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RESEARCH ARTICLE

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Identification of *Lr24* with targeted region amplified polymorphism (TRAP) analysis in wheat

Abstract This research is aimed at developing TRAP markers, as a probe for library screening, closely linked to or co-segregated with *Lr24*. Ninety TRAP primer pairs were used to test the resistant and susceptible parents, as well as the resistant bulk and the susceptible bulk in our study. The polymorphic TRAP primers of TcLr24 were employed to genotype the F_2 population from TcLr24×Thatcher subsequently. Ten of 90 TRAP primer pairs displayed polymorphism between TcLr24 and Thatcher, accounting for 11.11%. A further study found that primer ARBI1/RGA-2F generated a 161 bp fragment presented only in the resistance plants of F_2 population. Forty-five other wheat leaf rust resistant NILs and 30 diploid materials of wheat were also tested to detect the specificity of the primer. This specific band was amplified in TcLr19, TcLr29, TcLr38, TcLr42 and TcLr44, but absented in all the 30 diploid materials. It was concluded that this marker ARBI1/RGA-2F was closely linked to *Lr24*, which could be used to detect *Lr24* in the F_2 population of TcLr24×Thatcher, and be further used as a probe for cDNA and BAC library screening of TcLr24.

Keywords resistance genes, TRAP, TcLr24, wheat leaf rust

1 Introduction

Wheat leaf rust disease, incited by fungal pathogen *Puccinia triticina* (formerly *Puccinia recondita* f. sp. *tritici*), is one of the most destructive diseases on wheat (*Triticumm aestivum* L.) worldwide. More than 10% yield losses occur when heavy rust infection defoliates flag leaves during grain filling. Breeding for resistance is considered to be the most efficient, cost-effective and environment-friendly approach to prevent the losses caused by rust epidemics. More than 50 wheat leaf rust resistance genes have been designated and located on chromosomes. However, only a subset of the known genes are fine-mapped to more specific genetic locations, such as *Lr1* (Cloutier et al., 2007), *Lr10* (Feuillet et al., 2003), and *Lr21* (Huang et al., 2003).

Of the three resistance genes, *Lr10* and *Lr21* have been constructed with coiled coil (CC), nucleotide-binding-site (NBS), and leucine-rich-repeat (LRR) motifs (Feuillet et al., 2003; Huang et al., 2003). While *Lr1* is a member of a large psr567 gene family, it contains a CC-NBS-LRR domain. Therefore, the PCR-based approach cannot be used in cloning other wheat leaf rust resistance genes, and the map-based cloning approach is still the best choice. Developing and tagging closely linked or co-segregated molecular markers, and constructing fine maps for the target genes are the necessary promise for cloning the other wheat leaf rust resistance genes. Thus, molecular markers can also provide an effective screening tool for cloning resistance genes.

Strategies based on restriction fragment length polymorphism (RFLP), randomly amplified polymorphism DNA (RAPD), inter-simple sequence repeat (ISSR), simple sequence repeat (SSR) and amplified fragments length polymorphism (AFLP) have been successfully used in the research field of crop germplasm. A new random marker system called sequence related amplified polymorphism (SRAP) using primers with AT-or GC-rich cores to amplify intragenic polymorphisms was invented in 2001 (Li and Quiros, 2001). TRAP (Hu and Vick, 2003), which has been recently developed based on SRAP, is amplified by one fixed primer designed based on a target EST sequence in the database and a second primer of arbitrary sequence except for AT- or GC-rich cores that anneal with introns and exons, respectively. TRAPs are effectively used in assessing genetic diversity among wild sunflower (*Helianthus annuus* L.) accessions (Hu and Vick, 2003), in fingerprinting lettuce (*Lactuca sativa* L.) cultivars (Hu

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et al., 2005), in tagging a recessive branching gene in sunflower (Rojas-Barros et al., 2005), in mapping QTL in a wheat intervarietal recombinant inbred population (Liu et al., 2005), and in constructing genetic maps (Liu et al., 2007). Gene *Lr24* derives from *Thinopyrum ponticum*, which is the translocation of 3Ag/3D (Smith et al., 1968; Sears, 1973), and confers very strong resistance to wheat leaf rust in China. The virulent frequency of rust isolates is 0.0% to gene *Lr24*, found in 2002, and it is still a valuable and available leaf rust resistance gene. Molecular markers of *Lr24* have been acquired based on different genetic backgrounds (Schachermary et al., 1995; Dedryver et al., 1996; Gupta et al., 2006; Zhang et al., 2008), and validated worldwide (Stepien et al., 2002; Stepine et al., 2003; Blaszczyk et al., 2004; Singh et al., 2004; Liu, 2006). No molecular marker has been developed for *Lr24* based on TRAP till now. Here, TRAP was used in this study to search for corresponding markers of *Lr24*.

2 Materials and methods

2.1 Plant materials and *P. triticina* races

Forty-five Thatcher backgrounded wheat leaf rust resistant near isogenic lines (NILs) including TcLr24, one susceptible line Thatcher, and 141 individuals of the TcLr24 ×Thatcher F_2 progeny of the races 88-26-12-4 (BGQQ) and 82-F-13-2 (SHRT) avirulent to *Lr24* were used in this study. All seeds and the leaf rust races were kindly supplied by the Centre for Wheat Rust Disease Research of Agricultural University of Hebei, China. Thirty diploid materials of wheat were kindly supplied by the Chinese Academy of Agricultural Sciences (CAAS), China.

2.2 Primers

Combination of fixed primers and arbitrary primers were selected for TRAP analysis. The arbitrary primers with AT- or GC-rich cores to march with introns or extrons were the same as in SRAP. Also, the fixed primers with the conserved resistance gene domain CC-NBS-LRR were used in this study (Table 1).

2.3 Evaluation of leaf rust resistance

TcLr24×Thatcher F_2 seeds were sown in pots, the resistant parent TcLr24 and susceptible parent Thatcher were also planted as controls. They were inoculated with low virulent *P. triticina* to *Lr24* at one leaf stage, respectively. The inoculated plants were cultured at 16°C and 100% humidity for 24 h in the dark. The infection type was scored 14 days after being cultured at (24±5)°C, and 90% relative humidity with the 6–class phenotype recorder standard of Roelfs (1984): immune (0 IT), fleck (0; IT), small uredinia with necrosis (1 IT), and small uredinia with chlorosis (2 IT) were considered as resistance, while medium-sized uredinia with or without chlorosis (3 IT) and large uredinia without chlorosis (4 IT) were considered as susceptibility.

2.4 DNA extraction

A modified version of the cetyltrimethylammonium bromide (CTAB) method was used to extract genomic DNA (Prabhu et al., 1998) from leaves. For each accession, 0.5 g of ground leaf tissue was suspended in 2.5 mL of extraction buffer [20 mmoL·L^{-1} EDTA, 0.1 moL·L^{-1} Tris-HCl (pH 8.0), 1.4 moL·L^{-1} NaCl, 2% (w/v) CTAB and 5 μL of β-mercaptoethanol]. The suspension was well mixed, incubated at 60°C for 30 min, extracted by chloroform-isoamyl alcohol (24∶1), and then precipitated with 0.6 vol isopropanol at −20°C. The pellet formed after centrifugation at full speed for 5 min was washed with 70% (v/v) ethanol and 10 mmoL·L^{-1} NH_4OAc. The DNA was then suspended in TE buffer. The resulting DNA concentration was determined using a *UVI* DU6600, spectrophotometer (Beckman Coulter) by adjusting the ratio of $OD_{260/280}$ between 1.8 and 2.0 to 30–50 ng·μL^{-1} for PCR amplification.

Table 1 TRAP primers used for amplification

arbitrary primers	primer sequences (5'–3')	fixed primers	primer sequences (5'–3')
ARBI1	GACTGCGTACGAATTAAT	RGA-11F	AACCCAATTCCACCTCTTTTACA
ARBI2	GACTGCGTACGAATTGAC	RGA-11R	TTCCCCTTGCAATAGTCACCATAG
ARBI3	GACTGCGTACGAATTTGA	RGA-2F	CTATGGTGACTATTGCAAGGGGAA
ARBI4	GGAACCAAACACATGAAGA	RGA-2R	ATTGTGATTGATGGCATGTCTACG
ARBI5	TCATCTCAAACCATATACAC	CIN001	GGNGGNAT(T/C/A)GGIAA(A/G)ACIAC
ARBI6	TTCTTCTTCCCTGGACACTT	CIN004	NA(G/A)NGCIA(G/A)IGGIA(G/A)ICC
ARBI7	CTATCTCTCGGGACCAAAC	CIS001	GGNCA(A/G)GGNGGITT(T/C)GGI(A/T)(C/G)IG
ARBI8	TGAGTCCAAACCGGATA	CIS003	GGNCA(A/G)GGNGGITT(T/C)GGI(A/T)(C/G)IG
ARBI9	TGAGTCCAAACCGGTGC	P1	GGAATGGGNGGNGTNGGNAA(A/G)CANAC
	—	P3	A(A/G)NGCNA(A/G)(A/T)GG(A/C)A(A/G)NCC

2.5 PCR amplification

PCR amplification was conducted in a final reaction volume of 25 μL PCR reaction mixture in a T-gradient Thermal Cycler PCR (Bio-metra) with the following components: 50 ng of genomic DNA sample, 1×PCR buffer (20 mmoL·L^{-1} $MgCl_2$), 200 μmoL·L^{-1} of each dNTP, 0.2 μmoL·L^{-1} of primers and 1 U of *Taq* DNA polymerase. PCR conditions were predenaturing template DNA at 94°C for 3 min, then cycles at 94°C for 1 min, 35°C for 1 min, and 72°C for 1 min, followed by 35 cycles at 94°C for 1 min, at 50°C for 1min, and 72°C for 1 min. The final extension was at 72°C for 10 min before cooling to 4°C. PCR-amplified products were loaded onto 2.0% agarose gel and run at 120 V, photographed on *UVIpro*.

2.6 TRAP analysis of gene *Lr24*

Bulk of resistant gene (Br) and susceptible gene (Bs) were pooled by mix aliquot DNA from 10 resistance plants and susceptible plants of TcLr24×Thatcher F_2 population, respectively. TcLr24, Thatcher, Br and Bs were used to screen polymorphic primer combinations of *Lr24*. Further linkage demonstration of the primer was conducted using TcLr24×Thatcher F_2 population.

2.7 Isolation, cloning and sequencing of the specific fragments

The specific fragments were isolated from the gels and reclaimed by Kit according to the manufacturer's instructions. The isolated fragments were then ligated into the pGEM-T easy vector (Promage), and transformed into fresh competent cells of *Escherichia coli* strain DH5α. Positive clones were cultured at 37°C for 15 h and were sequenced by Shanghai Sangon Biological Engineering Technology & Service Co., Ltd.

3 Results

3.1 Phenotype segregation

One hundred and forty-one F_2 individuals derived from a cross between the line TcLr24 and Thatcher were scored for resistance by inoculation with BGQQ and SHRT. The infection type of the F_2 plants was either 0–0; like the resistance parent TcLr24 or 3–4 (susceptibility) like the susceptible parent Thatcher. 81 F_2 populations were segregated in 63 plants with IT 0; or 1, and 18 plants with IT 3 or 4 to BGQQ, which conformed to 3R:1S segregation ratio (χ^2= 0.7), and 60 F_2 populations were segregated in 44 plants with IT 0; or 1, and16 plants with IT 3 or 4 to SHRT, which conformed to 3R:1S segregation ratio (χ^2= 0.02). The 3R:1S segregation (Table 2) for a single inherited gene confirmed the dominant action of the *Lr24* resistance gene to the isolates BGQQ and SHRT, respectively.

3.2 Primers displayed polymorphism of *Lr24*

A total of 90 primer pairs were used to test for polymorphism between the resistant parent TcLr24 and susceptible parent Thatcher, the Br and Bs. Ten of them displayed polymorphism of TcLr24 accounting for 11.1%. The ten primers were further tested with the F_2 progeny of TcLr24×Thatcher. One specific fragment of 161bp produced by ARBI1/RGA-2F appeared in TcLr24 and all the resistance populations and no visible band appeared in Thatcher and all the susceptible population tested (Fig. 1). This indicated that the specific band was co-segregated with *Lr24*.

When forty-five other wheat leaf rust resistant NILs and 30 diploid materials of wheat were tested by this primer, this specific band was also amplified in TcLr19, TcLr29, TcLr38, TcLr42 and TcLr44 (Fig. 2) but absented itself in all the 30 diploid materials (Fig. 3). This result showed that the 161 bp fragment amplified by ARBI1/RGA-2F cannot be widely used in MAS in agricultural breeding programs but in screening *Lr24* in TcLr24×Thatcher generation.

3.3 Cloning and sequencing of target band

The TRAP fragments amplified in TcLr19, TcLr29, TcLr38, TcLr42 and TcLr44 were reclaimed and cloned for the sequence respectively; they all possessed the same length (161 bp) as in TcLr24 (Fig. 4). However, they were not compeletly identical. As shown in Fig. 4, three SNPs (single nucleartide polymorphism) were present in TcLr19 and TcLr29, one in TcLr38, two in TcLr42 and TcLr44, respectively. Also, the sequences of TcLr29 and TcLr44 were identical. An ORF was present in all the sequences, however, the integrality was unknown because of the short sequence. Also, 83% identity to BAC clone (21775–21897

Table 2 Segregation of TcLr24×Thatcher F_2 progenies infected by isolates BGQQ and SHRT

F_2 population	isolates	number of plants	infection type		segregation ratio	χ^2 value
			0 or 0 (resistant)	3 or 4 (susceptible)		
TcLr24×Thatcher	BGQQ	81	63	18	3:1	0.70
TcLr24×Thatcher	SHRT	60	44	16	3:1	0.02

Note: $\chi^2_{0.05,\ 1}$= 3.84.

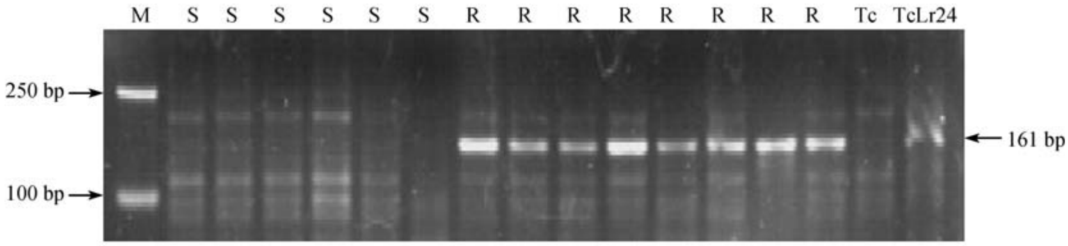

Fig. 1 161-bp fragments amplified with ARBI1/RGA-2F in parts of TcLr24×Thatcher F_2 progenies
Note: Tc, R, S and M represent Thatcher; resistant individuals from TcLr24×Thatcher F_2 generation; susceptible individuals from TcLr24×Thatcher F_2 generation and DL2000 Marker respectively.

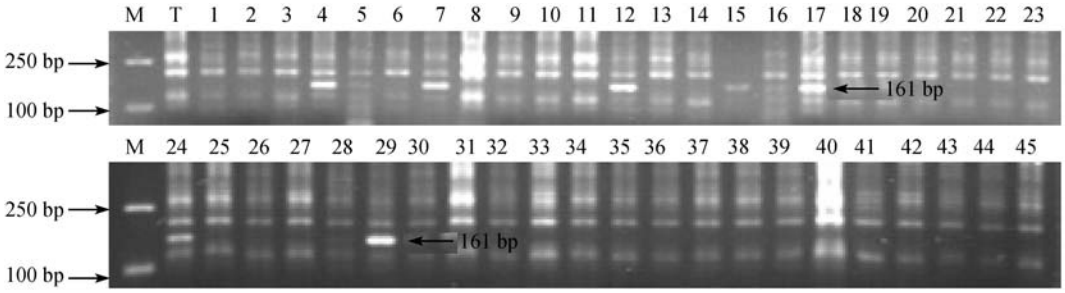

Fig. 2 Amplification of 45 NILs of different wheat leaf rust resistance gene by ARBI1/RGA-2F
Note: Tc-Thatcher, 1–45 represent 45 NILs respectively, in which, 4 stands for TcLr42, and 7 for TcLr38, 12 for TcLr29, 17 for TcLr19, 24 for TcLr44, 29 for TcLr24; the arrowhead shows the specific band amplified by ARBI1/RGA-2F; M: DL2000 DNA Marker.

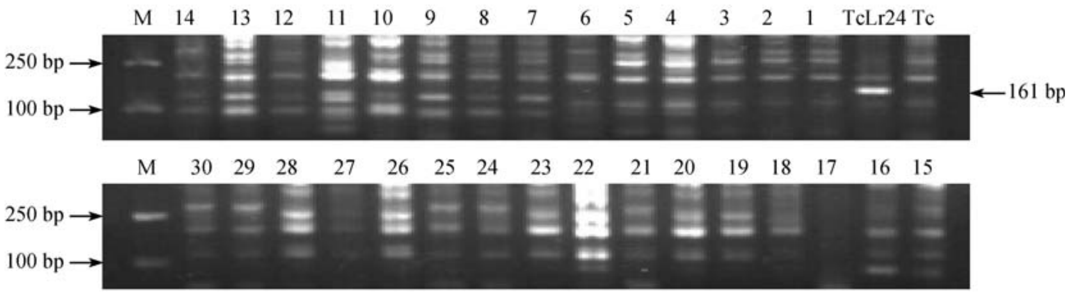

Fig. 3 Amplification of 30 diploid wheat materials by ARBI1/RGA-2F
Note: 1–30 represent the 30 diploid materials of wheat; the arrowhead shows the specific band amplified by ARBI1/RGA-2F; M represents DL2000 DNA marker.

in 1648_464) conferring leaf rust resistant protein of wheat leaf rust resistance gene *Lr1* was found by Blastn (Fig. 5).

4 Discussion

A simple and rapid PCR-based marker system, TRAP was recently developed based on SRAP, which can be widely used in wheat plasmid researches (Liu et al., 2005; Liu et al., 2007). One stable primer, ARBI1/RGA-2F, was acquired by screening in the F_2 population of TcLr24×Thatcher, in which one specific band of 161 bp was amplified in TcLr24, resistance population of TcLr24×Thatcher, but none appeared in Thatcher and the susceptible population, indicating that the specific band was cosegregated with *Lr24* and can be used to identify *Lr24* in F_2 progeny of Thatcher crossed with TcLr24. However, the specific test in forty-five other NILs of leaf rust resistance and 30 diploid materials of wheat indicated that the 161 bp band was not specific for *Lr24*, and this band was amplified in TcLr19, TcLr29, TcLr38, TcLr42 and TcLr44. The sequence analysis of these fragments showed their full length was 161 bp, and SNPs were detected, with frequency distributing from 0.62% to 3.73%. The amplification site of these materials may locate at the different site of the same chromosome or on a different chromosome, or may be at the same site of the same chromosome. In 45 wheat leaf rust resistant NILs, the

Fig. 4 Sequences and the SNPs of the 161 bp fragment in NILs

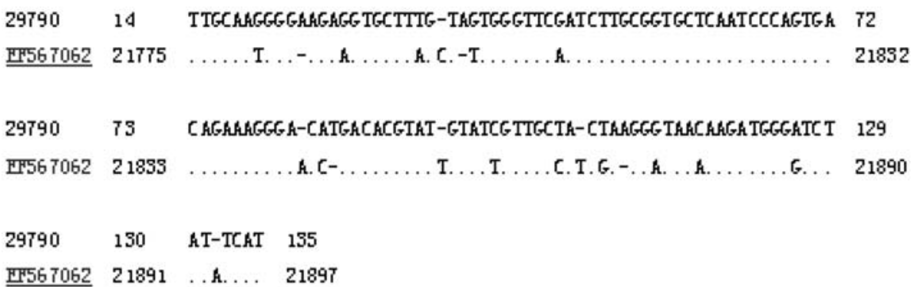

Fig. 5 Blastn analysis of 161 bp sequence amplified from TcLr24

TRAP discovered only few genes containing the 161 bp band with few SNPs, which indicated that the band was some specific for the *Lr19*, *Lr29*, *Lr38*, *Lr42* and *Lr44*. Putative specific marker was obtained for these genes, but there was no linkageship of the marker for these genes based on the F_2 populations of TcLr19×Thatcher, TcLr38×Thatcher, and TcLr42×Thatcher (data not shown).

There are two approaches to acquire markers for genes of interest, bulked segregant analysis (BSA) (Michelmore et al., 1991) and NILs. Closely linked marker to *Lr24* was acquired by TRAP analysis employed on BSA and the F_2 plants of TcLr24×Thatcher, non-specificity was found when validated on the NILs of wheat leaf rust resistant gene, which showed the advantage of the former on gene markers. Many molecular markers tightly linked to *Lr* genes of wheat had been developed, and some of them have been converted to SCAR or STS markers (Seyfarth et al., 1999; Cherukuri et al., 2003; Gupta et al., 2005; Ashwini et al., 2006), which can be used in marker assisted selection (MAS) and gene cloning. There were unspecific markers (Feuillet et al., 1995; Naik et al., 1998) at the same time, and they had little application on MAS. Exception was detected when genecloning of *Lr1*; the probe was used specifically in the segregation population. The fragment of 161 bp steadily appeared in various PCR amplifications. Therefore, TRAP should be further studied in germplasm verification, construction on genetic map, marking for important trait genes, fingerprinting of gDNA and cDNA, and map-based cloning, hence the TRAP marker in this study will have potential screening *Lr24* in the BAC library of TcLr24.

Specific primers for the sequence will be designed to acquire the full length cDNA of gene *Lr24*, and functional verification of the fragment should be further studied, which will lay a foundation for the location, cloning, and definition of resistance mechanism of gene *Lr24*.

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