Theor Appl Genet (1996) 92:263–266

H. Yang · S. S. Korban

Screening apples for OPD20/600 using sequence-specific primers

Received: 25 June 1995 / Accepted: 14 July 1995

Abstract Apple scab, caused by *Venturia inaequalis* (Cke.) Wint., is the most serious disease of apple trees in many areas of the world. Resistance to *V. inaequalis*, derived from the small-fruited species *Malus floribunda* 821, is determined by a major dominant gene, *Vf*. Using random decamer primers, we identified a RAPD marker, OPD20/600, which is linked to the *Vf* gene. OPD20/600 was then cloned and sequenced. Sequence-specific primers based on the marker were used to further screen *M. floribunda* 821, 7 scab-susceptible apple cultivars, 10 scab-resistant apple cultivars, and 28 scab-resistant Coop selections. The sequence-specific primers allowed identification of polymorphisms of OPD20/600 based on the presence or absence of a single band. The advantages of sequence-specific primers over decamer primers for developing genetic markers are discussed.

Key words Apple · *Malus* · *Venturia inaequalis* · Apple scab resistance · RAPD marker

Introduction

Apple scab caused by *Venturia inaequalis* (Cke) Wint. is the most serious disease of apple trees in many areas of the world. Genes for resistance to apple scab have been identified from several *Malus* species (Shay et al. 1953; Williams and Kuc 1969). The resistance derived from the small-fruited species *Malus floribunda* clone 821 is determined by a major dominant gene designated *Vf*, which is the gene that has been the most widely used in scab resitance breeding programs all over the world (Crosby et al. 1992), as it is resistant to all five previously known races of *V. inaequalis* and to a large degree the recently identified sixth race found in Ahrensburg, Germany (Parisi et al. 1993). The durability of the *Vf* gene from *M. floribunda* clone 821 has made this gene an important target for mapping efforts in order to isolate, clone, and transfer it to scab-susceptible commercial apple cultivars.

Early in this century, the first cross between *M. floribunda* 821 and the scab-susceptible cultivar 'Rome Beauty' was made by Dr. C. S. Crandall at the University of Illinois. In 1945, fruit breeders of the University of Illinois and Purdue University used two F_2 seedlings from this cross to begin the cooperative scab-resistance apple breeding program. The program later included Rutgers University as well. Through these cooperative efforts, 38 scab-resistant selections have been released for advanced testing, each being identified under the designation of "Coop" to emphasize the joint effort extended in this program (Williams et al. 1967, 1972, 1975; Dayton et al. 1977; Williams et al. 1984; Korban et al. 1990; Crosby et al. 1993). The Coop selections have become increasingly important in apple production as the interest in growing apples with reduced pesticides has gained wide acceptance. So far, several Coop selections have been named and released (Korban and Morrisey 1989; Crosby et al. 1992), while others remain under testing or are being used as parents in crosses.

The development of molecular markers for identifying apple cultivars has been reported (Nybom and Schaal 1990; Harada et al. 1993; Mulcahy et al. 1993; Koller et al. 1993). More recently, random amplified polymorphic DNA (RAPD) markers have been identified that are associated with the *Vf* gene (Yang and Krüger 1994) and which verify gene introgression of *M. floribunda* 821 into the cultivated apple (Durham and Korban 1994). These markers have been identified by screening numerous decamer random primers and by using a modification of the bulked-segregant analysis of Michelmore et al. (1991).

In this paper, we describe the development of marker-sequence-specific polymerase chain reaction (PCR) primers, demonstrate their use in screening apple cultivars and scab-resistant Coop selections, and determine

Communicated by J. MacKey

H. Yang · S. S. Korban (✉)
Department of Horticulture, University of Illinois, 310 Madigan Bldg., 1201 W. Gregory Drive, Urbana, IL 61801, USA

the distribution of this RAPD marker in scab-resistant genotypes.

Materials and methods

Plant material and genomic DNA extraction

The following genotypes were used in this study: *Malus floribunda* 821 (original source of *Vf* gene); 7 scab-susceptible cultivars including 'Gala', 'Golden Delicious', 'Jonagold', 'Jonathan', 'McIntosh', 'Red Delicious', and 'Wijcik', 10 scab-resistant cultivars including 'Dayton', 'Enterprise', 'Goldrush', 'Jonafree', 'Prima', 'Priscilla', 'Pristine', 'Redfree', 'Sir Prize', and 'Williams' Pride'; and 28 scab-resistant Coop selections.

Leaf tissue was collected from mature trees growing in the field at the Pomology Research Center at the University of Illinois at Urbana, Illinois. Leaves were stored at −70 °C until needed. DNA was extracted following the protocol of Colosi and Schall (1993). Leaf tissue was ground in liquid nitrogen along with three bearing balls in a 2-ml microfuge tube and vortexed vigorously. DNA was extracted using 2 × CTAB.

Cloning and sequencing OPD20/600

PCR-based random amplification of DNA was conducted as described earlier by Yang and Krüger (1994). After separation of DNA fragments by electrophoresis, OPD20/600 was excised from the agarose gel, and TE buffer was added to the gel slice. Then the gel slice was minced using a pipet tip and discarded following 2 min of centrifugation in an Eppendorf microcentrifuge. The prepared DNA was re-amplified using the primer OPD20. Products of re-amplification were directly used for blunt-end ligation, which inserted the fragment into the *Sma*I site of the pBluescript M13 plasmid vector (Stratagene, La Jolla, Calif). The ligation reaction, in a 50 µl volume, was initiated using approximately 500 ng of the PCR product, 100 ng of *Sma*I-cut pBluescript M13 vector, and 10 units of T4 DNA ligase (Boehringer Mannheim, Germany). The ligations were incubated at 22 °C for 16 h. Ten microliters of ligation mixture was diluted with 20 µl of dH_2O and used to transform *E. coli* strain DH10B. Following transformation, cells were dispersed on LB plates containing 50 µg/ml ampicilin (AMP), 25 µl isopropylthiogalactoside (IPTG) (20 mg/ml), and 25 µl of X-gal (2%). The IPTG and X-gal were added to LB/AMP plates 30 min prior to the plating of the transformants. Plates were incubated overnight at 37 °C. The identity of the cloned RAPD fragment was confirmed by screening colonies using PCR. Vector-specific primers were used.

Using the dideoxy-chain termination method of Sanger et al. (1977), we conducted double-stranded DNA sequencing using a Sequenase Version 2.0 T7 DNA Polymerase (USB, Ohio) and M13/pUC forward and reverse-sequencing primers 17 bp in length (Boehringer Mannheim, Germany). Isolation and purification of template plasmid DNA for sequencing was conducted as described by Goode and Fernstein (1992).

Primer design and analysis

Primers of 25 bp were synthesized at the Genetic Engineering Facility at the University of Illinois. Apple cultivars and Coop selections were screened using a pair of 25-er primers; this was done by amplifying genomic DNA in a standard PCR reaction as described by Yang and Krüger (1994) with two modifications. The annealing temperature was increased from 35 °C to 72 °C; the reaction was reduced from 40 to 25 cycles of 30 s at 94 °C followed by 2 min at 72 °C.

Results and discussion

OPD20/600, the RAPD fragment linked to the *Vf* gene, was successfully cloned using the pBluescript M13 plasmid. Transformation was conducted using *E. coli* strain DH10B cells made competent with $CaCl_2$. The identity of the cloned products was verified by PCR using vector-specific primers (Fig. 1). Out of ten colonies tested, four were identified as recombinant pBluescript M13 plasmids, five showed a small fragment of 100 bp, and one had an insert of less than 600 bp in size. One of the four positive recombinant pBluescript M13 plasmids was selected in order to sequence the RAPD fragment OPD20/600 using M13/pUC forward- and reverse-sequencing primers. A 150-bp region from each of the 5′ and 3′ ends of the fragment was sequenced.

On the basis of sequencing results, two oligonucleotides of 25 bp were designed and used as sequence-specific primers for DNA amplification (Table 1). Each primer contained the original 10 bases of the RAPD primer plus the next 15 internal bases from the 3′ end. According to homology analysis using the OligoNet 1.0, no complimentary sequences were found between the two oligonucleotides and the interoligonucleotide.

When the two 25-er primers were used to screen individual apple cultivars, Coop selections, and *M. floribunda* 821 at an annealing temperature of 72 °C, the polymorphisms of the RAPD marker, OPD20/600, were identified by the presence or absence of a single band (Fig. 2). All background DNA fragments, which could be otherwise amplified by the decamer primer OPD20, were no longer detected on the gel.

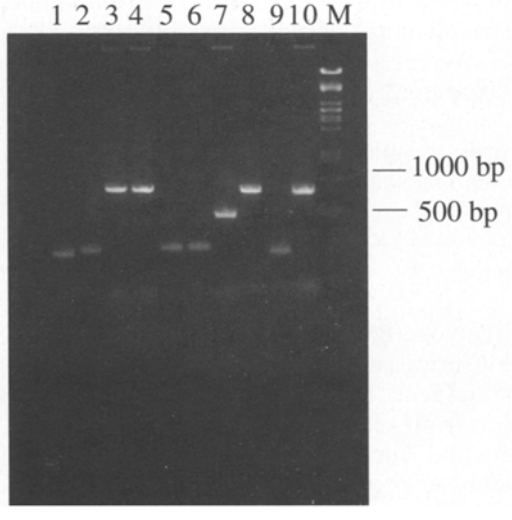

Fig. 1 Screening *E. coli* colonies for recombinant pBluescript M13 plasmid containing OPD20/600 inserts. *Lanes 1, 2, 5, 6, 9* no inserts, *lanes 3, 4, 8, 10* inserts of OPD20/600, *lane 7* insert of an unknown fragment, *lane M* λDNA restricted by *Pst*I

Table 1 Sequences of 25-er marker-sequence-specific primers

Primer	Nucleotide sequence
OPD20/600 I:	5′-ACC CGG TCA *CGC CCTCAC GGTTTTA*-3′
OPD20/600 II:	5′-ACC CGG TCA *CAC TGA AAA ATC TCTT*-3′

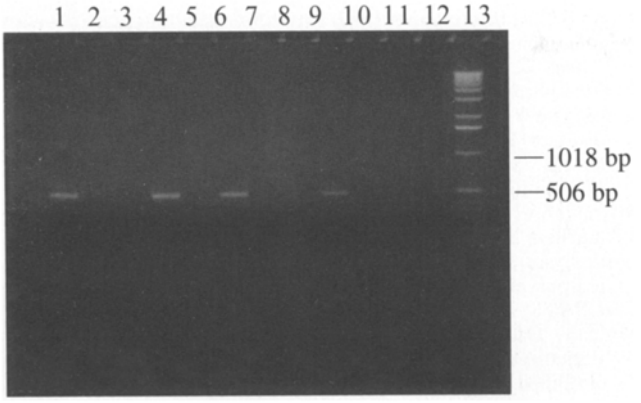

Fig. 2 Amplification of genomic DNA using a pair of 25-er primers. From *left* to *right*: *lanes 1–8* Coop 31–Coop 38, *lane 9 M. floribunda* 821, *lanes 10, 11, 12* 'Jonathan', 'Jonagold', and McIntosh', respectively, *lane 13* 1-kb ladder

For all of the scab-susceptible cultivars tested, including 'Gala', 'Golden Delicious', 'Jonathan', 'Jonagold', McIntosh', 'Red Delicious', and 'Wijcik', no amplification of OPD20/600 was detected. *M. floribunda* 821 showed the DNA fragment as a single band. The presence/absence of OPD20/600 in the genotypes tested is summarized in Table 2. Out of 28 Coop selections and 10 scab-resistant cultivars, 17 showed the presence of OPD20/600. This indicates that approximately 45% of all Coop selections/cultivars released so far carry this specific fragment and provides a strong evidence that the RAPD fragment OPD20/600 is linked to the *Vf* gene for scab-resistance derived from the wild species *M. floribunda*.

Since the Coop selections/cultivars carrying the *Vf* gene are at least five to six generations removed from the original source of *Vf*, *M. floribunda* 821, and assuming a 50% decrease in the effect of the gene locus after each hybridization cycle, the probability of a DNA fragment that is not being selected for to be retained in the Coop selections/cultivars should be about 3.7%. Therefore, the presence of the OPD20/600 in 45% of the Coop selections/cultivars suggests that OPD20/600 is associated with a common trait present in all Coop selections/cultivars, which is scab resistance.

Table 2 Presence/absence of OPD20/600 in Coop selections and cultivars developed from the scab-resistance apple breeding program[a]

Genotypes	Cultivars and selections with or without OPD20/600	
	+	−
Resistant cultivars	Dayton, Enterprise Jonafree, Prima, Redfree, Williams' Pride	Goldrush, Priscilla, Pristine, Sir Prize,
Resistant selections	Coops 1, 9, 12, 15 16, 24, 26, 29, 31, 34, 36	Coops 3, 6, 7, 8, 10 11, 14, 17, 18, 19, 20 25, 27, 28, 33, 35, 37,
Both groups	17	21

[a] All scab-susceptible cultivars did not contain the OPD20/600 fragment; *Malus floribunda* 821, original source of the scab-resistance gene *Vf* and used as parent in all these genotypes, contained the OPD20/600 fragment

It is interesting to note that Coops 35 and 37, which were OPD20/600-negative, were derived from a cross in which Coop 17 was one of the parents, which was also found to be OPD20/600-negative. Moreover, 'Pristine' (Coop 32), OPD20/600-negative, was a progenitor of Coop 10, which was also OPD20/600-negative. Durham and Korban (1994) identified a RAPD marker, OPA15/900, from *M. floribunda* 821, which was found to be introgressed into various Coop selections. The OPA15/900 was amplified in Coop 9, Coop 29, Coop 30, and Coop 36; all these genotypes also carried OPD20/600, therefore indicating that OPA15/900 might be also linked to the *Vf* gene.

It is known that RAPD techniques are sensitive to changes in reaction conditions, such as Mg^{++} concentration in the buffer and temperature conditions. These changes often do not allow accurate comparisons among results obtained in different laboratories. In addition, the production of background fragments in the agarose gel sometimes interfers with the identification of bands of interest. The use of 25-er marker-sequence-specific primers allows amplification of a single DNA fragment. This will improve the reliability of the RAPD fragment as a genetic marker (Paran and Michelmore 1993) and thus facilitates its application in marker-aided selection (Tanksley 1983). Therefore, OPD20/600 provides a useful genetic marker for the *Vf* gene for scab resistance.

Although several scab-resistance sources have been identified in different *Malus* species (Dayton and Williams 1968, 1970), the *Vf* gene has been most widely used in many breeding programs because it is believed to be the most stable (Hough 1944). Cultivars and selections carrying the *Vf* gene have been field-immune to apple scab for all five known races of *V. inaequalis* for over 50 years in the different countries where they have been grown. Since 1988, scab symptoms have been observed on the cultivar 'Prima' and a few *Vf* selections including Coops 7, 9, and 10 in Ahrensburg, Germany (Krüger 1988). Greenhouse inoculations with the German inoculum in France resulted in infection of some but not all cultivars and selections carrying the *Vf* gene (Parisi et al. 1993), indicating that the *Vf* resistance has been possibly overcome by the new race of *V. inaequalis*, designated as race 6. These events raise the urgency of diversifying the sources of resistance to *V. inaequalis* in the scab-resistant breeding material. Thus, additional efforts must be made to combine the independent sources of scab-resistance genes (Dayton and Williams 1968, 1970) in a new breeding strategy (Lespinasse 1989). Although scab resistance is a qualitative trait, screening a new progeny for the presence of different resistance genes based on phenotypic response requires time-

consuming testing procedures; moreover, the expression of the resistance gene(s) can be influenced by the environment and their reactions cannot be resolved (Lamb and Hamilton 1969). Therefore, DNA markers provide valuable tools for screening progenies for the presence of several different non-allelic scab-resistance genes.

Acknowledgements This project was supported in part by funds from the Illinois Agriculture Experiment Station, project no. 65-0325.

References

Colosi JC, Schaal BA (1993) Tissue grinding with ball bearing and vortex mixer for DNA extraction. Nucleic Acids Res 21:1051–1052

Crosby JA, Janick J, Pecknold PC, Korban SS, O'Connor PA, Ries SM, Goffreda J, Voordeckers A (1992) Breeding apples for scab resistance. Acta Hortic 317:43–70

Crosby JA, Janick J, Pecknold PC, Korban SS, Ries SM, Goffreda J, Voordeckers A (1993) Coop 32 to 38: seven disease-resistant apple selections released for advanced testing. Purdue Univ Agric Exp Sta Bull No. 658

Dayton DF, Williams EB, Janick J, Emerson FH, Hough LF, Bailey CH (1977) Coop 19, 20, 21, and 22: Four scab-resistant apple selections released for advanced testing. Purdue Univ Agric Exp Sta Bull No. 755

Durham R, Korban SS (1994) Evidence of gene introgression in apple using RAPD markers. Euphytica 79:109–114

Goode B, Feinstein SC (1992) Speedprep purification of template for double-stranded DNA sequencing. Bio Techniques 12: 374–375

Harada T, Matsukawa K, Sato T, Ishikawa R, Niizeki M, Saito K (1993) DNA-RAPDs detect genetic variation and paternity in *Malus*. Euphytica 65:87–91

Hough LF (1944) A survey of the scab resistance of the foliage on seedlings in selected apple progenies. Proc Am Soc Hortic Sci 44:260–271

Koller B, Lehmann A, McDermott JM, Gerrler C (1993) Identification of apple cultivars using RAPD markers. Theor Appl Genet 85:901–904

Korban SS, Morrisey JM (1989) Scab-resistant apple cultivars. Fruit Var J 43:48–50

Korban SS, O'Connor PA, Ries SM, Janick J, Crosby JA, Pecknold PC (1990) Coop 27, 28, 29, 30, and 31: five scab-resistant apple selections released for advanced testing. Ill Univ Agric Exp Sta Bull No. 789

Krüger J (1988) Beständigkeit der Schorfresistenz aus *Malus floribunda* 821 auf dem Versuchsfeld der Bundesforschunganstat für gartenbauliche Pflanzenzüchtung in Ahrensburg. Erwerbsobstbau 30:52

Lamb RC, Hamilton JM (1969) Environmental and genetic factors influencing the expression of resistance to scab (*Venturia inaequalis* (Cke.) Wint.) in apple progenies. J Am Soc Hortic Sci 94:554–557

Lespinasse Y (1989) Breeding pome fruits with stable resistance to diseases. In: Working group "Integrated plant protection in orchards", Subgroup "Integrated control of pome fruit diseases". Int Org Biol Integ Cont. WPRS Bull Vol 2, pp 100–115

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genome regions by using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Mulcahy DL, Cresti M, Sansavini S, Douglas GC, Linskens HF, Berganini MG, Vignani R, Pancaldi M (1993) The use of random amplified polymorphic DNAs to fingerprint apple genotypes. Sci Hortic 54:89–96

Nybom H, Schall BA (1990) DNA "fingerprints" applied to paternity analysis in apples (*Malus* × *domestica*). Theor Appl Genet 79:763–768

Paran I, Michelmore RW (1993) Development of relieble PCR-based markers linked to downy mildew resistance genes in lettuce. Theor Appl Genet 85:885–993

Parisi L, Lespinasse Y, Guillaumes J, Kruger J (1993) A new race of *Venturia inaequalis* virulent to apples with resistance due to the *Vf*-gene. Phytopathology 83:533–537

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Shay JR, Dayton DF, Hough LF (1953) Apple scab resistance from a number of *Malus* species. Proc Am Soc Hortic Sci 62:348–356

Tanksley SD (1983) Molecular markers in plant breeding. Plant Mol Biol Rep 1:3–8

Williams EB, Kuc J (1969) Resistance in *Malus* to *Venturia inaequalis*. Annu Rev Phytopathol 7:223–246

Williams EB, Janick J, Emerson FH, Dayton DF, Mowry JB, Hough LF, Bailey CH (1967) Five scab-resistant apple selections released for grower testing. Purdue Univ Agric Exp Sta Res Prog Rep No. 71

Williams EB, Janick J, Emerson FH, Dayton DF, Mowry JB, Hough LF, Bailey CH (1972) Six scab-resistant apple selections released for grower testing. Purdue Univ Agric Exp Sta Res Prog Rep No. 399

Williams EB, Janick J, Emerson FH, Dayton DF, Mowry JB, Hough LF, Bailey CH (1975) Coop 12–18: Seven scab resistant apple selections released for advanced testing. Purdue Univ Agric Exp Sta Bull No. 69

Williams EB, Janick J, Emerson FH, Korban SS, Dayton DF, Mehlenbacher SA, Hough LF, Bailey CH (1984) Coop 23, 24, 25, and 26: four scab-resistant apple selections released for advanced testing. Purdue Univ Agric Exp Sta bull No. 456

Yang HY, Krüger J (1994) Identification of an RAPD marker linked to the *Vf* gene for scab resistance in apples. Plant Breed 112:323–329