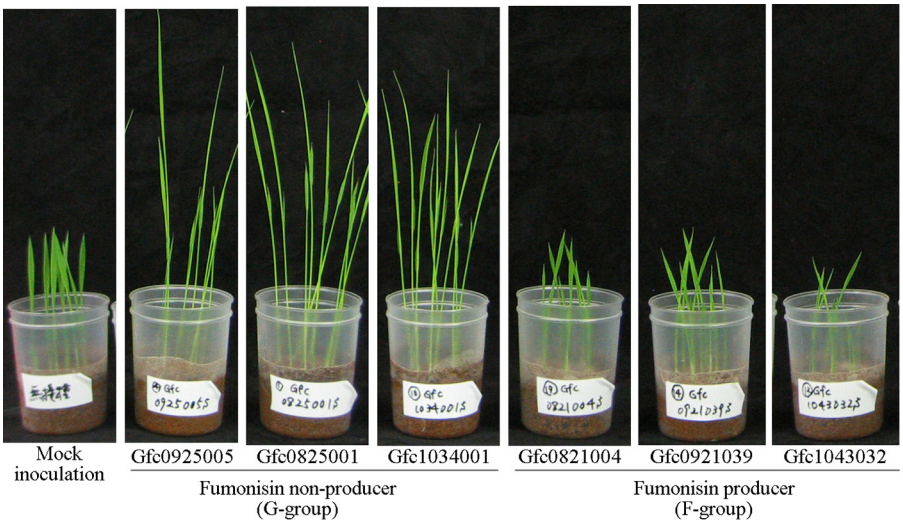

FIG 3 Symptoms of rice seedlings infected with *Fusarium fujikuroi* strains. The figure indicates part of the pathogenicity assays conducted on the fumonisin-producing and nonproducing strains. *F. fujikuroi* fumonisin producers and nonproducers are indicated as the F group and G group mentioned in the Discussion, respectively. Strain names are indicated under each picture.

grew on the medium, and all were considered to be TM sensitive (TMS) (Table 1), whereas 39 of the 44 fumonisin nonproducers grew on the medium and were considered to be TM resistant (TMR) (Table 1).

DISCUSSION

In the present study, we revealed genetic differences between fumonisin producers and nonproducers in Japanese *F. fujikuroi* by SNP and phylogenetic analyses. Fumonisin nonproducers produce more gibberellin and have higher pathogenicity with regard to rice seedlings than do fumonisin producers (Fig. 3 and Tables 1 and 2). Therefore, we designated fumonisin producers and nonproducers fumonisin (F) and gibberellin (G) groups, respectively.

The ability to produce certain secondary metabolites may have an impact on the evolution and ecological adaptations of fungi (25). *F. fujikuroi* competes with various microorganisms during its life cycle, and fumonisin is a potential antibiotic. Keyser et al. (26) demonstrated that the MIC values of fumonisin B_1 for four fungal species other than *F. fujikuroi* complex species were 0.25 to 10 mM, whereas they were higher than 40 mM for four *F. fujikuroi* complex species and *Aspergillus flavus*. Gibberellin production contributes to initial fungal colonization in rice roots (11). F-group strains have been detected in various plant species, including rice, but G-group strains have only been isolated in rice so far.

The cause of low-level or zero fumonisin production in G-group strains remains unclear. Recently, Rösler et al. (27) demonstrated that the low level of *FUM21* transcription, a local transcription factor in the fumonisin biosynthesis gene cluster, is a cause of low fumonisin production in the IMI58289 strain from Taiwan. Although IMI58289 retains the ability to produce fumonisin, a lack of the genes that are essential for fumonisin biosynthesis could cause a complete loss of fumonisin production. Fumonisin-nonproducing *F. verticillioides* strains isolated from bananas lack most of the fumonisin biosynthesis gene cluster and have been reclassified as a new sister species, *Fusarium musae* (28–30). Chiara et al. (31) also discovered the absence of seven genes from the fumonisin biosynthesis gene cluster in a fumonisin nonproducer strain of *F. fujikuroi* (FGSC 8932). In the present study, PCR amplification for FUM18_G51T was successful in all five F-group strains but failed in three out of the five G-group strains tested. PCR amplification was attempted repeatedly, but no amplification was observed for the three strains, even at low annealing temperatures (data not shown). These results

suggest that a part of the fumonisin biosynthesis gene cluster that includes the *FUM18* gene may be absent in these strains.

In the present study, we determined the genetic differences between the G and F groups of *F. fujikuroi* (Fig. 1 and 2). The F group is closer to *Fusarium proliferatum*, which is a fumonisin-producing species, than the G group, and is more genetically diverse than the G group. However, the bootstrap values between the groups in the phylogenetic trees were not large (Fig. 1 and 2), and reproductive isolation between the groups seems to have been absent, because crossing between an F-group strain (Gfc0825009) and a G-group strain (Gfc0801001) succeeded (data not shown). Recently, Niehaus et al. (24) indicated that two pathotypes of *F. fujikuroi* associated with secondary metabolites, a bakanae type, producing gibberellin but little or no fumonisin, and a stunting type, producing fumonisin but not gibberellin, are phylogenetically different, as observed in the F and G groups in the present study (Fig. 1 and 2).

TM resistance corresponded closely to the cluster of G-group strains in the phylogenetic tree (Fig. 1 and Table 1). In Japan, TM has been used intensively to control bakanae disease, and benzimidazole-resistant strains have emerged since 1984 in Japan (6). In China, benzimidazole-resistant strains of *F. fujikuroi* have also emerged, and amino acid mutations E198V and F200Y, which promote resistance, have been found in β2-tubulin (32). The prevalence of benzimidazole-resistant strains was 83% (432 out of 518 strains) in isolates from Japanese rice seeds between 1985 and 1989 (33) and 79% (46 out of 58 strains) in bakanae-diseased rice plants in 1993 (18), although at the time they were identified as *F. moniliforme*, not *F. fujikuroi*. These results suggest that TM-resistant strains prevail in the *F. fujikuroi* population in rice plants, possibly as a result of seed treatment. In the present study, 89% (39 out of 44 strains) of the G-group strains were TM resistant (Table 1). Most of the exceptional TM-sensitive strains in the G group were not Japanese, but were U.S. isolates, whereas none of the 51 F-group strains were TM resistant (Table 1). Therefore, strong selection could be exerted on the *F. fujikuroi* population in rice plants. This selection pressure could result in the comparatively low levels of genetic variation and branching found in the G group in the phylogenetic trees (Fig. 1), generally known as the bottleneck effect.

Our results show the tendency that fumonisin nonproducers have gibberellin high production and are pathogenic to rice seedlings (Table 2). This is consistent with the following published observations. Fumonisin production was not detected in 86% (50 out of 58 strains) of the *F. moniliforme* isolates with gibberellin production (1 to 74 ppm) (18). Little or no fumonisin was detected in seven strains of *F. fujikuroi* isolated from Nepalese rice with gibberellin production (240 to 1,590 ppm) (16). Little or no fumonisin production and high gibberellin production were observed in all 10 strains of *F. fujikuroi* isolated from Asian rice (from Nepal, Vietnam, China, and Bangladesh) (17) and from 8 strains of geographically diverse origin in the whole-genome sequences investigated (24). However, with regard to pathogenicity, caution and prudence were required for an assessment. Pathogenicity was assessed by plant height in the present study. It has been reported that fumonisin producers do not induce the typical symptoms induced by gibberellin in rice, such as stem elongation, but instead cause root rot and stunting (24, 34). Gene disruption of *Fusarium* cyclin C1 (*FCC1*) or *FUM1*, which play an important role in fumonisin biosynthesis in the fumonisin-producing strain B14, resulted in a significant reduction in stunting-type symptoms (24, 35). These studies indicate that fumonisin production contributes to stunting-type symptoms. Although the F-group strains with fumonisin production (8) did not incite typical stunting of rice in the present study, stunting-type symptoms may have been detected if another pathogenicity assay method had been used.

Among the SNPs investigated in the present study, FUM78_C41T, FUM8_G2834A, and CPR_C1152A were specific only to the F and G groups in the Japanese strains, whereas two SNPs (TEF_T618G and RPB2_2C3250T) were specific to the Japanese strains and to the eight strains from other countries (24). Niehaus et al. (24) demonstrated the specific PCR detection of *NRPS31* and *PKS51* in bakanae-type strains and stunting-type strains. These are possible markers for differentiation between the two

types of *F. fujikuroi*, although their robustness should be investigated in further studies because it has been reported that TEF_T618G is not associated with fumonisin production in *F. fujikuroi* isolated from grapes in the southern United States (10).

The significant bias of TM resistance and the low genetic variability observed in the G-group strains suggest that selection pressure by TM could have been exerted on the *F. fujikuroi* population in Japan. However, the discovery of genetically different bakanae and stunting types in *F. fujikuroi* by Niehaus et al. (24) suggests that the genetic differentiation of *F. fujikuroi* is not exclusive to Japan. It is possible that the F group/stunting type and G group/bakanae type of *F. fujikuroi* may have different host plant preferences, although they overlap in rice. This overlap makes the difference in their host plant preferences ambiguous. Both F- and G-group strains were isolated from rice in the present study, and PCR detection of *PKS51* was indicated in the B14 stunting type and the B20 bakanae type isolated from rice in South Korea (24). In some cases, we isolated both the F-group and G-group strains from a single rice plant with bakanae symptoms (data not shown). However, when crops other than rice were used as isolation sources, none of the G-group strains were isolated in the present study, and none of the bakanae-type strains were detected when maize isolates from South Korea were investigated (24). Further studies of the *F. fujikuroi* population in various crops from geographically diverse regions accompanied by the identification of the two types would reveal the ongoing global intraspecific evolution of *F. fujikuroi*.

MATERIALS AND METHODS

Fungal strains. The 95 *F. fujikuroi* strains investigated in the present study were the same as those previously subjected to fumonisin production analysis (Table 1) (8). All except 5 fumonisin producers and 5 nonproducers were Japanese isolates. Fifty-one strains, including MAFF235463 isolated from maize, strawberries, wheat, and rice, were fumonisin producers, and 44 strains, including MAFF235949 isolated from rice seeds, rice seedlings carrying bakanae disease, and from unknown sources, were fumonisin nonproducers.

Genomic DNA extraction. Genomic DNA was extracted from 3- to 4-day-old mycelia cultured in potato dextrose broth (PDB), as described previously (36). Each of the obtained DNA pellets was dissolved in 200 μl of water, and the DNA concentration was adjusted to 5 ng/μl based on a comparison with DNA of a known concentration conducted by agarose gel electrophoresis.

AFLP analysis. We used an AFLP microbial fingerprinting kit (Life Technologies, Carlsbad, CA, USA). Preselective and selective PCRs were performed in an iCycler thermal cycler (Bio-Rad Laboratories Hercules, CA, USA). Hi-Di formamide (9.95 μl; Life Technologies) and standard-size GeneScan 500 dye (ROX; 0.05 μl; Life Technologies) were added to a 1.5-μl sample after selective PCR. The samples were analyzed with a 3100 genetic analyzer, and the obtained data were processed using the GeneMapper software (Life Technologies) to produce the binary matrix for phylogenetic analysis. Detailed information for this analysis is available in the supplemental material.

PCR and sequencing. PCRs for *CPR* and *P450-4* were performed using AmpliTaq DNA polymerase (Life Technologies) and the following cycling parameters: 94°C for 2 min and 30 cycles of 94°C for 1 min, 68°C for 1 min, and 72°C for 1 min, using the P138-5/P138-6 and P450-4-GD1/P450-4-GD2 primer pairs, respectively (Table S1) (19). PCRs for *FUM1*, *FUM8* including the *FUM7*/*FUM8* intergenic region, and *FUM18* including the *FUM18*/*FUM19* intergenic region were performed using the HS398/HS399, HS576/HS577, and HS506/HS519 primer pairs, respectively (Table S1), under the same conditions described above except that the annealing temperatures were 63°C, 58°C, and 60°C, respectively, instead of 68°C. The PCR for *RPB2* was performed using rTaq (TaKaRa Bio, Inc., Ootsu, Japan) and the same cycling parameters described above except that the annealing temperature was 53°C instead of 68°C and the primer pair was 7cf/11ar (37). The PCR for *TEF1*α was performed according to Suga et al. (36). The PCR products were directly sequenced as previously described (36). We used BigDye Terminator version 3.1 with cycle sequencing kits (Life Technologies) and both or either of the primers used for the PCR, and the sequences were obtained using an ABI 3100 genetic analyzer (Life Technologies). The nucleotide sequence data were processed using ChromasPro (Technelysium Pty., Tewantin, Queensland, Australia) and Genetyx (Tokyo, Japan).

Phylogenetic analysis. The binary data for a total of 66 AFLP markers from 95 taxa were compiled into a single data matrix. Neighbor-joining (NJ) analysis (38) of the binary data was performed using PAUP* (version 4.0β10) (39).

The sequence data sets combined the data for *TEF1*α and *RPB2* from 35 taxa, including an outgroup (*Fusarium hostae* NRRL29889). The sequences were aligned with MAFFT (version 7). Phylogenetic trees were obtained using maximum parsimony (MP), maximum likelihood (ML), and Bayesian phylogenetic analyses. The best-fit evolutionary model was determined for each data set by comparing different evolutionary models for MP and ML analyses, and using the Bayesian information criterion (BIC) (40) for the Bayesian analysis. As a result of the calculations, the combined data set of the *TEF1*α and the *RPB2* regions was fitted to a J2ef model with a gamma-shaped distributed rate. MP analysis was carried out using PAUP*. The best tree topology of the MP trees was established using the Kishino-Hasegawa

likelihood test (41) on PAUP*. The ML analysis was performed by the likelihood ratchet method (42). The strength of the internal branches from the resulting tree was tested by bootstrap (BS) analysis (43) using 10,000 replications in NJ, MP, and ML analyses. Bayesian phylogenetic analyses with the selected evolutionary model were carried out using MrBayes (version 3.2.5) (44). Detailed information for this analysis is available in the supplemental material.

Single nucleotide polymorphism analysis. PCR-restriction fragment length polymorphism (PCR-RFLP) experiments for TEF_C447A, FUM1_G423A, FUM78_C41T, FUM8_G2834A, FUM18_G51T, and RPB2_C3250T were performed under conditions similar to those used for HIS PCR-RFLP (8). The derived cleaved amplified polymorphic sequence (dCAPS) primers (HS435, HS482, and HS834) for PCR-RFLP were designed using dCAPS Finder 2.0 (Table S1) (45). PCR sequencing for CPR_C1152A and P4504_C842T was performed as described above.

A multiplex PCR comprising *TEF1*α*/FUM* or *CPR/P450-4* was performed to prepare template DNA for allele-specific primer extension (ASPE) reactions. The PCR was performed in an iCycler thermal cycler (Bio-Rad Laboratories). The ASPE reactions were performed according to the manufacturer's instructions using Platinum Genotype Tsp DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA). The biotin-labeled products were sorted by hybridization with polystyrene microspheres coated with the anti-tag sequences. The microspheres were finally resuspended in buffer containing streptavidin-R-phycoerythrin (Invitrogen Life Technologies). The median fluorescence intensity (MFI) of 100 microspheres was measured with a Luminex 100 flow cytometer (Luminex Corporation, Austin, TX, USA). SNPs were determined from more than 100 MFI values after subtracting the background MFI value obtained using water instead of the extension product. SNPs were determined based on ratios of more than twice the MFI values between the paired microspheres corresponding to SNPs. The SNPs of the *F. fujikuroi* strains used by Niehaus et al. (24) were obtained from the BioProject at the NCBI, as described below. Detailed information for this analysis is available in the supplemental material.

Pathogenicity assay. A dwarf rice cultivar, Tanginbozu, was used to assess the pathogenicity of the strains. The rice seeds were soaked in water at 60°C for 10 min to disinfest them of possible natural pathogens and then were cooled with running water immediately before use. The strains were cultured in potato dextrose agar (PDA) for 1 week at 25°C, and a conidial suspension in sterile water was obtained. The rice seeds (weighting 1 g before hot-water disinfestation) were soaked in a petri dish containing a 1×10^5/ml conidial suspension and incubated at 30°C for 48 h in the dark. The control seeds were soaked in sterile water instead of the conidial suspension. Ten budding seeds were transferred to a filter paper, and superfluous mycelia that had stuck to the seed surfaces were removed. The seeds were then sown in pots (5.5 cm diameter, 7 cm height) containing wetted nursery soil (Grand-sol no. 11; Sumitomo Chemical, Tokyo, Japan). The pots were placed in a transparent plastic box covered with cellophane. The box was placed in an MIR-154 incubator (Panasonic Healthcare, Tokyo, Japan), and maintained at 30°C with continuous lighting for 4 days. The cellophane was then removed from the box, and the plants were watered. The incubator conditions were changed to 23°C with a 16-h light/8-h dark cycle. The heights (in millimeters) of the three tallest individual plants per pot were measured 3 days after the change of incubator conditions. The disease level was scored as the ratio of the mean height of the inoculated plants to the mean height of the control (noninoculated) plants, and the mean result from three pots (replicates) was considered to represent the pathogenicity of the strain.

Fumonisin analysis. The strains were grown on sterile rice. Five grams of rice grain was soaked in 4 ml water prior to autoclaving in an Erlenmeyer flask. Each flask was inoculated with a piece of culture grown on PDA and kept at 25°C for 10 days. The experimental cultures were repeated in triplicate. Fumonisins were extracted with 25 ml methanol-water (3:1 [vol/vol]) by reciprocal shaking for 30 min. The extract filtered through paper was purified by a strong anion-exchange column (Sep-Pak Accell Plus QMA short cartridge [360 mg]; Waters, Milford, MA, USA), and the concentrations of the fumonisins (FB_1, FB_2, and FB_3) were quantified by ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis using an Acquity UPLC system coupled to a Xevo quadrupole time of flight (QTof) mass spectrometer (Waters). The fumonisins FB_1 (Enzo Life Sciences, Lausen, Switzerland), FB_2 (Enzo Life Sciences), and FB_3 (Iris Biotech, Marktredwitz, Germany) were used as a standard. Working solutions containing FB_1, FB_2, and FB_3 at concentrations between 0.05 and 5.0 mg/liter (FB_1/FB_2, 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 mg/liter, and FB_3, 0.05, 0.1, 0.2, 0.4, 0.6, and 1.0 mg/liter, respectively, in 6 bottles) were used to create a calibration curve. The concentration (*x*) (FB_1, FB_2, and FB_3) and corresponding peak area ratio (*y*) were plotted for the 6 bottles of the working solution. The limits of quantification (LOQs) for the fumonisin analysis were the lowest concentration (FB_1/FB_2, 0.1 mg/liter; FB_3, 0.05 mg/liter) on the calibration curves for FB_1, FB_2, and FB_3. In cases in which the concentration of the sample exceeded the range given by the calibration curve, the sample was diluted 10 times and reanalyzed. Detailed information for this analysis is available in the supplemental material.

Gibberellin analysis. The strains were grown in 10% ICI (Imperial Chemical Industries Ltd., UK) medium (46) for 7 days on a reciprocal shaker. The gibberellins in the culture filtrate were analyzed by thin-layer chromatography (TLC) or by UPLC-MS/MS. The gibberellins GA_3 (Wako Pure Chemicals Ind., Ltd., Osaka, Japan), GA_1, GA_4, and GA_7 (Olchemim Ltd., Olomouc, Czech Republic) were used as standards. TLC for GA_3 and GA_4/$_7$ was performed according to Linnemannstöns et al. (47).

The concentrations of the gibberellins (GA_1, GA_3, GA_4, and GA_7) were quantified by UPLC-MS/MS analysis with the same equipment as fumonisin analysis. The experimental cultures were repeated in triplicate, and the resulting broths were subjected to UPLC-MS/MS analysis. Working solutions containing GA_1, GA_3, GA_4, and GA_7 at concentrations of 0.5 to 5.0 mg/liter (GA_3/GA_7, 0.5, 1.0, 2.0, and 5.0 mg/liter; GA_1/GA_4, 0.5, 1.0, 1.5, and 2.0 mg/liter, in four bottles) were used to create a calibration curve. The concentration (*x*) (GA_1, GA_3, GA_4, and GA_7) and corresponding peak area ratio (*y*) were plotted for the four

bottles of the working solution. The LOQ for the gibberellin analysis was the lowest concentration (0.5 mg/liter) on the calibration curves for GA_1, GA_3, GA_4, and GA_7. In cases in which the concentration of the sample exceeded the range given by the calibration curve, the sample was diluted 4 and 10 times and reanalyzed. Detailed information for this analysis is available in the supplemental material.

TM resistance. The strains were grown on PDA for 1 week. Disks (4 mm in diameter) were transferred to PDA amended with TM (water-soluble Topsin M powder containing 70.0% TM; Nihon Nohyaku, Tokyo, Japan) at a final concentration of 100 μg/ml to discriminate between TM-resistant (TMR) and TM-sensitive (TMS) strains. This was done because the MIC of benomyl that is a benzimidazole fungicide differs between resistant (MIC, $\geq$100 μg/ml) and sensitive (MIC, $\leq$12.5 μg/ml) strains of *Fusarium moniliforme* (33).

Data availability. The sequences obtained in the present study are available with the accession numbers LC133052 to LC133070 at the DDBJ/EMBL/GenBank database. The sequences of the *TEF1*α and *RPB2* genes of the *F. fujikuroi* strains used by Niehaus et al. (24) were obtained from the BioProject PRJEB14872 (ID: 412609) at the National Center for Biotechnology Information (NCBI), and the other sequences were obtained from the DDBJ/EMBL/GenBank database with the accession numbers shown in Table S2.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02414-18.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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