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

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Accepted 20 December 2000.

Scab caused by the fungal pathogen *Venturia inaequalis* is the most common disease of cultivated apple (*Malus × domestica* Borkh.). Monogenic resistance against scab is found in some small-fruited wild *Malus* species and has been used in apple breeding for scab resistance. *Vf* resistance of *Malus floribunda* 821 is the most widely used scab resistance source. Because breeding a high-quality cultivar in perennial fruit trees takes dozens of years, cloning disease resistance genes and using them in the transformation of high-quality apple varieties would be advantageous. We report the identification of a cluster of receptor-like genes with homology to the *Cladosporium fulvum* (*Cf*) resistance gene family of tomato on bacterial artificial chromosome clones derived from the *Vf* scab resistance locus. Three members of the cluster were sequenced completely. Similar to the *Cf* gene family of tomato, the deduced amino acid sequences coded by these genes contain an extracellular leucine-rich repeat domain and a transmembrane domain. The transcription of three members of the cluster was determined by reverse transcription-polymerase chain reaction to be constitutive, and the transcription and translation start of one member was verified by 5' rapid amplification of cDNA ends. We discuss the parallels between *Cf* resistance of tomato and *Vf* resistance of apple and the possibility that one of the members of the

gene cluster is the *Vf* gene. *Cf* homologs from other regions of the apple genome also were identified and are likely to present other scab resistance genes.

Additional keyword: durable resistance.

Almost all of our daily fruit supply comes from perennial fruit trees. Consumers demand fruit that is perfectly spotless, but want it produced with the least amount of pesticides. To combine high-quality fruit traits with disease resistance is, therefore, the aim of most fruit-tree breeding programs today (Laurens 1996). As a result of the long juvenile period of perennial fruit trees, breeding programs with this aim take dozens of years to accomplish. *Vf* apple scab resistance is a typical example: *Vf* resistance, inherited as a monogenic trait, has been introgressed in the cultivated apple (*Malus × domestica* Borkh.) from the small-fruited, wild *Malus* spp. accession *Malus floribunda* 821 through a breeding program that started in 1914 (Crandall 1926), although did not produce the first varieties of acceptable quality and fruit size until 1970 (Dayton et al. 1970).

Another peculiarity of perennial fruit trees is the long replanting cycle of fruit tree orchards (typically 15 to 20 years). Therefore, durable disease resistance is much more important in fruit trees than in any herbaceous crop. Durable disease resistance can be achieved by combining more than one disease resistance gene in a single fruit variety to reduce the risk of a pathogen overcoming a single disease resistance gene. In fact, *Vf* apple scab resistance has been overcome in recent years in many apple-growing areas in Europe (Bénaouf and Parisi 2000; Parisi et al. 1993). Other scab resistance genes are available in wild relatives of apple (Williams and Kuc 1969). Combining these resistance genes with *Vf* resistance would be advantageous. Marker-assisted selection (MAS) with the use of molecular markers for scab resistance could be of great help for such "pyramiding" of scab resistance genes. Because apple is self-incompatible and, therefore, highly heterozygous, all genes will be newly recombined in each gen-

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Nucleotide and amino acid sequences of HcrVf1, HcrVf2 and HcrVf3 have been submitted to the EMBL database as accession nos. AJ297739, AJ297740, and AJ297741, respectively.

eration and individuals carrying the resistance genes as well as all other requested quality traits will have to be selected. Even if such an individual is found, it always will be a new variety that has to face the difficult task of replacing the already well-established, popular varieties on the market. Transferring multiple scab resistance genes by gene engineering could help to overcome this problem.

Apple scab is caused by the ascomycete *Venturia inaequalis*, and the interaction between this pathogen and its host has been studied since breeding for apple scab resistance began (Boone 1971; Gessler 1989; Williams and Kuc 1969). Boone (1971) and, more recently, Sierotzki et al. (1994; 1998) and Koch et al. (2000) have shown that every apple variety has resistance genes against all genotypes of *V. inaequalis*, except those missing the corresponding avirulence alleles. In nature, however, the scab inoculum always is a mixture of virulent and avirulent genotypes. Consequently, these resistances are not evident in the field and, for this reason, are called “ephemeral” (Koch et al. 2000). One of these genes, *Vg*, determining ephemeral resistance recently has been mapped (Durel, *personal communication*). *Vf* scab resistance and other apple scab resistance genes with broad action against most races of *V. inaequalis* also have been mapped (Cheng et al. 1998; Gardiner et al. 1999; Patocchi et al. 1999a). The identified molecular markers of these genes can be used in MAS but, to date, no scab resistance gene has been cloned. We recently reported the construction of an apple bacterial artificial chromosome (BAC) library (Vinatzer et al. 1998), physical mapping of the *Vf* gene region (Patocchi et al. 1999a), and the construction of a BAC contig spanning the entire *Vf* locus (Patocchi et al. 1999b). The constructed BAC contig is 550 kb and encompasses the *Vf* region between the molecular markers M18 on one side of the gene and AM19 on the other side. The possible location of the *Vf* gene was restricted in this contig to 350 kb covered by a minimum of five BAC clones (Patocchi et al. 1999b).

When the first plant disease resistance genes had been cloned in Arabidopsis, flax, and tobacco, a remarkable similarity in the sequences coding for a nucleotide binding site-leucine-rich repeat (NBS-LRR) motifs was observed within some of the genes (Staskawicz et al. 1995). Designing heterologous primers on conserved regions of these genes, resistance gene analogs (RGA) (Leister et al. 1996) that cosegregated with resistance loci were identified in many plant species (Aarts et al. 1998; Kanazin et al. 1996; Leister et al. 1996; Meyers et al. 1998; Yu et al. 1996). This approach did not lead to the identification of NBS-LRR-class RGAs on the *Vf* BAC contig (*unpublished data*), suggesting a different mechanism to control resistance in the case of *Vf* resistance. In this paper, we report the identification on the *Vf* BAC contig of a cluster of homologs of the *Cladosporium fulvum* (*Cf*) resistance genes of tomato (Dixon et al. 1996; Jones et al. 1994; Parniske et al. 1997; Thomas et al. 1997). We show the complete genomic sequence of three genes of the cluster and the analysis of their transcription. We discuss the phenotypic similarities of *Cf* and *Vf* resistance and the possibility that one of the members of the *Cf* homolog gene cluster of the *Vf* region is the *Vf* gene. We also report that other loci in the apple genome hybridize to the *Cf* homologous sequences. These loci could correspond to ephemeral resistance loci or loci for broad-range apple scab resistance genes that could be com-

bined with *Vf* resistance to create durable apple scab resistance.

RESULTS

Identification of a cluster of *Cf* homologs at the *Vf* locus.

Previously we constructed two BAC contigs covering the entire *Vf* locus, one contig consisting of BAC clones derived from the chromosome carrying the allele of the *Vf* locus conferring resistance forming the “resistant” contig and one consisting of BAC clones derived from the chromosome carrying the *vf* allele forming the “susceptible” contig (Patocchi et al. 1999b). Within the 550-kb resistant contig, we identified five clones (M18-2, M18-5, M18-6, M18-7, and M18-8) that cover 350 kb and compose the minimum “tiling path” between the two markers closest to *Vf*, which are separated from the *Vf* locus by recombinants in our mapping population (Fig. 1A). Their inserts were used as probe to screen 750,000 clones of a cDNA library of leaves of the apple cultivar Florina (inoculated with *V. inaequalis*) in order to identify candidate *Vf* genes. A total of 101 cDNAs hybridizing to the five BAC inserts were identified and sequenced from the poly(A) tail, and 50 different sequences were identified. The corresponding cDNAs also were sequenced from the 5' end, and the 5' and 3' sequences were compared to GenBank. Although they were not full length, three cDNA clones (cDNAs 8-1, 10-5, 15-4), which were very similar to each other, presented homology to the *Cf* resistance genes of tomato (Dixon et al. 1996; Jones et al. 1994; Parniske et al. 1997; Thomas et al. 1997). Their deduced amino acid sequence was 56% similar and 36% identical to the *Cf9 C. fulvum* disease resistance gene of tomato (Jones et al. 1994). No other cDNA clones with homology to known disease resistance genes were identified.

The longest of the three cDNA clones, 8-1, was used as probe on a Southern blot of *Eco*RI-digested BAC clones of the whole resistant and susceptible BAC contigs (including the clones not cosegregating with *Vf* resistance). The probe hybridized to multiple bands on all but two clones of the contig, comprising the clones not cosegregating with resistance on the resistant and the susceptible chromosome. The high number of hybridizing bands indicated that a gene cluster of many *Cf* resistance gene homologs is present in the *Vf* region. We called the members of the cluster “*HcrVf* genes” (homologs to *C. fulvum* resistance genes of the *Vf* region), in analogy to Parniske et al. (1999). This meant that the three *Cf* homolog cDNA clones (8-1, 10-5, and 15-4) could be derived from any of the BAC clones of the *Vf* region (from either the resistant or susceptible chromosome) or from genomic regions other than the *Vf* locus, in analogy to the presence of various *Cf* gene clusters in tomato (Parniske et al. 1997) and because apple is a species of ancient allotetraploid origin, which was confirmed by the finding that most restriction fragment length polymorphism markers hybridize to two loci located on two different chromosomes (Maliepaard et al. 1998).

Sequence assembly and genetic mapping of *HcrVf* genes.

To concentrate our effort in finding candidate *Vf* genes exclusively on *Cf* homologs from the resistant chromosome at the *Vf* locus, we constructed *Sau*3AI and *Eco*RI subclone libraries of the five BAC clones used for the cDNA library screening and probed them with cDNA 8-1. After partially

sequencing the subclones hybridizing to cDNA 8-1, we realized that many sequences were so similar to each other that it was impossible to assign them to different open reading frames (ORFs). Only two *Eco*RI subclones of BAC clone M18-2 could be assigned to one single ORF because these two subclones corresponded in length to the two *Eco*RI fragments of M18-1 (BAC clone overlapping with M18-2) that had hybridized to cDNA 8-1 in the Southern blot experiment described above. This ORF was called *HcrVf1*. We were able to align the other subclone sequences with *HcrVf1*, but as a result of the high homology between sequences, it was still impossible to assign the subclones to separate genes. We observed, however, that homology between various subclone sequences continued upstream of the putative start codon and downstream of the putative stop codon of *HcrVf1*. We used primers designed on these conserved sequences upstream and downstream of *HcrVf1* and were able to amplify two complete ORFs (named *HcrVf2* and *HcrVf3*) in addition to *HcrVf1*. Figure 1B shows the region of the resistant contig from which the *HcrVf* genes were amplified. Because *HcrVf1* and *HcrVf2* are located on BAC clone M18-2, which has one end separated from the *Vf* locus by two recombinants in our mapping populations, it was necessary to determine whether *HcrVf1* and *HcrVf2* cosegregate with *Vf* resistance. We determined that *HcrVf2* cosegregates with *Vf* resistance but were unable to find primers polymorphic for *HcrVf1* in our crosses. It has thus not yet been determined whether *HcrVf1* cosegregates with *Vf* resistance.

To determine whether there are more *HcrVf* genes on the five BAC clones cosegregating with *Vf* resistance, we performed a Southern blot of *Eco*RI–*Dra*I-digested BAC clones of the *Vf* region probed with a 118-bp polymerase chain reaction (PCR) fragment amplified with primers designed on the most conserved region among the three *HcrVf* genes that is not digested by either *Eco*RI or *Dra*I (data not shown). Five different restriction fragments of the five BAC clones spanning the *Vf* region hybridized to this probe (Fig. 2). Thus, this is the minimum number of *HcrVf* genes on these five BAC clones, although because some *HcrVf* genes may have *Eco*RI–*Dra*I fragments of the same size, this number may be higher.

Transcription analysis of *HcrVf1*, *HcrVf2*, and *HcrVf3*.

Because the sequences of cDNAs 8-1, 10-5, and 15-4 did not correspond to *HcrVf1*, *HcrVf2*, or *HcrVf3*, we used reverse transcription (RT)-PCR to determine their transcription. We used *HcrVf1*-specific primers, bulked the RNA extracted from leaves harvested at different time points after inoculation with *V. inaequalis*, and obtained three RT-PCR bands from the RNA of Prima and Florina, two scab-resistant cultivars that are heterozygous at the *Vf* locus. One of the three bands was missing when the scab-susceptible cultivar Golden Delicious was used as template. Cloning and sequencing of this product from Prima and Florina showed a 100% match with each other as well as with *HcrVf1*, which is therefore transcribed. We used *HcrVf2*- and *HcrVf3*-specific primers and obtained single

Fig. 1. A, Schematic representation of the *Vf* region. Dark-gray bars indicate the bacterial artificial chromosome (BAC) clones derived from the chromosome carrying the *Vf* allele at the *Vf* locus ("resistant" chromosome), whereas light-gray bars indicate the BAC clones derived from the homologous chromosome ("susceptible" chromosome). The *Vf* locus was mapped to a 350 kb region covered by a minimum of five BAC clones, indicated in black (Patocchi et al. 1999b). Markers M18, AM19, and AL07 were the starting points of the chromosome walk that led to the construction of the BAC contigs. BAC clones are not drawn to scale. **B,** Relative positions (on the resistant contig) of the four characterized *HcrVf* genes (homologs of *Cladosporium fulvum* resistance genes of the *Vf* region). Hatched horizontal bars represent the part of the *Vf* region that does not contain *HcrVf* genes.

bands in the two *Vf* cultivars that were missing in the susceptible Golden Delicious. The sequences of the *HcrVf2* RT-PCR products matched the genomic *HcrVf2* sequence and confirmed transcription of *HcrVf2*. The *HcrVf3* RT-PCR products showed a 97% match with *HcrVf3*. Alignment with the sequenced BAC subclones, however, showed a 100% match with a 5,000-bp *EcoRI* subclone of M18-6. Therefore, transcription of this additional gene, *HcrVf4*, was shown. *HcrVf3* may be a pseudogene or have a very low transcription level compared with *HcrVf4*. Figure 1B shows the approximate location of *HcrVf4* in the BAC contig.

We then performed RT-PCR experiments on the RNA extracted from uninfected leaves of Florina and Prima and determined that *HcrVf1*, *HcrVf2*, and *HcrVf4* also are transcribed in the absence of a pathogen attack.

Determination of transcription and translation start of the *HcrVf* genes.

We used 5' rapid amplification of cDNA ends (RACE) on *HcrVf1* and *HcrVf2* to verify the predicted translation start for the ORFs of the *HcrVf* genes. By cloning and sequencing several 5' RACE products obtained with primers designed in regions common to *HcrVf1* and *HcrVf2*, we identified two clones showing a perfect match with the genomic sequence of *HcrVf2*, comprising the first 244 bases of the ORF and the 36 bases upstream of the putative start codon, indicating that the leader sequence of *HcrVf2* might be 36 bases. A putative TATA box 26 bases from the presumed transcription start and a CAAT box 22 bases upstream of the TATA box were identified. Upstream of the presumed transcription start, a 26-base, CT-rich sequence was found with no correspondence to the upstream sequence of *HcrVf2*. According to Roche (Rotkreuz, Switzerland), CT-rich sequences can be RACE-PCR artifacts as a result of the slippage of the poly(T) primer during amplification. From our data, it is not possible to determine whether this hypothesis is correct. The presence of a 280-bp perfect match between the RACE clones and the genomic sequence of *HcrVf2*, however, strongly supports the idea that the sequence of the two RACE clones is the 5'-end of the *HcrVf2* transcript and, as a result of the high homology between the members of the *HcrVf* cluster, the ORFs that we predict for *HcrVf1* and *HcrVf3* also should be correct.

HcrVf1 through *HcrVf3* are predicted to be membrane-bound glycoproteins with an extracellular leucine-rich repeat domain.

The deduced amino acid sequences of *HcrVf1*, *HcrVf2*, and *HcrVf3* are 1,015, 980, and 916 amino acids, respectively (Fig. 3). Although the DNA sequences of the sequenced ORFs of the *HcrVf* genes have, on average, only 44% identity to the tomato *Cf9* *C. fulvum* disease resistance, the proteins predicted for the *HcrVf* genes show the same domains as those predicted for the *Cf9* resistance gene (Jones et al. 1994). A signal peptide and its cleavage site is predicted to be present at the N terminus of the three proteins (domain A). Domain B is thus the N terminus of the mature protein, once the signal peptide has been cleaved. The C domains of the *HcrVf* genes 1 through 3 contain 30, 29, and 26 imperfect LRRs, respectively. The average length of the LRRs is 24 amino acids, and each LRR contains the consensus sequence LxxLxxLxxLxLSxNxLxGxIP. The glycine in the consensus sequence indi-

cates that the LRRs are of extracellular nature (Jones et al. 1994). As for the *Cf* proteins, no function could be assigned to domain D, which is composed of 27 amino acids. Domain F, composed of 36 hydrophobic amino acids, is predicted to be the transmembrane domain of the protein. Domains E and G, flanking the hydrophobic domain, are rich in polar amino acids, which is consistent with their predicted role of orienting and anchoring the protein to the cell membrane. Many putative N-glycosylation sites are present in domains B to D of *HcrVf1* (26 sites), *HcrVf2* (23 sites), and *HcrVf3* (23 sites), respectively. Therefore, similar to the *Cf* resistance genes, the *HcrVf* genes probably encode extracytoplasmic glycoproteins anchored to the cell membrane, with the majority of the extracytoplasmic domain consisting of LRR motifs.

At the amino acid level, the three complete ORFs compared two by two show, on average, 87% identity. The most conserved region among the three ORFs is domain A and the region spanning from domain D to G, presenting, on average, 95 and 96% identity, respectively. The more variable domains are B and C, presenting, on average, 84 and 86% identity, respectively. Furthermore, the variability is not distributed equally within domain C, which can be divided into subdomain C2 (89% identity between the three considered *HcrVf*

Fig. 2. At least five *HcrVf* genes are located on the bacterial artificial chromosome (BAC) clones cosegregating with *Vf* resistance. A 118-bp probe derived from the highly conserved domains E and F of *HcrVf2* was hybridized to *EcoRI*-*DraI* double-digested BAC clones spanning the *Vf* region. The probe hybridizes to five bands of the BAC clones from the "resistant" chromosome cosegregating with *Vf* resistance and to an additional five of the corresponding BAC clones from the "susceptible" chromosome. Each of the bands corresponds to at least one *HcrVf* gene (because some *HcrVf* genes may have *EcoRI*-*DraI* fragments of the same size, this number may be higher). Other *HcrVf* sequences were found on BAC clones AM19-1 through AM19-4 and AL07-1 through AL07-3 (not cosegregating with *Vf* resistance; data not shown).

genes) and C1 (82% identity between the three considered *HcrVf* genes). The lower level of identity in C1 is not only a result of the difference in the number of LRRs (with reference to *HcrVf1*, *HcrVf2* lacks two and *HcrVf3* lacks five LRRs, respectively), but also to the higher number of amino acid differences observed within a single LRR.

***Hcr* genes are present in multiple loci in the apple genome.**

Comparing the *HcrVf* genomic sequences with the BAC end sequences isolated during the construction of the *Vf* BAC contig (Patocchi et al. 1999b), we noticed that the AL07-2-Sp6 BAC end probe, which had hybridized to most BAC clones of the *Vf* contig, including the BAC clones of the distinct AL07 contig separated from the *Vf* locus by 1.2 cM (Patocchi et al. 1999b), showed 93% identity with the *HcrVf* genes. This probe was used to screen the apple BAC library, and 11 clones were identified that did not map to the *Vf*-region: 15J14, 40O10, 43F22, 49J15, 55I7, 57G7, 62H3, 72M18, 76G16, 90N4, 94H24. This result shows that *Hcr* sequences also are present in other loci of the apple genome.

DISCUSSION

Identification of candidate *Vf* genes with homology to the tomato *Cf* resistance gene family.

We identified a cluster of *Cf* homologs at the *Vf* locus. ORFs of three members of the cluster, *HcrVf1*, *HcrVf2*, and *HcrVf3*, which were completely sequenced, a fourth ORF

(*HcrVf4*) that has been partially sequenced, and the presence of at least one more *HcrVf* gene, which can be deduced from the Southern blot experiment (Fig. 2). *HcrVf1*, *HcrVf2*, and *HcrVf4* are transcribed genes, and *HcrVf3* is probably a non-expressed pseudogene. *HcrVf2*, *HcrVf3*, and *HcrVf4* co-segregate with *Vf* resistance in our mapping populations (2,071 plants), whereas the map position of *HcrVf1* has yet to be determined. Because *HcrVf3* is a pseudogene, to date only *HcrVf2* and *HcrVf4* are candidate *Vf* genes.

The comparison of the deduced amino acid sequences of *HcrVf1* through *HcrVf3* with the deduced amino acid sequence of the tomato *Hcr9* genes clearly shows the same type of overall protein structure (Fig. 3). It is possible to divide the predicted apple *HcrVf* proteins and the tomato *Hcr9* proteins in the same domains. Additionally, the level of conservation and variability of the different domains between the members of each of the two families is similar. Domains A, D, E, F, and G are very conserved, whereas domains B and C are more variable. In both gene families, domain C can be divided into two subdomains, C1 and C2, on the basis of degrees of conservation, with C1 being more variable than C2. Parniske et al. (1997) propose that the region of the *Hcr9* genes corresponding to the sixteen LRRs closest to the N terminus (domain C1 in the *HcrVf* gene family) encodes the region of the proteins involved in the recognition of the avirulence product of the pathogen. For this reason, the region is evolving more rapidly and is, therefore, less conserved, probably to adapt to the evolution of the pathogen.

Cf-9

LxxLxxLxxLxLxSxNkLxGxIP

MDCVKLVFLMLYTLFCLQALSSS

LPHLCQPDQALSLIQ

NKMTPTNFNASDQYDRTYVDITQSY

PRTLxwNkTSCCSDMWGCHDCTTQQ

VIALDQKFSQLOGLSFPSS

LPQLSNKLRLDLSFVNNLAWQ

FGFESSLTHLDSHSSPTOLIPSE

ICHLSLHLVRLCDQYGLSLVPPNFELL

LKLNLTQRLANLSSSSSLTIPS

TMPSHLLTQLSGTEHLGQIPF

VFLNLSNLGLSLNFPQVTPFPTPK

WMSQSSASLTVYVSDVNIADIPKPS

FSHLTLSELMYGRNLSNPTPKP

LWNLTNIVFLHGDHNLGEPISH

FTPIPKKRLSLVNNNDGQGLF

ISGLQNLRLDLSNLTGPTIPNS

ISGLQNLRLDLSNLTGPTIPNS

TFSLPSVLVLDLSNTPSGKIQEF

SKSTLSAVTLQNKLRGIPNS

LLNQNLKMLLSSHNLGHSSISA

ICNLNKLTLDDLGSNNLGTIPQCV

VERNEVLSHLDSNLGLSTGPTPK

FVGNVLRVLSLHGNKLTKGVPS

MINCKYTLTLDDLGNMNLSTPFW

LGVLQPKTLSLSLKNSLGTIPKSSN

TNLPMGQILDLSSNGPSNLGPERI

LNLQTKMLDESGTPEPISDSEY

DIYNYLTPITSTGQDQVDSAL

LDNSMTNLISKAREBOHPSI

IGDLVGLRTITNLNHLBGHPSA

FOGLVLSLSDLSNKSISGETPOK

LASLTFLVLVNLNHNHVGKIPKG

KQFDSFGNTSYQNGDLRGFLPSKLOG

EDQVTPPAELDQEEERD

SPMISWQGVLVGYCGVLIGLSLVI

YWNSTQFPAWFS

RMDLKLHILITTKMKHKKERY

HcrVf1

LxxLxxLxxLxLxSxNkLxGxIP

MERTMRVILLIRFLAIATITFSIGLSNG

NEPWPFLCKESRQALLMFQDKLDP

ANKRLASWAEREDSDCCSGVGVCDHTTGH

HELHxNkSDHMDPESFFGGKINPS

LLSLKHLPDLDSYNNFSGCTQIPF

FGSMSTLTHALGFSMPQGVFIHN

QGLSLKLYLISFVNNFVNNLAWQ

ISGLSLKHLLDLSVNLGKASDWLQV

TNMLPSLVLVLDMSQGLQYIPLP

TNPTLPSLVLVLDLSRNFVNNLMPRW

VPSLKNLVSLHLRCQFGQGPPIST

WFLNKLARELDSNEMSLDQIPK

WLPNQKDLALSLSKNSQGLQIPK

FNMTGLKVLNLSNDFVNNLTPKW

LYGLANLSELLLVSYNALRGESISS

IGMTSLVNLVLENNOLOGLIPNS

LGHCKLKLAVLDLSNHFVARRSPFESLS

CGCPGLKLSLRYTLISGPTFMS

LQNLSLSEKLDLSGNHFGNCTFV

IQGLKMLDLDLSYNNFPGVSVSE

FSNLKXKHFVAKNSPILKTSRD

WVPFQLELRLDLSNHLGKFWPMW

LRVYQLELRLDLSNHLGKFWPMW

WNLTPFWWVNLSHNGVQYIPLP

NIIVAGRSVVDLSGNQPTAL

PIVPTSLVLDLSNHSFGSGVHFPCDR

PDEPKLILYLHGLNPLFKGVPCD

WMSWPLQGLVNLNGLKGVNPS

MG ELPS

LQGLTMLSVLDLSNHSFGSGVHFPCDR

GKSLSNVNLNLSNKFEGDIPNE

VCYLSQLQLDLAHLNLSGMI PCFPH

LSALADSSKSSFTPTGTNDGVSGLT

ENALVTPKTEMEY

SKLGLVNLNLSNKFEGDIPNE

LTGLALQYVNLNLSNKFEGDIPNE

IGMAQLSELDLSNHLGKFWPMW

MTMLTFLSHNLSYNLGRILE

STQLQSLDQSSFGVGNELGAPLNRCS

ENGVIPTPTVEHDGGGYRLLEDS

WYFVTLGVGFFGTGFWTLVGLSLVN

MPWILLSQLLN

RVLKMYHIVETV*

HcrVf2

LxxLxxLxxLxLxSxNkLxGxIP

MERTMRVILLIRFLAIATITFSIGLCNG

NGPWPFLCKVSERRALLMFQDKLDP

VNRLASWAEREDSDCCSGVGVCDHTTGH

HELHxNkSEYSDWEPFNFFGGKINPS

LLSLKHLPDLDSNNDFNCTQIPF

FGSMSTLTHALGFSYVLTGPIPK

LQGLSLKLYLISFVNNFVNNLAWQ

ISGLSLKHLLDLSVNLGKASDWLQV

TNMLPSLVLVLDMSQGLQYIPLP

TNPTLPSLVLVLDLSRNFVNNLMPRW

VPSLKNLVSLHLRCQFGQGPPIST

WFLNKLARELDSNEMSLDQIPK

LQGLSLKLYLISFVNNFVNNLAWQ

ICONTGTTLTNLGNNGFNSTLPM

LYSNNLSELLLGNALRGESISS

IGN

LQGLSLKLYLISFVNNFVNNLAWQ

FSNLKXKHFVAKNSPILKTSRD

IQGLKMLDLDLSYNNFPGVSVSE

FSNLKXKHFVAKNSPILKTSRD

WVPFQLELRLDLSNHLGKFWPMW

LRVYQLELRLDLSNHLGKFWPMW

WNLTPFWWVNLSHNGVQYIPLP

NIIVAGRSVVDLSGNQPTAL

PIVPTSLVLDLSNHSFGSGVHFPCDR

PDEPKLILYLHGLNPLFKGVPCD

WMSWPLQGLVNLNGLKGVNPS

MG ELPS

LQGLTMLSVLDLSNHSFGSGVHFPCDR

GKSLSNVNLNLSNKFEGDIPNE

VCYLSQLQLDLAHLNLSGMI PCFPH

LSALADSSKSSFTPTGTNDGVSGLT

ENALVTPKTEMEY

SKLGLVNLNLSNKFEGDIPNE

LTGLALQYVNLNLSNKFEGDIPNE

IGMAQLSELDLSNHLGKFWPMW

MTMLTFLSHNLSYNLGRILE

STQLQSLDQSSFGVGNELGAPLNRCS

ENGVIPTPTVEHDGGGYSLVEDE

WYFVTLGVGFFGTGFWTLVGLSLVN

MPWILLSQLLN

RVLKMYHIVETV*

HcrVf3

LxxLxxLxxLxLxSxNkLxGxIP

MERTMRVILLIRFLAIATITFSIGLCNG

NGPWPFLCKESRQALLMFQDKLDP

VNRLASWAEREDSDCCSGVGVCDHTTGH

HELHxNkTDPFLDLKSSFGPGKINPS

LLSLKHLPDLDSNNFYFPTQIPF

FGSMSTLTHALGFSRGQVFIHN

ISGLSLKHLLDLSVNLGKASDWLQV

TNMLPSLVLVLDMSQGLQYIPLP

TNPTLPSLVLVLDLSRNFVNNLMPRW

VPSLKNLVSLHLRCQFGQGPPIST

WFLNKLARELDSNEMSLDQIPK

WLPNQKDLALSLSKNSQGLQIPK

FNMTGLKVLNLSNDFVNNLTPKW

LYSNNLSELLLGNALRGESISS

RCGPQGLKLSLRYTLISGPTFMS

FNMLSSLKLDSVNLNGLTGVPCD

IQGLKMLDLDLSYNNFPGVSVSE

FSNLKXKHFVAKNSPILKTSRD

WVPFQLELRLDLSNHLGKFWPMW

LRVYQLELRLDLSNHLGKFWPMW

WNLTPFWWVNLSHNGVQYIPLP

NIIVAGRSVVDLSGNQPTAL

PIVPTSLVLDLSNHSFGSGVHFPCDR

PDEPKLILYLHGLNPLFKGVPCD

WMSWPLQGLVNLNGLKGVNPS

MG ELPS

LQGLTMLSVLDLSNHSFGSGVHFPCDR

GKSLSNVNLNLSNKFEGDIPNE

VCYLSQLQLDLAHLNLSGMI PCFPH

LSALADSSKSSFTPTGTNDGVSGLT

ENALVTPKTEMEY

SKLGLVNLNLSNKFEGDIPNE

LTGLALQYVNLNLSNKFEGDIPNE

IGMAQLSELDLSNHLGKFWPMW

MTMLTFLSHNLSYNLGRILE

STQLQSLDQSSFGVGNELGAPLNRCS

TNGVIPPPTVEQDGGGYRLLEDS

WYFVTLGVGFFGTGFWTLVGLSLVN

MPWILLSQLLN

RVLKMYHIVETV*

Fig. 3. Comparison of the predicted amino acid sequences of *Cf9*, *HcrVf1*, *HcrVf2*, and *HcrVf3* (*HcrVf4* is not shown because its sequence is incomplete). The *Cf-9* resistance gene was divided into seven domains. Domain A is predicted to be a signal peptide, B is the presumed NH₂ terminus of the mature protein, C is a leucine-rich repeat (LRR) domain, D is of unknown function, E is an acidic domain, F is hydrophobic and predicted to be a trans-membrane domain, and G is a basic domain representing the C terminus (Jones et al. 1994). The same seven domains also are predicted for the three *HcrVf* genes. C1 and C2 are the two subdomains of domain C. The putative N-glycosylation sites are underlined.

Is one member of the *HcrVf* gene cluster the *Vf* gene?

After sequencing 50 different cDNAs hybridizing to the five BAC clones of the *Vf* locus, we cannot exclude that there are other genes in the region. An indication that the 750,000 cDNAs screened may not have been sufficient to detect all the genes of the region is the fact that we did not detect the cDNAs of *HcrVf1*, 2, and 4, which are transcribed genes. The way to determine the total number of genes present at the *Vf* locus will be to completely sequence the five BAC clones of the region. Therefore, we cannot exclude that there are candidate *Vf* genes besides the *HcrVf* genes in the *Vf* region. Anyway, in addition to the homology of the *HcrVf* genes at the *Vf* locus of apple with the tomato *Hcr9* genes, there are other similarities between *Vf* apple scab resistance and *Cf* tomato *C. fulvum* resistance that cause us to believe that one of the members of the *HcrVf* gene cluster may be the determinant of *Vf* resistance. On the host side, *Vf* apple scab resistance is influenced by the genetic background and has different resistance phenotypes, ranging from no symptoms to restricted sporulating lesions (Chevalier et al. 1991; Gessler et al. 1989). Also, the *Cf* resistance phenotype depends on the genetic background (Hammond-Kosak et al. 1994). Scab resistance is found on older leaves of all apple varieties (ontogenic resistance) (Gessler and Stumm 1984). Parniske et al. (1997) reported that certain *Hcr9* genes in tomato give resistance in older leaves but not in younger leaves. By analogy, we can hypothesize that ontogenic resistance of apple involves the *HcrVf* homologs present at the *Vf* locus and at other loci of all apple cultivars. On the pathogen side, *C. fulvum* and *V. inaequalis* are extracellular pathogens, which makes it plausible that an extracellular LRR recognition domain is involved in *Vf* resistance as it is in *Cf* resistance. It also may be possible that more than one member of the *HcrVf* gene cluster at the *Vf* locus has LRR domains that recognize the same (or different) avirulence protein(s) of *V. inaequalis*.

Because the *HcrVf* alleles from the resistant chromosome at the *Vf* locus are derived from the wild apple species *M. floribunda* and those from the susceptible chromosome are derived from *Malus × domestica* cultivars such as Golden Delicious, the two homologous chromosomes at the *Vf* locus are highly polymorphic. Thus, it is not possible to identify a single mutation in one of the *HcrVf* alleles on the resistant chromosome compared with the corresponding *HcrVf* allele on the susceptible chromosome to determine which *HcrVf* gene is the *Vf* gene. Therefore, the absence of RT-PCR products for *HcrVf1*, *HcrVf2*, or *HcrVf3* in Golden Delicious cannot be interpreted as an indication that one of these genes determines *Vf* resistance. The ultimate answer to the identity of the *Vf* gene will be obtained only from the transformation, now in progress, of susceptible apple cultivars with the single *HcrVf* genes that are transcribed and cosegregate with *Vf* resistance. Moreover, if we obtain scab-resistant transformants from high-quality commercial cultivars, we will have identified the *Vf* gene and, at the same time, created scab-resistant, high-quality apple cultivars.

Can *Hcr9* homologs contribute to the creation of durable scab resistance in apple?

A *Vf* scab-resistant cultivar has a limited value because *Vf* scab resistance has already been overcome in some regions of Europe (Bénaouf and Parisi 2000; Parisi et al. 1993). There-

fore, it is necessary to combine other scab resistance genes with *Vf* resistance. We have shown that the apple genome contains several *Hcr* genes in the *Vf* region (on the resistant and susceptible chromosomes) as well as in other regions that are different from the *Vf* locus. These *Hcr* genes could represent the ephemeral resistance genes present in all apple cultivars that are effective against monoclonal isolates of *V. inaequalis* and/or other broad-range apple scab resistance genes from wild relatives of the cultivated apple (Williams and Kuc 1969). Some of these genes have been mapped with molecular markers (Cheng et al. 1998; Gardiner et al. 1999). The sequence information of the *HcrVf* genes can now be used in an approach similar to the candidate gene approach used with the NBS-LRR genes in other plant species (Aarts et al. 1998; Kanazin et al. 1996; Leister et al. 1996; Meyers et al. 1998; Yu et al. 1996) to map and identify candidate genes for apple scab resistance genes. This could accelerate scab resistance gene cloning and, consequently, the creation of durable disease-resistant apple varieties as a result of scab resistance gene pyramiding.

MATERIALS AND METHODS

Plant material and plant inoculation.

Plants of the scab-resistant Florina (heterozygous for the *Vf* gene) budded on M9 root stock were inoculated with a conidial suspension of *V. inaequalis*, as described by Gianfranceschi et al. (1996). The inoculum was collected from susceptible seedlings of different crosses at Wädenswil (FAW, Swiss Federal Research Station). The first three leaves on each shoot were collected 24 or 48 h after incubation at 18°C and 100% humidity. They were dipped immediately in liquid nitrogen and stored at -80°C until use. Noninoculated leaves also were collected and stored in the same way.

cDNA library construction and screening.

mRNA was extracted from 0.5 g of apple leaves with the Poly(A)Pure mRNA isolation kit (Ambion, Austin, TX, U.S.A.), following the manufacturer's instructions. mRNA quality was tested by gel electrophoresis in accordance with Sambrook et al. (1989). cDNA was synthesized with the cDNA Synthesis Kit (Stratagene, La Jolla, CA, U.S.A.). The kit's instructions were followed, except rather than using the kit's columns, cDNA was size selected on a 1% agarose mini gel in 1× TAE. After a 30-min run at 5 V per cm, the marker lane was stained with ethidium bromide and the cDNA between the loading slot and the 500-bp marker band was cut from the gel. The cDNA was purified with the GENECLAN kit (Bio101, Joshua Way, CA, U.S.A.). cDNA was then cloned and packaged, following the manufacturer's instructions.

For library screening, phages were plated at a density of 20,000 PFU per 150-mm plate. Plaques were transferred to Circular Hybond N+ nylon filters, following standard procedures (Sambrook et al. 1989).

To probe the cDNA libraries with the BAC clones spanning the *Vf* region, BAC DNA inserts were isolated by digesting the BAC DNAs with *EcoRI* (the enzyme used for cloning). The restriction fragments were run on an 0.8% agarose gel, the vector band was cut from the gel, the voltage was inverted, and the insert fragments were cut from the gel, once they had again nearly reached the loading slot. The fragments were

extracted from the gel with the GENECLAN kit, and 200 ng of BAC DNA inserts were labeled with ^{32}P dCTP in a 200- μl vol with the random priming labeling kit (GIBCO BRL, Rockville, MD, U.S.A.). The probe was added to the plaque filters that had been prehybridized for 24 h at 68°C in hybridization solution (5 $\times$ SSC [1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate]; 0.5% SDS [sodium dodecyl sulfate]; 25 mM potassium phosphate buffer, pH 6.5; and 5 $\times$ Denhardt's). Hybridization was then carried out for 24 h at 68°C. Filters were washed once at 68°C for 15 min in 2 $\times$ SSC, 0.1% SDS, and twice for 30 min at 68°C in 0.5 $\times$ SSC, 0.1% SDS. Filters were exposed for 3 days to X-ray films at -80°C. Plaque regions corresponding to signals detected on the X-ray film were subjected to secondary screening after separate in vivo excisions for each of the plaque regions, and 200 colonies from each plaque region were used for the secondary screen. Positive colonies from the secondary screen were confirmed by an additional hybridization. DNA extracted from positive clones was sequenced with M13 primers with the BigDye kit (PE Applied Biosystems, Foster City, CA, U.S.A.) and run on an ABI 373 DNA sequencing system (PE Applied Biosystems). Sequences were analyzed with GCG software (Genetics Computer Group, Madison, WI, U.S.A.) and compared with GenBank by BLASTX and Fasta (Altschul et al. 1997).

BAC subcloning and screening.

BAC DNA was digested with either *Sau3AI* or *EcoRI* for 3 h, and *Sau3AI* digests were cloned directly in pGEM-4z vector (Promega, Madison, WI, U.S.A.). *EcoRI* digests were size selected on an 0.8% agarose gel in order to increase the representation of clones with large inserts, and three different slices (fragments longer than 4,000 bp, between 4,000 and 500 bp, and shorter than 500 bp) were gel purified with the Qiagen gel purification kit (Basel, Switzerland) and ligated separately to the pGEM-4z vector. Ligations were electroporated into *Escherichia coli* DH10 cells (GIBCO BRL), *Sau3AI* and *EcoRI* subclones were transferred to 384-well plates, and high-density colony filters were prepared with a Beckmann 2000 (Fullerton, CA, U.S.A.) automated workstation, in accordance with Zhang et al. (1996). Prehybridization, hybridization, and washes of subclone filters were performed as described for the phage filters. A gel-purified PCR product amplified from cDNA clone 8-1 was used as probe. Positive subclones were sequenced, and sequences were analyzed as described for the cDNA clones. Primers developed on subclone sequences were designed with the Primer 3.0 program (Whitehead Institute of Biomedical Research, Cambridge, MA, U.S.A.) and synthesized from Microsynth (Balgach, Switzerland). Primers *HcrVf1*-for (GCGGTCTCATGCCGTAAAG) and *HcrVf1*-rev (TACTGGCATATTCGTCGAG) were used to amplify the complete genomic sequence of *HcrVf1*, *HcrVf2*, and *HcrVf3* from the BAC clones spanning the *Vf* genomic region. The annealing temperature of both primers was 60°C.

Estimation of the number of *HcrVfs* at the *Vf*-region.

Southern blots carrying *EcoRI* and *DraI* double-digested BAC DNA, extracted in accordance with Patocchi et al. (1999b), were hybridized with a probe developed in the highly conserved domains E and F of *HcrVf1*, 2, and 3. The probe was amplified from *HcrVf2* with the primers domainE-for

(GGTTCTACGTGAGCTTGGGA) and domainF-rev (AGC ACTATCCTATTCAGCAACTG).

RT-PCR.

Leaves from cultivars Florina (*Vf/f*), Prima (*Vf/f*), and Golden Delicious (*vf/f*) were inoculated with *V. inaequalis*, as described by Gianfranceschi et al. (1996), and harvested 0, 12, 24, 48, and 96 h postinoculation. Total RNA was extracted from approximately 25 mg of leaf tissue with the Promega SV Total RNA Isolation System, following the manufacturer's instructions. The Qiagen OneStep RT-PCR kit was used to perform RT-PCR, following the manufacturer's instructions. The templates for RT-PCR (three bulks, one for each cultivar) were set up by mixing the same volumes of 1:50 dilutions of extracted total RNA of leaves harvested at different times, without previous quantification. To check for DNA contamination, control reactions were performed by inhibiting reverse transcriptase (15 min at 95°C). Primers RT1-for (CAATGCCT TACGTGGTGAAA) and domainF-rev (AGCACTATCTAT TCAGCAAYYSTG) were used to amplify *HcrVf1* RT-PCR products. Primers RT1-for and RT2-rev (CAGGGATTCCA GCCAATCTA) were used for the amplification of *HcrVf2* RT-PCR products, whereas primers RT3-for (TCACGGTTCAA AGACCATCC) and RT3-rev (GCAGGGATCCCAGATAT TGC) were used for *HcrVf3*. The annealing temperature of all primers was 60°C. The primers used for transcription analysis of *HcrVf2* also were used for its genetic mapping.

Single RT-PCR products were purified with the Qiagen QIAquick PCR purification kit and sequenced. Sequencing and sequence analysis were performed as described above. When RT-PCR reactions produced several bands, the bands of the expected size were gel purified and cloned into pGEM-T vector with the Promega pGEM-T vector system.

RACE.

RACE experiments were performed with the Roche 5'-3' RACE kit in combination with the Roche Expand High Fidelity PCR System, following the manufacturer's instructions. Florina RNA bulk, prepared for the RT-PCR experiments, was used as template. The three specific RACE primers, SP1 *HcrVf* (CAACCAGTCAGATGCTTTG), SP2 *HcrVf* (TGC TTTAACTGAGCAAAGAAG), and SP3 *HcrVf* (TAT TAAGGTGCAGCTCGTGG), necessary for the amplification of the 5' gene ends, were designed in regions common to the genomic sequences of *HcrVf1* and *HcrVf2*. RACE products were run on a 1% 0.5 $\times$ Tris-borate-EDTA agarose gel, and bands of the expected size were gel purified and blunt-end cloned with the Roche PCR cloning kit.

ORF analysis and protein prediction.

ORFs were predicted with ORF Finder. Putative signal peptide cleavage sites and putative transmembrane domains of the *HcrVf* genes were predicted with the CBS prediction server. N-glycosylation sites were predicted in accordance with Alberts et al. (1990).

ACKNOWLEDGMENTS

The authors are grateful for the financial support made available by the Laimburg Center for Agricultural and Forestry Experimentation, Autonomous Province of Bolzano-Bozen, Italy, and the Swiss Federal Institute of Technology, grant 0-20-354-96.

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