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Full Length Research Paper

Identification of random amplified polymorphic DNA (RAPD) marker of Ph-3 gene for late blight resistance in tomato

Qingdao Zou, Haitao Li*, Zijun Zhang*, Shuwen Lu, Yongcheng Wang, Guodong Yang, Xinyu Zhang and Yipeng Qiu

Vegetable Research Institute of Liaoning Academy of Agricultural Sciences, Shenyang, Liaoning 110161, China.

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Late blight is a highly destructive disease of tomato worldwide. Host resistance is the most effective method for disease control. The application of molecular markers is an efficient way to identify host resistance for breeding programs. In this study, bulked segregant analysis (BSA) was used to search for random amplified polymorphic DNA (RAPD) markers linked to the late blight resistance gene Ph-3, using an F_2 population (147 individuals) derived from a cross of tomato lines CLN2037 (resistant) and T2-03 (susceptible). Two hundred and thirty decamer primers with arbitrary sequences were chosen for polymerase chain reaction amplification. One RAPD marker CCPB272-03740 (primer sequence GGTCGATCTG) was found to be tightly linked to the resistance gene Ph-3 and was located 5.8 cm from the resistance gene. Marker CCPB272-03740 is the first marker of gene Ph-3 based on PCR reaction.

Key words: Tomato, late blight, random amplified polymorphic DNA (RAPD) marker, gene Ph-3.

INTRODUCTION

Late blight, caused by Phytophthora infestans (Mont.) de Bary, is a major threat to tomato production. The pathogen can infect fruits, leaves, petioles, stems and seeds (Rubin et al., 2001a; Rubin and Baisder 2004a) and cause serious yield loss. It is one of the most important diseases of tomato in China, due to the lack of resistant cultivars, and farmers apply fungicides frequently and follow cultural practices for disease control. However, resistance of the pathogen to fungicides (example metalaxyl) often hampers disease control (Gisi and Cohen, 1996). Breeding resistant hybrids is an economical and efficient method for late blight control.

Several highly resistant accessions have been identified by AVRDC (The World Vegetable Center) for resistance breeding, including accession L3708 of Solanum pimpinellifolium, which has shown high levels of resistance in most locations in the world (Black et al., 1996a, b). By crossing with susceptible variety 'Moneymaker' and line CLN657, AVRDC has developed late blight-resistant inbred lines CLN2037B and CLN2037E, which give the Ph-3 gene from resistant parent L3708. Resistance gene Ph-3 is a single partially dominant allele mapped to chromosome 9 (Chunwongse et al., 2002).

Molecular markers are a highly efficient way to identify resistance genes for breeding programs as compared to traditional screening methods. Molecular mapping studies have identified Ph-3 in L3708 and confirmed its tight linkage with restriction fragement length polymorphism (RFLP) Marker TG591 (Chunwongse et al., 2002). Since the early 1980's, RFLPs has been used successfully for a wide range of plant species (Beckmann and Soller, 1983). However, for some species, lack of polymorphism revealed by RFLPs has retarded progress. Furthermore, the RFLP assay is time consuming and labour intensive. The RAPD assay has alleviated some of the technical problems associated with RFLP, and has been widely used to resolve problems in plant breeding and genetics (Waugh et al., 1992).

This report describes the development of a RAPD

*Corresponding author. E-mail: qingdaozou@hotmail.com.

marker closely linked to the Ph-3 gene, which is resistant to late blight in tomato.

MATERIALS AND METHODS

Plant materials and evaluation for resistance to late blight

Late blight-resistant line CLN2037B was provided by AVRDC, and late blight-susceptible line T2-03 was provided by the Liaoning Academy of Agricultural Sciences (LAAS). An F_2 population segregating for late blight resistance was generated by crossing susceptible line T2-03 as the female parent with resistant line CLN2037B. The F_2 seeds were produced by self-pollination of F1 plants. A total of 147 F_2 plants and 30 plants of each parent were evaluated individually for reaction to P. infestans in a growth room in LAAS. Plants were grown in 6 cm diameter plastic pots containing a 1:1 (v/v) mixture of peat-lite and perlite for six weeks.

The pathogen isolate, race T1.2, was isolated from a diseased tomato plant collected from Shenyang, China. Inoculum was prepared from 12 to 14 day-old rye A agar Petri plate cultures grown under darkness at 20°C. The sporangial suspension was diluted in sterile water to 2.5 × 104 sporangia per ml and incubated at 12°C for 2 h to induce zoospore release for artificial inoculation. Plants were inoculated using a paint sprayer to atomize the zoospore suspension onto the foliage to the point of run-off, and incubated at 20°C. Leaf wetness was maintained for the first 24 h by keeping the plants in the dark at 100% RH. Thereafter, plants were maintained at 70 to 90% RH and a daily 14 h photo-period.

Plants were scored individually for disease severity 7 and 10 days after inoculation on a disease severity rating (DSR) of 0 to 6, where 0 = no of symptoms; 1 = <5% leaf area affected and small (<2 mm) lesions; 2 = 6 to 15% leaf area affected and restricted (<4) mm lesions; 3 = 16 to 30% leaf area affected and/or few superficial small stem lesions; 4 = 31 to 60% leaf area affected and/or few small penetrating stem lesions; 5 = 61 to 90% leaf area affected and/or deep expanding stem lesions; 6 = 91 to 100% leaf area affected, extensive stem damage or plant death. Plants were classified into three categories based on DSR, 1 to 2 = resistant; 3 to 4 = intermediate; and 5 to 6 = susceptible. Chi-square analysis was performed to test the goodness of fit of 1 resistant, 2 intermediate and 1 susceptible ratio in the F_2.

DNA extraction and RAPD analysis

DNA extraction from plants of parental lines and hybrids was done as described by van der Beek et al. (1992). DNA was isolated from the young leaves of tomato seedling. DNA concentration was determined by spectrophotometry and visual observation in 1% agarose gel (Beek et al., 1992).

For BSA, equal amounts of DNA from 20 resistant F_2 plants and 20 susceptible F_2 plants were pooled into an R-pool and S-pool, respectively (Michelmore et al., 1991). DNA from plants with intermediate resistant phenotypes were not used.

Two hundred and thirty primers of single 10-mer kits (TaKaRa Technologies Inc.) were used for polymerase chain reaction (PCR). The 25 µl reaction mixture contained 2.0 µl dNTP, 2.0 µl Mg2+, 0.25 µL Taq polymerase, 5 µl DNA (5 ng/µL), 2.5 µl Primer (10 µl/L), 2.5 µl10×Buffer and 10.75 µl water. The reaction mixture was overlaid with 10 µl of mineral oil. Amplification was performed for 45 cycles. After initial denaturation for 5 min at 94°C, each cycle consisted of 1 min at 94°C (denaturation), 1 min at 32°C and 1.5 min at 72°C, followed by the final extension of 10 min at 7°C. Amplification products were resolved by electrophoresis in 1.5% agarose gel with 1 x TBE buffer for 3 h at 4 V/cm and revealed by ethidium bromide staining.

Linkage analysis

From the 147 individual plants of F_2 population, 38 resistant plants and 34 susceptible plants were selected for linkage analysis of the RAPD marker and Ph-3 locus, the intermediate resistant plants were not selected for linkage analysis.

Linkage analysis was performed on molecular data and inoculation data of the selected F_2 plants. Plants with the same amplified fragments as resistant parent were recorded “a” and with the same amplified fragments as susceptible parent were recorded “b”. According to artificial inoculation results, the resistant plants were recorded “a” and susceptible plants were recorded “b”. The genetic distance between the RAPD marker and the Ph-3 locus could be assessed after entering the molecular data and artificial inoculation data of each plant into software MAPMAKER 3.0b (Lander and Green, 1987).Linkage was considered significant with a LOD score value above 3.0 and a distance below 40 Cm. Recombination fractions were converted into Kosambi centimorgans (cM).

RESULTS

Evaluation for resistance to late blight

Sporangia were cultured for inoculation. Parents and F_2 plants were classified as resistant, susceptible or intermediate based on their disease severity scores 10 days after inoculation. Mean disease severity scores were 1.0 for the resistant parent CLN2037B and 6.0 for the susceptible parent T2-03. Of the 147 F_2 plants, 38 were resistant (DSR 0-2), 75 were intermediate (DSR 3-4) and 34 were susceptible (DSR 5 to 6).The fitness test, $X2C = 2.39 < X20.05 = 3.84$, indicated the segregation in the F_2 population for a single partially dominant gene conditioning late blight resistance in CLN2037B.

Identification of RAPD markers linked to the Ph-3 region

Out of the 230 primers tested on the 2 bulks and 2 parents, 19 could generate polymorphism bands between the resistant parent and the susceptible parent. However, the polymorphism disappears between the resistant bulk and the susceptible bulk. Only primer CCPB272-03 (sequence: GGTCGATCTG) generated a dominant marker (~740 bp) amplified in the resistant parent and resistant bulk (Figure 1).

Six resistant individuals and 6 susceptible individuals of the F_2 population were used to test the polymorphism. The 6 resistant individuals generated the same amplified fragment to the resistant parent, and the 6 susceptible individuals generated the same amplified fragment to the susceptible parent. Thus, the amplified fragment, with a molecular weight of 740 bp, was a RAPD marker linked to Ph-3 locus (Figure 2). The marker was named as CCPB272-03740 by the name of primer and the size of

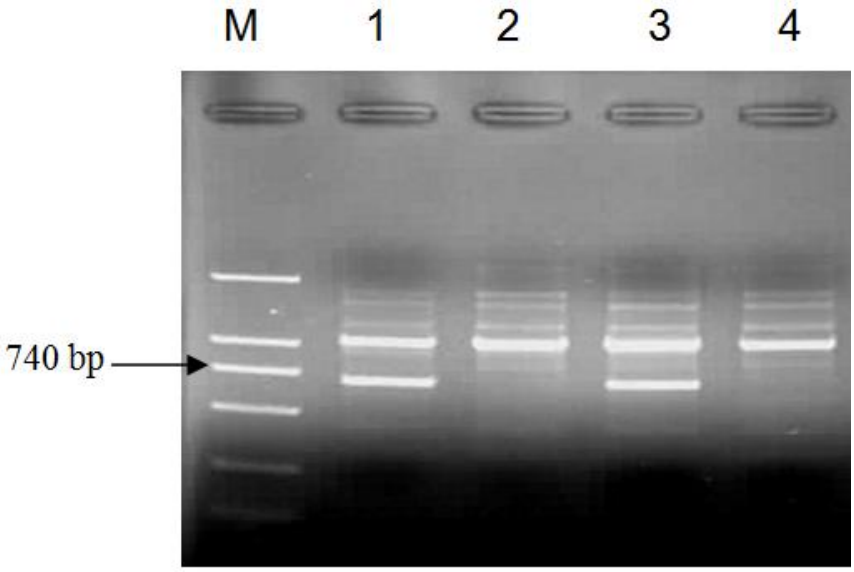

Figure 1. PCR amplification with primer CCPB272-03 in parents, resistant and susceptible bulks. Lane M: Marker; lane 1, resistant parent; lane 2: susceptible parent; lane 3; resistant bulk; lane 4: susceptible bulk.

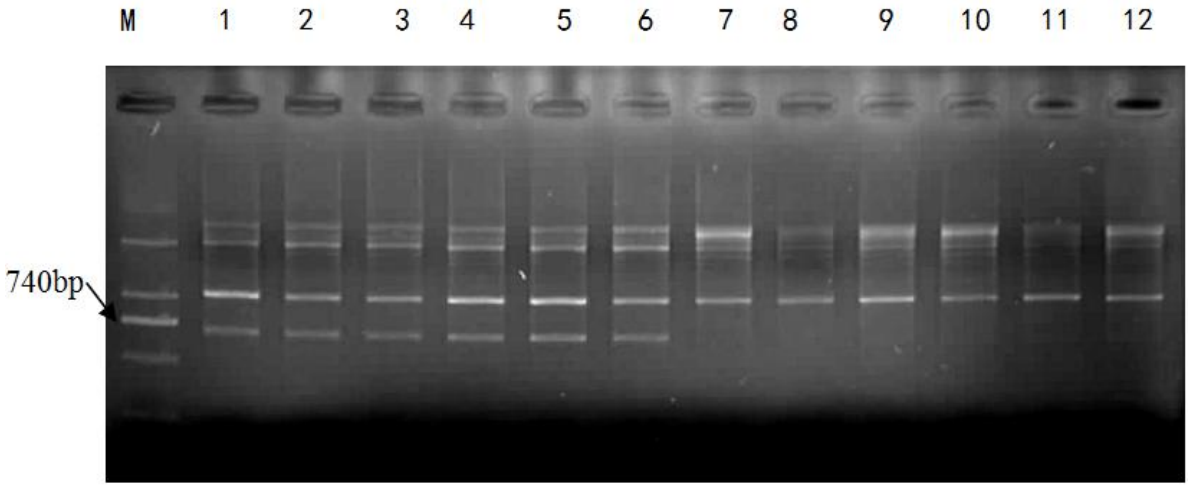

Figure 2. PCR amplification with primer CCPB272-03 in individuals of resistant and susceptible bulks. Lane M: Marker; lanes 1 to 6: resistant individual; lanes 7 to 12: susceptible individual.

the amplified band linked to the Ph-3 locus.

This primer has been used to amplify the DNA of 38 resistant plants and 34 susceptible plants from F_2 population. Four individuals have been observed to be recombinant between CCPB272-03740 marker and Ph-3 locus with an exchange value of 5.44%. For other individuals, the molecular data was consistent with artificial inoculation data (Figure 3). Based on our analysis using MAPMAKER3.0b software, marker CCPB272-03740 is tightly linked to the resistance gene Ph-3 and is located 5.8 cM from the resistance gene.

DISCUSSION

In this study, we developed a RAPD marker linked to late blight resistance Ph-3 in tomato .The marker is 5.8 cM from resistance gene by linkage analysis of molecular data and artificial inoculation data, this is a first RAPD marker of resistance gene Ph-3 and will be helpful for marker-assisted selection (MAS) of late blight resistance breeding in tomato.

Marker-assisted selection allows for faster and more reliable identification of resistant plants in breeding

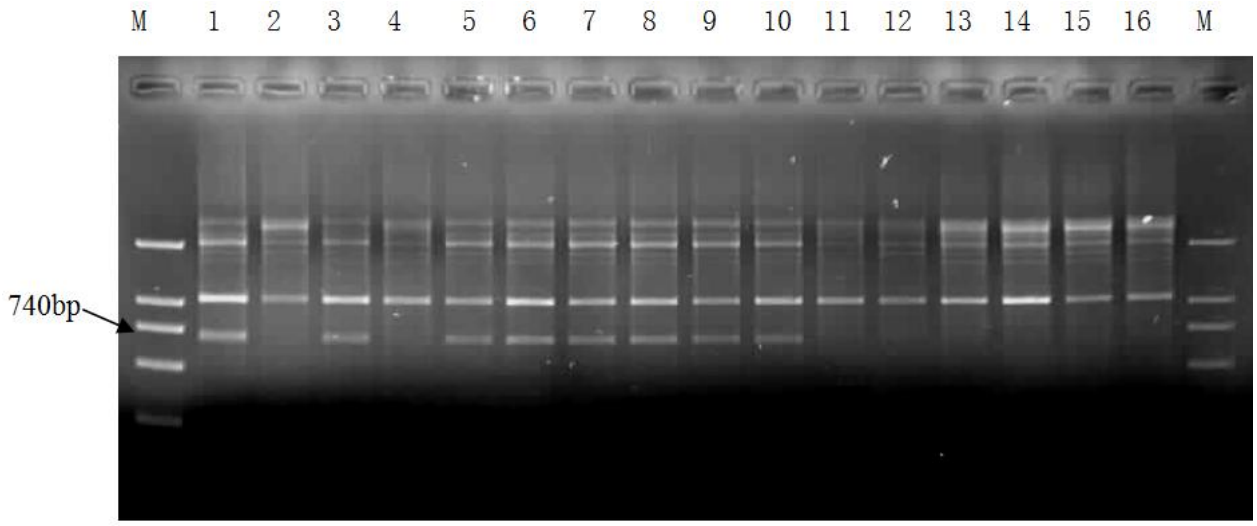

Figure 3. PCR amplification with primer CCPB272-03 in parents, resistant and susceptible bulks and F_2 population. Lane M: Marker; lane 1: resistant parent; lane 2: susceptible parent; lane 3: resistant bulk; lane 4: susceptible bulk; lanes 5 to 10: resistant individual; lanes 11 to 16: susceptible individual.

programmes. The Ph-3 gene was previously mapped to the long arm of chromosome 9, which is linked to RFLP marker TG591 (Chunwongse et al., 2002). As compared to RFLP marker, RAPD marker is more efficient and has reduced labour in MAS (Waugh et al., 1992). Comparison of the genetic distances generated by different molecular markers in diversity studies have been reported by several authors (Hahn et al., 1995; Russell et al., 1997; Yang et al., 1996) and have revealed only moderate agreement between genetic distance estimates made using RFLP and RAPD markers.

Late blight is a worldwide disease in tomato, and the most efficient way for controlling the destructive disease is resistance breeding. A few race-specific major resistance genes (example, Ph-1, Ph-2 and Ph-3) and several race-nonspecific resistance quantitative trait locus (QTLs) have been reported for late blight, Ph-3 is a strong resistance gene and has been incorporated into many breeding lines of Fresh Market and Processing Tomato (Foolad et al., 2008). Advanced breeding lines in combinations with Ph-2 and Ph-3 are being developed recently, two inbred lines NC 1 CELEBR and NC 2 CELEBR had been develop by Fresh Market Tomato Breeding Group in North Carolina State University, which incorporated combined early blight resistance (Campbell 1943 and PI 126445 origin) and late blight resistance (Ph-2 and Ph-3), also resistant to Verticillium wilt (Ve gene) and races 1 and 2 of Fusarium wilt (Gardner and Panthee, 2010).

Genetic variability for late blight resistance exists in Solanum. Resistance seems to be common in many Solanum habrochaites accessions, including AVRDC accessions L3683 and L3684, and LA1033. It seems likely that LA1033 has late blight resistance alleles complementary to Ph-3.

Considering the history of late blight resistance in tomato and potato, it is doubtful that single-gene late blight resistance will be durable. Pyramiding late blight resistance genes may offer the best way to obtain stable and high levels of resistance. Host plant resistance, coupled with cultural methods such as removing and destroying infected tomato or potato plants, eliminating all tomato or potato cull piles in the vicinity of the tomato field, and careful irrigation and moisture management, can offer farmers a better means to control P. infestans.

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