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FULL RESEARCH PAPER

The development of a PCR-based method for detecting *Puccinia striiformis* latent infections in wheat leaves

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Abstract Stripe rust of wheat caused by *Puccinia striiformis* f. sp. *tritici* is one of the most important diseases on wheat worldwide, especially in temperate regions with cool moist weather conditions. A rapid and reliable detection of the pathogen in latent infected wheat leaves during overwintering of the fungus in the dormant stage will contribute to determine the initial inoculum potential and thus to predict early outbreak and to improve effective management of the disease. To achieve this aim, a PCR-based method was developed for specific and sensitive detection of *P. striiformis*. Specific primers were designed according to a genome-specific sequence of *P. striiformis*. To evaluate the specificity of the primers, seven different isolates and races of *P. striiformis* as well as six other pathogens of wheat were tested. All isolates of *P. striiformis* yielded a distinct band of a fragment of 470 bp, while using DNA of the other wheat pathogens as a template no amplification product was detected. The sensitivity of the primers was tested using serial dilutions of total DNA from *P. striiformis*; the limit of detection was 10 pg of DNA. Using extracts from *P. striiformis*-infected wheat leaves, the fungus could be determined in the leaves before symptoms appeared. The stripe rust could also be detected in the dormant stage by the PCR assay in samples of wheat leaves taken during the winter season. The application of the PCR assay may be useful for rapid and reliable detection of *P. striiformis* in latent infected leaves of overwintering wheat plants.

Keywords Wheat · Stripe rust · *Puccinia striiformis* · Molecular diagnosis · PCR

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Introduction

Stripe rust (or yellow rust) of wheat (*Triticum aestivum*), caused by *Puccinia striiformis* f. sp. *tritici* (an obligate biotrophic organism), is one of the most important diseases of wheat worldwide. The disease predominantly occurs in temperate regions with cool moist weather condition, including Asia, Europe, North America, South America, Middle East and Africa. In numerous wheat-growing areas yield losses caused by stripe rust can vary from 10 to 70% depending on the cultivars grown and favourable weather conditions (Saari and Prescott 1985). China presents the largest

wheat-growing area in the world affected by stripe rust and severe epidemics of stripe rust occurred in 1950, 1964, 1990 and 2002 (Li and Shang 1989; Wan et al. 2004). The major cause of the widespread epidemic in 2002, as in the previous epidemics, could mainly be attributed to the three factors: cultivation of wheat cultivars with ineffective race-specific resistance over large areas, widely distributed virulent races with a broad virulence spectrum overcoming the race-specific resistance and favourable weather conditions (Li 1999; Wan et al. 2004). Because of the rapid evolution of new races, cultivars with race-specific resistance often become susceptible soon after they have been introduced in practice. Integrated measures are essential to control the disease effectively. These measures comprise, for instance, reducing the initial inoculum, accurate forecasting methods of weather conditions and fungal spread, as well as cultivation of resistant cultivars and timely applied fungicides.

Practical experiences have proven that control and management of stripe rust should persist in principle in "giving the first place to prevention, and then comprehensive curative measures" (Li 1999). In China, forecast and prediction systems for stripe rust have been established since many years. However, because in overwintering regions, wheat crops infected by *P. striiformis* show no symptoms in the dormant stage of the pathogen, it is difficult to determine the primary infection potential. Therefore, it seems important to develop a sensitive and rapid method to detect *P. striiformis* in the dormant stage in young wheat plants in overwintering regions of the pathogen.

Molecular methods, especially the polymerase chain reaction (PCR), have been developed in the last years for specific, sensitive and rapid detection of plant pathogenic fungi. For instance, in infected host tissues, *Phytophthora nicotianae* (Grote et al. 2002; Ippolito et al. 2002; Lacourt and Duncan 1997), *Phytophthora infestans* (Judelson and Tooley 2000), *Phytophthora parasitica* (Goodwin et al. 1990), *Fusarium solani* (Li and Hartman 2003), *Phakopsora pachyrhizi* (Frederick et al. 2002), *Colletotrichum gloeosporioides* (Mills et al. 1992) and *Leptosphaeria korrae* (Tisserat et al. 1991) were successfully detected using the PCR assay.

Studies of Zheng et al. (2000a, b) showed that *P. striiformis* f. sp. *tritici* has genome specificity and genetic stability, this *P. striiformis* repeat (PSR) sequence allowed the development of genome-specific primers. The objective of this study was to develop a specific and rapid PCR assay to detect *P. striiformis* in latent infected wheat plants.

Materials and methods

Multiplication of rust fungi and cultivation of other wheat pathogens

Seven different isolates of *P. striiformis* (Table 1) including three single-spore isolates as well as the races CY31, CY32, CY23 and the pathotype Shuiyuan were selected and individually propagated on seedlings of the wheat cv. Mingxian 169, which is susceptible to all known races of *P. striiformis* f. sp. *tritici* (Wang et al. 1986). Urediospores were collected for DNA analysis and pure samples of urediospores were frozen in liquid nitrogen and stored at −70°C until DNA extraction. The isolates of *Puccinia graminis* and *Puccinia recondita* as well as the isolates of *Erysiphe graminis* were also propagated on the wheat cv. Mingxian 169. The urediospores of the stem rust and leaf rust isolates as well as the mycelium and conidia of the powdery mildew isolate collected were also frozen in liquid nitrogen and stored at −70°C for DNA extraction. The wheat pathogens (*Bipolaris sorokiniana*, *Fusarium graminearum* and *Rhizoctonia cerealis*) were cultivated on potato dextrose liquid medium at 25°C for 6 days. After incubation, mycelium of the different fungal species was collected on filter paper and thoroughly washed with distilled water. Mycelium was frozen in

Table 1 Isolates of different fungi used to screen primer specificity

Fungal isolates	Source	Origin	Number
Puccinia striiformis	Wheat, cv. MingXian169	Yangling (Shaanxi)	7
Bipolaris sorokiniana	Wheat	Yangling (Shaanxi)	1
Fusarium graminearum	Wheat	Yangling (Shaanxi)	1
Rhizoctonia cerealis	Wheat	Yangling (Shaanxi)	1
Erysiphe graminis	Wheat, cv. MingXian169	Yangling (Shaanxi)	1
Puccinia graminis	Wheat, cv. MingXian169	Shenyang	1
Puccinia recondita	Wheat, cv. MingXian169	Shenyang	1

liquid nitrogen and stored at −70°C until DNA extraction. All pathogens were collected from the regions where stripe rust (or yellow rust) occurred frequently.

DNA extraction from pathogens and wheat leaves

The urediospores (25 mg) were transfered into a 2 ml tube, 500 μl extraction buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 100 mM EDTA) was added and homogenized with a plastic pestle attached to an electric drill. Then, 5 μl proteinase K (1 mg ml^{-1}) was added and the suspension was filled up to 1 ml with extraction buffer. After incubation for 30 min at 65°C, the mixture was divided into two 1.5-ml microfuge tubes and extracted with phenol/chloroform/isoamyl alcohol (25:24:1, pH=8.0) and chloroform respectively. The top aqueous phase was transferred to a clean tube, and an equal volume of cold isopropanol was added. After 1 h of incubation at −20°C, the solution was centrifuged for 20 min at 12,000 rpm at 4°C to precipitate the nucleic acid. The pellet was rinsed twice with cold 70% ethanol, dried and dissolved in 0.1 ml of TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0). One μl of ribonuclease at 10 mg ml^{-1} was added (final concentration 20 μg ml^{-1}) and kept at 4°C overnight to completely digest the RNA. The DNA was reprecipitated, rinsed with cold 70% ethanol, dried, and dissolved in 50 μl of TE. DNA extraction of the other pathogens of wheat leaves (Table 1) was also performed according to the protocol described above. DNA was quantified using a spectrophotometer (Eppendorf AG22331, Germany) according to the manual instructions.

Primer design

The study on *P. striiformis* repeat (PSR) sequence (Zheng et al. 2000a, b) suggested that this sequence represented useful targets for specific detection of this pathogen by PCR. Potential sequences of primers were analyzed for specificity by using the software of primer premier 5.0. Two pairs of primers were designed, and primers were compared with sequences stored in the Genbank and EMBL database to determine whether the primer sequences were unique for *P. striiformis*. The two oligonucleotide primers specific to *P. striiformis* had the following sequences: Pst1: 5′>ATGCT GGCAGTGTGGTTG 5′>ACATTTCCACGAA TCTCCTTG; Pst2: 5′>GTCT GTA AGATGTTAG ATGC 5′>ATGCTGGCAGTGTGGTTG. Primers were synthesized by Shanghai Shenggong Biological Engineering Technology and Service Co., Ltd., China.

Establishment and optimization of PCR

Purified genomic DNA from *P. striiformis* was used for optimization of PCR amplification. Amplification was carried out in volumes of 25 μl containing 20 ng DNA template, 2.5 μl 10× reaction buffer [750 mM Tris–HCl, 200 mM $(NH_4)_2SO_4$, 0.1% Tween-20], 25 mM $MgCl_2$, 2.5 mM each of dATP, dCTP, dGTP, dTTP, 0.2 μM primer, and one unit Taq polymerase, and the volume was made up with water to 25 μl. In this study, different concentration proportions of $MgCl_2$/dNTP were prepared to optimize PCR amplification. All PCR assays were performed in a thermal cycler (PTC400, Bio-rad): initial denaturation at 94°C for 3 min, followed by 34 cycles of amplification. Each amplification cycle consisted of denaturation at 94°C for 50 s, primer annealing at 50 to 60°C for 90 s and primer extension at 72°C for 2 min. After amplification, a final extension step was done at 72°C for 10 min. The amplification products (each 10 μl) were injected into wells of a 1.5% agarose gel and electrophoresed at 10 V/cm for 1 to 1.5 h, along with a molecular size marker (GL2000, TaKaRa Biotechnology Co., Ltd) in 1×TAE buffer.

Primer specificity and sensitivity

To determine the species specificity of the primers, DNA extracted from urediospores of three single isolates as well as the races CY23, CY31, CY32 and the pathotype Shuiyuan of *P. striiformis* was used as a template for PCR amplification under the conditions described above. In order to verify the specificity of the primers, purified genomic DNA of different pathogens of wheat (Table 1) was included in the PCR assay. Reaction was carried out using the optimum PCR conditions and cycling parameters as described above. Sensitivity of both primers was assessed using serial dilutions of purified genomic DNA (20 ng μl^{-1}); the following dilutions were tested: 20, 10, 1, 0.1, 0.01 and 0.001.One μl of each dilution was added to the PCR reaction and PCR amplification was performed using optimal conditions at annealing temperatures of the 51°C with the Pst1 primer and

54°C with the Pst2 primer. The experiment was carried out in triplicate to determine the consistency of the level of detection by this method.

Detection of *P. striiformis* in inoculated wheat leaves

Wheat seedlings of cv. Mingxian169, susceptible to all known *P. striiformis* races, were grown in the greenhouse and the primary leaves were inoculated with race CY31. Leaf samples were collected for PCR assay from the first to the sixth day after inoculation (at the sixth day, leaf symptoms of stripe rust appeared).

For detection of *P. striiformis* in wheat leaves from the field, first leaves of wheat seedlings were collected randomly from different commercial wheat-growing sites at growth stage 21–26 (Zadoks et al. 1974), where *P. striiformis* overwinters and oversummers successfully in China, for the dormant survival of yellow rust; 500 leaves in the field were sampled in 2003 and 2004. Each leaf was cut into two pieces: one part was prepared for DNA extraction, and the other was inoculated for rust detection.

Sequencing of PCR detection products

The PCR products were purified using an agarose Gel Extraction Kit (QIAGEN); the fragments were cloned into T-easy vector (Promega) for sequencing and the sequence aligned with the original sequence. The result showed that the PCR detection product had the same sequence as the original sequence (results not shown).

Results

Optimization of PCR amplification

The concentration proportion of Mg^{2+}/dNTP is important for specificity and sensitivity of the PCR assay; therefore, nine concentration proportions of Mg^{2+}/dNTP were used to optimize for PCR amplification. The PCR amplification results showed that the 2.0 μl/1.5 μl ratio of Mg^{2+}/dNTP was the most suitable proportion. PCR was performed at different annealing temperatures from 50 to 60°C. The optimum annealing temperatures for the primers PST1 and PST2 were 51 and 54°C, respectively.

Sensitivity and selection of primers

Sensitivity of the two primers was tested using a tenfold serial dilution from 20 to 0.001 ng μl^{-1} of total DNA extracted from *P. striiformis* f. sp. *tritici*. The PCR amplification was performed according to the above mentioned annealing temperatures. The results revealed that the detection limits of both primers used varied. The lowest amount of DNA amplified for primer PST1 was 0.1 ng μl^{-1} and for primer PST2 0.01 ng μl^{-1} (Figs. 1 and 2). The amplification product of primer PST1 had the predicted size of 450 bp and that of primer PST2 470 bp. The PST2 primer was used in the following experiments for detecting *P. striiformis.*

Universality and specificity of the primer PST2

To test the universality of the PST2 primer, purified DNA from different physiological races and single-spore isolates of *P. striiformis* f. sp. *tritici* was amplified. The predicted amplification product of 470 bp and the same intensity of bands for all *P. striiformis* isolates were obtained, indicating that the primer PST2 was none-race specific and suitable for detecting the stripe rust pathogen in wheat (Fig. 3).

DNA samples extracted from wheat and six other aerial fungal pathogens of wheat (e.g. *P. recondita*, *P. graminis*, *B. sorokiniana*, *F. graminearum*, *R. cerealis* and *E. graminis*) were used as negative controls to determine the specificity of the *P. striiformis* primer. The primer displayed a high specificity and distinguished clearly between DNA as a template from *P. striiformis* and the other wheat pathogens as well from wheat leaves using each 20 ng μl^{-1} genomic DNA from different sources. Clear bands were only detected against *P. striiformis* f. sp. *tritici*. Using the primer PST2 the different isolates of *P. striiformis*

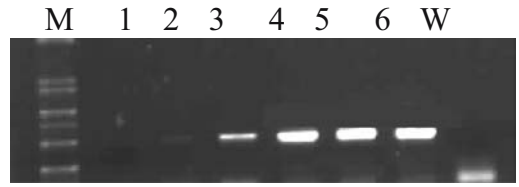

Fig. 1 Sensitivity of PCR assays detected by agarose gel electrophoresis using PST1primer. *Lanes from left to right*: *M* molecular weight markers; *1* 0.001 ng μl^{-1}; *2* 0.01 ng μl^{-1}; *3* 0.1 ng μl^{-1}; *4* 1.0 ng μl^{-1}; *5* 10 ng μl^{-1}; *6* 20 ng μl^{-1} DNA as a template and *w* sterile water

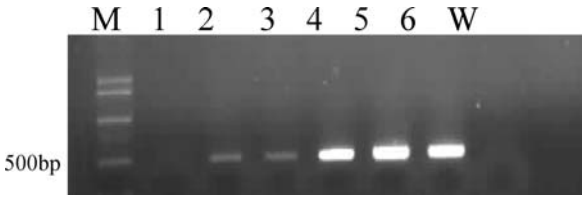

Fig. 2 Sensitivity of PCR assays detected by agarose gel electrophoresis using PST2 primer. *Lanes from left to right*: *M* molecular weight markers; *1* 0.001 ng μl^{-1}; *2* 0.01 ng μl^{-1}; *3* 0.1 ng μl^{-1}; *4* 1.0 ng μl^{-1}; *5* 10 ng μl^{-1}; *6* 20 ng μl^{-1} DNA as a template and *w* sterile water

produced the same amplification product of 470 bp at the same amount, and this confirms the species-specificity of the primer (Fig. 4).

Detection of *P. striiformis* f. sp. *tritici* in inoculated wheat leaves

To test whether the PCR assay could detect DNA of *P. striiformis* in leaves of wheat plants, leaves inoculated with the pathogen were sampled at different times after inoculation and the DNA was extracted. DNA of wheat leaves treated with sterile water was used as a negative control. The PCR amplification results showed that DNA from infected leaves produced a 470 bp specific band; *P. striiformis* DNA could be detected in latent infected leaves 3 days after inoculation. On the other hand, no amplification product was obtained from plant DNA (Fig. 5).

From 1,000 samples collected in the field, only in 16 samples could the pathogen *P. striiformis* be detected by the PCR assay. From the leaf samples inoculated on agar medium for 2 weeks in a growth chamber at 14~17°C, 100% RH and a photoperiod of 16 h, urediospores of *P. striiformis* developed only in 16 samples.

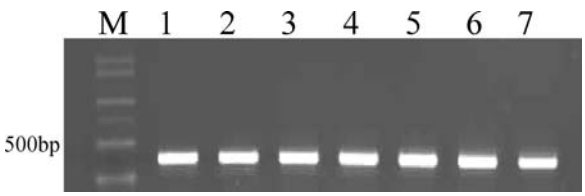

Fig. 3 Gel electrophoresis of PCR-amplified products using DNA from different races of *P. striiformis* f. sp. *tritici*. *Lanes from left to right*: *M* molecular weight markers, *1* race CY31, *2* race CY32; *3*, *4*, *5* different single-spore isolates of race CY30; *6* race CY23 and *7* pathotype Shuiyuan

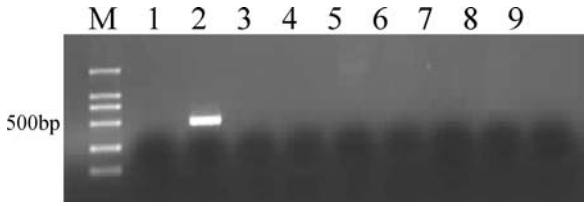

Fig. 4 Specificity test of primer PST2 using DNA from different wheat pathogens. Gel electrophoresis of PCR-amplified products of the different wheat pathogens. *Lanes from left to right*: *M* molecular weight markers; *1* healthy wheat leaves; *2 P. striiformis*; *3 P. recondita*; *4 P. graminis*; *5 B. sorokiniana*; *6 F. graminearum*; *7 R. cerealis*; *8 E. graminis*; *w* sterile distilled water (no template DNA). 20 ng DNA of each sample was used as a template

Discussion

Extraction of genomic DNA from urediospores of *P. striiformis* f. sp. *tritici*, possessing a thick cell wall, is difficult and the protocol used for extracting DNA from urediospores is rather complicated (Chen et al. 1993). Therefore, development of a simple method for extracting DNA from urediospores and *P. striiformis*-infected wheat leaves was necessary. Compared to Chen's protocol, the modified method constitutes a relatively simple procedure yielding both the quantity and quality of DNA of *P. striiformis*. The PCR-based assay is relatively rapid and simple to apply for detection compared with other methods. Fraaije et al. (2001) developed a multiplex PCR-based diagnostic assay using the β-tubulin gene as a target for detection of *P. striiformis* and *P. recondita*. However, *P. graminis* was not mentioned in their studies and the molecular marker used was not specific for the detection of *P. striiformis*. On the other hand, in our study, a moderately genome-specific repetitive sequence of *P. striiformis* (PSR) has been cloned by Shan et al. (1998) showing high specificity and stability (Zheng et al.

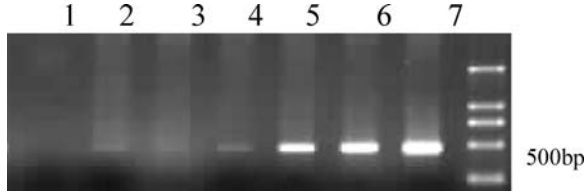

Fig. 5 PCR-detection of *P. striiformis* f. sp. *tritici* in leaves of wheat seedlings of cv. Xiaoyan 22 at different times after inoculation using primer PST2 and an annealing temperature of 54°C; 20 ng DNA from different samples was used as a template. *Lanes from left to right*: *1* water-treated wheat leaves; *P. striiformis*-inoculated wheat leaves: *2* 1; *3* 2; *4* 3; *5* 4; *6* 5 and *7* 6 days after inoculation

2000a, b). Potentially, the PSR could be a reliable molecular marker for the detection of *P. striiformis*. Therefore, two primers in different sequence regions of the PSR were designed. The two primer sets, PST1 and PST2, revealed that the targets were well conserved within the repetitive DNA sequence of *P. striiformis*. The genome sequences used for primer design are not race-, but species-specific for detecting races of *P. striiformis* f. sp. *tritici*.

The PCR assay developed was highly suitable to detect accurately *P. striiformis* from infected wheat leaves in the latent stage 3–4 days after inoculation of the pathogen. These results suggest that the PCR method could be applied to detect *P. striiformis* in wheat plants at an early stage of infection or in latent infected leaves. Reliable diagnosis of the stripe rust pathogen in the dormant overwintering stage in young wheat plants would be important to determine the portion of primary infected leaves, so that appropriate control measures and eradication procedures may be included in management strategies for stripe rust of wheat.

Long-distance dispersal of urediospores by wind has been responsible for wide spread epidemics of stripe rust. Regular spread of urediospores of *P. striiformis* from the overwintering mountainous areas into the eastern epidemic region in China has been reported (Wan et al. 2004). The PCR assay developed could also be applied in conjunction with conventional air spore samplers to monitor the air-borne inoculum of *P. striiformis*. The data obtained could be incorporated in forecast systems established for stripe rust of wheat. The potential use of the PCR assay to detect the air-borne inoculum of *Leptosphaeria maculans* and *Pyrenopeziza brassicae* has been described by Calderon et al. (2002).

A further increase in sensitivity of the classical PCR method might be possible by including a nested-PCR assay. For instance, Silvar et al. (2005) showed that sensitivity of detection of *Phytophthora capsici* could be significantly increased using nested-PCR compared to a conventional PCR assay. While in conventional PCR, the lowest amount of amplified DNA was 5 pg for the primers used, in the nested-PCR, the detection limit was 0.5 fg of template DNA. The application of more sensitive nested-PCR will be particularly relevant when the target DNA concentration is low (e.g. low % of *P. striiformis* infected plants showing no symptoms) or PCR inhibitory constituents (e.g. plant DNA) are present. In further studies, nested-PCR will be performed to analyze the sensitivity of the stripe rust pathogen in the dormant stage in wheat plants.

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