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Full Length Article

Development of a Specific SCAR Marker to Race 21C3CTH of *Puccinia graminis* f. sp. *tritici* in China

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Abstract

Wheat stem (black) rust is caused by *Puccinia graminis* f. sp. *tritici* (*Pgt*). In China, the incidence of the rust has decreased mainly owing to close pathogen race monitoring which guided the wheat breeding for resistance. Because of the rapid virulence evolution of this pathogen, continuous monitoring work is necessary. Traditional methods for race identification are time-consuming, environment dependent and costly, and thus novel molecular methods which can overcome the above disadvantages and replace traditional ones are expected. Race 21C3CTH is important in China in that it is prevalent and notable for its virulence on *Sr11* and therefore, in this study was chosen as a target race to develop the novel method. Using the random amplified polymorphic DNA (RAPD) protocol, a total of 156 random primers were used to screen for the specific marker from 6 *Pgt* races occurred in China. One of the primers, S92 (5′-CAGCTCACGA-3′), amplified a specific band to race 21C3CTH, but not to any of other five races. This specific band was purified and cloned with the pGM-T vector system and sequenced. The resultant 782 bp amplicon was isolated and a pair of sequence characterized amplified region (SCAR) primers as a specific marker was designed using Primer Premier 5.0 software. This pair of primers was used to amplify all genomic DNAs of isolates of 21C3CTH and the other five races. The expected band consistently appeared in all isolates of 21C3CTH, but not in any isolate of the other races. Further validation using 53 isolates collected from three provinces and one municipality in China confirmed that the marker is highly reliable and robust for specifically identifying race 21C3CTH. The molecular marker developed has advanced into race specific level rather than species specific and is rapid, cheap and accurate for race identification. The results will also establish the foundation on developing more new markers to characterize races of *Pgt*. © 2015 Friends Science Publishers

Keywords: *Puccinia graminis* f. sp. *tritici*; molecular identification; RAPD; SCAR marker

Introduction

Puccinia graminis Pers.: Pers. f. sp. *tritici* Erikss. and E. Henn. (*Pgt*), the causal agent of wheat stem (black) rust, historically caused severe losses to wheat (*Triticum aestivum* L.) production worldwide, including China (Singh *et al*., 2011; Pardey *et al*., 2013; Rehman *et al*., 2013). Owing to extensive race and virulence surveillance of *Pgt* populations, the disease has been successfully controlled in the past few decades, mainly through the breeding and release of resistant cultivars (Li and Zeng, 2002; Raloff, 2005; Singh *et al*., 2011, Pardey *et al*., 2013). Race identification, which began approximately 100 years ago, was traditionally performed only on wheat hosts of differential genotypes, including Stakman's set (Stakman and Premeisel, 1917) or Stakman's set plus supplementary hosts, or in recent years 5 sets of different known stem rust resistance gene (*Sr*) line hosts (Roelfs and Martens, 1988; Jin *et al*., 2009; Cao and Chen, 2010). However, these race identification protocols are somewhat time consuming, laborious, expensive and require specific environmental conditions. Thus, it is necessary to develop a simple, rapid and effective technique as an alternative method to conventional race differentiation procedures, it would be helpful for more effective race characterization of *Pgt*.

Advances in molecular technology have provided an opportunity for developing new approaches for studying *Pgt* to differentiate races or virulence among strains. In particular, several attempts have been made to develop specific or robust molecular markers for identifying different pathogen types. The internal transcribed spacer (ITS) region was analyzed to determine the phylogenetic relationships among strains and formae speciales of *P. graminis* (Zambino and Szabo, 1993). A rapid amplified polymorphic DNA (RAPD) approach was used to differentiate races of *P. striiformis* f. sp. *tritici* in North America (Chen *et al*., 1993; Chen *et al*., 1995). RAPD primers were used to identify 64 single-uredinial isolates of *P. triticina* collected from wheat in different regions of Canada and 15 molecular phenotypes were distinguished

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(Kolmer *et al*., 1995). The RAPD and amplified fragment length polymorphism (AFLP) markers linked to avirulence loci in *P. graminis* were reported (Zambino *et al*., 2000). Eleven RAPD primers were used to test 131 single-pustule isolates of *P. triticina* that were collected from different countries and 82 RAPD phenotypes were described (Kolmer and Liu, 2000). AFLP was used to investigate 23 physiological races of *P. triticina* collected from wheat-growing areas of China and Mexico and a sequence characterized amplified region (SCAR) marker for race MFR was developed (Pu, 2004). Sequence-tagged microsatellite profiling was used to develop 110 microsatellites for *P. graminis* f. sp. *tritici* (Keiper *et al*., 2006). Twenty four simple sequence repeat (SSR, microsatellite) markers for *P. graminis* were developed (Szabo, 2007). Eighteen SSR markers were developed for the phytopathogenic rust fungus *Puccinia triticina* (Szabo and Kolmer, 2007). Cao *et al*. (2008) developed a pair of SCAR markers for the rapid identification and detection of *P. striiformis* f. sp. *tritici*. Specific RAPD-SCAR markers were developed for detecting the predominant *P. striiformis* f. sp. *tritici* races CYR32, CYR33 and Su11 in China, respectively (Hao *et al*., 2010; Wang *et al*., 2010). The fast isolation method using AFLP sequences containing repeats (FIASCO) was applied to construct a microsatellite-enriched genomic library of *P. graminis* f. sp. *tritici* and two pairs of specific SSR primers were developed (Wang *et al*., 2011; Liu *et al*., 2014).

In China, although extensive race survey of *Pgt* populations has been conducted in the last two decades, very few races have been encountered because of the poor incidence of the disease throughout the nation. Historically, the race cluster 21C3--- ('---'refers to three specific letters depending on individual race or pathotype) was very common in China. In 1993, *Sr11*-virulence types, including 21C3CTX or 21C3CPX ('X' refers to 'H' or 'R'), were first identified within the '21C3---' cluster and subsequently, race 21C3CTH continued to increase up to the year 2007, when it became predominant with a frequency of 72.9% (Yao *et al*., 1993a, b; 1995; 1997; Yao and Cao, 2001; Han *et al*., 2010).

Therefore, the objective of this study was to develop a specific marker for the rapid identification of race 21C3CTH in China.

Materials and Methods

Purification and Reproduction of Test Races

The target race used in this study for marker development was 21C3CTH of *Pgt* and races 21C3CPH, 21C3CFH, 34MKG, 34C2MKK and 34C2MKR, which were common in the last two decades in China, were used for comparison. These races were collected from Sichuan, Hubei, Henan, Liaoning, and Heilongjiang provinces from 2007 to 2011. Five mono-pustule isolates were established for each race and grew on primary leaves of the susceptible Little Club (LC) wheat cultivar planted in 9 cm internal diameter pots in a greenhouse (20±1°C; humidity 60%–70%). The race identity and purity of each isolate were performed on the basis of sets of 21 differential varieties, including partial Stakman's set, 5 Chinese supplementary varieties and 12 single *Sr* gene lines (Cao and Chen, 2010), the procedures were described by Roelfs and Martens (1988). Urediniospores of each isolate were collected, dried at room temperature, frozen in liquid nitrogen and immediately stored at −80°C for later use.

DNA Extraction

Genomic DNA of each isolate was extracted using a combination system of glass beads oscillation with the cetyltrimethyl ammonium bromide (CTAB) method, as described by Sun *et al*. (2010). Twenty milligrams of urediniospores of each isolate was crushed for 4 min with glass beads in 600 µL CTAB extraction buffer (0.8% CTAB, 140 mmol/L D-sorbitol, 30 mmol/L N-lauroyl-sarcosine, 0.8 mol/L NaCl, 20 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1 mol/L Tris-HCl, pH 8.0, 0.1% Polyvinylpyrrolidone (PVP), 10 mg/mL proteinase K). After 90 min incubation at 65°C, the lysate was extracted and precipitated with an equal volume of chloroform/isopropanol alcohol (24:1) and then treated with 40 µL RNase A (10 µg/µL) for 30 min (37°C). DNA was extracted again with chloroform/isopropanol alcohol (24:1). The DNA was precipitated by adding dehydrated ethanol and centrifuged for 10 min at maximum speed in a microcentrifuge (12,000 × *g*). The DNA pellets were washed in 70% EtOH 2–3 times, air-dried and re-suspended in 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA extraction of healthy wheat leaves was performed as described by Aldrich and Cullis (1993).

RAPD Analysis

A total of 156 10-bp random primers were synthesized by Sangon Biotech (Shanghai, China). RAPD amplifications were conducted using 60 ng genomic DNA in a 25 µL volume containing 2.5 µL 10× polymerase chain reaction (PCR) buffer (Mg^{2+} free), 1.5 µL $MgCl_2$ (25 mM), 0.625 µL dNTPs (10 mM), 0.75 µL primer (10 µM) and 0.3 µL rTaq (5 U/µL; Takara Biotechnology; Dalian, China). An S1000 Thermal Cycler (BIO-RAD; USA) was programmed to implement the following cycling conditions: one denaturing cycle of 94°C for 5 min; 45 cycles of 94°C for 30 s, 36°C for 40 s and 72°C for 90 s, with a final 10-min extension step at 72°C and holding at 4°C. Aliquots of each PCR product from these amplifications were separated by electrophoresis on 1.2% (w/v) agarose gel in 1× TBE buffer (5× TBE: 54 g Tris-base, 27.5 g boric acid, 20 mL 0.5 mol/L EDTA, pH 8.0) and visualized using an ultraviolet (UV) transilluminator.

Isolation, Cloning and Sequencing of the Unique RAPD Fragment of 21C3CTH

The 21C3CTH-specific band was excised from gels under UV light and purified using a DNA Agarose Gel Extraction Kit (Sangon; Shanghai, China). The recovered DNA fragments were cloned in the pGM-T vector system (TIANGEN; Beijing, China). The ligation reaction included 1 μL 10× T4 ligation buffer, 1 μL pGM-T vector (50 ng), 5 μL PCR product and 1 μL T4 DNA ligase (3 Weiss units/μL) in a total volume of 10 μL. The reaction was incubated overnight at 16°C. The ligation reaction was then transformed into *Escherichia coli* strain DH5α competent cells by adding 100 μL instantly thawed competent cells to a sterile 1.5 mL Eppendorf tube containing 10 μL of each ligation reaction on ice. Recombinants were identified as white colonies on Luria-Bertani (LB) plates with X-gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside) and IPTG (Isopropyl-β-D-thiogalactopyranoside). Each white colony was cultured in 5 mL liquid LB medium containing 100 μg/mL ampicillin overnight and recombinant plasmids were extracted using the Plasmid Extraction Kit (Sangon; Shanghai, China). The size of the cloned RAPD fragment was verified by PCR using the corresponding RAPD primer and universal primers T7 (5'-TAATACGACTCACTATAGGG-3') (or SP6 (5'-ATTTAGGTGACACTATAG-3')), or digested by the *Eco*RI restriction enzyme in the multiple clone sites of the vector. The reaction system was 20 μL containing 1 μL plasmids (0.5 μg/μL), 1 μL *Eco*RI, 2 μL 10× *Eco*RI buffer and 16 μL nuclease-free water (Sambrook and Russell, 2001). The expected fragment was sequenced with a 3730xl DNA Analyzer (Sangon Biotech; Shanghai, China).

Development and Validation of Specific SCAR Marker

Based on the sequence data of the cloned RAPD fragments, a specific pair of SCAR primers for 21C3CTH was designed from both ends of the sequence using Primer Premier 5.0 software and synthesized by Sangon Biotech (Shanghai, China).

The reaction conditions were optimized for the pair of SCAR primers. The SCAR primers were used to test 5 mono-pustule isolates for each of 21C3CTH and the other 5 races. PCR using sterilized distilled water in place of the *Pgt* DNA template was used as a negative control.

Comparison of the SCAR Marker and Differential Test Results

The uredial sample accessions used in the marker test included 23 asexual isolates collected from commercial wheat field and breeding plots maintained by rust research scientists and cooperators nationwide and 30 sexual uredio-pustule isolates produced by aeciospore infection. A total of 53 isolates were used for validation (Table 1).

Each isolate was also tested on the sets of 21 differential varieties, including partial Stakman's set, 5 Chinese supplementary varieties and 12 single gene lines (provided by A.P. Roelfs) for race identity and spore purity. The identification was performed using standard procedures as previously described (Roelfs and Martens, 1988). These isolates were used to determine the presence and absence of the SCAR marker.

Results

The Specific DNA Band Identified for 21C3CTH

Of 156 RAPD primers tested with the target and 5 reference races, primer S92 (CAGCTCACGA) produced a specific RAPD band in 21C3CTH, but not in the other 5 races (Fig. 1). The result was consistent over three replicate experiments.

Cloning and Sequence Analyses of the 21C3CTH-Specific Band

The 21C3CTH-specific band was isolated from the gel, cloned to pGM-T and sequenced from both ends of the insertion using T7 forward and SP6 reverse primers. The nucleotide sequence (Fig. 2) was 782-bp long and was deposited in GenBank under the accession number KF131680. No significant sequence similarity was found in the National Center for Biotechnology Information database.

Design and Test of a SCAR Marker for 21C3CTH

The sequences of the specific pair of the SCAR primers were as follows:

21C3CTH-f: 5'-CAGCTCACGATAAACCAGTTACA-3';

21C3CTH-r: 5'-GACTGCGAACAACAGGAAGTC-3'.

The optimized reaction conditions for the pair of SCAR primers were as follows: each PCR mix consisted of 2.5 μL 10× PCR buffer (Mg^{2+} free), 1.5 μL $MgCl_2$ (25 mM), 2.5 μL dNTPs (10 mM), 1 μL each primer (10 μM), 1 μL DNA (40 ng), 0.3 μL rTaq (5 U/μL) and 15.2 μL ddH_2O. Amplification was carried out using the following conditions: one cycle of 3 min at 94°C; 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s; followed by a final elongation step of 72°C for 10 min and holding at 4°C.

The specificity of the SCAR primers was tested using genomic DNA extracted from the single-pustule isolates of 21C3CTH and the 5 other races. The expected band was only observed in the isolates of race 21C3CTH, but not in those of the other races (Fig. 3).

Table 1: Comparison of SCAR marker identification with differential host identification using the 53 isolates of *P. graminis* f. sp. *tritici* collected in 2012–2013

Isolate	Origin	Year	Race	Avirulence/Virulence formulae[a]	21C3CTH[b]
Ab2	Sichuan	2013	21C3CTH	5,21,9e,36,30,31,38/7b,11,6,8a,9g,9b,17,9a,9d,10,Tmp,24,McN	+
Ab12	Sichuan	2013	21C3CTH	5,21,9e,36,30,31,38/7b,11,6,8a,9g,9b,17,9a,9d,10,Tmp,24,McN	+
Ab5	Sichuan	2013	21C3CTH	5,21,9e,36,30,31,38/7b,11,6,8a,9g,9b,17,9a,9d,10,Tmp,24,McN	+
Ab1	Sichuan	2013	21C3CTQ	5,21,9e,36,30,31,38/7b,11,6,8a,9g,9b,17,9a,9d,10,Tmp,24,McN	-
Ab3	Sichuan	2013	21C3CTQ	5,21,9e,30,17,Tmp,31,38/7b,11,6,8a,9g,36,9b,9a,9d,10,24,McN	-
Ab14	Sichuan	2013	21C3CTQ	5,21,9e,30,17,Tmp,31,38/7b,11,6,8a,9g,36,9b,9a,9d,10,24,McN	-
Ab15	Sichuan	2013	21C3CTQ	5,21,9e,30,17,Tmp,31,38/7b,11,6,8a,9g,36,9b,9a,9d,10,24,McN	-
Ab7	Sichuan	2013	21C3CTT	5,21,9e,Tmp,24,31,38/7b,11,6,8a,9g,36,9b,30,17,9a,9d,10,McN	-
Ab8	Sichuan	2013	21C3CTT	5,21,9e,Tmp,24,31,38/7b,11,6,8a,9g,36,9b,30,17,9a,9d,10,McN	-
Ab9	Sichuan	2013	21C3CTT	5,21,9e,Tmp,24,31,38/7b,11,6,8a,9g,36,9b,30,17,9a,9d,10,McN	-
Ab11	Sichuan	2013	21C3CTT	5,21,9e,Tmp,24,31,38/7b,11,6,8a,9g,36,9b,30,17,9a,9d,10,McN	-
Ab4	Sichuan	2013	21C3HTT	5,9e,31,38/21,7b,11,6,8a,9g,36,9b,30,17,9a,9d,10,Tmp,24,McN	-
Ab6	Sichuan	2013	21C3HTT	5,9e,31,38/21,7b,11,6,8a,9g,36,9b,30,17,9a,9d,10,Tmp,24,McN	-
Ab10	Sichuan	2013	21C3HTT	5,9e,31,38/21,7b,11,6,8a,9g,36,9b,30,17,9a,9d,10,Tmp,26,McN	-
Ab13	Sichuan	2013	21C3HTT	5,9e,31,38/21,7b,11,6,8a,9g,36,9b,30,17,9a,9d,10,Tmp,24,McN	-
XN3-1	Shaanxi	2013	34MKG	21,9e,11,36,30,17,10,Tmp,31,38/5,7b,6,8a,9g,9b,9a,9d,24,McN	-
N2	Beijing	2012	34MKG	21,9e,11,36,30,17,10,Tmp,31,38/5,7b,6,8a,9g,9b,9a,9d,24,McN	-
YT5	Sichuan	2013	34MRG	21,9e,8a,36,30,17,10,Tmp,31,38/5,7b,11,6,9g,9b,9a,9d,24,McN	-
YT2	Sichuan	2013	34MRG	21,9e,8a,36,30,17,10,Tmp,31,38/5,7b,11,6,9g,9b,9a,9d,24,McN	-
YT3	Sichuan	2013	34MRG	21,9e,8a,36,30,17,10,Tmp,31,38/5,7b,11,6,9g,9b,9a,9d,24,McN	-
XN11-3	Shaanxi	2013	34MRG	21,9e,8a,36,30,17,10,Tmp,31,38/5,7b,11,6,9g,9b,9a,9d,24,McN	-
YT8	Sichuan	2013	34MRG	21,9e,8a,36,30,17,10,Tmp,31,38/5,7b,11,6,9g,9b,9a,9d,24,McN	-
XN19-1	Shaanxi	2013	34MRG	21,9e,8a,36,30,17,10,Tmp,31,38/5,7b,11,6,9g,9b,9a,9d,24,McN	-
YT6	Sichuan	2013	34MRG	21,9e,8a,36,30,17,Tmp,31,38/5,7b,11,6,9g,9b,9a,9d,10,24,McN	-
YT4	Sichuan	2013	34MRG	21,9e,8a,36,30,17,Tmp,31,38/5,7b,11,6,9g,9b,9a,9d,10,24,McN	-
XN18	Shaanxi	2013	34MTG	21,9e,36,30,17,Tmp,31,38/5,7b,11,6,8a,9g,9b,9a,9d,10,24,McN	-
XN20-3	Shaanxi	2013	34MTG	21,9e,36,30,17,Tmp,31,38/5,7b,11,6,8a,9g,9b,9a,9d,10,24,McN	-
Q4	Liaoning	2012	34MTG	21,9e,36,30,17,Tmp,31,38/5,7b,11,6,8a,9g,9b,9a,9d,10,24,McN	-
XN3-2	Shaanxi	2013	34Oro2MTG	21,9e,36,30,17,Tmp,31,38/5,7b,11,6,8a,9g,9b,9a,9d,10,24,McN	-
XN15-1	Shaanxi	2013	34Oro2MRG	21,9e,8a,36,30,17,10,Tmp,31,38/5,7b,11,6,9g,9b,9a,9d,24,McN	-
XN9-3	Shaanxi	2013	34Oro2MRG	21,9e,8a,36,30,17,10,Tmp,31,38/5,7b,11,6,9g,9b,9a,9d,24,McN	-
XN2-1	Shaanxi	2013	34Oro2MRG	21,9e,8a,36,30,17,10,Tmp,31,38/5,7b,11,6,9g,9b,9a,9d,24,McN	-
XN16-P	Shaanxi	2013	34Oro2MRG	21,9e,8a,36,30,17,10,Tmp,31,38/5,7b,11,6,9g,9b,9a,9d,24,McN	-
XN5-2	Shaanxi	2013	34Oro2MRG	21,9e,8a,36,30,17,10,Tmp,31,38/5,7b,11,6,9g,9b,9a,9d,24,McN	-
XN5-3	Shaanxi	2013	34Oro2MRG	21,9e,8a,36,30,17,10,Tmp,31,38/5,7b,11,6,9g,9b,9a,9d,24,McN	-
XN5-1	Shaanxi	2013	34Oro2MRG	21,9e,8a,36,30,17,10,Tmp,31,38/5,7b,11,6,9g,9b,9a,9d,24,McN	-
XN8-2	Shaanxi	2013	34C3RKG	9e,11,36,30,17,10,Tmp,31,38/5,21,7b,6,8a,9g,9b,9a,9d,24,McN	-
XN8-3	Shaanxi	2013	34C3RKG	9e,11,36,30,17,10,Tmp,31,38/5,21,7b,6,8a,9g,9b,9a,9d,24,McN	-
XN9-4	Shaanxi	2013	34C3RKG	9e,11,36,30,17,10,Tmp,31,38/5,21,7b,6,8a,9g,9b,9a,9d,24,McN	-
XN11-2	Shaanxi	2013	34C3RKG	9e,11,36,30,17,10,Tmp,31,38/5,21,7b,6,8a,9g,9b,9a,9d,24,McN	-
XN8-1	Shaanxi	2013	34C3RKG	9e,11,36,30,17,10,Tmp,31,38/5,21,7b,6,8a,9g,9b,9a,9d,24,McN	-
XN22-1	Shaanxi	2013	34C3RKG	9e,11,36,30,17,Tmp,31,38/5,21,7b,6,8a,9g,9b,9a,9d,10,24,McN	-
XN4-b	Shaanxi	2013	34C3RKG	9e,11,36,30,17,Tmp,31,38/5,21,7b,6,8a,9g,9b,9a,9d,10,24,McN	-
XN22-3	Shaanxi	2013	34C3RKG	9e,11,36,30,17,Tmp,31,38/5,21,7b,6,8a,9g,9b,9a,9d,10,24,McN	-
XN10-3	Shaanxi	2013	34C3RKG	9e,11,36,30,17,Tmp,31,38/5,21,7b,6,8a,9g,9b,9a,9d,10,24,McN	-
XN21-3	Shaanxi	2013	34C3RKG	9e,11,36,30,17,Tmp,31,38/5,21,7b,6,8a,9g,9b,9a,9d,10,24,McN	-
XN19	Shaanxi	2013	34C3RTG	9e,36,30,17,10,Tmp,31,38/5,21,11,7b,6,8a,9g,9b,9a,9d,24,McN	-
XN16-m	Shaanxi	2013	34C3RTG	9e,36,30,17,10,Tmp,31,38/5,21,11,7b,6,8a,9g,9b,9a,9d,24,McN	-
XNw-1	Shaanxi	2013	34C3RTG	9e,36,30,17,10,Tmp,31,38/5,21,11,7b,6,8a,9g,9b,9a,9d,24,McN	-
XN10-2	Shaanxi	2013	34C3RTG	9e,36,30,17,10,Tmp,31,38/5,21,11,7b,6,8a,9g,9b,9a,9d,24,McN	-
XN36-30	Shaanxi	2013	34C3RTG	9e,36,30,17,10,Tmp,31,38/5,21,11,7b,6,8a,9g,9b,9a,9d,24,McN	-
XN15-2	Shaanxi	2013	34C3RTG	9e,36,30,17,10,Tmp,31,38/5,21,11,7b,6,8a,9g,9b,9a,9d,24,McN	-
XN20-1	Shaanxi	2013	34C3RTG	9e,36,30,17,10,Tmp,31,38/5,21,11,7b,6,8a,9g,9b,9a,9d,24,McN	-

[a] monogenic lines

[b] + indicates present and – indicates absent

Comparison of PCR Detection of the SCAR Marker and Race Identification using Differential Tests

In order to validate the SCAR marker and evaluate its usefulness for specifically identifying the isolates of 21C3CTH, 53 *Pgt* isolates from Liaoning, Shaanxi and Sichuan provinces and Beijing (municipality) collected during 2012–2013 were tested using the differential hosts and the SCAR marker. All isolates identified as 21C3CTH consistently produced the specific 782-bp band and those identified as other races (21C3CTQ, 21C3CTT, 21C3HTT, 34MKG, 34MRG, 34Oro2MTG, 34Oro2MRG, 34C3RKG, 34C3RKG and 34C3RTG) did not produce any band (Table 1, Fig. 4). These data indicated that the SCAR marker can be used to specifically identify or detect race 21C3CTH.

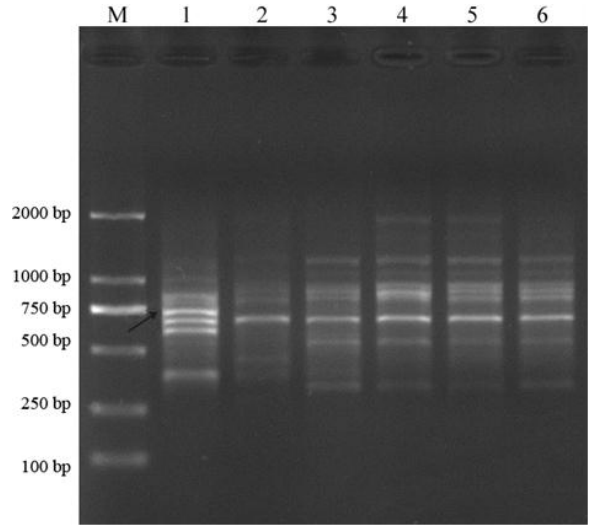

Fig. 1: Random amplified DNA polymorphisms of 6 lanes representing 6 different races of *P. graminis* f. sp. *tritici* amplified with primer S92. The arrow shows the specific RAPD marker, a 782-bp band only present in the lane of 21C3CTH. Lane M: DNA ladder DL2000. Lanes 1–6 are races 21C3CTH, 21C3CPH, 21C3CFH, 34MKG, 34C2MKK, and 34C2MKR, respectively

1	CAGCTCACGA	TAAACCAGTT	ACATTTTACA	CCACAACAAC	ACACTACACG
51	ACAATATTAC	TACCCACCGG	TGGATCAACC	ATTTTGAGTA	ATATTGATGT
101	TAGAGCGAGC	GTTACTCGAT	TAGCTAATGA	TCAAGTTCTT	CCAACTCGAC
151	AGATTGATTC	AATACCAGAA	CGTGAATCCA	TTCCTCTTGA	TTTACCTAGA
201	AACAATAACA	ATGGTAATGC	TGAAGTAACA	ACTTTATCAT	CTTCAATTTC
251	ACCACCATCT	AGATATGATT	ATGGGACTAA	TAGAGTAGAG	GTTGTCAGTA
301	CAGTAAACAT	TCACCGAACT	CACGAAACTG	ATCCTCTCTT	GACTTATGAG
351	ACATCACTAA	CTTATTTAAC	AACTTATTTT	GATCAAACTA	AGCCGATTCG
401	AACTGAGACT	AGACGAGAAA	CTGTTACAGA	AATACGTCGA	AATACATTGA
451	TTAATCCATC	AGTGACAAGA	AAACCATTGA	TTATTCGTCG	AACACGTTTA
501	CGAGGTGCAG	CTGCAAGGGA	CATTTCATTA	GATCCTAGAG	ATGGATCACA
551	TTTAACCCGT	AAACCAGTCG	TTGTGATTAC	TAAAAAGCCA	ATCATTGAAC
601	CAACAGTCAT	TTTAGATCGA	CCTTTTGCCT	CTGACCATTT	TCCTCGTGAT
651	TTTAATCGCC	ACCGTGTCTC	TCATGTTGAT	GAACTTGGCC	TGGCTCCAAC
701	TCCTGTTACT	TATTATACAA	CTTTCACTCA	CTTTACAACC	GAATTGGTTG
751	GTGGACTTCC	TGTTGTTCGC	AGTCGTGAGC	TG	

Fig. 2: Nucleotide sequence of the specific band of race 21C3CTH of *P. graminis* f. sp. *tritici*

Discussion

The great success of varieties bred for disease resistance and their proper deployment has resulted in negligible wheat production losses caused by wheat stem rust since the 1970s in most countries (Singh *et al*., 2011). The crucial contribution of theoretical guidance provided by information obtained via the continual surveillance of *Pgt* races cannot be overstated for this success. In China, the differential hosts and nomenclature used for racial analysis of *Pgt* basically follow those adopted in the United States typified at different periods of time by three representative reports (Tu, 1934; Wu and Huang, 1987; Yao *et al*., 1993b).

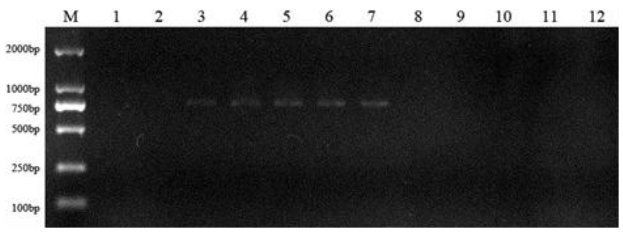

Fig. 3: Agarose gel electrophoretic profiling showing the bands of a sequence characterized amplified region (SCAR) marker (21C3CTH-f)/(21C3CTH-r) with 5 different single-pustule isolates of 21C3CTH and no bands produced with the other 5 races and controls. Lane M: DNA ladder DL2000. Lanes 1–2: reaction solution without DNA. Lanes 3–7: different single-pustule isolates of 21C3CTH. Lanes 8–12: 21C3CPH, 21C3CFH, 34MKG, 34C2MKK, and 34C2MKR, respectively

Nevertheless, there is still much room for improvement in identification methods. The host-based identification methods are rather inflexible, time consuming and expensive and are therefore not ideal for continuous monitoring of pathogenic populations. Molecular identification or detection methods, especially those using PCR-based technologies, have shown great promise as alternatives (Miller *et al*., 2009; Capote *et al*., 2012). Therefore, we attempted to develop specific molecular identification markers for *Pgt* races. Although several molecular detection studies have been carried out for plant pathogenic fungi at the species level or above, few such markers have been developed at the race level.

This study was conducted to develop a race-specific, highly reliable PCR based molecular identification method for the predominant *Pgt* race 21C3CTH. We chose the RAPD technique because this race's genome is highly rich in polymorphisms and then converted the obtained RAPD marker into a SCAR marker to overcome the disadvantages of low repeatability and specificity associated with the use of RAPD markers. Indeed, SCAR markers are considered to be more suitable and are generally preferred over RAPD markers for identification because of their good specificity, reproducibility, reliability and robustness (Huh and Bang, 2006). In this research, a 782-bp specific DNA fragment of 21C3CTH was found by amplifying the genomic DNA of *Pgt* races with a RAPD primer S92. Following this, a pair of primers was designed and tested. The new SCAR marker of proper size w**as** amplified successfully from all the isolates of 21C3CTH used in this research, whereas no products were generated from other races tested. The SCAR marker developed in this study was shown to be reliable for characterizing race 21C3CTH.

Compared with differential host testing, the molecular marker method for race identification is rapid, cheap and accurate and does not rely on the strict environmental conditions required for testing directly on wheat differentials. Further studies will be needed to develop

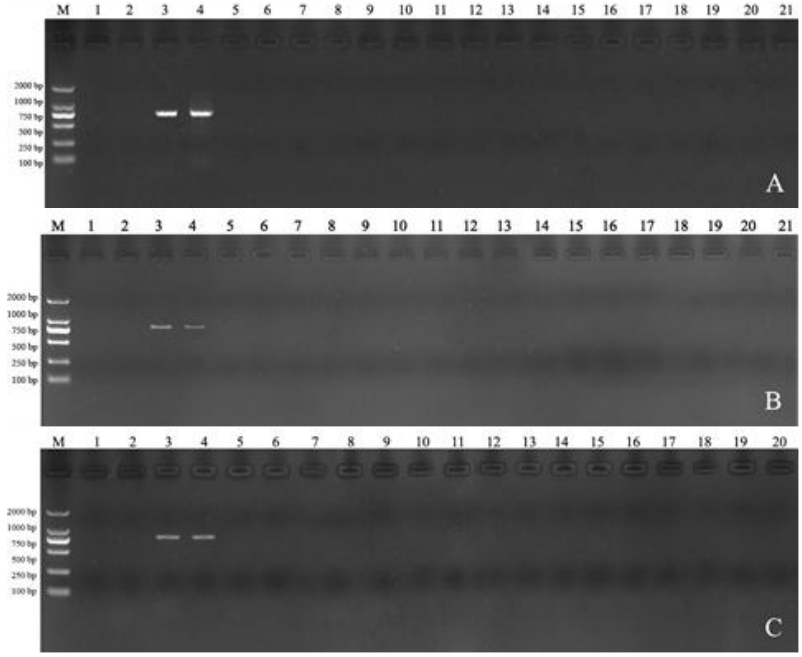

Fig. 4: Agarose gel electrophoretic profiling showing the presence or absence of 21C3CTH based on a sequence characterized amplified region (SCAR) marker, (21C3CTH-f)/(21C3CTH-r) using 53 isolates of *P. graminis* f. sp. *tritici* collected from Shaanxi, Sichuan, Liaoning, and Beijing in 2012–2013 (A, B, C) . Lane M: DNA ladder DL2000. Lane 1: reaction solution without DNA. Lane 2: genomic DNA of wheat genotype Little Club. Lane 3: positive CK of 21C3CTH. Lanes 4–21 in A: Ab2(21C3CTH), Ab1(21C3CTQ), Ab3(21C3CTQ), Ab14(21C3CTQ), Ab15(21C3CTQ), Ab7(21C3CTT), Ab8(21C3CTT), Ab9(21C3CTT), Ab11(21C3CTT), Ab4(21C3HTT), Ab6(21C3HTT), Ab10(21C3HTT), Ab13(21C3HTT), XN3-1(34MKG), N2(34MKG), YT5(34MRG), YT2(34MRG), YT3(34MRG). Lanes 4–21 in B: Ab12(21C3CTH), XN11-3(34MRG), YT8(34MRG), XN19-1(34MRG), YT6(34MRG), YT4(34MRG), XN18(34MTG), XN20-3(34MTG), Q4(34MTG), XN3-2(34Oro2MTG), XN15-1(34Oro2MRG), XN9-3(34Oro2MRG), XN2-1(34Oro2MRG), XN16-P(34Oro2MRG), XN5-2(34Oro2MRG), XN5-3(34Oro2MRG), XN5-1(34Oro2MRG), XN8-2(34C3RKG). Lanes 4–20 in C: Ab5(21C3CTH), XN8-3(34C3RKG), XN9-4(34C3RKG), XN11-2(34C3RKG), XN8-1(34C3RKG), XN22-1(34C3RKG), XN4-b(34C3RKG), XN22-3(34C3RKG), XN10-3(34C3RKG), XN21-3(34C3RKG), XN19(34C3RTG), XN16-m(34C3RTG), XNw-1(34C3RTG), XN10-2(34C3RTG), XN36-30(34C3RTG), XN15-2(34C3RTG), XN20-1(34C3RTG)

specific markers for other *Pgt* races in China with the purpose of having a clear picture of race composition in the pathogen virulence and monitoring in the early season of the rust population.

Conclusion

The results of the SCAR marker validation indicated that the SCAR marker consistently amplified the specific bands in all isolates of 21C3CTH, but not in those of other reference races tested. The marker specificity was further demonstrated using isolates collected across different locations in China in 2012–2013. The characterization of 21C3CTH with the SCAR marker was completely consistent with the results of the race identification with the set of differential wheat genotypes. Therefore, the marker is highly reliable for identification of 21C3CTH among the *Pgt* races in China.

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References

Aldrich, J. and C.A. Cullis, 1993. RAPD analysis in flax: Optimization of yield and reproducibility using Klen *Taq* 1 DNA polymerase, chelexl 100, and gel purification of genomic DNA. *Plant Mol. Biol. Rep*., 11: 128–141

Cao, L.H., S.C. Xu, R.M. Lin, T.G. Liu and W.Q. Chen, 2008. Early molecular diagnosis and detection of *Puccinia striiformis* f. sp. *tritici* in China. *Lett. Applied Microbiol*., 46: 501–506

Cao, Y.Y. and W.Q. Chen, 2010. Stepwise shift of differential hosts and racial designation of *Puccinia graminis* f. sp. *tritici*. *J. Triticeae Crops*, 30: 167–172

Capote, N., A.M. Pastrana, A. Aguado and P. Sánchez-Torres, 2012. Molecular tools for detection of plant pathogenic fungi and fungicide resistance. In: *Plant Pathol*, pp: 151–202, C. Cumagen (ed), InTech Publishers, Shinghai, China

Chen, X.M., R.F. Line and H. Leung, 1993. Relationship between virulence variation and DNA polymorphism in *Puccinia striiformis*. *Phytopathology*, 83: 1489–1497

Chen, X.M., R.F. Line and H. Leung, 1995. Virulence and polymorphic DNA relationships of *Puccinia striiformis* f. sp. *hordei* to other rusts. *Phytopathology*, 85: 1335–1342

Han, J.D., Y.Y. Cao and Z.G. Sun, 2010. Race dynamics of *Puccinia graminis* f. sp. *tritici* in China and the virulence on CIMMYT wheat germplasm resistant to Ug99. *J. Triticeae Crops*, 30: 163–166

Hao, B.J., B.T. Wang, Q. Li, G.B. Li, F. Wang and B. Zhang, 2010. RAPD analysis and SCAR marker establishment of Su11 group of *Puccinia striiformis* f.sp.*tritici* in China. *Acta Phytopathol. Sin.*, 40: 1–6

Huh, M.K. and K.H. Bang, 2006. Identification of *Atractylodes japonica* and *A. macrocephala* by RAPD analysis and SCAR markers. *Silvae Genet*., 55: 101–105

Jin, Y., L.J. Szabo, M.N. Rouse, T. Fetch, Jr., Z.A. Pretorius, R. Wanyera and P. Njau, 2009. Detection of virulence to resistance gene *Sr36* within the TTKS race lineage of *Puccinia graminis* f. sp. *tritici*. *Plant Dis*., 93: 367–370

Keiper, F.J., M.S. Haque, M.J. Hayden and R.F. Park, 2006. Genetic diversity in Australian populations of *Puccinia graminis* f. sp. *avenae*. *Phytopathology*, 96: 96–104

Kolmer, J.A. and J.Q. Liu, 2000. Virulence and molecular polymorphism in international collections of the wheat leaf rust fungus *Puccinia triticina*. *Phytopathology*, 90: 427–436

Kolmer, J.A., J.Q. Liu and M. Sies, 1995. Virulence and molecular polymorphism in *Puccinia recondita* f. sp. *tritici* in Canada. *Phytopathology*, 85: 276–285

Li, Z.Q. and S.M. Zeng, 2002. *Wheat Rusts in China*. Sinauer Associates, China Agricultural, Beijing, China

Liu, T.G., X. Wang, L. GAO, B. Liu, W.Q. Chen and W.S. Xiang, 2014. A FIASCO-based approach for detection and diagnosis of *Puccinia graminis* f.sp.*tritici* in China. *J. Integ. Agric*., 13: 2438–2444

Miller, S.A., F.D. Beed and C.L. Harmon, 2009. Plant disease diagnostic capabilities and networks. *Annu. Rev. Phytopathology*, 47: 15–38

Pardey, P.G., J.M. Beddow, D.J. Kriticos, T.M. Hurley, R.F. Park, E. Duveiller, R.W. Sutherst, J.J. Burdon and D. Hodson, 2013. Right-sizing stem-rust research. *Science*, 340: 147–148

Pu, Z.G., 2004. Development of a molecular detection assay for the physiological race MFR of *Puccinia triticina*. *Acta Phytopathol. Sin*., 34: 449–454

Raloff, J., 2005. Wheat warning-new rust could spread like wildfire. *Science News Online*, 168: 13. http://www.sciencenews.org/articles/20050924/food.asp (22.10.2007)

Rehman, A.U., M. Sajjad, S.H. Khan and N. Ahmad, 2013. Prospects of wheat breeding for durable resistance against brown, yellow and black rust fungi. *Int. J. Agric. Biol*., 15: 1209–1220

Roelfs, A.P. and J.W. Martens, 1988. An international system of nomenclature for *Puccinia graminis* f. sp. *tritici*. *Phytopathology*, 78: 526–533

Sambrook, J. and D.W. Russell, 2001. *Molecular Cloning: A Laboratory Manual*, 3rd edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA

Singh, R.P., D.P. Hodson, J. Huerta-Espino, Y. Jin, S. Bhavani, P. Njau, S. Herrera-Foessel, P.K. Singh, S. Singh and V. Govindan, 2011. The emergence of Ug99 races of the stem rust fungus is a threat to world wheat production. *Annu. Rev. Phytopathol*., 49: 465–481

Stakman, E.C. and J. Premeisel, 1917. A new strain of *Puccinia graminis*. *Phytopathology*, 7: 73

Sun, Z.G., Y.Y. Cao, J.D. Han and W.S. Chen, 2010. Comparison of diferent methods for extraction of DNA of wheat stem rust urediospores. *Hubei Agric. Sci*., 49: 1281–1284

Szabo, L.J., 2007. Development of simple sequence repeat markers for the plant pathogenic rust fungus, *Puccinia graminis*. *Mol. Ecol. Notes*, 7: 92–94

Szabo, L.J. and J.A. Kolmer, 2007. Development of simple sequence repeat markers for the plant pathogenic rust fungus *Puccinia triticina*. *Mol. Ecol. Notes*, 7: 708–710

Tu, C., 1934. Physiologic forms of *Puccinia graminis tritici* in Kwangtung, Southern China. *Phytopathology*, 24: 423

Wang, B.T., X.P. Hu, Q. Li, B.J. Hao, B. Zhang, G.B. Li and Z.S. Kang, 2010. Development of race-specific SCAR markers for detection of Chinese races CYR32 and CYR33 of *Puccinia striiformis* f. sp. *tritici*. *Plant Dis*., 94: 221–228

Wang, X., T.G. Liu, W.S. Xiang and W.Q. Chen, 2011. Development of a SSR molecular marker for *Puccinia graminis* f. sp. *tritici*. *Sci. Agric. Sin*., 44: 4593–4599

Wu, Y.S. and Z.T. Huang, 1987. Racial identification and dynamics analysis of *Puccinia graminis* f. sp. *tritici* for past 20 years in China. *J. Shenyang Agric. Uni*., 18: 105–138

Yao, P. and Y.Y. Cao, 2001. Race population trend of *Puccinia graminis* f. sp. *tritici* in China (1995–1999). *The Plant Protection Development Strategy of the 21st Century*, pp: 667–670. Beijing, China's Science and Technology Publisher

Yao, P., Y.Y. Cao, W.Z. Liu and Y.S. Wu, 1995. Race dynamic of *Puccinia graminis* f. sp. *tritici* in China in 1993. *Acta Phytophylacica Sin*., 22: 303–308

Yao, P., Y.Y. Cao, W.Z. Liu and Y.S. Wu, 1997. Race population trend of *Puccinia graminis* f. sp. *tritici* in 1990–1994 in China. *Acta Phytopathol. Sin*., 24: 297–302

Yao, P., Y.Y. Cao, X.L. Jia and Z.J. Zhou, 1993a. Race identification of *Puccinia graminis* f. sp. *tritici* in China in 1992. *Plant Protect.*, 19: 6–8

Yao, P., Y.Y. Cao and Y.S. Wu, 1993b. Race identification of *Puccinia graminis* f. sp. *tritici* in China in 1990. *Acta Phytopathol. Sin.*, 20: 65–70

Zambino, P.J., A.R. Kubelik and L.J. Szabo, 2000. Gene action and linkage of avirulence genes to DNA markers in the rust fungus *Puccinia graminis*. *Phytopathology*, 90: 819–826

Zambino, P.J. and L.J. Szabo, 1993. Phylogenetic relationships of selected cereal and grass rusts based on rDNA sequence analysis. *Mycologia*, 85: 401–414

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