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RESEARCH ARTICLE

Comparative genomics provide a rapid detection of *Fusarium oxysporum* f. sp. *conglutinans*

LING Jian*, ZHANG Ji-xiang*, ZENG Feng, CAO Yue-xia, XIE Bing-yan, YANG Yu-hong

Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing 100081, P.R.China

Abstract

Fusarium oxysporum f. sp. *conglutinans* (*Foc*) is the causal agent of *Fusarium* wilt disease of *Brassica oleracea*. A rapid, accurate, and reliable method to detect and identify plant pathogens is vitally important to integrated disease management. In this study, using a comparative genome analysis among *Fusarium oxysporum* (*Fo*), we developed a *Foc*-specific primer set (Focs-1/Focs-2) and established a multiplex-PCR assay. In the assay, the Focs-1/Focs-2 and universal primers for *Fusarium* species (W106R/F106S) could be used to detect *Foc* isolates in a single PCR reaction. With the optimized PCR parameters, the multiplex-PCR assay showed a high specificity for detecting *Foc* and was very sensitive to detect as little as 100 pg of pure *Foc* genomic DNA or 1 000 spores in 1 g of twice-autoclaved soil. We also demonstrated that *Foc* isolates were easily detected from infected plant tissues, as well as from natural field soils, using the multiplex-PCR assay. To our knowledge, this is a first report on detection *Fo* by comparative genomic method.

Keywords: *Fusarium oxysporum*, wilt disease, cabbage, multiplex-PCR, molecular detection, comparative genomics

1. Introduction

Fusarium wilt disease, caused by *Fusarium oxysporum* (*Fo*), is an important fungal vascular disease of crops worldwide and can infect more than 100 economically important crops (Forsyth *et al.* 2006). *Fo* has a remarkably broad host range, and can be divided into ~120 *formae speciales* (f. sp.), some of which can be further divided into several physiological races (Nelson *et al.* 1983). *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*) can cause *Fusarium* wilt in cabbage, resulting in heavy production losses in northern China (Li *et al.* 2003). *Foc* has two physiological races, race 1 (ATCC 52557, *Foc1*) and race 2 (ATCC 58385, *Foc2*), which show different levels of pathogenicity to their hosts (Ramirez-Villupadua *et al.* 1985).

Currently, few effective, economical and safe treatments have been developed to protect *Brassica oleracea* from *Fusarium* wilt disease (Fravel *et al.* 2003). Resistant plant cultivars have been developed; however, breeding for resistance is ineffective when new races develop (Ramirez-Villupadua *et al.* 1985; del Mar Jiménez-Gasco *et al.* 2004). For this reason, a rapid and reliable method for the early detection and identification of the pathogen is vitally important in integrated disease management (Lin *et al.* 2008, 2010). Traditionally, methods for discriminating *Foc* from other *formae speciales* were based on morphological characteristics, and identification generally depended on pathogenicity testing against the corresponding host (Reddy *et al.* 2012). While the results

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LING Jian, E-mail: lingjian2005@126.com;
Correspondence YANG Yu-hong, Tel: +86-10-82109545, E-mail: yangyuhong@caas.cn
* These authors contributed equally to this study.

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of the conventional methods are reliable and accurate, they are time-consuming and destructive, and require a great knowledge of *Fusarium* taxonomy. In addition, it is inefficient and difficult to identify *Fusarium* pathogens in plant tissues or infected soil using such microscope-based methods (Yergeau *et al.* 2005). Therefore, new diagnostic methods for the rapid, reliable, sensitive and economical detection and identification of these pathogens are needed.

In recent years, techniques based on various molecular markers have been increasingly applied to the rapid detection and discrimination of fungi. In addition, even when fungal mycelia are not visible under the microscope, molecular methods, such as PCR, can be used for direct identification of pathogens in complex mixtures because of their sensitivity and specificity (Jurado *et al.* 2006). Multiple genotyping techniques, including restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and sequence characterized and amplified region (SCAR), are effective in detecting and identifying different isolates from sub-specific groups of *Fo* (Kelly *et al.* 1998; Chiocchetti *et al.* 2001; Alves-Santos *et al.* 2002; Liu *et al.* 2006; Lievens *et al.* 2007; Pasquali *et al.* 2007). However, the random locations of these markers in the genome require enough available data for comparisons with other sequences in the public databases. Therefore, extensive strains are needed to validate the stability of the marker (Lievens *et al.* 2008).

A greater number of genome resources for *Fo* are now available, including the *Fusarium* comparative database (http://www.broadinstitute.org/) that has currently published 12 *Fo* genomes, from which the genome assembly and annotation data can also be obtained from the National Center for Biotechnology Information (NCBI). These genome resources allow for comparative genomic analyses between different *formae speciales* of *Fo*, and facilitate the development of specific primer sets to detect different *formae speciales* and even different physiological races (Huang *et al.* 2005). In this study, using a comparative genome analysis, we developed a *Foc*-specific primer set (Focs-1/Focs-2) that can amplify a 436-bp *Foc*-specific sequence fragment. We established a multiplex-PCR assay based on this primer set, and a universal primer set for *Fusarium* species (W106R/F106S) to detect *Foc* isolates. Our multiplex-PCR assay showed a high specificity for detecting *Foc* sensitively and *Foc* isolates easily from infected plant tissues as well as from natural field soils.

2. Results

2.1. Identification of *Foc*-specific genome fragments

Foc is consisting of *Foc1* (ATCC 52557) and *Foc2* (ATCC 58385). The genome of *Foc2* was published in NCBI. We compared the genome sequence of *Foc2* with those of the other 11 *Fo* genomes downloaded from NCBI database. As a result, we obtained a total of 355 candidate *Foc*-specific genome fragments (accounting for 817 kb) whose lengths were larger than 1 kb (Appendix A). There were 19 fragments whose lengths were larger than 5 kb, with the largest being 7 382 bp. To test these candidate fragments, we designed primer pairs to amplify the fragments in both *Foc1* and *Foc2*, and in the other *Fo* listed in Table 1. Our experiments verified that the 7 382-bp fragment located in supercontig 1.67 of *Fo PHW808* could be amplified in *Foc1* and *Foc2*, but not in the other *Fo*, which indicated that the fragment was specific to *Foc.* We termed the fragment as $Focs_{7382}$. We designed a series of primer pairs based on the sequence of $Focs_{7382}$. We further filtered the primer pairs by PCR to select the best primer pair, which could amplify a clear and specific band for *Foc* in the range of 300–1 000 bp and did not interfere the result of multiple PCR with the primer pair and universal primers of *Fusarium* species (W106R/F106S). As a result, we obtained a suitable *Foc*-specific primer pair (Focs-1/Focs-2) whose product size was 436 bp.

2.2. Multiplex-PCR system development

To reduce the false positive of the PCR system, we developed a multiplex-PCR system that used the primer sets Focs-1/Focs-2 and W106R/F106S simultaneously. W106R/F106S is specific to *Fusarium* speices and can amplify a 729-bp *Fusarium*-specific fragment in a PCR system. As shown in Fig. 1, the multiplex-PCR system was able to amplify a 729-bp fragment using the all 57 *Fo* isolates and four other *Fusarium* species as templates, but the fragment cannot be amplified with other non-*Fusarium* pathogens (Fig. 1-B, C). Furthermore, the multiplex-PCR system amplified two fragments, a 729-bp and a 436-bp, only from 23 *Foc* isolates (Fig. 1-A). Our results showed that the multiplex-PCR system could be used as a molecular detection method for *Foc.*

2.3. Multiplex-PCR system sensitivity test

To test the sensitivity of the multiplex-PCR system, genomic DNA (gDNA) of a *Foc* isolate (ATCC 52557) was serially diluted from 10 to 10^{-3} ng. The dilution set was used as PCR templates and the products were detected by a gel image analyzing system. The signal intensity, reflected by the brightness of the bands, decreased continuously as the template concentrations decreased (Fig. 2-A). The multiplex-PCR system was able to amplify the 436 and 729-bp fragments in as little as 100 pg (10^{-1} ng) of gDNA in a 20 μL reaction mixture. To determine the sensitivity of

Table 1 Isolates of *Fusarium oxysporum* and other fungal and oomycota used in this study

Number	Strains	Scientific name	Hosts	Sources[1)]
1	ATCC 52557	*Fusarium oxysporum* f. sp. *conglutinans*	Cabbage	ATCC
2	ATCC 58385	*F. oxysporum* f. sp. *conglutinans*	Cabbage	ATCC
3	FoSY-12	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Shanxi, China
4	FoHB-1	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Shanxi, China
5	FoTY-1	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Shanxi, China
6	FGL-13-8	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Shanxi, China
7	FGL-15	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Shanxi, China
8	FCH-12-1	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Shanxi, China
9	FGL-13-1	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Shanxi, China
10	FGL-12-46	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Shanxi, China
11	FOSY-2-1	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Shanxi, China
12	FGL-03-6	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Shanxi, China
13	FoYQ-1	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Beijing, China
14	FoYQ-2	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Beijing, China
15	FoYQ-3	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Beijing, China
16	FoYQ-4	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Beijing, China
17	FoYQ-5	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Beijing, China
18	FoYQ-6	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Beijing, China
19	FoGS-1	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Gansu, China
20	FoGS-2	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Gansu, China
21	FoGS-3	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Gansu, China
22	FoGS-4	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Gansu, China
23	FoGS-5	*F. oxysporum* f. sp. *conglutinans*	Cabbage	Gansu, China
24	ATCC 16601	*F. oxysporum* f. sp. *raphani*	Radish	ATCC
25	R1	*F. oxysporum* f. sp. *melonis*	Muskmelon	Fujian, China
26	F-melonis-FJ1	*F. oxysporum* f. sp. *melonis*	Muskmelon	Fujian, China
27	F-melonis-FJ2	*F. oxysporum* f. sp. *melonis*	Muskmelon	Fujian, China
28	F-melonis-FJ3	*F. oxysporum* f. sp. *melonis*	Muskmelon	Fujian, China
29	F-banana-1	*F. oxysporum* f. sp. *cubense*	Banana	Guangdong, China
30	F-banana-4	*F. oxysporum* f. sp. *cubense*	Banana	Guangdong, China
31	F-egg plant-2	*F. oxysporum* f. sp. *melongenae*	Eggplant	Fujian, China
32	F-egg plant-FJ1	*F. oxysporum* f. sp. *melongenae*	Eggplant	Fujian, China
33	F-egg plant-FJ2	*F. oxysporum* f. sp. *melongenae*	Eggplant	Fujian, China
34	Fuw-BN4	*F. oxysporum* f. sp. *cucumerinum*	Cucumber	Beijing, China
35	Fuw-YQ	*F. oxysporum* f. sp. *cucumerinum*	Cucumber	Beijing, China
36	F-cucumber-GD	*F. oxysporum* f. sp. *cucumerinum*	Cucumber	Guangdong, China
37	Fuc-BN-8	*F. oxysporum* f. sp. *cucumerinum*	Cucumber	Fujian, China
38	F-tomato-ZJ	*F. oxysporum* f. sp. *lycopersici*	Tomato	Zhejiang, China
39	F-tomato-BJ	*F. oxysporum* f. sp. *lycopersici*	Tomato	Beijing, China
40	F-tomato-FJ	*F. oxysporum* f. sp. *lycopersici*	Tomato	Fujian, China
41	F-pepper-FJ	*F. oxysporum* f. sp. *capsicum*	Pepper	Fujian, China
42	F-pepper-BJ	*F. oxysporum* f. sp. *capsicum*	Pepper	Beijing, China
43	ATCC 16608	*F. oxysporum* f. sp. *tracheiphilum*	Beans	ATTC
44	F-cowpea-FJ	*F. oxysporum* f. sp. *cowpea*	Cowpea	Fujian, China
45	Fu-soybean	*F. oxysporum* f. sp. *glycines*	Soybean	Jilin, China
46	F-gourd-FJ	*F. oxysporum* f. sp. *lagenariae*	Gourd	Fujian, China
47	Fuw-BN3	*F. oxysporum* f. sp. *niveum*	Watermelon	America
48	Fuw-BN1-2	*F. oxysporum* f. sp. *niveum*	Watermelon	Beijing, China
49	Fuw-BN1-3	*F. oxysporum* f. sp. *niveum*	Watermelon	Beijing, China
50	MYA 3040	*F. oxysporum* f. sp. *lactucae*	Lettuce	ATCC
51	ATCC52422	*F. oxysporum* f. sp. *chrysanthemi*	Chrysanthemum	ATCC
52	Foc-mh5-3	*F. oxysporum* f. sp. *vasinfectum*	Cotton	Jiangsu, China
53	Foc-mh2-2	*F. oxysporum* f. sp. *vasinfectum*	Cotton	Jiangsu, China
54	ATCC 744009	*F. oxysporum* f. sp. *fragariae*	Strawberry	ATCC
55	F-castor	*F. oxysporum* f. sp. *lini*	Castor	Jilin, China
56	Foc-w	*F. oxysporum* f. sp. *benincasae*	Wax gourd	Beijing, China

(Continued on next page)

Table 1 (Continued from preceding page)

Number	Strains	Scientific name	Hosts	Sources[1)]
57	F-momordica-FJ2	*F. oxysporum* f. sp. *momordica*	Balsam pear	Fujian, China
58	CGMCC3.5840	*F. solani*	–	CGMCC
59	CGMCC3.4599	*F. lateritium*	–	CGMCC
60	CGMCC41483	*F. moniliforme*	–	CGMCC
61	Fv-1	*F. verticillioides*		Beijing, China
62	*Colletotrichum*	*Colletotrichum*	–	Beijing, China
63	*Rhizoctonia solani*	*Rhizoctonia solani*	–	Beijing, China
64	*Botrytis cinerea*	*Botrytis cinerea*	–	Beijing, China
65	Fulvia	*Fulvia fulva* (Cooke) Ciferri	–	Beijing, China
66	JO-TS	*Phytophthora capsici*	Pepper	Fujian, China

[1)] ATCC, American Type Culture Collection; CGMCC, China General Microbiological Culture Collection Center.

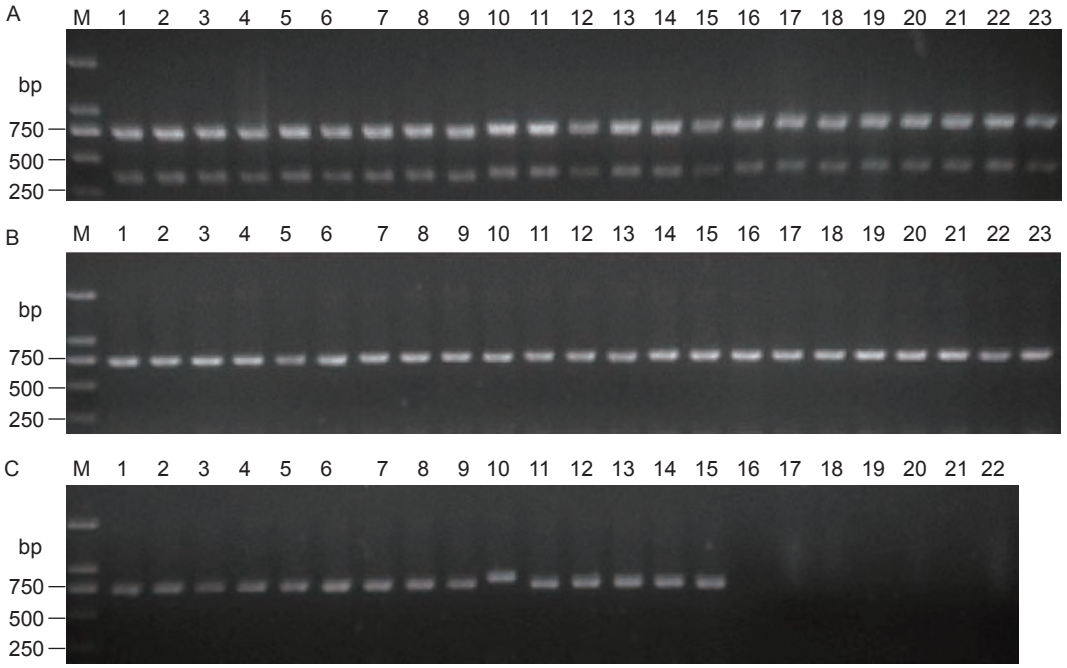

Fig. 1 Multiplex-PCR amplification system used to detect *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*). A, 1–23, 23 *F. oxysporum* f. sp. *conglutinans.* The corresponding strains were listed in Table 1. Two DNA bands (729 and 436 bp) can be detected. M, molecular markers of Trans2K DNA marker. B, 1–23, 23 isolates of other formae specials of *F. oxysporum.* The corresponding strains were listed in Table 1. Only one DNA band (729 bp) can be detected. C, 1–11, 11 isolates of other formae specials of *F. oxysporum.* The corresponding strains were listed in Table 1. Only one DNA band (729 bp) can be detected; 12–15, other four *Fusarirm species* (58–61 in Table 1). Only one DNA band (729 bp) can be detected; 16–20, four other fungi and one oomycete (62–66 in Table 1); 21–22, negative control using sterile dH_2O as the template.

the PCR detection system using primer sets Focs-1/Focs-2 and W106R/F106S against fungal spores presented in soil samples, gDNAs were extracted from the mixtures of *Foc* spores (at various spore suspensions, from 10^5 to 10^2) and from 1 g of twice-autoclaved soil samples. The results showed that the 436-bp and 729-bp fragments were detected in the 10^5, 10^4 and 10^3 suspensions, but not in 10^2 or the negative control (Fig. 2-B). Therefore, the multiplex-PCR system was able to detect as a few as 1 000 spores in 1 g of twice-autoclaved soil.

2.4. Detection in plant tissues and in field soil samples

To test the practicality of our multiplex-PCR assay, we detected *Foc* in plant tissues and in field soil samples. The isolated strains from plant tissues and field soil samples were confirmed by pathogenicity tests (Fig. 3-A and D). Using our multiplex-PCR assay, we detected *Foc* in diseased cabbage cultured in our laboratory and in naturally *Foc*-infected plant tissues (leaves and roots) collected from

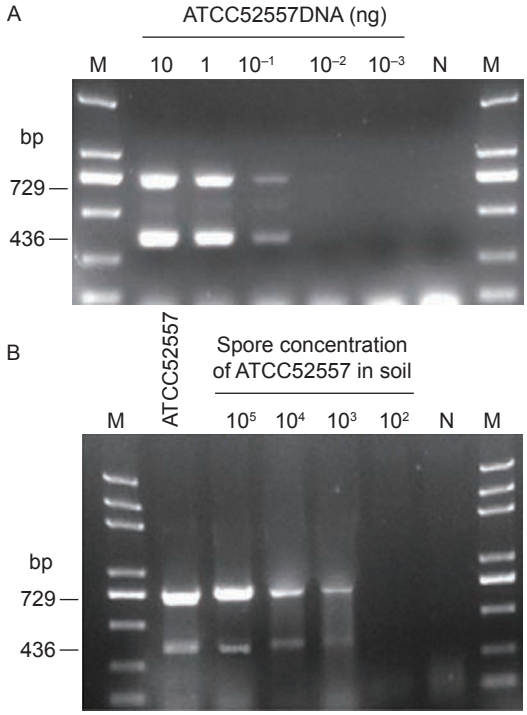

Fig. 2 Detection sensitivity of the multiplex-PCR system. A, detection sensitivity of the multiplex-PCR system amplified fragments in genomic DNA of ATCC 52557. A serial of dilutions of ATCC 52557 genomic DNA (gDNA) ranging from 10 to 10^{-3} ng were used as templates. The locations of the 436-bp and 729-bp size DNA bands are indicated on the left. B, detection sensitivity of the multiplex-PCR system amplified fragments in gDNA of soil samples. 1 μL of gDNA extracted from the mixtures of *Foc* spores and twice-autoclaved soil samples were used as templates. The locations of the 436-bp and 729-bp size DNA bands are indicated on the left. N, negative control using sterile ddH_2O as the PCR template.

Beijing City, Gansu and Shanxi provinces, China. The DNA of healthy cabbage tissues and ddH_2O were used as negative controls. As shown in Fig. 3-B, the multiplex-PCR assay succeeded in amplifying a 729-bp and a 436-bp fragment from the diseased cabbage cultured in our laboratory, and also succeeded in amplifying the two fragments from the diseased plant tissues collected from field samples. The results showed that the multiplex-PCR assay could be used as a pathogen detection method for diseased plants.

We used the multiplex-PCR assay to detect pathogens in field soil samples collected from diseased fields in Beijing, Gansu and Shanxi. As displayed in Fig. 3-C, the multiplex-PCR assay succeeded in amplifying a 729-bp and a 436-bp fragment from the *Foc*-infected soil samples and the positive control. It was noted that the disease-free field sample also contained a 729-bp fragment, indicating that other *formae speciales* of *Fo* might exist in the field. The results showed that the multiplex-PCR assay could be used as a pathogen detection method in field soils.

3. Discussion

In this study, we report a multiplex-PCR assay to detect *Foc* easily in naturally infected plant tissues, as well as in field soils. Compared with the weeks-long traditional methods to isolate *Foc* from soil or plant tissues, the multiplex-PCR reported here can definitively detect *Foc* in soil samples and plant tissues within 4 h. Furthermore, the method described here was very easy to conduct and did not require detailed knowledge of the morphological characteristics of the pathogens. To our knowledge, this is the first report of the detection of *Foc* isolates using a multiplex-PCR assay.

Because of its rapidity and efficiency, the PCR-based method has been widely used to detect *Fo* species (Parry and Nicholson 1996; Yoder and Christianson 1998; Möller *et al.* 1999; Wilson *et al.* 2004; Zhang *et al.* 2005; Dita *et al.* 2010; Li *et al.* 2012). The specific primer sets design is the most important in PCR-based molecular detection technology. These species-specific primers are mostly designed based on housekeeping genes that can differentiate *Fusarium* species but fail to detect different *formae speciales* of *Fo* (van der Does and Rep 2007). Therefore, the ideal approach to distinguish various *formae speciales* should be developed based on the screening of specific virulence genes and genomic fragment sequences from different *formae speciales* (Lievens *et al.* 2008). However, technically, it is very difficult and time-consuming to find specific genes or genomic fragments if the corresponding genome is not available. With the decreasing cost of genome sequencing, more and more *Fo* genomes are being published. In total, 12 *Fo* genomes are available in the *Fusarium* comparative database, which provides a resource to identify *formae speciales*-specific genome fragments (Ling *et al.* 2015). In this study, comparative genome method was used to identify specific genome regions in *Foc*. Based on the *Foc*-specific sequences, we developed successfully the *Foc*-specific primer set for *Foc* detection. To our knowledge, this is a first report on detection *Fo* by comparative genome method.

Although it is very convenient, the false positives are often revealed in conventional PCR-based method due to non-specific amplification. To reduce the false positives, multiplex-PCR assay was widely used in detection of pathogens (Gao and Wang 2010). In this study, we established multiplex-PCR assay that includes a universal primer set for *Fusarium* specices and a specific primer set (Focs-1/Focs-2) for *Foc*. Although we confirmed the specificity of these two primers sets by electronic PCR in NCBI database, the risk of false positives for each primer set still exists. Therefore, using these two primers in a PCR assay, and detecting *Foc* isolates from other *Fo formae speciales* could reduce greatly the false positives in the detection of *Foc*. In addition, the sensitivity of PCR assays is an important index

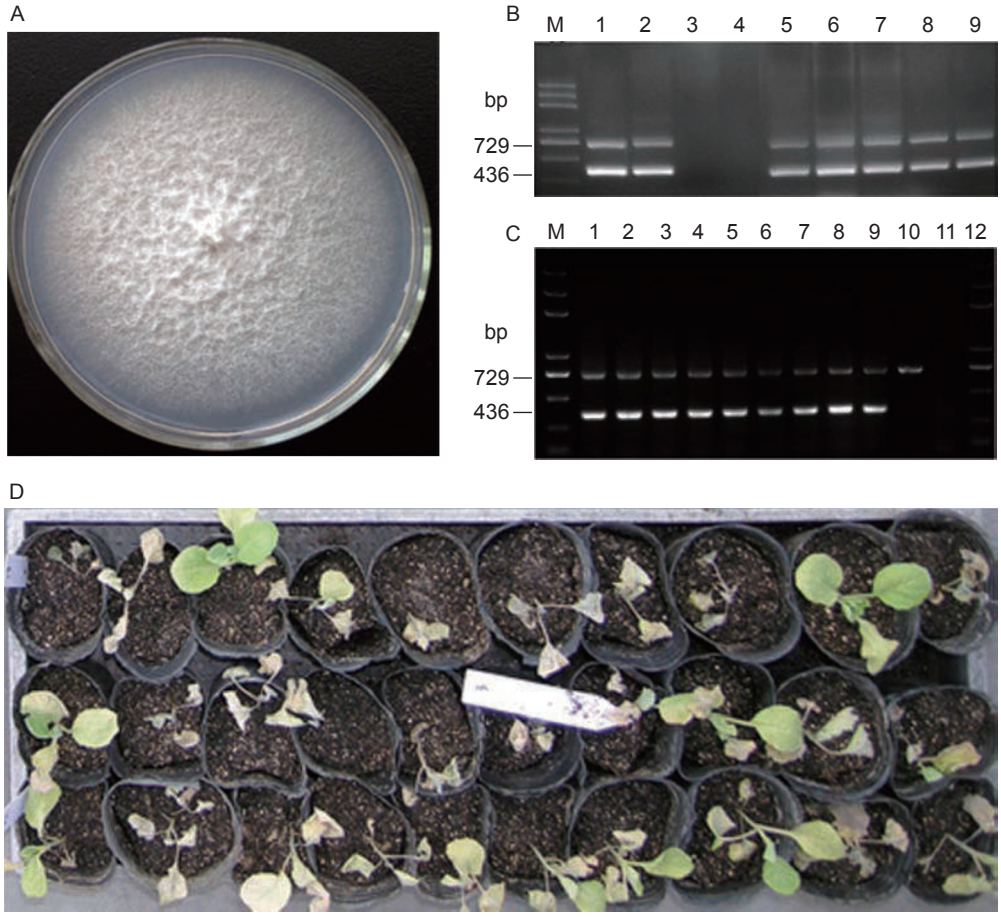

Fig. 3 Multiplex-PCR system was used to detect *Foc* in plant tissues and in field soil samples. A, the mycelium morphology of *Foc* isolated from plant tissues and field soil samples. B, multiplex-PCR amplification of gDNA from infected plant tissues. 1, ATCC 52557; 2, diseased cabbage tissues collected from our lab; 3, healthy cabbage tissues used as a negative control; 4, ddH_2O used as a negative control; 5–6, diseased cabbage leafs collected from Gansu Province; 7, diseased cabbage leafs collected from Beijing; 8, diseased cabbage roots collected from Gansu Province; 9, diseased cabbage roots collected from Shanxi Province. C, multiplex-PCR amplification of gDNA from infected field soil. 1, ATCC 52557; 2, DNA of pathogen isolated from diseased soil samples; 3–7, total DNA of soil samples from Gansu; 8–9, total DNA of soil samples from Beijing; 10, DNA of undiseased plants rhizospheric soil; 11, negative control (ddH_2O). D, disease symptoms induced by isolated *Foc* on Chinese cultivar Zhonggan 21.

in the molecular detection of plant pathogens. In a 20-μL reaction, our multiplex-PCR system in this study was able to detect 100 pg of pure *Foc* gDNA, or 1 000 spores in 1 g of twice-autoclaved soil. The sensitivity of our PCR assay was comparable to those reported for *F. oxysporum* f. sp. *niveum*, the causal agent of watermelon *Fusarium* wilt by conventional PCR (Zhang *et al.* 2005).

On the other hands, other methods such as real-time PCR and loop-mediated isothermal amplification (LAMP) were also used in *Fo* detection (Lin *et al.* 2012; Haegi *et al.* 2013; Zhang *et al.* 2013; Li *et al.* 2014; Peng *et al.* 2014; Raju *et al.* 2015). Compared with conventional PCR, real-time PCR assay is more sensitive for the diagnosis of toxoplasmosis, with detection limit being 10–100 times lower than that of conventional PCR (Lin *et al.* 2012). However, expensive device and reagents are required for real-time PCR detection, limiting its usage in detection. In addition, although it is high sensitive, the false-positive of real-time PCR assay is higher than that of conventional PCR (Peng *et al.* 2014). LAMP method can enable a single-step amplification and production detection in less than 1 h, compared with 2–3 h in real-time PCR and conventional PCR, showing a higher versatility in pathogen detection. But LAMP shows a slightly lower sensitivity compared with PCR methods (Peng *et al.* 2013).

A rapid and effective detection method for soil pathogens would lead to more effective diseases controls. In this study, we collected soil samples with different physico-chemical properties from three north provinces in China. Our multiplex-PCR system can detect successfully *Foc* in all soil samples, indicating that our method is unaffected by soil types. It was noted that the population of *Fo* shows

significantly difference between different natural soil types (Edel *et al.* 2001). However, the cultivated crops could contribute to the reduction in the diversity within population of *Fo,* causing the occurrence of a dominant population in the soil (Edel *et al.* 1997). Cabbages were planted successively in the fields of Beijing, Gansu and Shanxi, causing the wilt disease of cabbage and having become more and more serious. Zhang *et al.* (2013) reported that the population of *Foc* will increase continuously after the continuous cropping of cabbage. Furthermore, the pathogenicity of *Foc* would increase following the increment of proportion of dominant pathogenic isolates, indicating the concentration in soil having impacts on *Fo* pathogenicity. Our multiplex-PCR system was very sensitive to detect as few as 1 000 spores in 1 g of soil, indicating that the method can be used for the early diagnosis to *Foc*, which can lead to effective management practices, such as usage of proper fungicide or crop rotation.

4. Conclusion

In this study, using a comparative genome analysis among *Fusarium* species, we identified a total of 817-kb *Foc*-specific genome fragments, with the largest being 7 382 bp. Based on the 7 382 bp genome fragment, we developed a *Foc*-specific primer set (Focs-1/Focs-2) and established a multiplex-PCR assay using Focs-1/Focs-2 and universal primers for *Fusarium* species (W106R/F106S) to detect *Foc* isolates, in a single PCR reaction. The developed multiplex-PCR assay showed a high specificity for detecting *Foc* and was very sensitive, detecting as little as 100 pg of pure *Foc* genomic DNA or 1 000 spores in 1 g of twice-autoclaved soil. The developed multiplex-PCR assay also can detect *Foc* isolates easily from infected plant tissues, as well as from natural field soils.

5. Materials and methods

5.1. Fungal isolates and growth condition

A total of 23 strains of *Foc* were used in our study. All strains were confirmed by Koch's postulates as follows: the isolated strains were grown on Pichia adenine dropout (PAD) plates. When the strains appeared as white mycelia on PAD plates, colonies were isolated and examined by microscopy for the presence of the diagnostic macroconidia and microconidia of *Fo*. If isolated strains could infect cabbage and caused typical vascular wilt and root rot on cabbage, these strains were *Foc*. In addition, 34 isolates of other nineteen formae specials of *Fo* and other four *Fusarium* species were also used in this study (Table 1). The genomic DNA (gDNA) from four other fungal pathogens (*Colletotrichum*, *Rhizoctonia solani*, *Botrytis cinerea* and *Fulvia fulva* (Cooke) Ciferri) and one oomycete (*Phytophthora capsici*) were used as control (Table 1).

A single spore culture of each tested fungal isolate was grown on potato dextrose agar (PDA) plates (200 g L^{-1} of potato extracts, 1% glucose, and 2% agar). Single colonies of *P. capsici* were grown on V8 plates (100 mL V8 juice L^{-1}, 3 g $CaCO_3$ L^{-1}). The strains used in this study were friendly provided by Xiao Rongfeng of Fujian Academy of Agriculture Sciences, China, Lin Biyun of Guangdong Academy of Agriculture Sciences, China, Yan Hong of Beijing Academy of Agriculture and Forestry Sciences, China, Zhang Shihong of Jilin University and Zhang Zhengguang of Nanjing Agricultural University, China.

5.2. Plant materials

Chinese cultivar cabbage Zhonggan 21, which is susceptible to *Foc*, was used in this study. The cabbage was grown in plastic pots with autoclaved mixture of vermiculite and turf (1:1, v/v). Pots were maintained in the greenhouse at 25°C, 16 h photoperiod until at the 2–3 true leaves stage.

5.3. Preparation and inoculation of soil

In order to detect *Foc* in soil-based samples, 1 mL titers of serially diluted *Foc* spore suspension (10^5–10^2 spores mL^{-1}) was inoculated onto 1 g of twice-autoclaved soil in 15-mL conical tubes with three replicates. The tubes were vortexed at maximum speed for 1 min, freeze-dried for 20 d, ground in liquid nitrogen to produce a fine powder, and stored at –80°C for next analysis (Zhang *et al.* 2005).

5.4. Isolation of *Fusarium* isolates from naturally infested field soil

Soil samples were collected from the *Foc*-outbreak fields in Beijing City, Gansu and Shanxi provinces of China, and the soil types were listed in Table 3 according to the soil information system of China (Shi *et al.* 2004). Take 1 g of naturally infected field soil samples into 10 mL physiological saline in 15 mL conical tubes. The tubes were vortexed at 160 r min^{-1} for 30 min. After that, dilution series (from 10^{-1}–10^{-4}) of soil solutions were prepared for next step. 100 µL of soil solutions in every concentration were applied on Komada plates (K_2HPO_4 (1 g), KCl (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), Fe-Na-EDTA (0.01 g), L-asparagin (2 g), D-galactose (20 g), 1 000 mL water) (Komada 1975). The plates were incubated at 25°C in the dark, and observations were made after 5 d. The *Fusarium*-like colony-forming units (CFU) were

selected and then purified on PDA plates at 25°C for one week. *Fusarium* colonies were examined microscopically to confirm the species using standard literature (Nelson *et al.* 1983) and stored at 4°C for following steps.

5.5. Isolation of *Fusarium* isolates from infected plant tissues

Fungi isolates were isolated from infected plant tissues collected from Beijing, Shanxi and Gansu by traditional protocol (Dita *et al.* 2010; Lin *et al.* 2013), and the isolated *Foc* strains were also confirmed by Koch's postulates. At least 10 plants were collected in each sampling point. A 5 mm×3 mm tissue was taken from the junction of healthy and illness, sterilized in 70% ethanol for 2 min, and dried by airing after rinsed in sterile water for three times. After that, the colonies around the patch were purified in another new PDA plate, and incubated under the same condition for seven days and then stored at 4°C for next steps.

5.6. Genomic DNA isolation

Genomic DNA of all fungi isolates was extracted according to the procedure as described previously (Lin *et al.* 2008, 2012). Total genomic DNA of infected plant tissues was extracted using NuClean Plant Gen DNA Kit (Transgen Biotech Co., Ltd., Beijing), and infected soil DNA was extracted using FastDNA® Spin Kit (MP Biomedicals, USA).

5.7. Identification of specific genome fragments for *Foc*

The genome sequences of various *Fo* were obtained from NCBI assembly (http://www.ncbi.nlm.nih.gov/assembly/), and their information was listed in Table 2. *F. oxysporum PHW808* is *Foc* race 2 (58385). We compared *PHW808* genome sequence with that of all other *F. oxysporum* using Blastn with e-value being $1e^{-10}$. The sequences of *PHW808* that had no similarity with all other *F. oxysporum* were considered as candidate specific fragments of *Foc*. After bioinformatically analyzing, we designed primer pairs to test the candidate specific fragments of *Foc*. Those fragments that amplified in both 52557 and 58385, but not in other *Fo* tested, we considered to be *Foc*-specific fragments. All PCR experiments were replicated at least for three times.

5.8. Primer design and PCR amplification

Primer 5.0 software was used to design a series of primer pairs according to the sequence of the largest *Foc*-specific fragments, whose length is 7 382 bp, designed as Foc7382. The designed primer pairs were further tested by PCR to ensure that they can amplify clear and specific band, and the size of the product should be between 300 bp and 1 kb. A primer pair Focs-1/Focs-2 that meets our requirement was used in all experiments, whose product was 436 bp. In addition, to reduce the false positive of the PCR assay,

Table 2 The genomes of *Fo* used in this study

	Strain	Host	Genome-size (Mb)	Number of genes
1	*F. oxysporum CL57*	*Lycopersicum*	49.3	18238
2	*F. oxysporum Cotton*	Cotton	52.9	18905
3	*F. oxysporum Fo47*	Soil	49.6	18191
4	*F. oxysporum Fo5176*	*Arabidopsis*	54.9	21087
5	*F. oxysporum 4287*	*Lycopersicum*	61.3	20925
6	*F. oxysporum Melonis*	*Muskmelon*	54.0	19661
7	*F. oxysporum HDV247*	*Pisum*	55.1	19623
8	*F. oxysporum II5*	*Musa*	46.5	16634
9	*F. oxysporum MN25*	*Lycopersicum*	48.6	17931
10	*F. oxysporum NRRL32931*	*Homo*	47.9	17280
11	*F. oxysporum PHW808*	*Brassica*	53.5	19854
12	*F. oxysporum PHW815*	*Raphanus*	53.4	19306

Table 3 The soil samples collected in this study

Sample sources	Soil type	pH	Organic matter (%)	Total nitrogen (%)	Quick-acting potassium (ppm)
Yanqing, Beijing	Yellow earths	8.0–8.5	<1.0	0.5	125–200
Changping, Beijing	Cinnamon soil	7.3–8.5	<1.0	0.064	90–120
Shunyi, Beijing	Tidal cinnamon soil	8.2–8.4	0.95–1.3	0.065–0.088	120
Taiyuan, Shanxi	Yellow-brown earths	6.5–6.8	7.27	0.235	150
Shouyang, Shanxi	Light salinity tidal soil	8.2–8.4	1.19	0.063	121
Lanzhou, Gansu	Gray-black earths	7.9–8.0	11.17	0.455	190
Dingxi, Gansu	Black gunny soil	8.4	1.95	0.130	193

another primer set W106R/F106S which can generate the 729-bp DNA fragment from all *F. oxysporum* isolates was used as internal reference (Li *et al.* 2012). The primer pairs are listed below:

Focs-1: 5′-TCAATGATAGTGACAAGGGTTT-3′
Focs-2: 5′-AATTTGCTGTGATAGGTGGAT-3′
W106R: 5′-GCAGTCGTACGTCATCGACC-3′
F106S: 5′-CCATGGCAGATGGCGAGTCA-3′

The PCR was carried out using 20 μL reaction mixtures, which consists of 1 μL of genomic DNA, 2 μL of 10×PCR buffer, 0.5 μL of dNTPs (10 mmol), 0.4 μL of Eas*Taq* polymerase (5 U μL^{-1}), 0.8 μL of each primer (10 μmol L^{-1}) of corresponding primer sets, and ddH_2O to final volume. The parameters for PCR were denatured at 94°C for 5 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 63°C for 30 s, and polymerising at 72°C for 1 min, and with a final extension at 72°C for 10 min. All PCR experiments were replicated at least three times. PCR products were subjected to electrophoresis in 1.5% agarose gels.

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Appendix associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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