Theor Appl Genet (1994) 89: 661–664

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Identification of a codominant amplified polymorphic DNA marker linked to the verticillium wilt resistance gene in tomato

Received: 7 March 1994 / Accepted: 29 April 1994

Abstract Resistance to verticillium wilt, a vascular disease causing yield losses in many crops, is conferred in tomato by a single dominant allele, *Ve*. A population segregating for the *Ve* allele was generated using near-isogenic tomato lines. Analysis of the parental tomato DNA using the polymerase chain reaction and 400 random primers, each 10 deoxyribonucleotides in length, produced 1,880 amplified DNA fragments. Of the four polymorphisms observed between the resistant and susceptible parental genotypes, only one was linked to the *Ve* gene. No recombination was observed between this DNA marker and the *Ve* locus, indicating that the linkage is less than 3.5±2.7 cM. The marker detected both the susceptible and resistant alleles, producing amplified DNA fragments of approximately 1,300 and 1,350 bp, respectively. The sequence of the primer, determined from cloned amplified products, was 5′ CTCACATGCA 3′ instead of the expected 5′ CTCACATGCC 3′. The marker will be of value to tomato breeding programs because of the tight linkage, codominant nature, and analytical procedure utilized.

Key words *Verticillium dahliae* · Random amplified polymorphic DNA (RAPD) · Codominant DNA polymorphism · *Lycopersicon esculentum*

Introduction

Verticillium wilt is an important vascular disease causing severe yield losses in many crops. Although resistant germplasm is available, the development of resistant commercial varieties has been hindered by relatively slow and subjective screening procedures. A well characterized, race-specific allele conferring high levels of resistance against verticillium wilt has been reported in tomato, *Lycopersicon esculentum* Mill. (Schaible et al. 1951) and mapped using restriction fragment length polymorphisms (RFLPs) to chromosome 7 (Juvik et al. 1991). Identification of a random amplified polymorphic DNA (RAPD) marker for such resistance would enable efficient screening for the trait without relying on inoculations with the verticillium wilt pathogen.

Until recently, the identification of DNA markers linked to genes controlling important traits has relied on the development of RFLPs. The use of the polymerase chain reaction (PCR) to amplify discrete fragments of DNA and quickly detect polymorphisms offers several advantages for the identification and use of markers linked to important loci (Williams et al. 1990). The procedure is simple, relatively inexpensive, requires only a few nanograms of DNA, and results can be obtained within 24 h. The RAPD procedure has been used to identify DNA markers for resistance to bacterial speck of tomato (Martin et al. 1991), downy mildew of lettuce (Paran et al. 1991), oat stem rust (Penner et al. 1993), and rust of common bean (Miklas et al. 1993).

Our objective was to identify a RAPD marker for verticillium wilt resistance that would minimize the need for subjective and time-consuming screening procedures and assist with further characterization of the resistance. We report here the identification of an amplified DNA polymorphism linked to the verticillium wilt resistance gene in tomato. This is the first report of a RAPD marker for resistance to verticillium wilt and describes a tightly linked marker that detects both the resistant and susceptible alleles.

Communicated by A. L. Kahler

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Materials and methods

Plant materials

The tomato near-isogenic lines (NILs) used in this study, 'Ailsa Craig' and 'Craigella', differ in that 'Craigella' has the dominant *Ve*

allele that confers resistance to verticillium wilt and the recessive unlinked *u* allele responsible for uniform ripening (Maxon Smith and Ritchie 1983). The *Ve* allele originates from a small-fruited, wild Peruvian cherry tomato, 'Utah 665' (Schaible et al. 1951). At least five crosses, the initial cross and four backcrosses, were made to transfer the *Ve* allele into the recurrent parent and develop the NILs. Verticillium wilt-resistant lines were selected in each segregating generation and then followed by two selfing generations to establish homozygosity (Darby et al. 1978).

Susceptible 'Ailsa Craig' and resistant 'Craigella' (GCR-151) were grown from seed (Horticulture Research International, Littlehampton, UK) and reciprocal crosses made. The seed was planted, and the resulting heterozygous F_1 plants selfed to produce an F_2 population, derived from a single fruit, consisting of 76 individuals segregating for the *Ve* allele. All F_2 individuals were evaluated for disease reaction, and DNA of 49 selected individuals was examined using the polymerase chain reaction. The genotype of each resistant F_2 individual examined by RAPD analysis was verified by the disease reaction of 10–15 derived F_3 progeny.

Disease evaluation

Conidia were collected from 10 to 14 day old cultures of a tomato strain of *Verticillium dahliae* Kleb. race 1 (Petoseed Co., Woodland, Calif.) grown at room temperature with shaking (100 RPM) in potato dextrose broth. Cultures were filtered through cheesecloth, and conidia were concentrated from the filtrate by centrifugation at 5,000 *g* for 25 min and then diluted with sterile deionized water to give a concentration of approximately 5×10^7 spores/ml.

Plants were screened for resistance to *V. dahliae* using a modified procedure of Huang and Hanna (1991). Plants were grown in 32 cell Rootrainers (Spencer Lemaire, Edmonton, Canada) under a 16:8 h light:dark 22°:18°C cycle. At the three leaf stage, plants were removed from the Rootrainer, the lower half of the root ball was removed, and the remaining root ball submerged for 10 min in the *V. dahliae* spore suspension. For each screening, parental and F_1 genotype controls were also inoculated with either *V. dahliae* or sterile deionized water. After 21 days, plants were rated as either susceptible to the pathogen, as indicated by chlorosis and necrosis of the lower leaves and stunting of the plant, or as resistant, showing no symptoms and appearing similar to the uninoculated plants.

PCR reactions

DNA was extracted from fresh leaves using the CTAB procedure described by Bernatzky and Tanksley (1986). The PCR reactions were performed in a 25 µl volume of 10 m*M* TRIS-HCl, pH 8.3, 50 m*M* KCl, 1.9 m*M* $MgCl_2$, 0.001% gelatin, 0.1 m*M* of each dNTP, 0.2 µ*M* of primer, 1.0 U *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn.), and 25 ng of template DNA. The 400 random decamer primers (UBC100 through UBC500) used to screen the DNA from the two parental genotypes were obtained from Dr. J. Carlson, University of British Columbia (Vancouver, Canada). Amplification was performed with an Ericomp DNA thermal cycler using 45 cycles, each cycle consisting of 1 min at 94°C, 1 min at 35.5°C, 15 s at 45°C, and 1.75 min at 72°C. Following the final cycle, the amplified product was subjected to a final extension of 10 min at 72°C. Amplified products were separated by electrophoresis at 3 V/cm for 4 h in a 2% agarose gel. Gels were subsequently stained with 10 mg/l of ethidium bromide for 30 min. Linkage and standard error were calculated by the product method (Immer and Henderson 1943).

Cloning and sequencing of RAPD

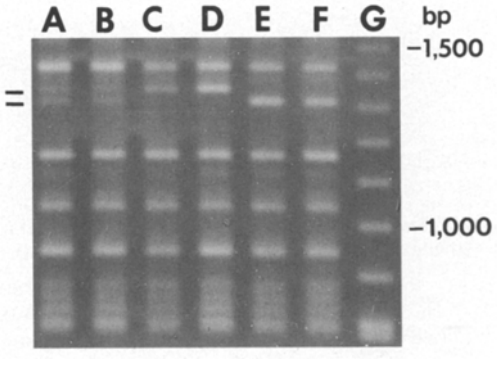
Amplified products of the linked RAPD were excised from the agarose gels, and the DNA purified as described by Thuring et al. (1975). The purified DNA was ligated into pBluescript II SK (+) vector (Stratagene, La Jolla, Calif.) that had been restricted with *Eco*RV using the T-A overhang cloning procedure described by Marchuk et al. (1991). The DNA sequence of the cloned PCR fragments was determined by the dideoxy-chain termination method with the M13 universal and T3 primers. The deoxyribonucleotide sequence determined from the termini of each amplified DNA fragment was used to resynthesize the deoxyribonucleotide primer linked to the verticillium wilt resistance locus (Operon Technologies, Alameda, Calif.).

Results

Susceptible tomato plants inoculated with race 1 of *V. dahliae* were stunted with chlorosis and necrosis evident on the lower leaves. All 20 F_1 heterozygotes tested were resistant. A 3:1 segregation ratio was observed in the F_2 population with 56 resistant and 20 susceptible plants [chi square (3:1)=0.07; $P>0.7$].

The 400 primers used to screen the NILs produced a total of 1,880 amplified DNA fragments ranging in size from approximately 400 to 2,500 bp. The number of amplified products observed with any 1 primer ranged from 0 to 16. Of the 400 primers examined, 4 produced an amplified DNA fragment in one parental genotype but not the other. Examination of the DNA from all 20 susceptible plants and 29 randomly chosen from the 56 resistant plants from the F_2 population revealed that only 1 primer, UBC458, produced a DNA fragment that cosegregated with resistance. The size of the amplified DNA fragment linked to the *Ve* allele was approximately 1,350 bp (Fig. 1). This particular primer also produced a smaller DNA fragment of approximately 1,300 bp that was observed in all susceptible F_2 and some of the resistant F_2 plants (Fig. 1). The smaller amplified DNA fragment in F_1 and resistant F_2 plants was always accompanied by the larger DNA fragment. As expected, 18, or approximately two-thirds, of the 29 resist-

Fig. 1 Linkage of the RAPD and *Ve* locus in tomato plants inoculated with *Verticillium dahliae* race 1. Individuals from an F_2 population segregating for the *Ve* allele were examined using primer UBC458 and the RAPD procedure. The disease reaction and genotype of the individual tomato plants are: *A* and *B* resistant heterozygote; *C* and *D* resistant homozygote; *E* and *F* susceptible homozygote. *Lines* in the *left margin* show the location of the amplified DNA linked to *Ve* (*above*) and *ve* (*below*) alleles. *Lane G* contains a 100 bp DNA ladder



ant F_2 individuals were heterozygotes and produced both the large and small amplified DNA fragments.

Recombination was not observed between the amplified DNA fragment produced by primer UBC458 and the *Ve* gene, indicating that they are tightly linked. Linkage between UBC458 and the *Ve* locus was less than the 3.5±2.7 cM estimate expected if a single recombination had occurred in the absence of recombination suppression due to an alien background. Primer UBC458 represents a particularly useful molecular marker because of its close linkage to the *Ve* gene and its ability to detect both alleles. The relatively close proximity of the RAPD marker to the *Ve* locus also makes it a suitable starting site for chromosome walking and isolation of the *Ve* gene.

Resynthesis of the UBC458 primer using the reported sequence, 5′ CTCACATGCC 3′, resulted in a completely different RAPD profile than that produced by the original primer. Terminal sequences of the cloned RAPD products produced with the original UBC458 primer were 5′ CTCACATGCA 3′. A primer synthesized using this sequence produced the original RAPD profile including the DNA polymorphism linked to the *Ve* gene.

Discussion

This study identified a RAPD marker that can be used to screen for verticillium wilt resistance in tomato. The marker will be particularly useful for tomato breeding programs because of its close linkage to the *Ve* gene, its codominant nature, and the technically simple and fast analytical procedure utilized. The ability to efficiently screen for resistance without relying on inoculations with the pathogen dramatically increases both the numbers that can be screened and the reliability of the results.

The amount of linked and unlinked donor DNA in the NILs that has been removed by backcrossing can vary considerably (Young and Tanksley 1989). The expected rate of recovery for the recurrent parent DNA is $1-(0.5)^t$, where t is the number of backcross generations plus the selfing generation (Martin et al. 1991). Thus, approximately 1.6% of the donor parent DNA would remain following the five backcrosses and selfing used to develop the *Ve* NILs. However, only four polymorphisms were observed within the 1,880 amplified fragments, indicating a lower percentage of donor DNA or a higher than expected similarity in the introgressed region of DNA.

Since no crossovers were observed, the estimated distance between the RAPD marker and the *Ve* locus is less than 3.5±2.7 cM. Martin et al. (1990) calculated the probability of finding a DNA marker within an expected distance of the selected gene as $y/2(nx+1)$, and the distance at a 95% confidence level as $(y/2)\ (1-0.05^{1/nx})$, where y=genome size of 1,500 cM, n=number of primers, and x=average number of DNA products per primer. We estimate that 400 primers producing an average of 5 amplified products each would yield a marker within an expected distance of 0.4 cM and an upper 95% confidence limit of 1.1 cM from the *Ve* gene in tomato. Although variability in the relationship between map units and physical distance in different regions of the genome is likely, in tomato these distances correspond to 175 kb and 525 kb, respectively, based on a C value of 700 megabases (Galbraith et al. 1983). These distances are within the insert size of commercial vectors that can be used for chromosome walking and cloning of the gene (Sambrook et al. 1989).

This is the first report of an amplified polymorphic DNA marker for resistance to verticillium wilt and of a codominant RAPD marker for disease resistance. As with the RAPD markers developed for resistance to oat stem rust (Penner et al. 1993) and rust of common bean (Miklas et al. 1993), recombination between the polymorphism and resistance locus was not observed. These results indicate the propensity of RAPD analysis for developing tightly linked DNA markers that are readily adapted for use by breeding programs. In addition to improving disease resistance screening, the identified RAPD marker facilitates further characterization of verticillium wilt resistance and eventual cloning of the gene.

Acknowledgements The authors thank Dr. G. Hobson of Horticulture Research International, Littlehampton, UK for tomato seeds of the parental genotypes and Dr. J. Watterson of Petoseeds Co., Woodland, Calif. for providing a culture of *V. dahliae* race 1. This research was supported by the Alberta Agriculture Research Institute Farming for the Future Project no. 920117 and the Science and Technology Council of Canada. LRS contribution 3879386.

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