Theor Appl Genet (2004) 109: 231–239
DOI 10.1007/s00122-004-1624-x

ORIGINAL PAPER

P. Baldi · A. Patocchi · E. Zini · C. Toller · R. Velasco · M. Komjanc

Cloning and linkage mapping of resistance gene homologues in apple

Received: 26 October 2003 / Accepted: 29 January 2004 / Published online: 30 March 2004
© Springer-Verlag 2004

Abstract Apple (*Malus x domestica* Borkh.) sequences sharing homology with known resistance genes were cloned using a PCR-based approach with degenerate oligonucleotide primers designed on conserved regions of the nucleotide-binding site (NBS). Sequence analysis of the amplified fragments indicated the presence of at least 27 families of NBS-containing genes in apple, each composed of several very similar or nearly identical sequences. The NBS-leucine-rich repeat homologues appeared to include members of the two major groups that have been described in dicot plants: one possessing a toll-interleukin receptor element and one lacking such a domain. Genetic mapping of the cloned sequences was achieved through the development of CAPS and SSCP markers using a segregating population of a cross between the two apple cultivars Fiesta and Discovery. Several of the apple resistance gene homologues mapped in the vicinity, or at least on the same linkage group, of known loci controlling resistance to various pathogens. The utility of resistance gene-homologue sequences as molecular markers for breeding purposes and for gene cloning is discussed.

Introduction

Plant disease resistance genes have been cloned and characterized from a number of species, including both mono- and dicotyledonous plants (Hammond-Kosack and Jones 1997; Martin 1999). Most of the resistance genes cloned so far belong to the leucine-rich repeat (LRR) family and encode proteins containing an LRR domain near the C-terminus. The LRR domains show hypervariability and are thought to confer recognition specificity. In addition to the LRR domain, many resistance genes possess a nucleotide-binding site (NBS) near the N-terminus and additional structures such as a leucine zipper or a toll-interleukin receptor (TIR) (Bent 1996). The NBS-LRR class resistance genes are proposed to act as receptors in signal-transduction pathways that are triggered in response to pathogen attack (Hammond-Kosack and Jones 1997). The NBS regions of known resistance genes contain several highly conserved motifs, in spite of the diversity of pathogens against which they act. The P-loop and the kinase-2 domains have been described as ATP- and GTP-binding sites (Meyers et al. 1999; Traut 1994). Additional motifs found in the NBS region of plant LRR resistance genes are the kinase-3a and the GLPL (also called “hydrophobic domain”), a putative membrane-spanning domain.

A polymerase chain reaction (PCR)-based strategy for cloning NBS-LRR resistance gene homologues (RGHs) has been devised, using degenerate primers designed on the conserved sequence motifs within the NBS region (Kanazin et al. 1996; Leister et al. 1996). This approach has been successfully applied in different plant species such as soybean (Kanazin et al. 1996; Yu et al. 1996), potato (Leister et al. 1996), barley and rice (Leister et al. 1998), wheat (Seah et al. 1998), and *Arabidopsis thaliana* (Aarts et al. 1998). In many cases the genomic locations of the RGH sequences were obtained using molecular linkage maps, and at least some RGHs showed close genetic linkage with known resistance genes. Therefore, genomic regions likely to contain resistance genes may be identified by cloning and mapping RGHs. Moreover, the RGH sequences themselves may represent candidate resistance genes, as for example in *A. thaliana* (Aarts et al. 1998).

Communicated by H. Nybom

P. Baldi (✉) · E. Zini · C. Toller · R. Velasco · M. Komjanc
Istituto Agrario San Michele a. Adige,
via E. Mach 1,
38010 San Michele all’Adige, TN, Italy
e-mail: paolo.baldi@mail.ismaa.it
Tel.: +39-0461-615378
Fax: +39-0461-650956

A. Patocchi
Plant Pathology, Institute of Plant Science, Swiss Federal Institute of Technology, ETH Zurich,
Zurich, Switzerland

Several diseases and pests constrain apple production worldwide, affecting the yield and forcing heavy use of chemicals to control the pathogens. It is therefore a primary target to obtain resistant cultivars in order to drastically reduce the amount of fungicides applied to crops and introduced in the environment. One of the most important diseases affecting apple is apple scab, caused by the fungus *Venturia inaequalis* (Cooke) Wint. Although several major scab-resistance genes have long been known (Williams and Kuç 1969), only one of them, the *Vf* gene, has been successfully incorporated into commercially available cultivars. Already a low deployment of this single major apple scab gene has caused a high selection pressure on the pathogen population. This has led to the natural selection of two races of scab that have overcome the *Vf* resistance. These two races are nowadays present in some European locations (Parisi et al. 1993).

A possible strategy to reduce the risk of resistance breakdown and to achieve durable resistance could be the combination of different major and minor resistance genes in a single cultivar so that, to be able to infect the plant, the pathogen has to simultaneously circumvent several resistance mechanisms. It is therefore important to identify new sources of resistance within the apple genome, conferred by new and still unknown major resistance genes, or by genes conferring quantitative or partial resistance against pathogens. In apple, quantitative resistances have been described for mildew (Janse et al. 1994; Seglias and Gessler 1997) and scab (Liebhard et al. 2003a). Moreover, several known scab-resistance genes (i.e., *Vb* and *Va*) have not yet been mapped, and probably new sources of resistance are still undiscovered in the apple genome. Therefore, obtaining a collection of RGH sequences could be an effective strategy to identify genomic regions linked to disease resistance (Leister et al. 1996, 1998; Yu et al. 1996). Here we report the cloning and characterization of 30 apple resistance genes homologues (ARGHs) by the use of degenerate primers designed to bind the P-loop, kinase-2, and GLPL elements of the NBS region. We show that multiple and diverse ARGHs exist in the apple genome. Eighteen of these were mapped using a linkage map of the apple cultivars Fiesta and Discovery (Liebhard et al. 2003b).

Materials and methods

PCR amplification of RGH sequences

Total genomic DNA was extracted from leaves of the apple cultivar Florina, carrying *Vf* resistance against scab, according to the method described by Aldrich and Cullis (1993). Three different pairs of degenerate primers were used for PCR amplifications: (1) B1: 5′-GGIGGIRTIGGIAARACIAC-3′ (forward) (Rivkin et al. 1999) and B2: 5′-WTIARIGYIARIGGIARICC-3′ (reverse) (Rivkin et al. 1999); (2) P-loop: 5′-GAATTCGGNGTNGGNAAGACAAC-3′ (forward) (based on Shen et al. 1998) and LDD-AS: 5′-CCAIACATCATCWAGSACAA-3′ (reverse) (Leister et al. 1999); and (3) BP2f: 5′-GGNGGDGTDGGSAARAC-3′ (forward) and BP2r: 5′-GCTAGTGGCAMNCCWCC-3′ (reverse).

The PCRs were carried out in a total volume of 50 µl with 100 ng template DNA and 0.25 µM of each primer in 10 mM Tris HCl (pH=8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 0.1 mM dNTPs, and 0.5 U of *Taq* polymerase (Biolyne). Amplification included initial denaturation for 3 min at 94°C, followed by 40 cycles of 94°C for 1 min, 50°C for 45 s, and 72°C for 1 min, then a final extension of 72°C for 8 min. After electrophoresis through a 2% agarose gel in 0.5× TAE, amplification products were visualized by staining with ethidium bromide.

Cloning and analysis of PCR products

Bands of the expected size of each amplification were excised from gels, eluted, and cloned using the pGEM-T Easy Vector System following the manufacturer's instructions (Promega, Madison, Wis.). Individual clones were digested using the *Alu*I enzyme and resolved on a 2% agarose gel. All the clones showing different restriction patterns and also several showing very similar restriction patterns were chosen for sequencing. Sequencing reactions were prepared using the Perkin Elmer Big Dye DNA sequencing kit (Perkin Elmer, Foster City, Calif.). Sequencing reactions were cleaned using the Centri-sep spin columns (Princeton Separations, Adelphia, N.J.) and read using an ABI PRISM 310 Genetic Analyzer (Perkin Elmer). Sequence analysis and alignments were carried out using the online version of CLUSTALW (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). Phylogenetic analysis was performed using the neighbor-joining and UPGMA methods with PAUP (Sinauer, Sunderland, Mass.) software.

Marker development and ARGH mapping

To develop PCR-based markers for genetic mapping of RGH sequences, regions that were divergent among clones were identified by multiple-sequence alignment and subsequently used for designing specific primers. Computer software Oligo 4.0 was used to facilitate the identification of sequence segments with desirable internal stability curves as priming sites and to avoid potential 3′ dimer or hairpin formation (Rychlik 1995). Primer length ranged from 20 to 24 bases in order to obtain similar melting temperatures. PCR reactions were prepared and run essentially as described above, but with 35 cycles of 94°C for 45 s, 55°C for 1 min, and 72°C for 1 min. The amount of template DNA varied from 5 ng to 100 ng.

Initially each pair of primers was evaluated for amplification and polymorphism with genomic DNA from Fiesta and Discovery—the seed and pollen parents of the population used to map the RGHs. Restriction digestions required to reveal polymorphism between the two parents were performed using 8 µl (for polyacrylamide gels) or 15 µl (for agarose gels) of each PCR reaction. PCR products and their digestions were separated on 2% agarose gels or nondenaturing polyacrylamide gels and detected by staining with ethidium bromide or silver staining (Sanguinetti et al. 1994), respectively. Markers defined by a pair of specific primers and restriction-enzyme digestion are referred to as cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel 1993). Markers defined by a pair of specific primers, restriction enzyme digestion, and separation on nondenaturing polyacrylamide gels are referred to as single strand conformation polymorphism (SSCP) markers (Orita et al. 1989).

ARGH mapping was performed using a subset of the Fiesta × Discovery population comprising 44 individuals (Liebhard et al. 2003b). Mapping procedure was the same as that employed by Liebhard et al. (2003b), but the LOD score used was decreased from 4.0 to 3.5. The software used was Joinmap 2.0 (Stam and van Ooijen 1995) in connection with JMDesk 3.6 (http://www.ecogenics.ch/software-e.html).

Results

Cloning and characterization of RGH sequences

All three primer combinations used showed a band of the expected size after PCR amplification (Fig. 1). Primer combinations II and III each generate a band of around 500 bp, while primer combination I produces a band of approximately 300 bp, due to the reverse primer being designed on the kinase-2 domain. The bands of interest were excised from the gel and cloned into the pGEM-T vector. Restriction analyses of PCR-amplified inserts of randomly selected clones indicated that the 500-bp and the 300-bp DNA bands contained heterologous fragments. All the clones showing different restriction patterns, as well as several clones showing very similar restriction patterns, were identified and sequenced. A total of 114 clones were sequenced, and 52 showed significant homologies with known RGHs after searching in GenBank using the BLASTX algorithm. Nevertheless, many sequences turned out to be very similar or nearly identical, so for nucleotide-translation analysis purposes, all the sequences sharing more than 95% homology were considered to represent a single family of ARGHs. In this way 30 different families have been identified (Table 1) and deposited in GenBank with accession numbers AY369209–AY369235 and AY378695–AY378697.

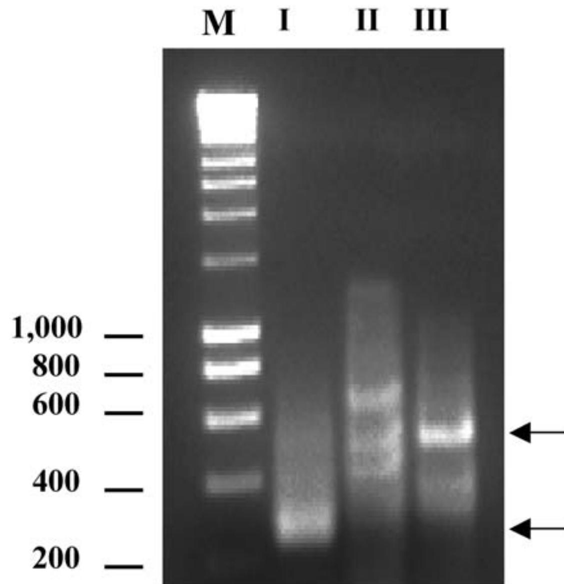

Fig. 1 PCR amplification of genomic DNA using three primer combinations. *M* Marker; *I* P-loop forward, LDD-AS reverse; *II* BP2f forward, BP2r reverse; *III* B1 forward, B2 reverse. The bands cloned are indicated by *arrows*. The size of marker DNA fragments is indicated to the *left* of the picture in base pairs

Sequences were translated to look for motifs characteristic of plant NBS regions (Meyers et al. 1999). Some ARGH sequences (ARGH15, ARGH19, and ARGH43) contained stop codons or frame-shift mutations that resulted in distorted amino acid sequences; therefore, these clones were excluded from further analysis. Figure 2 shows the amino acid alignments of the functional motifs of ARGH families together with six known resistance genes. The P-loop and the GLPL motifs, or the kinase-2 motif for the sequences cloned with primer combination I, were included in the degenerate primers and, therefore, excluded from the sequence data analysis. The encoded amino acid sequences showed all the characteristic motifs of plant NBS regions, such as kinase-2 and kinase-3a domains. Translated sequences were also analyzed visually to search for the additional motifs found by Meyers et al. (1999). Two of these motifs are useful for separating plant NBS genes into two major classes: TIR-NBS and non-TIR-NBS. The RNBS-A-non-TIR motif (see Fig. 2) occurs in the NBS region of resistance genes that do not contain the TIR domain, and was found in ARGH04, ARGH13, ARGH20, ARGH21, ARGH31, ARGH32, ARGH34, ARGH35, and ARGH42, suggesting that these sequences correspond to NBS regions of non-TIR-NBS-LRR genes. All the other ARGHs contained the RNBS-A-TIR motif, suggesting that the corresponding genes belong to the TIR-NBS-LRR family that possesses the TIR domain (Meyers et al. 1999). Also the last residue of the kinase-2 domain can be used to predict with 95% accuracy whether an RGH would belong to the TIR-NBS or the non-TIR-NBS family. As expected, a tryptophan residue (W) was found in all the sequences carrying the RNBS-A-non-TIR motif, while an aspartic acid residue (D), with the only exception of ARGH16 carrying a glutamic acid (E), was found in the sequences presenting the RNBS-A-TIR motif. For all the sequences cloned with

Table 1 Degenerate primers used in the cloning of resistance gene homologue (RGH) sequences in apple and apple resistance genes homologues (ARGH) families found for each primer combination

Primer combinations	Degenerate primers		ARGH clones
	Forward	Reverse	Family[a]
I	P-loop	LDD-AS	ARGH 20*, ARGH21, ARGH30, ARGH 31*, ARGH32, ARGH34, ARGH35, ARGH39, ARGH41, ARGH42, ARGH43*
II	BP2f	BP2r	ARGH15*, ARGH16, ARGH17, ARGH19*, ARGH22, ARGH23, ARGH25, ARGH26
III	B1	B2	ARGH02*, ARGH04, ARGH06, ARGH08, ARGH09, ARGH11, ARGH12, ARGH13*, ARGH37, ARGH40, ARGH46

[a]Some families are represented by only one sequence (indicated by *), while others contain several sequences with high sequence similarity (homology ≥95%)

```
                RNBS-A-TIR                      Kinase-2     Kinase-3a              RNBS-C

ARGH39   FKGSSFIENIRETADRPNGLVQIQKQLLFDIL--26aa--LL -------------------------------------------------
ARGH41   FERSSFIENINERADRPNGLVQIQMQLLSDIL--26aa--LL -------------------------------------------------
L6       FDCCCFIDNIRETQEK-DGVVVLQKKLVSEIL--28aa--LVVLDDVD--17aa--QSRFIITSRS --14aa--YEVGSMSKPRSLELFSKHAF
ARGH06   FEVPCFLENVREASRADPTLIRLQETLLSPML--23aa--LLVLDDVD--17aa--GSRIIITTRN --12aa--HNVQVLDKGAALALFSLHAF
ARGH11   FEVHCFLENVREVSNVD-----LQKRLLRPML--26aa--LLVLDDVD--17aa--GSRIIITTRN --12aa--HNVQVLDKGAALALFSLHAF
ARGH08   FEVHCFLGNVREVSKINRTLIGLQKRLLFPML--26aa--LLVLDDVD--17aa--GSRIVITTRN --12aa--YKMRGLNDCEALELFSLNAF
ARGH12   FEGSSFLANVRGVCSKDG-IAHLQKQLLSEIL--26aa--LLILDDVD--17aa--GSRIVITTRD --12aa--YEVKPLTQDEALFLFCRNAF
ARGH02   FEACCFLDNVKEEFAYG--AVHMQEKFLSRIL--26aa--LIVLDDVD--17aa--GSRIIITTRD --10aa--YSPKVLSGDGALELFSQYAF
N        FDGACFLKDIKEN-KRG--MHSLQNALLSELL--25aa--LIVLDDID--18aa--GSRIIITTRD --10aa--YEVTALPDHESIQLFKQHAF
ARGH17   FQFKSFLADVRDATSKHG-LVDLQNKLISDIL--25aa--LVIMDNID--17aa--GSRIILTTRD --12aa--YPAQKFNEGEALELFSWHAF
ARGH09   FEFKSFLDDVNDTTSRHG-LVYLQKKLISDIL--25aa--LVIMDNID--17aa--GSRIIITTRD --10aa--YPAQKLKDRESLELFSWHAL
ARGH22   FEFKSFLTDVSDTGNKHG-MVHLQEKLIFDIL--25aa--LVIIDNVD--17aa--GSRIIITTRD --10aa--YPAQKLNKEEALELLSWHAF
ARGH37   FQFKSFLHDISNTASKHG-LVHLQEKLISDIL--25aa--LVIMDNID--17aa--GSRIIITTRD --10aa--YPAQKLNEREALGLFSWHAL
ARGH16   FQFKCFLADVSDTVSKYG-LIYLQEQLVSNIL--27aa--LLVIDDVE--17aa--GSIIIITTRD --12aa--YPTWVMNEEEALELFSWHAF
ARGH23   FQFKCHLGDVSDTEHRYG-LVYLQKQLVSNML--27aa--LIVIDNVD--17aa--GSIIIITTRD --12aa--YPAKEMNEEEALELFSWYTF
ARGH46   FEGKSFLQNVR----ERK-LVQVQEQLLFDIL--26aa--LAIVDDID--17aa--GSRIIITTRD --12aa--YELPVMNREEALMLLSWHAF
ARGH40   FAGKSFLANVR----EEK-LVDVQNILLYDVL--26aa--LVIVDGVD--17aa--GSRIIITTRD --12aa--CHLPAMNEKEALELLSRRAN
ARGH25   FDGASFLENVR----EKK-LVDLQEQFLSDIL--26aa--LVVIDDLD--17aa--GSRIIVTSRN --12aa--CPLPSMSEREALELLSLHAF
ARGH26   FSRQCYLEEVRS--KKKK-MVSLQEQLLRDIL--26aa--LIVVDDID--17aa--GSRIIITTRD --12aa--YKAQEMTNEEAFELFSWHAF

         RNBS-A-nonTIR                          Kinase-2     Kinase-3a              RNBS-C

ARGH13   FEKRVWVCVSDPF-----------36aa-----------LLVLDNVW--18aa--Truncated----------------------------
ARGH20   FQKRIWVCVSEPF-----------36aa-----------LL -------------------------------------------------
ARGH04   FELKVWVSVSDDF-----------39aa-----------LIVLDDIW--23aa--GSKIIVTTRD --12aa--HNLECMANDDCLEIFERHAF
ARGH32   FDTNAWVCVSEQY-----------39aa-----------LL -------------------------------------------------
ARGH42   FDTNAWVCVSEQY-----------39aa-----------LL -------------------------------------------------
ARGH31   FDMNAWVCVSEQY-----------39aa-----------LF -------------------------------------------------
ARGH21   FDVRAWACVSEDF-----------39aa-----------LF -------------------------------------------------
I2C-2    FDLKAWFCVSEAY-----------39aa-----------LIVLDDVW--19aa--GSKIIVTTRK --11aa--ISMGNLSTEASWSLFQRHAF
ARGH34   FDCYAWITASQSY-----------46aa-----------IV -------------------------------------------------
RPM1     FESYAWVTISKSY-----------46aa-----------IVVLDDVW--17aa--GSRVMMTTRD --14aa--HEIELLKEDEAWVLFSNKAF
ARGH35   FKPRAWITVSQSF-----------46aa-----------LI -------------------------------------------------
MLA6     FDCRAFVPVGQNP-----------41aa-----------LVIIDDIW--18aa--GSRLITTTRI --15aa--YQMEPLSVDDSRMLFSKRIF
RPS2     YDVLIWVQMSREF—----------39aa—----------LLLLDDVW--17aa--KCKVMFTTRS —-12aa--LRVEFLEKKHAWELFCSKVW
ARGH30   LPFSGMREEEEQF-----------40aa-----------LI
```

Fig. 2 Amino acid alignment of functional motifs from apple resistance gene homologues (ARGHs) and six known resistance genes: tobacco *N*, tomato *I2C-2*, *Arabidopsis RPM1* and *RPS2*, flax *L6*, and barley *MLA6*

primer combination I, it was not possible to determine the last amino acid of the kinase-2 domain as it was included in the degenerate primer used for the PCR amplification. A region similar to the consensus sequence for the RNBS-C motif (Meyers et al. 1999) was found in all of the sequences obtained with primer combinations II and III, confirming that they belong to the NBS-LRR family.

In order to further evaluate the relationship among apple ARGHs and known plant resistance genes, a phylogenetic analysis was performed. The deduced amino acid sequences of representative members of 27 ARGH families and the NBS domain of six known resistance genes were aligned and a neighbor-joining tree was generated from the alignment (Fig. 3). The dendrogram divided the sequences with high bootstrap values into two broad subfamilies: all the TIR-NBS sequences were present in one major cluster and the non-TIR-NBS in a second one. The two major branches in both cases were divided into at least three secondary groups. ARGH30 appeared to be rather distant from all the other ARGHs. The majority of the tree nodes were supported with >70% of the 1,000 replicates in bootstrap analysis.

Marker development and linkage mapping

To facilitate genetic mapping of the cloned ARGH sequences and an assessment of their potential linkages with disease-resistance genes in apple, 27 primer pairs were designed on the divergent DNA sequence regions (Table 2). When genomic DNA of the two cultivars Fiesta and Discovery was amplified with all 27 primer pairs, only a primer pair designed on ARGH42 showed a presence/absence polymorphism on 2% agarose gel. A single band of the same, expected size was observed for all the other ARGHs. To reveal polymorphisms, a panel of restriction enzymes (mainly enzymes with four-base recognition sites) was used to digest the PCR products. Polymorphism was observed in only a few cases (ARGH02, ARGH12, and ARGH23) between Fiesta and Discovery after digestion and electrophoresis on 2% agarose gel, allowing the development of CAPS markers. All the other ARGH sequences were therefore separated (after digestion) on nondenaturing acrylamide gel. In 14 out of 24 cases a polymorphism was shown, thereby allowing the development of SSCP markers. ARGH04, ARGH06, ARGH09, ARGH11, ARGH16, ARGH21, ARGH24, ARGH38, ARGH40, and ARGH41 did not show any polymorphism, even after electrophoresis on acrylamide gel, and therefore were not mapped. In Table 2 the specific primers used for the development of CAPS and SSCP markers are shown, together with the enzymes presenting polymorphisms and the resulting marker type. ARGH23 was the only sequence to show two different polymorphisms: the first on agarose gel after digestion with *Alu*I and the second (ARGH23b) on acrylamide gel after digestion with *Cfr*13I. Genetic

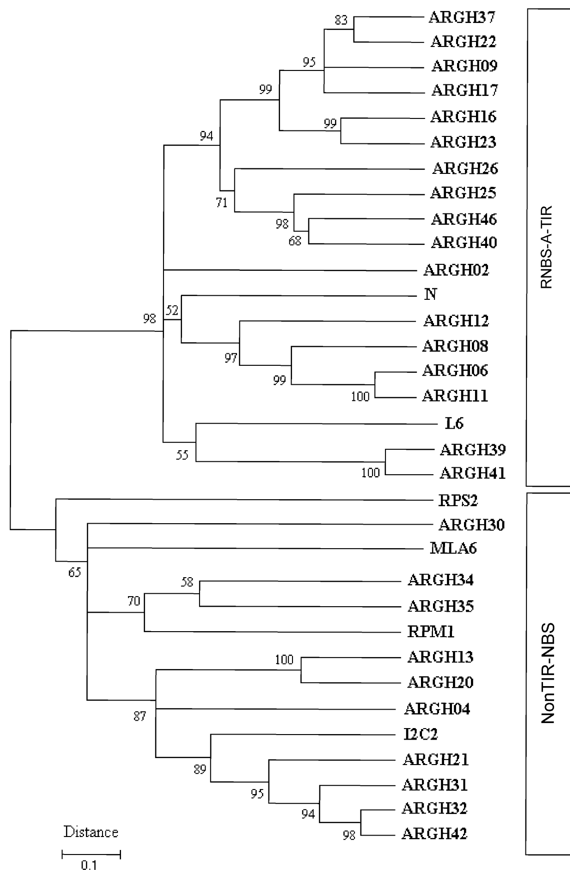

Fig. 3 Phylogenetic tree based on the alignment of amino acid sequences from ARGH clones and six known resistance genes. Bootstrap values based on 1,000 replications are indicated on the *branches*

mapping of members of the different ARGH classes was performed with 44 individuals from the Fiesta × Discovery cross described in Liebhard et al. (2003b). The ARGH loci mapped both singly and in clusters and were widely distributed among the apple genome, being present in 12 out of 17 linkage groups (Fig. 4). In some cases, the ARGH sequences were located in the proximity of known loci containing genes or QTLs for disease resistance. Particularly ARGH34 mapped on linkage group 1, very close to the *Vf* locus. On linkage group 2, three ARGH sequences were found; ARGH17 and ARGH37 were placed at the same position, about 4 cM from the SSR marker CH02c02a (Fig. 4). Recently *Vr2*, a gene conferring resistance against apple scab, was found to cosegregate with the same SSR marker (Patocchi, unpublished data). ARGH20 was placed on linkage group D11, very close to E37M34-0094a. In that region, a QTL for leaf and fruit scab resistance has been found (Liebhard et al. 2003a). QTLs for scab resistance have also been found in linkage groups 6, 7, and 10 (Liebhard et al. 2003a). In all cases, an ARGH was also present in the same linkage group, ARGH35, ARGH39, and ARGH08, respectively.

ARGH02 and ARGH30 mapped to the same position on linkage group F17; all the other ARGHs mapped singly on different linkage groups, with the exception of ARGH31 and ARGH42, which were located on linkage group D3 at about 2 cM from one another (Fig. 4).

Discussion

Improvement of disease resistance is one of the priorities in apple breeding. The use of molecular techniques, such as the development of DNA markers, can significantly expedite the process. PCR approaches with degenerate oligonucleotide primers designed from the NBS region of cloned disease-resistance genes have led to the cloning of similar sequences from many plant species, not only soybean (Kanazin et al. 1996; Yu et al. 1996), rice and barley (Leister et al. 1998), and *A. thaliana* (Aarts et al. 1998), but also tomato (Pan et al. 2000a), pea (Timmerman-Vaughan et al. 2000), and melon (Brotman et al. 2002). Correlation or cosegregation of some of these sequences with known disease-resistance gene loci has been documented. In this study, we have isolated more than 50 genomic fragments homologous to NBS-LRR genes, thus allowing a first sampling of this family in apple. However many sequences showed little or no variation (<5%) and might represent alleles of homologous genes or even polymerase errors. Therefore the ARGHs were divided into 30 families sharing less than 95% nucleotide sequence homology. Probably many more similar sequences are present in the apple genome as only three primer combinations (based on the sequences of known resistance genes) were used. In fact the NBS-LRR class of resistance genes has been proven to be a very large family in plants; in *Arabidopsis* it has been estimated that about 1% of the entire genome may be represented by NBS-LRR-like genes (Meyers et al. 1999). Large sets of related sequences with different recognition specificities were found within a single plant species; this may reflect an ongoing competition between pathogens and plants that causes rapid evolution of such genes (Parniske et al. 1997).

NBS-LRR sequences are usually divided into two main groups (Meyers et al. 1999; Pan et al. 2000b): type I contains the TIR element (toll-interleukin-1 receptor-like domain) and has been found only among dicots, while type II lacks the TIR domain and has been found both in dicots and monocots. The partial sequence of the NBS portion is usually sufficient to assign a given gene to either type I or II. Therefore it was possible to divide the cloned ARGH sequences into TIR and non-TIR-NBS according to the sequence data analysis. Seventeen of the ARGH fragments clustered with typical TIR genes such as tobacco *N* and flax *L6*, while ten sequences clustered with *Arabidopsis RPS2* and *RPM1,* tomato *I2C-2*, and barley *MLA6*, which belong to the non-TIR group (Fig. 3). Moreover ARGH-TIR sequences seemed to be divided into at least three different groups. Also in other species, phylogenetic analyses clustered TIR-NBS gene homologues into different major groups (Cordero and Skinner 2002). However it is not known whether this divergence is functionally significant.

Table 2 Polymorphisms identified with ARGH-derived primers and the corresponding type of marker obtained. All polymorphisms were detected between the two parental lines, cultivars Fiesta and Discovery. In some cases, more than one enzyme could produce polymorphism, but the segregation was always the same. The only exception was ARGH23

RGH clones	Primers		Polymorphisms	Marker type
	Forward	Reverse		
ARGH 2	GTTCATGAGAAAATTGCTTGTC	TCATAGTCTCTTTTGGGTTGC	*Mbo*I	CAPS
ARGH 4	GTATTCAACAACAAAAATGATGC	CTGGTTTTCCACTATTAACTTC	None	
ARGH 6	TTCTAGAGCAGACCCTACTCT	TGCGCCTTTATCTAACACTTG	None	
ARGH 8	CTAAAATAAACCGTACTCTAATTG	GTCTTTCTTAGGTTGTTCTTTC	*Tru*I	SSCP
ARGH 9	CTTGATGACGTTAACGACACT	TATCAGGCCAACTATTTCCAAG	None	
ARGH 11	GTTAGAGAGGTTTCTAACGTTG	CTGCGCCTTTATCTAACACTTG	None	
ARGH 12	GTTTACGAGAGAATTTCTCATG	GAGAAAAAGAGCTTCATCTTGG	*Alu*I	CAPS
ARGH 16	AACAAAATTCATCACAACTTCCA	ATGATATTCTTCATTAGGCAAAC	None	
ARGH 17	TTGCCGACGTTCGTGATGCT	GATATCCTTTGTTTGGACAACC	*Rsa*I	SSCP
ARGH 20	GTTAGTCTATAATGATGCCAATG	CAAGGACAAGAAGAAACTTTAC	*Hin*6I	SSCP
ARGH 21	TCAGCTCCTTTACAATAATGAC	ATCATCAAGGACAAATAGAAATC	None	
ARGH 23	CATTGCTTCCAGTTTAAATGTC	CAAATGTATACCAACTGAAAAGC	*Alu*I	CAPS
ARGH 23b	CATTGCTTCCAGTTTAAATGTC	CAAATGTATACCAACTGAAAAGC	*Cfr*13I	SSCP
ARGH 24	ATTTATAACCAGGTTTATCATAAG	GATATCCTTCATTCGGATAACTA	None	
ARGH 25	CAAACATCATCGTAATTTTGACG	CATACTCTTCATGAGGATAATTC	*Cfr*13I	SSCP
ARGH 26	CAATTATCAGCAAAGTTTTAGTC	TTCGTTGGGACTAGGATTTTC	*Cfr*13I	SSCP
ARGH 30	GAGTAAAGGGTAAGTTTAATCC	ACAATCAGATACCTCTTACCAG	*Rsa*I	SSCP
ARGH 31	CCCAAGTCCTTTACAATGATG	ATCATCCAGCACAAATAAAAATG	*Mbo*I	SSCP
ARGH 32	CTCAACTGCTTTACAATGATGG	CATCCAGGACAAGTAAAAATC	*Taq*I, *Rsa*I	SSCP
ARGH 34	TGTATGACCAGCCGAAGGTG	CCAGGACAACAATGTACCTC	*Taq*I	SSCP
ARGH 35	GTCTACGATGATGATGAAGTG	TCAGGTACCTCCTTTTCTGC	*Taq*I, *Cfr*13I	SSCP
ARGH 37	TGCACGACATTAGCAACACTG	GAAACAACTTCTTTGAGAGTTC	*Alu*I	SSCP
ARGH 38	TATAATGATAAGAGGGTAGTGC	AATCTGCTTGGGAATCAGAAC	None	
ARGH 39	AACTTTAGAAGCTTTAAAGGAAG	TCTTCTAGAGCTTATGGCACTT	*Alu*I	SSCP
ARGH 40	TTTTCAACAAATATCCGGATAAG	GTAGATGACATATCCTATCCG	None	
ARGH 41	AAATTTTATTGGCTTTGAAAGAAG	TTTTCTAGAGCTTATGGCGCTT	None	
ARGH 42	ACGATGAACAAGTTACAGAGC	CTCTTTCCTATCAATTGTTGCC	P/A[a]	
ARGH 46	AATATCAGGATACGTTTGAAGG	CCACAGTAGTCAGCAGTTTC	*Tru*I	SSCP

[a] *P/A* Indicates presence or absence of the DNA band

The development of molecular markers using the cloned ARGH sequences was not an easy task. When specific primers designed on the cloned sequences were used to amplify genomic DNA from the two cultivars, in only one case (ARGH42) could we detect a presence/absence polymorphism. In all other cases, a single band was visible on a 2% agarose gel and no polymorphism was detected. Even after digestion with a panel of four-cutter restriction enzymes, only 3 out of 26 ARGHs showed polymorphisms on agarose gel, indicating that very few differences were present at the Fiesta and Discovery alleles. To obtain greater resolution, the digested PCR products were denatured and loaded onto nondenaturing polyacrylamide gel. Under these conditions, the single-strand DNA fragments migrate according to their primary nucleotide sequence and not their length (Orita et al. 1989). DNA fragments with sequences that differ by one or few nucleotides usually migrate differently, allowing very similar alleles to be distinguished. Using the SSCP technique, a polymorphism between Fiesta and Discovery could be identified in 14 additional sequences.

When mapped, the ARGHs appeared to be widely distributed in the apple genome, covering 12 out of 17 linkage groups. In many species, the RGH sequences have been shown to map in clusters that are sometimes composed of several related genes. In soybean, one reported gene cluster contained two phenotypically defined resistances against *Phytophtora* and powdery mildew, and five RGH clones (Kanazin et al. 1996). The fact that different resistance genes are organized in clusters could be of evolutionary importance, as clusters of resistance genes and their homologues may undergo unequal crossover and generate new alleles. We also found that in apple, several of the cloned RGHs appeared to map in clusters. At the *Vf* locus, Vinatzer et al. (2001) identified a cluster of genes homologous to the *Cf* gene family of tomato called *HcrVf* (homologue to the *Cladosporium fulvum* resistance genes of the *Vf* region). These genes encode proteins containing an extracellular LRR

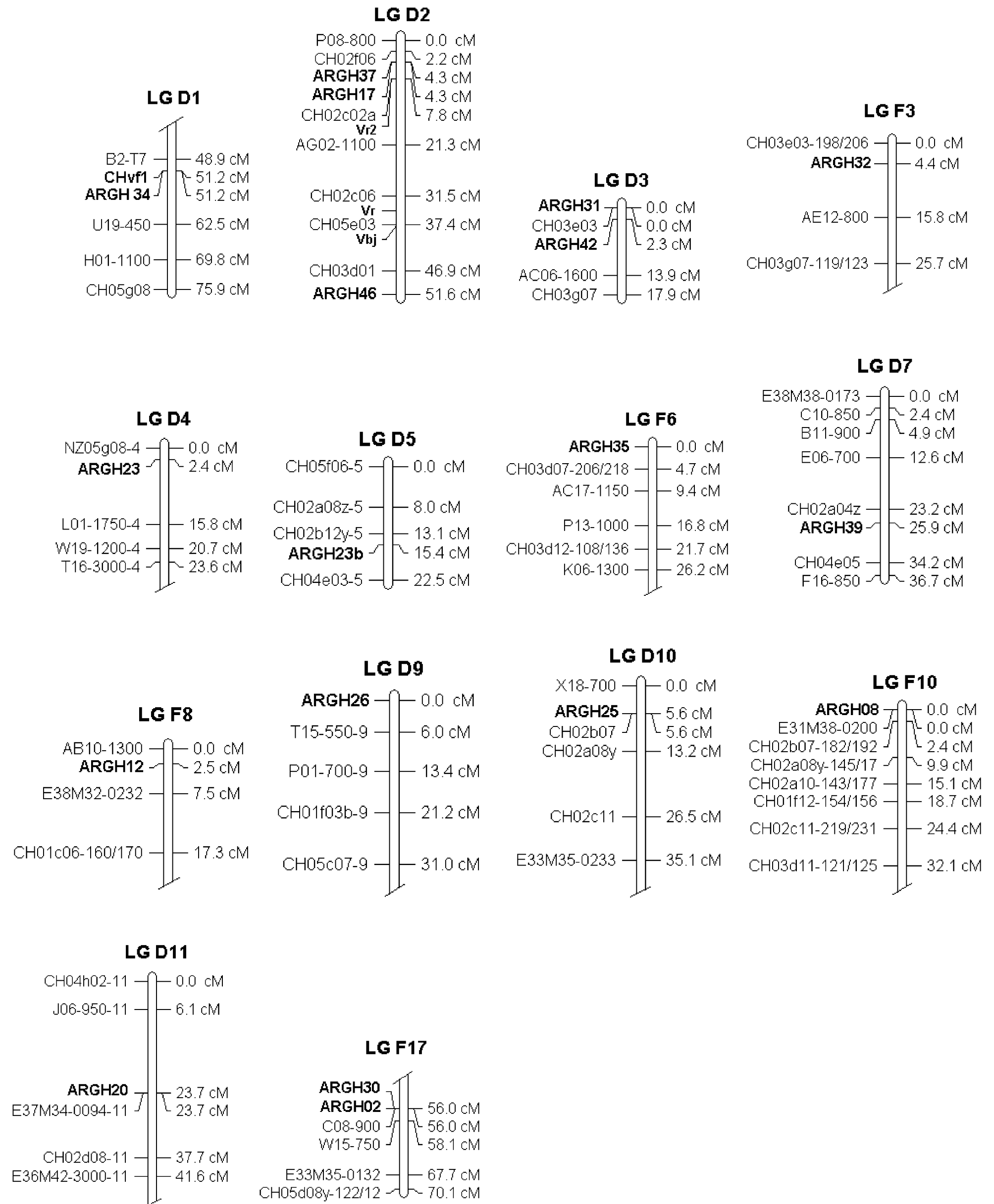

Fig. 4 Linkage maps showing the position of RGH sequences on apple linkage groups. Mapping was obtained using a progeny of cultivars Fiesta (F) × Discovery (D). Linkage groups are numbered from *F1 to F17* and from *D1 to D17* for Fiesta and Discovery, respectively. Map positions of *Vbj*, *Vr*, and *Vr2* are deduced

domain and a transmembrane domain, but not the NBS region. An NBS-containing gene cluster could be represented by ARGH17 and ARGH37, which were placed in the same position on linkage group 2 and are very similar to each other (Fig. 3). ARGH30 and ARGH02 cannot be considered members of a gene cluster due to their phylogenetic distance, in spite of the fact that they are tightly linked on the map. Nevertheless the presence of members of two different ARGH families in the same position could indicate the presence of a "hot spot" on the apple genome containing multiple NBS-LRR sequences.

Finally, ARGH31 and ARGH42 were placed on linkage group 3. Even if from our data these ARGHs are not tightly linked, only 2 cM separated the two phylogenetically similar sequences, indicating the possible presence of a cluster of genes.

Genomic analysis of clusters with multiple LRR gene members could be used to identify functional genes responding to different signals. In tomato, the *Pto* gene belongs to a complex locus consisting of five to seven genes. While *Pto* confers resistance to *P. syringae*, a tightly linked homologue, *Fen*, confers sensitivity to an organophosphate insecticide (Martin et al. 1993, 1994). Examples of related genes that have acquired different roles can be found in several gene families involved in the regulation of floral identity, light reception, and cell differentiation (Pickett and Meeks-Wagner 1995).

In some cases, the ARGH sequences were located in the proximity of loci known to contain genes or QTLs for disease resistance. On linkage group 2, several apple scab-resistance genes have been mapped; *Vbj* co-segregates with the SSR CH05e03 (Gygax, personal comunication), *Vr* has been mapped between the SSRs CH05e03 and

CH02c06 (Hemmat et al. 2002), and *Vr2* has been found to cosegregate with CH02c02a (Patocchi, unpublished). Therefore none of the three ARGHs mapped on this linkage group can be considered as candidate apple scab-resistance genes (Fig. 4). On linkage group 11, a powdery mildew resistance gene was mapped (Seglias 1997); however, the gene was mapped close to the SSRCH04H02 and therefore, also in this case, the NBS ARGH20 cannot be considered a candidate resistance gene. Nevertheless our mapping data indicate that in some cases, RGH sequences are present near sources of resistance on the apple genome. If this observation can be confirmed by further studies, these gene families could become a possible source of markers associated to disease-resistance genes.

QTLs for leaf and fruit scab resistance were found on linkage groups 6, 7, 10, 11, 12, 15, and 17 by Liebhard et al. (2003a) and on linkage group 1 and again on linkage groups 11 and 17 by Durel et al. (2003). In almost all cases, an ARGH was also present on the same linkage group (Fig. 4). Two ARGHs (ARGH20 and ARGH34) mapped inside the QTL regions. ARGH20 maps on linkage group D11 in the region of a QTL for leaf scab resistance (Liebhard et al. 2003a), while ARGH34 maps near the *Vf* locus in a region that could be associated with a QTL for scab resistance (Durel et al. 2003). Further studies are, however, necessary to prove this association.

In apple, relatively few genes conferring resistance against the most common pathogens have been studied and characterized, and only for *Vf* has the positional cloning led to the cloning of the first scab resistance gene, *HcrVf2* (Belfanti et al. 2004; Patocchi et al. 1999a, 1999b; Vinatzer et al. 2001). Locating NBS-LRR homologues, as well as RGH appertaining to other classes, on apple maps may therefore help to accelerate the identification of genomic regions containing functional resistance genes.

Acknowledgements This work was supported by the project "Advanced Biology" funded by the Fondazione delle Casse di Risparmio di Trento e Rovereto, Trento, Italy. The authors thank Luigi Cattivelli and Eve Silfverberg-Dilworth for critical reading of the manuscript and Andrea Gandolfi for technical assistance.

References

Aarts MGM, te Lintel Hekkert B, Holub EB, Beynon JL, Stiekema WJ, Pereira A (1998) Identification of R-gene homologous DNA fragments genetically linked to resistance loci in *Arabidopsis thaliana*. Mol Plant Microbe Interact 11:251–258

Aldrich J, Cullis CA (1993) RAPD analysis in flax: optimization of yield and reproducibility using Klen 1 DNA polymerase, Chelex 100 and gel purification of genomic DNA. Plant Mol Biol Rep 11:128–141

Belfanti E, Silfverberg-Dilworth E, Tartarini S, Patocchi A, Barbieri M, Zhu J, Vinatzer BA, Gianfranceschi L, Gessler C, Sansavini S (2004) The *HcrVf2* gene from a wild apple confers scab resistance to a transgenic cultivated variety. Proc Nat Acad Sci USA 101:886–890

Bent AF (1996) Function meets structure in the study of plant disease-resistance genes. Plant Cell 8:1757–1771

Brotman Y, Silberstein L, Kovalski I, Perin C, Dogimont C, Pirat M, Klingler J, Thompson GA, Perl-Treves R (2002) Resistance gene homologues in melon are linked to genetic loci conferring disease and pest resistance. Theor Appl Genet 104:1055–1063

Cordero JC, Skinner DZ (2002) Isolation from alfalfa of resistance gene analogues containing nucleotide-binding sites. Theor Appl Genet 104:1283–1289

Durel CE, Parisi L, Laurens F, Van der Weg WE, Liebhard R, Jourjon MF (2003) Genetic dissection of partial resistance to race 6 of *Venturia inaequalis* in apple. Genome 46:224–234

Hammond-Kosack KE, Jones JDG (1997) Plant disease-resistance genes. Annu Rev Plant Physiol Plant Mol Biol 48:575–607

Hemmat M, Brown SK, Weeden NF (2002) Tagging and mapping scab-resistance genes from R12740-7A apple. J Am Soc Hortic Sci 127:365–370

Janse J, Verhaegh JJ, Den Nijs APM (1994) Early selection of partial resistance to powdery mildew, *Podosphaera leucotricha* (Ell. et EV.) Salm. in apple progenies. Euphytica 77:7–9

Kanazin V, Marek LF, Shoemaker RC (1996) Resistance-gene analogs are conserved and clustered in soybean. Proc Natl Acad Sci USA 93:11746–11750

Konieczny A, Ausubel FM (1993) A procedure for mapping *Arabidopsis* mutations using codominant ecotype-specific markers. Plant J 4:403–410

Leister D, Ballvora A, Salamini F, Gebhardt C (1996) A PCR-based approach for isolating pathogen-resistance genes from potato with potential for wide application in plants. Nat Genet 14:421–429

Leister D, Kurth J, Laurie DA, Yano M, Sasaki T, Devos K, Graner A, Schulze-Lefert P (1998) Rapid reorganization of resistance gene homologues in cereal genomes. Proc Natl Acad Sci USA 95:370–375

Leister D, Kurth J, Laurie DA, Yano M, Sasaki T, Graner A, Schulze-Lefert P (1999) RFLP- and physical mapping of resistance gene homologues in rice (*O. sativa*) and barley (*H. vulgare*). Theor Appl Genet 98:509–520

Liebhard R, Koller B, Patocchi A, Kellerhals M, Pfammatter W, Jermini M, Gessler C (2003a) Mapping quantitative field resistance against apple scab in a 'Fiesta' × 'Discovery' progeny. Phytopathology 93:493–501

Liebhard R, Koller B, Gianfranceschi L, Gessler C (2003b) Creating a saturated reference map for the apple (*Malus x domestica* Borkh.) genome. Theor Appl Genet 106:1497–1508

Martin GB (1999) Functional analysis of plant resistance genes and their downstream effectors. Curr Opin Plant Biol 2:273–279

Martin GB, Brommonschenkel S, Chunwongse J, Frary A, Ganal MW, Spivey R, Wu T, Earle ED, Tanksley SD (1993) Map-based cloning of a protein-kinase gene conferring disease resistance in tomato. Science 262:1432–1436

Martin GB, Frary A, Wu T, Brommonschenkel S, Chunwongse J, Earle ED, Tanksley SD (1994) A member of the tomato *Pto* gene family confers sensitivity to fenthion resulting in rapid cell death. Plant Cell 6:1543–1552

Meyers BC, Dickerman AW, Michelmore RW, Sivaramakrishnan S, Sobral BW, Young ND (1999) Plant disease-resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. Plant J 20:317–332

Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformational polymorphisms. Proc Natl Acad Sci USA 86:2766–2770

Pan QL, Liu YS, Budai-Hadrian O, Sela M, Carmen-Goren L, Zamir D, Fluhr R (2000a) Comparative genetics of nucleotide binding site-leucine rich repeat resistance genes homologues in the genome of two dicotyledons: tomato and *Arabidopsis*. Genetics 155:309–322

Pan QL, Wendel J, Fluhr R (2000b) Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genome. J Mol Evol 50:203–213

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

Parniske M, Hammond Kosack KE, Golstein C, Thomas CM, Jones DA, Harrison K, Wulff BBH, Jones JDG (1997) Novel disease-resistance specificities result from sequence exchange between tandemly repeated genes at the *Cf-4/9* locus of tomato. Cell 91:821–832

Patocchi A, Gianfranceschi L, Gessler C (1999a) Towards the map-based cloning of *Vf*: fine and physical mapping of the *Vf* region. Theor Appl Genet 99:1012–1017

Patocchi A, Vinatzer BA, Gianfranceschi L, Tartarini S, Zhang HB, Sansavini S, Gessler C (1999b) Construction of a 550-kb BAC contig spanning the genomic region containing the apple resistance gene *Vf*. Mol Gen Genet 262:884-891

Pickett FB, Meeks-Wagner DR (1995) Seeing double: appreciating genetic redundancy. Plant Cell 7:1347–1356

Rivkin MI, Vellejos CE, McClean PE (1999) Disease resistance-related sequences in common bean. Genome 42:41–47

Rychlik W (1995) Selection of primers for polymerase chain reaction. Mol Biotechnol 3:129–134

Sanguinetti CJ, Dias Neto E, Simpson AJ (1994) Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques 17:914-921

Seah S, Sivasithamparam K, Karakousis A, Lagudah ES (1998) Cloning and characterisation of a family of disease resistance gene analogs from wheat and barley. Theor Appl Genet 97:937–945

Seglias NP, Gessler C (1997) Genetics of apple powdery mildew resistance from *Malus zumi* (Pl2). IOBC/WPRS Bull 20:195–208

Shen KA, Meyers BC, Islam-Faridi MN, Chin DB, Stelly DM, Michelmore RW (1998) Resistance-gene candidates identified by PCR with degenerate oligonucleotide primers map to clusters of resistance genes in lettuce. Mol Plant Microbe Interact 11:815–823

Stam P, van Oijen JW (1995) JOINMAP ver 2.0: software for the calculation of genetic linkage maps. CPRO-DLO, Wageningen, The Netherlands

Timmerman-Vaughan GM, Frew TJ, Weeden NF (2000) Characterization and linkage mapping of R-gene analogous DNA sequences in pea (*Pisum sativum L.*). Theor Appl Genet 101:241–247

Traut TW (1994) The function and consensus motifs of nine types of peptide segments that form different types of nucleotide-binding sites. Eur J Biochem 222:9–19

Vinatzer BA, Patocchi A, Gianfranceschi L, Tartarini S, Zhang HB, Gessler C, Sansavini S (2001) Apple contains receptor-like genes homologous to the *Cladosporium fulvum*-resistance gene family of tomato with a cluster of genes cosegregating with *Vf* apple scab resistance. Mol Plant Microbe Int 14:508–515

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

Yu YG, Buss GR, Maroof MAS (1996) Isolation of a superfamily of candidate disease-resistance genes in soybean based on a conserved nucleotide binding site. Proc Natl Acad Sci USA 93:11751–11756