Invasion and Colonization Pattern of *Fusarium fujikuroi* in Rice

Chieh-Yi Chen,¹ Szu-Yu Chen,¹ Chun-Wei Liu,¹ Dong-Hong Wu,^{2,3} Chien-Chih Kuo,⁴ Chun-Chi Lin,⁵ Hau-Ping Chou,⁶ Yu-Yao Wang,⁶ Yi-Chen Tsai,⁷ Ming-Hsin Lai,² and Chia-Lin Chung^{1,†}

¹ Department of Plant Pathology and Microbiology, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei City 10617, Taiwan

² Crop Science Division, Taiwan Agricultural Research Institute, Council of Agriculture, No. 189, Chung-Cheng Road, Wufeng, Taichung City 41203, Taiwan

³ Department of Agronomy, National Chung Hsing University, No. 145, Xingda Rd., South District, Taichung City 40227, Taiwan

⁴ Crop Environment Section, Taichung District Agricultural Research and Extension Station, Council of Agriculture, No. 370, Song Hwai Road, Tatsuen Hsiang, Changhua County 51544, Taiwan

⁵ Crop Environment Section, Taitung District Agricultural Research and Extension Station, Council of Agriculture, No. 675, Chunghua Rd., Sec. 1, Taitung City 95055, Taiwan

⁶ Crop Environment Section, Kaohsiung District Agricultural Research and Extension Station, Council of Agriculture, No. 2-6 Dehe Rd., Dehe Village, Changjhih Township, Pingtung County 90846, Taiwan

⁷ Crop Environment Section, Hualien District Agricultural Research and Extension Station, Council of Agriculture, No. 150, Sec. 2, Ji'an Rd., Ji'an Township, Hualien County 97365, Taiwan

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ABSTRACT

Bakanae disease in rice can cause abnormal elongation of the stem and leaves, development of adventitious roots, a larger leaf angle, and even death. Little is known about the infection, colonization, and distribution of *Fusarium fujikuroi* in rice plants across different growth stages. In this study, microscopic observation and quantitative real-time PCR were combined to investigate the pathogenesis of bakanae, using artificially inoculated seedlings of a susceptible rice cultivar, Zerawchanica karatals (ZK), a resistant cultivar, Tainung 67 (TNG67), naturally infected adult field plants (cultivars Kaohsiung 139, Taikeng 2, and Tainan 11), and an *F. fujikuroi* isolate expressing green fluorescent protein. In rice seedlings, *F. fujikuroi* hyphae were found to directly penetrate the epidermis of basal stems and roots, then extend inter- and intracellularly to invade the vascular bundles. Occlusion of vascular bundles and radial hyphal expansion from vascular bundles to surrounding parenchyma were observed in adult plants.

Analysis of consecutive 3-cm segments of the whole plant revealed that *F. fujikuroi* was largely confined to the embryo, basal stem, and basal roots in seedlings, and distributed unevenly in the lower aerial parts (including nodes and internodes) of adult plants. The elongation and development of adventitious roots did not necessarily correlate with the amount of *F. fujikuroi* in diseased plants. Treatment of rice seeds with gibberellic acid-3 (GA₃) at 0.5 mg/liter resulted in significantly more elongation of ZK than TNG67 seedlings, suggesting that the susceptibility of ZK to bakanae is associated with its higher sensitivity to GA₃.

Keywords: adventitious roots, bakanae disease, fluorescence microscopy, *Fusarium fujikuroi*, gibberellic acid, mycology, pathogenesis, real-time quantitative PCR, vascular bundles

Bakanae disease of rice, caused by *Fusarium fujikuroi* Nirenberg [teleomorph *Gibberella fujikuroi* (Sawada) Ito] (Ito and Kimura 1931; Nirenberg 1976; O'Donnell et al. 1998), was first identified in 1828 in Japan and spread widely to all major rice cultivation areas (Ito and Kimura 1931; Jeon et al. 2013; Karov et al. 2009; Kim et al. 2015; Singh and Sunder 1997). It can lead to great yield losses ranging from 20 to 50% (Ito and Kimura 1931; Ou 1985). In recent years, increasing incidence and severity of bakanae disease has been reported in Italy, Pakistan, Malaysia, Indonesia, Bangladesh, India, and South Korea (Gupta et al. 2014; Haq et al. 2011; Khan et al. 2000; Kim et al. 2015; Matić et al. 2017; Zainudin et al. 2008). In Taiwan, a bakanae disease epidemic was first reported in the eastern region, and then became commonly observed throughout Taiwan (Chen et al. 2016). In eastern Taiwan in 2009, two main cultivars,

Kaohsiung 139 (KH139) and Taikeng 2 (TK2), showed a disease incidence of 10 to 15% (Huang and Chu 2009). In central Taiwan in 2012, disease incidences of 30.4% for the cultivar Taikeng 8 and 14.5% for Taikeng 9 (Zheng et al. 2016), and 12 to 14% for Tainung 71, and 6 to 8% for Taikeng 16 (Kuo et al. 2014) were reported.

Bakanae disease can cause various types of disease symptoms, ranging from blight, slenderness, stunting, a large leaf angle, crown rot, root rot, sterility and discoloration of grains (Desjardins et al. 2000; Huang and Chu 2009; Ou 1985; Sun and Snyder 1981; Webster and Gunnell 1992). The most characteristic symptoms are etiolation, hypertrophy and excessive or abnormal elongation (Gupta et al. 2014). Kurosawa (1926) first proposed that a chemical produced by the pathogen may be responsible for the elongation of diseased plants. Identified from the culture filtrate of the bakanae pathogen (Yabuta and Sumiki 1938), gibberellic acid (GA) was found to be a key factor causing anomalous elongation symptoms in the infected plants (Matić et al. 2017).

Bakanae is considered a seedborne and soilborne disease (Kanjansoon 1965; Lin 2015; Ou 1985). *F. fujikuroi* can produce microconidia, macroconidia, and ascospores (Leslie and Summerell 2006; Sun 1975). Rice seeds contaminated by ascospores during flowering or by conidia during harvesting (Sun 1975) are the main inoculum in the following season (Matić et al. 2017). Macroconidia and thick-walled hyphae in plant debris and soil can also serve as the inoculum (Kanjansoon 1965; Lin 2015; Ou 1987; Watanabe 1974). Due to the lack of chlamydospores, *F. fujikuroi* populations

[†]Corresponding author: C.-L. Chung; clchung@ntu.edu.tw

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in the field diminish quickly after host debris is decomposed (Leslie and Summerell 2006; Sun 1975).

The infection, colonization, and distribution of *F. fujikuroi* in rice have been investigated in a few studies. By introducing the spores of *F. fujikuroi* to rice seeds and various parts (wounded and nonwounded) of 2- to 4-cm height seedlings, Sun (1975) suggested that *F. fujikuroi* can infect plants through the seeds, stem base, and root tip, but not the stem, leaf blade, or leaf axil. Sunani et al. (2019) proved that inoculation of rice seeds, seedlings, and florets can lead to systemic infection and seed transmission of *F. fujikuroi*. By performing scanning electron microscopy, Sunani et al. (2019) also observed different types of infectious structures (i.e., swollen tip hyphae, infectious hyphae, appressoria, and infection cushions) and the intra- and intercellular colonization of *F. fujikuroi*. Researchers have used fluorescent *F. fujikuroi* isolates to observe hyphal interactions with the biocontrol agent *Talaromyces* sp. KNB-422 (Kato et al. 2012) and the early root colonization of a GA-producing wild-type and a GA-deficient mutant strain (Wiemann et al. 2013). By conducting hand sectioning and tissue isolation using the internodes of diseased field plants approaching the heading stage, Nisikado and Kimura (1941) discovered a wide distribution of *F. fujikuroi* hyphae and microconidia within vascular vessels and neighboring parenchyma cells in rice culms. Naito et al. (2008) also observed colonization of *F. fujikuroi* in the vascular system in naturally infected rice seedlings at the transplanting stage.

As an emerging threat to rice production, bakanae disease has drawn increasing attention over the last few decades (Gupta et al. 2015; Matić et al. 2017). Research efforts have been directed toward uncovering the whole genome sequences (Jeong et al. 2013; Wiemann et al. 2013) and population genetics of *F. fujikuroi* (Chen et al. 2016; Valente et al. 2017), fungal metabolites responsible for bakanae symptoms (Niehaus et al. 2016), and rice resistance against *F. fujikuroi* (Chen et al. 2019; Hur et al. 2015; Matić et al. 2016; Volante et al. 2017). In addition, SYBR green and TaqMan quantitative real-time PCR (qPCR) techniques have been developed for detection and quantification of *F. fujikuroi* in rice seeds and seedlings (Amatulli et al. 2012; Carneiro et al. 2017). To provide new insights into the interplay between rice and *F. fujikuroi*, the objectives of this research were to (i) investigate the infection and colonization of *F. fujikuroi* in the seedlings of resistant and susceptible rice cultivars; (ii) examine the spatial distribution of

F. fujikuroi in diseased seedling and adult plants; and (iii) assess the relationship between bakanae symptoms and the level of *F. fujikuroi* colonization in rice. The infection and colonization processes of *F. fujikuroi* were examined in more detail and at a better resolution using a newly generated isolate of *F. fujikuroi* expressing green fluorescent protein (GFP). The amounts of *F. fujikuroi* in consecutive 3-cm segments of the whole plant were precisely quantified by SYBR green-based qPCR. The effect of GA sensitivity on resistance or susceptibility was also investigated.

MATERIALS AND METHODS

Plant materials. Artificially inoculated seedlings and naturally infected adult field plants were used for microscopic observation and qPCR analysis in this study. Inoculations were conducted using a susceptible cultivar, Zerawchanica karatala (ZK), and a resistant cultivar, Tainung 67 (TNG67). These two cultivars were selected from the Rice Diversity Panel 1 (Ali et al. 2011; Eizenga et al. 2014) based on their consistent susceptibility and resistance performance in repeated experiments performed by Chen et al. (2019). Seeds were provided by Ming-Hsin Lai and Dong-Hong Wu at the Crop Science Division, Taiwan Agricultural Research Institute. Field samples of three commercial cultivars, KH139, TK2, and Tainan 11 (TN11), were collected from Taitung and Taichung from March to May 2019 (Table 1). Plants of an unknown cultivar at the booting stage were also collected from Kangding, Pingtung on 17 April 2019. These field plants were collected between the late tillering and milk stages. The plants were 30 to 84 cm in height, with three to six nodes on the stem.

Inoculation of *F. fujikuroi*. *F. fujikuroi* preserved on dry filter paper was gently smeared on half-strength potato dextrose agar (1/2 PDA) (19.5 g of PDA [HiMedia, India] and 7.5 g of agar [BioShop, Canada] per 1 liter) and cultured for 4 to 7 days at 25°C under a 12/12-h light/dark photoperiod. The spores were collected in sterile distilled water using a sterile tip, filtered through Kimwipes (Kimtech Science, U.S.A.), and then adjusted to 1×10^5 spores/ml for inoculation (a few plants used for microscopic examination were inoculated with 1×10^6 spores/ml). Rice seeds were immersed in 60°C sterile distilled water for 10 min for sterilization, and then soaked in sterile distilled water (replaced every day) for 4 days at a temperature of 32°C/28°C day/night. The germinated seeds were

TABLE 1. Adult field plants used in this study

Cultivar	Plant ID ^a	qPCR ^b		Number of nodes ^c	Number of nodes with adventitious roots ^c	Collection site	Collection date	Growth stage
		3 cm segment	Node and nearby internodes					
Kaohsiung 139	KH139-1-1	V		–	0	Chishang, Taitung	2019-03-28	Late tillering
	KH139-2-1	V		–	0			
	KH139-3-1	V		5	3	Chishang, Taitung	2019-05-23	Milk
	KH139-3-2	V		5	4			
	KH139-4-1	V		4	3			
	KH139-5-1		V	3	1			
	KH139-5-2		V	4	3			
	KH139-6-1		V	3	2			
	TN11-1-1		V	5	4	Holi, Taichung (field 1)	2019-05-08	Heading
Tainan 11	TN11-2-1		V	6	3			
	TN11-3-1		V	4	2	Holi, Taichung (field 2)	2019-05-08	Heading
	TN11-4-1		V	4	3			
	TN11-5-1	V		5	2	Holi, Taichung (field 1)	2019-05-24	Milk
	TN11-5-2	V		6	5			
	TN11-6-1	V		5	2			
	TN11-6-2	V		4	4			
	TN11-7-1	V		5	4			
	TK2-1-1	V		–	–	Guanshan, Taitung	2019-04-08	Late tillering
Taikeng 2	TK2-2-1	V		–	–			

^a Plant ID: cultivar-hill-plant.

^b V, qPCR analysis was conducted to determine the amount of *Fusarium fujikuroi* in consecutive 3-cm segments or the node and nearby internode segments of the plant.

^c –, not recorded.

soaked in the spore suspension or sterile distilled water (control) and shaken overnight (14 to 18 h). The inoculated seeds were transplanted into 288-cell plug trays filled with Akadama soil (one seed per cell; each cell was $L \times W \times H = 1.75 \times 1.75 \times 2.54$ cm) and cultivated in a growth chamber (32°C/28°C day/night temperature, 12/12-h light/dark photoperiod, luminous intensity 7,000 to 8,000 lx). Bakanae disease severity was evaluated at 21 days postinoculation (dpi) using a 0 to 3 rating scale, and the overall disease severity index was calculated (Chen et al. 2015). The rating scale was defined as follows: 0 = no symptoms; 1 = seedling showing one of the following symptoms: stem or internode elongation, large leaf angle, or slenderness and paleness; 2 = seedling showing complex symptoms; and 3 = seedling death (Chen et al. 2015). The height of each plant was measured (from the base to top of the aerial part). For each cultivar, *F. fujikuroi*-induced elongation was calculated as: the height of the diseased plant minus the average height of the mock-inoculated plants. Phenotypic differences were analyzed by Tukey's honestly significant difference test at $P < 0.05$ using GraphPad Prism version 7 (GraphPad Software).

Generation of a GFP-expressing strain of *F. fujikuroi*. Plasmid pPK2HPHGFP (Michielse et al. 2009) carrying the *GFP* gene was provided by Ying-Lien Chen from the Department of Plant Pathology and Microbiology, National Taiwan University. Protoplasts of *F. fujikuroi* isolate Ff266 (Chen et al. 2016; GenBank accession numbers MT431665 and MT431666) were prepared and used for polyethylene glycol-mediated transformation following the protocols of Hou et al. (2002). Briefly, 10 µg of pPK2HPHGFP linearized with *Hind*III was mixed with 200 µl of protoplast suspension (10^7 to 10^8 protoplasts/ml). Transformed protoplasts were regenerated on TB3 medium (0.3%, wt/vol, yeast extract; 0.3%, wt/vol, casamino acids; and 20%, wt/vol, sucrose) containing 0.7% (wt/vol) low melting agarose and hygromycin at 250 µg/ml. Randomly selected transformants were cultured on V8 medium (20%, wt/vol, V8 juice; 0.2%, wt/vol, CaCO_3 ; and 1.5%, wt/vol, agar) containing hygromycin at 250 µg/ml for three successive generations, and then subjected to single spore isolation. The transformants were checked for fluorescence intensity under a fluorescence microscope (Olympus BX53) and evaluated for virulence by inoculating ZK and TNG67. Inoculation and disease evaluation were conducted as described above in three independent trials, with 10 plants per cultivar per transformant in each trial. One of the transformants, *F. fujikuroi* G3-1, was chosen for subsequent experiments.

Hand sectioning and microscopic examination. Hand-cut cross sections and longitudinal sections of different tissues were examined under a fluorescence microscope (OLYMPUS BX53), either under bright field or with a U-MWIBA3 filter (OLYMPUS, excitation filter 460 to 495 nm, barrier filter 510 to 550 nm) for GFP detection. Microscopic observation was conducted using infected seedlings collected at 0 to 33 dpi from five independent inoculation trials. In each trial, one to three plants were sampled per day, and 20 to 60 evenly distributed cross sections per tissue sample (stem, root, embryo, and leaf) were examined per plant. In one of the trials, 10 longitudinal sections per tissue sample were examined. Hand sectioning and microscopy were also conducted on 15 adult field plants: 13 plants collected from the four fields listed in Table 1, as well as two plants (unknown cultivars at the booting stage) collected from Kangding, Pingtung on 17 April 2019. From each field at each collection time, two to three plants were sampled, and 100 to 160 cross sections of the stem and ~20 cross sections of the leaves were examined for each plant. Longitudinal and cross sectioning were also performed to check for internal discoloration, using an additional two KH139 plants (heading stage) collected from Chishang, Taitung on 28 March 2019. Some sections were stained with 0.1% (wt/vol) trypan blue staining solution (trypan blue in a 2:1:1 mixture of lactic acid, glycerol, and distilled water) for 7 min and de-stained with a 1:1 solution of glycerol and 0.3 M HCl (Roth et al. 2018).

DNA extraction. *F. fujikuroi* mycelium or rice tissues were collected in a 2-ml microtube with two stainless steel beads (5/32-inch diameter) and stored at -80°C. The sample was homogenized using a 2010 Geno/Grinder (SPEX SamplePrep). Genomic DNA was extracted from *F. fujikuroi* and rice following a standard cetyltrimethylammonium bromide extraction protocol (Doyle and Doyle 1987). A NanoDrop ND-1000 spectrophotometer (Thermo Scientific) was used to determine the quality and quantity of DNA.

Quantification of *F. fujikuroi* by qPCR. Each qPCR reaction contained 6 µl of Fast SYBR Green Master Mix (2×) (Applied Biosystems), 0.5 µM forward and reverse primers, and 100 or 25 ng DNA in a total volume of 12 µl. DNA (100 ng) was used for qPCR analysis of the aerial parts and roots of seedlings and the aerial parts of the adult plant; 25 ng of DNA was used for the seedling root samples because the amount of DNA that could be extracted from the 3-cm segment samples (described below) was only 1/3 to 1/8 that extracted from the aerial parts of the seedlings. The primers TqF2 (5'-GGCGCGTTTTGCCCTTCCT-3') and TqR (5'-AGCGGCTTCCTATTGTGCGAA-3') targeting the translation elongation factor 1- α gene in *F. fujikuroi* (Carneiro et al. 2017) were used. Amplification was performed in three technical replicates with the StepOnePlus Real-Time PCR System (Applied Biosystems). The thermal cycling parameters were 1 cycle of 95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Standard curves were generated for inoculated seedlings by mixing healthy plant DNA (extracted from the aerial parts) and a fivefold serial dilution of *F. fujikuroi* G3-1 DNA (10 ng to 0.0032 ng) with a total of 100 ng of DNA per reaction. For adult field plants, standard curves were generated by mixing healthy plant DNA (extracted from the aerial parts) and a fivefold serial dilution of *F. fujikuroi* Ff266 (50 to 0.016 ng), with a total of 100 ng of DNA per reaction.

The amounts of *F. fujikuroi* in different parts of the inoculated seedlings were quantified by qPCR. Four independent inoculation trials were conducted, with three plants per cultivar per time point. In trials one and two, consecutive 3-cm segments of the whole plant were collected at 3, 7, 10, 14, 17, and 21 dpi. In trials three and four, the embryo and the first 0- to 3-cm segments of the base of the aerial part and basal roots were collected at 7, 14, and 21 dpi. In the first trial, the embryo was cut into half and combined with the first 0- to 3-cm segment of the basal root and the base of the aerial part. To understand the precise distribution of *F. fujikuroi*, the embryo, stem, and root were collected separately in trials two to four. Differences among the quantities of *F. fujikuroi* in different segments of ZK or TNG67 at different time points were analyzed by Tukey's honestly significant difference test at $P < 0.05$ using GraphPad Prism version 7 (GraphPad Software). Pearson correlation analysis was used to analyze the correlation between seedling height and total biomass index (defined as the sum of the amount of *F. fujikuroi* [ng/100 ng of total DNA] in all the segments of a plant).

qPCR was also used to quantify the amounts of *F. fujikuroi* in consecutive 3-cm segments of 12 adult field plants (Table 1). To determine the distribution of *F. fujikuroi* in nodes, internodes, and adventitious roots, qPCR analysis was conducted for each node, the 0 to 1 cm and 1- to 2-cm-internode segments above/below the node, and the emerged adventitious roots of seven plants (Table 1).

Gibberellic acid treatment. Rice seeds were immersed in 60°C sterile distilled water for 10 min for sterilization, and then soaked in sterile distilled water (replaced every day) for 4 days at 32°C day/28°C night. Germinated seeds were treated with gibberellic acid-3 (GA_3) at 0.01, 0.5, or 25 mg/liter according to the method of Ma et al. (2008). Seeds immersed in sterile distilled water were used as a control (mock treatment). After 16 h of incubation at room temperature, the seeds were sown in pots ($L \times W \times H = 3.5 \times 3.5 \times 4.5$ cm) containing Akadama soil. Seedling heights were measured 14 days after treatment. The experiment was conducted in three independent trials, each with 10 seedlings per cultivar per treatment. Student's *t* test was performed to analyze the height differences between GA_3 - and mock-treated seedlings.

RESULTS

Generation of a fluorescent *F. fujikuroi* isolate, G3-1.

The mycelia and spores of *F. fujikuroi* G3-1 consistently exhibited bright green fluorescence (Fig. 1A). The morphological characteristics of G3-1 were similar to those of the wild-type strain Ff266. Both isolates formed orange to pink colonies on 1/2 PDA (Fig. 1B). ZK and TNG67 inoculated with *F. fujikuroi* G3-1 and Ff266 showed typical bakanae symptoms including elongated stems and leaves and a wider leaf angle (Fig. 1C). In susceptible ZK, no significant difference was found between the disease severity indexes of plants infected by G3-1 (53.6%) and Ff266 (60.3%). TNG67 plants infected with G3-1 had a lower disease severity index (7.1%) than those infected with Ff266 (23.6%) (Fig. 1D).

Colonization patterns of *F. fujikuroi* in rice seedlings.

Two types of *F. fujikuroi* colonization patterns were observed in both seedlings and adult plants. The hyphae aggregated (Fig. 2A) or grew around the cells (Fig. 2B). Both intracellular (Fig. 2C) and intercellular (Fig. 2D) hyphal extension were observed in shoot and root tissues. In a few sections, the hyphae were found to constrict before moving to the adjacent cell (Fig. 2E).

Pathogenesis of *F. fujikuroi* in rice seedlings. Sections of the leaves, roots, stems, and embryos of inoculated ZK and TNG67

were examined at different time points. *F. fujikuroi* spores were found to attach on the leaf surface at 1 to 3 dpi. However, from over 1,000 sections examined, leaf infection was not observed until 21 dpi. In root tissues, the *F. fujikuroi* infection process was similar in susceptible ZK and resistant TNG67. *F. fujikuroi* spores attached on the root surface at 1 to 3 dpi (Fig. 3A) and invaded the epidermis at 3 to 5 dpi (Fig. 3B). The hyphae penetrated the exodermis and sclerenchyma layer at 6 to 8 dpi (Fig. 3C) and grew toward the central cylinder (Fig. 3D). The mesodermis was colonized at ~14 dpi (Fig. 3E), then the hyphae penetrated the endodermis and proliferated in the vascular bundle after 21 dpi (Fig. 3F; the phenomenon was generally observed, but here we present a more clear and representative image from a ZK seedling inoculated with 10^6 [instead of 10^5] *F. fujikuroi* spores/ml). The infection was first observed mainly in the root tissues near the embryo. Later on, the hyphae spread to the entire root system but only a few hyphae were found in the root tips and lateral roots.

In the stem, a slight delay in initial infection and colonization of *F. fujikuroi* was observed in resistant TNG67. The spores germinated then invaded the surface of the basal stem (Fig. 3G) at 1 to 3 dpi and 1 to 7 dpi in ZK and TNG67, respectively. The hyphae then developed in the stem tissues (Fig. 3H to J) from 7 to 14 dpi and 10 to 14 dpi in ZK and TNG67, respectively. In both cultivars, the

Fig. 1. Comparison of *Fusarium fujikuroi* isolates G3-1 and Ff266. **A**, The hyphae and spores of the transformant G3-1 showed green fluorescence. **B**, Colony morphology of transformant G3-1 and wild-type Ff266 on 1/2 potato dextrose agar. **C**, Typical bakanae symptoms of Zerawchanica karatals (ZK) and Tainung 67 (TNG67) plants at 21 days postinoculation (dpi) with *F. fujikuroi* G3-1 or Ff266. **D**, Disease severity at 21 dpi. Data are presented as mean $\pm$ standard error. Bars labeled with different letters are significantly different as determined by Tukey's honestly significant difference test at $P < 0.05$.

epidermal and ground tissues of coleoptile were destroyed by a mass of hyphae after 17 to 20 dpi (Fig. 3K). Although the hyphae heavily colonized the basal stem, the pathogen was absent from the surrounding leaf sheath (Fig. 3L; the phenomenon was generally observed, but here we present a more clear and representative image from a ZK seedling inoculated with 10^6 *F. fujikuroi* spores/ml). Until 21 dpi, *F. fujikuroi* was mainly restricted to within ~3 cm from the base in both cultivars.

In the embryo, no difference was observed in the infection and colonization of ZK and TNG67 by *F. fujikuroi*. Only a few hyphae colonized the embryo tissues at 1 to 3 dpi. An expanding hyphal network was formed at 7 to 10 dpi (Fig. 3M and N), and the embryo was fully colonized after 14 dpi (Fig. 3O).

Distribution of *F. fujikuroi* in rice seedlings. The coefficient of determination (R^2) values for qPCR standard curves for ZK and TNG67 (Supplementary Fig. S1A) and for KH139, TK2, and TN11 (Supplementary Fig. S1B) were all >0.99. In repeated qPCR assays, *F. fujikuroi* DNA was detected specifically (based on melting curve analysis) and quantified accurately at a minimum concentration of 0.016 ng per 100 ng of total DNA, with a threshold cycle around 33 to 34. Therefore, *F. fujikuroi* was determined to be undetectable when the estimated amount was below 0.016 ng in this study.

The distribution of *F. fujikuroi* in ZK and TNG67 seedlings was evaluated by qPCR at 3, 7, 10, 14, 17, and 21 dpi. The results from inoculation trial 1 (Supplementary Fig. S2) and trial 2 (Fig. 4A) showed that in both ZK and TNG67, *F. fujikuroi* primarily infected the embryo and the first 0- to 3-cm segment of basal roots (R1) and the base of the aerial part (A1; containing only stem tissues) from 0 to 21 dpi. In general, *F. fujikuroi* was detected first in R1 and the embryo at 3 dpi, and then in A1 at 7 dpi (Fig. 4A). After 14 dpi, small amounts of *F. fujikuroi* (0.016 to 1.78 ng) were detected along the roots and occasionally in different segments of the aerial part of the plant (Fig. 4A). Great variation in the amount of *F. fujikuroi* was

observed among individual plants. In inoculation trials one and two, elongation symptoms were first observed in ZK at 10 dpi, and in TNG67 at 14 dpi (Supplementary Fig. S3A). The elongation growth of infected ZK and TNG67 at 14 dpi and 21 dpi was not significantly correlated with the total biomass of *F. fujikuroi* in the whole plant (Supplementary Fig. S3B).

To assess the tissue and cultivar preference of *F. fujikuroi*, the quantities of *F. fujikuroi* in R1, A1, and the embryo at 7, 14, and 21 dpi were examined in two additional trials (Fig. 4B). Based on combined data from inoculation trials two and four, no significant difference was found between R1 and A1 or between ZK and TNG67 at all three time points. Compared with R1 and A1, the embryo contained a similar amount of *F. fujikuroi* at 7 dpi and significantly higher amounts of *F. fujikuroi* at 14 and 21 dpi. The amount of *F. fujikuroi* in the embryo greatly increased from 0.69 ng at 7 dpi to 14.3 ng at 14 dpi in ZK, and from 4.38 ng at 14 dpi to 12.24 ng at 21 dpi in TNG67.

Sensitivity of ZK and TNG67 to GA₃. To determine if the different symptoms of ZK and TNG67 were caused by different sensitivities to GA₃, GA₃-induced elongation growth was evaluated (Fig. 5). With the addition of GA₃ at 0.01 mg/liter, no difference was observed between GA₃- and mock-treated plants for both cultivars. Compared with the mock-treated controls, the heights of ZK seedlings increased by 24.6% ($P < 0.001$) and 44.4% ($P < 0.001$) with the addition of GA₃ at 0.5 and 25 mg/liter, respectively; the heights of TNG67 seedlings increased by 13.4% ($P = 0.015$) and 51.4% ($P < 0.001$) with the addition of GA₃ at 0.5 and 25 mg/liter, respectively. The height of ZK seedlings was affected to a greater extent than that of TNG67 seedlings under GA₃ at 0.5 mg/liter, which suggested that ZK was more sensitive to GA₃.

Colonization patterns of *F. fujikuroi* in adult field plants. An adult field plant (KH139) showing bakanae symptoms and one that was healthy in appearance were examined macroscopically.

Fig. 2. *Fusarium fujikuroi* hyphal expansion and colonization. **A**, Aggregation of hyphae. **B**, Hyphae growing around the cells. **C**, Hyphae extending intracellularly. **D**, Intercellular hyphae (arrows). **E**, A hypha that constricted (arrow) when passing through the rice cell wall.

Cross sectioning of a 0- to 4-cm segment at the base of the stem revealed that the surface and inner tissues were green in the healthy plant and partially necrotic in the diseased plant (Supplementary Fig. S4A). Longitudinal sectioning of a diseased plant showed

discoloration in the lower node and internode segments. The upper part of the diseased plant remained green (Supplementary Fig. S4B). Whitish mycelia were observed in both the node and internode segments.

Fig. 3. Infection and colonization of rice seedlings by *Fusarium fujikuroi*. **A to E**, Pathogenesis of *F. fujikuroi* in the root. **A**, Spores adhering on the root surface. **B**, Hyphae penetrating the epidermis. **C**, Hyphae invading the exodermis and sclerenchyma layer. **D**, Hyphae growing toward the central cylinder. **E**, Hyphae colonizing the mesodermis and reaching the endodermis. **F**, Hyphae heavily colonizing the vascular cylinder. **G to L**, Pathogenesis of *F. fujikuroi* in the basal stem. **G**, Infection starting from a spore attached to the stem surface. **H to I**, Hyphal proliferation in the ground tissues. **J**, Hyphal growth from the stem to surrounding coleoptile. **K**, A mass of hyphae destroyed the epidermis and ground tissues of the coleoptile. **L**, Hyphae heavily colonized the stem but not the surrounding leaf sheath. **M to O**, Expanding hyphae network in the embryo. Plant materials in **A to D**, **F**, **H to I**, **K**, **L**, and **N**: ZK; and **E**, **G**, **J**, **M**, and **O**: TNG67. Sections in **F** and **L** were from plants inoculated with 10^6 *F. fujikuroi* spores/ml, and the rest of the sections were from plants inoculated with 10^5 *F. fujikuroi* spores/ml. co, coleoptile; en, endodermis; ep, epidermis; ex, exodermis; sp, spore; me, mesodermis; sc, sclerenchyma layer; sh, leaf sheath; and va, vascular bundle.

In symptomatic plants of the KH139, TK2, and TN11 cultivars between the late tillering and milk stages, *F. fujikuroi* colonization was found mainly within the vascular bundles in both the roots and stem (Fig. 6C, D, F, G, and I). The hyphae were observed in the xylem vessels of the root (Fig. 6C and D). In the stem, *F. fujikuroi*

colonized the xylem vessels, phloem vessels, and air space of central vascular bundles (Fig. 6F and G) and small vascular bundles (Fig. 6I). Browning was observed in some vascular bundles (Fig. 6G). Leaves were almost free of the pathogen. *F. fujikuroi* hyphae were observed in the vascular bundle of the leaf (Fig. 6J), but only in

Fig. 4. qPCR analysis of the distribution of *Fusarium fujikuroi* in rice seedlings. **A**, The amounts of *F. fujikuroi* in consecutive 3-cm segments of Zerawchanica karatals (ZK) and Tainung 67 (TNG67) plants inoculated with *F. fujikuroi* G3-1 (inoculation trial 2). A#, the segment number of the aerial part of the plant; R#, the segment number of the root part; and E, embryo. The aerial segment A1 contains only stem tissues, the segments above the dashed line contain only leaf tissues, and the remaining aerial segments may contain both stem and leaf tissues. **B**, The amounts of *F. fujikuroi* in A1, R1, and the embryo of ZK and TNG67 (inoculation trials two to four). Data are presented as mean $\pm$ standard error. Different letters indicate significant differences in means as determined by Tukey's honestly significant difference test at $P < 0.05$.

one out of 200 leaf sections examined in this study. In TN11 plants collected from two different fields in Holi, Taichung, the hyphae were found spreading out from vascular bundles to nearby parenchyma cells (Fig. 6K), which caused necrosis and browning of the parenchyma cells (Fig. 6L). Abundant hyphae and microconidia were also observed in the parenchyma (Fig. 6M).

Distribution of *F. fujikuroi* in adult field plants. The longitudinal distributions of *F. fujikuroi* in KH139, TK2, and TN11 plants were evaluated by qPCR analysis. Among rice plants in the same hill, *F. fujikuroi* was undetectable in asymptomatic plants and detectable in the plants showing bakanae symptoms (data not shown). The distribution patterns shown in Figure 7A suggested that *F. fujikuroi* mainly colonized the lower parts of the diseased plants. *F. fujikuroi* was distributed unevenly along the plants, and the maximum colonization was detected at the middle of the *F. fujikuroi*-colonized aerial part rather than at the base of the infected plants.

qPCR was also performed to understand the pathogen distribution in each node, nearby internodes, and adventitious roots (Fig. 7B). In general, lower amounts of *F. fujikuroi* were detected in the node than in the nearby internodes (1 and 2 cm above/below the specific node). In some plants (i.e., KH139-5-1, KH139-6-1, TN11-3-1, and TN11-4-1) adventitious roots were present on nodes without detectable *F. fujikuroi* colonization. In addition, the plants showing more nodes with adventitious roots did not necessarily contain higher amounts of *F. fujikuroi*. Examples included KH139-4-1 and TN11-5-1 (Fig. 7A) and TN11-4-1 (Fig. 7B).

DISCUSSION

This study combined light/fluorescent microscopy and qPCR analysis to qualitatively and quantitatively examine the pathogenesis and distribution of *F. fujikuroi* in rice. Time-course analysis of rice seedlings revealed that within 21 dpi, *F. fujikuroi* was largely confined to the embryo, basal stem, and basal roots. The remarkably high level of *F. fujikuroi* colonization in the embryo could be due to increased levels of nutrients (mainly sugars) derived from

germinating rice seeds (Yu et al. 2015). The distribution pattern of *F. fujikuroi* hyphae in adult plants suggested that the pathogen expands vertically through vascular bundles. Notably, leaf infection was scarcely detected. We observed colonization of the vascular bundle of the leaf (Fig. 6J), and Nisikado and Kimura (1941) observed the presence of *F. fujikuroi* in the leaf sheath arising from the node where *F. fujikuroi* reached. Although the leaf does not serve as an initial infection site (Sun 1975), *F. fujikuroi* could extend systemically to the leaf at later stage of the disease. At both the seedling and adult stages, while great variation in *F. fujikuroi* biomass was found in different individual plants, *F. fujikuroi* was almost always detectable in the basal stem and basal roots, which makes these two sites good targets for pathogen isolation and investigation of direct interactions between rice and *F. fujikuroi*. Interestingly, while *F. fujikuroi* mainly colonized the stem within 3 cm from the base in seedlings, the maximum colonization of *F. fujikuroi* in adult field plants was found in the middle portion of the stem. One explanation for this might be that in the paddy field, the temperature and oxygen concentration in the rice tissues submerged under water may be relatively low (Setter et al. 1987). Whether these conditions are unfavorable for *F. fujikuroi* needs further investigation.

In the basal stem and roots of rice seedlings, the hyphae of *F. fujikuroi* were found to directly penetrate the epidermal cells at 3 to 6 dpi, extend in rice tissues through the formation of inter- and intracellular mycelium, then enter into and colonize the vascular bundles. We observed a slight delay in initial infection and colonization of *F. fujikuroi* in the stem tissues of TNG67 compared with those of ZK. This could be due to the resistance of TNG67 and/or the reduced virulence of *F. fujikuroi* G3-1 on TNG67. In adult field plants, we observed occlusion of vascular bundles with hyphae and microconidia, and radial hyphal expansion from vascular bundles to surrounding parenchyma cells. The invasion and colonization patterns of *F. fujikuroi*, including direct hyphal penetration and expansion of hyphae inter- and intracellularly to invade xylem and phloem tissues, have been widely observed in other phytopathogenic *Fusarium* spp., such as *F. oxysporum* f. sp.

Fig. 5. Gibberellic acid-3 (GA₃)-induced elongation growth of Zerawchanica karatals (ZK) and Tainung 67 (TNG67). **A**, Seeds of ZK and TNG67 were treated with three concentrations of GA₃ (0.01, 0.5, and 25 mg/liter) and ddH₂O (CK). Rice seedlings were photographed at 14 days posttreatment. **B**, Plant heights (mean $\pm$ standard error) at 14 days posttreatment. Asterisks denote significant differences between GA₃-treated and CK plants as determined by Student's *t* test (*, 0.01 < *P* < 0.05; **, 0.001 < *P* < 0.01; and ***, *P* < 0.001).

cubense causing Panama disease of banana (Dong et al. 2019), *F. oxysporum* f. sp. *radicis-lycopersici* causing tomato foot and root rot (Lagopodi et al. 2002), *F. oxysporum* f. sp. *fragariae* causing strawberry wilt (Fang et al. 2012), and *F. graminearum* causing head

blight of wheat and root, stem, and ear rot of maize (Nguyen et al. 2016). Although appressoria and infection cushions were reported for *F. graminearum* (Boenisch and Schäfer 2011) and *F. fujikuroi* (Sunani et al. 2019), we did not observe any specialized infection

Fig. 6. Colonization of *Fusarium fujikuroi* in adult field plants. **A and B**, Clean xylem vessels in healthy root tissues without pathogen colonization. **C**, Hyphae (arrows) blocking the xylem vessels of the root tissues. **D**, Infected root tissues were partially brown. **E**, A healthy central vascular bundle in the stem. **F**, Hyphae (arrows) colonizing the xylem vessels, phloem vessels, and air space of central vascular bundles in the stem. **G**, A browning vascular bundle in the colonized stem. **H**, A healthy small vascular bundle in the stem. **I**, Hyphae blocking a small vascular bundle in the stem. **J**, Hyphae in the vascular bundle of the leaf. Leaf infection was observed in only one out of 200 leaf sections examined. **K**, Hyphae spreading out from the vascular bundle in the stem to nearby parenchyma cells. **L**, Necrosis and browning of infected parenchyma cells in the stem. **M**, Hyphae and microconidia in the parenchyma of the stem. Plant materials in A, C, E, and G to I are Tainan 11 plants collected from Holi, Taichung in April and May 2019; B and F are Kaohsiung 139 plants collected from Chishang, Taitung in March 2019; and D is an unknown cultivar collected from Kanding, Pingtung in April 2019. Samples in B, D, E, F, H, I, K, and L were stained with trypan blue. as, air space; br, browning; hy, hyphae; and sp, spore.

structures despite careful examination. The discrepancy between our observations and the findings of Sunani et al. (2019) may be due to the different rice cultivars and microscopy techniques used.

Some intracellular hyphae of *F. fujikuroi* were found to be constricted when passing through the rice cell wall (Fig. 2E). The

hyphae of rice blast fungus *Magnaporthe oryzae* were reported to constrict to ~500 nm in diameter to cross the cell wall (Kankanala et al. 2007). Since plasmodesmatal channels in plants are 30 to 50 nm in diameter (Cook et al. 1997), Kankanala et al. (2007) hypothesized that *M. oryzae* might manipulate the structure of

Fig. 7. qPCR analysis of the distribution of *Fusarium fujikuroi* in adult field plants. **A**, The amounts of *F. fujikuroi* in consecutive 3-cm segments of Kaohsiung 139 (KH139), Taikeng 2 (TK2), and Tainan 11 (TN11) plants. A#, the segment number of the aerial parts of the plant; and R#, the segment number of the root part. **B**, The amounts of *F. fujikuroi* in the nodes, internodes, and adventitious roots. #N: the node number, counting from the base of the plant; N-1 or N-2: 1 or 2 cm below the node; N+1 or N+2: 1 or 2 cm above the node; AR: adventitious roots emerged from the node. Plant ID: cultivar-hill-plant (details listed in Table 1). All plants showed typical symptoms of bakanae infection: elongation, slenderness, and yellowing. Adventitious roots were observed in all plants except KH139-1-1 and KH139-2-1 (labeled with *).

plasmodesmata for cell-to-cell movement. It was recently discovered that a mitogen-activated protein kinase in *M. oryzae*, Pmk1, controls hyphal constriction and the expression of effectors implicated in suppression of host immunity and plasmodesmatal conductance (Sakulkoo et al. 2018). Based on our observations, the constricted hypha of *F. fujikuroi* measured ~800 nm in diameter, which is also larger than the diameter of plasmodesmata. The mechanism of cell-to-cell invasion in *F. fujikuroi* needs further exploration.

It was observed that the degree of symptom severity did not necessarily correlate with the quantity of *F. fujikuroi*, both in the stem and roots and at the seedling and adult plant stages. While ZK and TNG67 showed distinct susceptible and resistant symptoms, microscopic observation and qPCR results suggested similar infection processes and *F. fujikuroi* biomasses within 21 dpi in these two cultivars. Among individual diseased seedlings of ZK or TNG67 showing similar elongation symptoms, the amounts of *F. fujikuroi* greatly varied (Supplementary Fig. S3). In diseased adult plants displaying similar symptoms, the longitudinal extension of *F. fujikuroi* could vary from approximately 6 to 84 cm above the stem base for TN11, 24 to 42 cm for TK2, and 3 to 72 cm for KH139 (Fig. 7A) (comparisons are based on plants collected from the same field at the same time). Previous studies also reported that susceptible-looking cultivars did not always contain more *F. fujikuroi* (Carneiro et al. 2017; Chen et al. 2019), and previously the expansion of *F. fujikuroi* in the stems of 11 diseased plants approaching the heading stage (collected from the same field) ranged from 10 cm to 105 cm above the base (Nisikado and Kimura 1939). Moreover, in a panel of 231 diverse rice lines, Chen et al. (2019) observed a weak correlation ($r = 0.498$) between disease severity index and colonization rate, and identified distinct QTLs for the two traits by genome-wide association analysis.

The formation of adventitious roots at the nodes is a distinct bakanae symptom observed in *F. fujikuroi*-infected adult plants. In deepwater rice, adventitious roots growing on the submerged nodes can support the main root system, which loses its function owing to the limited oxygen under flooded conditions (Steffens et al. 2006). Because the colonization of *F. fujikuroi* can cause dysfunction of vascular bundles in the roots and stem, whether the induction of adventitious roots plays a role in facilitating gas exchange and stress tolerance is worth further investigation. In this study, qPCR assays revealed that the appearance of adventitious roots does not reflect the quantity or the colonization site of *F. fujikuroi* in a rice plant. Even a small amount of *F. fujikuroi* colonization in the lower part of the plant could trigger the development of adventitious roots from the upper nodes with no infection. The emergence and growth of adventitious roots could be regulated by the interactions between GA, ethylene, and abscisic acid (Steffens et al. 2006).

Secondary metabolites produced by *F. fujikuroi*, such as GA, fusaric acid, and fumonisins, are key factors responsible for the abnormal elongation or stunting of infected host plants (Kurosawa 1926; Niehaus et al. 2016). We observed more elongation growth of ZK compared with TNG67 seedlings under treatment with GA₃ at 0.5 mg/liter, which indicated that the resistant and susceptible bakanae phenotypes of ZK and TNG67 are associated with their high and low sensitivities to GA₃, respectively. A highly significant correlation between the response to GA₃ (50 mg/liter) and elongation symptoms caused by the bakanae pathogen was also revealed in 14 out of 24 rice genotypes carrying different dwarf and semidwarf genes (Ma et al. 2008); notably, the rice genotypes sensitive to GA₃ were not always susceptible to bakanae disease (exceptions included the rice lines carrying *d29*, *sdq(t)* and two wild-type tall-plant genotypes, Nan-jing No.6 and Bei-ken-zi). For resistance breeding and disease control, considering the complex mechanisms underlying the development of bakanae symptoms, evaluation of not only morphological changes but also the sensitivity to GA and the level of *F. fujikuroi* colonization will

help to more accurately assess the resistance and susceptibility of rice cultivars.

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