

The *HcrVf2* gene from a wild apple confers scab resistance to a transgenic cultivated variety

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The *Vf* gene from the wild species *Malus floribunda* 821 is the most studied apple scab resistance gene. Several molecular markers mapping around this gene were the starting point for a positional cloning project. The analysis of the bacterial artificial chromosome clones spanning the *Vf* region led to the identification of a cluster of genes homologous to the *Cladosporium fulvum* resistance gene family of tomato. One of these genes, *HcrVf2* (homologue of the *C. fulvum* resistance genes of the *Vf* region), was used to transform the susceptible apple cultivar Gala. Four independent transformed lines resistant to apple scab were produced, proving that *HcrVf2* is sufficient to confer scab resistance to a susceptible cultivar. The results show that direct gene transfer between cross-compatible species can be viable when, as in apple, the use of backcrosses to introduce resistance genes from wild species cannot exactly reconstitute the heterozygous genotype of clonally propagated cultivars.

Apple scab, which is caused by the ascomycete *Venturia inaequalis* (Cke.) Wint., is the most important disease in all apple-growing districts with high spring and summer rainfall. Its control in commercial orchards can require up to 15 fungicide treatments per year. An alternative approach is the use of resistant cultivars (cvs.), and sources of scab resistance have been found in small-fruited wild species (1). However, the transfer of these genes by classical breeding to cultivated apples is difficult because of the long juvenile phase, self-incompatibility, and the impossibility of exactly reproducing the heterozygous state of cultivated varieties. Starting from the wild species *Malus floribunda* 821 carrying the *Vf* gene, breeders have developed several scab-resistant apple cvs. (2), but not one has met with commercial success. Indeed, when compared with such commercially popular cvs. as Golden Delicious and Gala, the main horticultural and fruit-quality traits of these scab-resistant cvs. are notably different and undoubtedly less acceptable. The direct transfer of the *Vf* gene to apple cvs. thus is seen as a potential strategy for disease control. Moreover, the fact that two *Vf*-virulent races of *V. inaequalis* have been identified makes *Vf* resistance a good system in which to study gene-for-gene interaction between apple and scab (3–5).

The mode of *Vf* inheritance corresponds to a single dominant gene or to the combined action of a cluster of tightly linked genes (1). However, the *Vf* gene is not viewed strictly as dominant, because homozygous *Vf* plants are usually more resistant than heterozygous ones (6, 7). When inoculated with scab, *Vf*-carrying apple plants show varying resistance reactions, ranging from the absence of symptoms to chlorotic/necrotic, clearly sporulating lesions. Plants without the *Vf* gene do not develop a defense reaction, and heavily sporulating, unrestricted lesions are visible. It has been proposed (8, 9) that the variation in resistance reactions of *Vf*-carrying plants may be due to the presence and action of modifier genes.

To elucidate the *Vf* gene's complex mode of action, positional cloning was performed, and the steps leading to the identification of a cluster of resistance genes encoding receptor-like proteins have been reported (10–13). The amino acid sequence

of these genes [homologues of *Cladosporium fulvum* resistance genes of the *Vf* region (*HcrVf*) (11)] contains an extracellular leucine-rich repeat domain and a putative transmembrane domain similar to those of the *Cf* resistance genes of tomato (14). The cluster is composed of at least four *HcrVf* genes, all transcribed but one producing only truncated mRNA (15). Fully transcribed genes *HcrVf1*, -2, and -4 cosegregated with apple scab resistance in >2,000 assayed plants and thus are viewed as candidate *Vf* resistance genes. This article reports the complete evidence that the *HcrVf2* gene derived from a wild *Malus* species can induce apple scab resistance in transformed cv. Gala plants and thus reports the cloning of a resistance gene from a woody plant.

Materials and Methods

Construction of the Plasmid Binary Vector. The entire ORF of *HcrVf2* (GenBank accession no. AJ297740) and the constitutive cauliflower mosaic virus 35S promoter were cloned into the binary vector pCambia-2301 (GenBank accession no. AF234316). Clones were verified by sequencing, and the clone pCORF2 was chosen because, except for one base pair change in the signal peptide, it revealed complete identity with *HcrVf2* (16). This clone was subsequently transferred into the *Agrobacterium tumefaciens* EHA105 strain containing the helper plasmid pCH32 (17) by following the methods of Höfgen and Willmitzer (18).

Plant Material and Apple Transformation. The *HcrVf2* gene was introduced into the scab-susceptible apple cv. Gala by *A. tumefaciens*-mediated transformation using the engineered EHA105 strain. Plantlets grown *in vitro* were preconditioned by vacuum infiltration in liquid regeneration media TN505 (16) or TN22 [Murashige and Skoog (MS) medium supplemented with 2 mg·liter⁻¹ naphthalene acetic acid and 2 mg·liter⁻¹ thidiazuron; P. Negri, personal communication]. Infection and cocultivation of leaves (19) and internodes (20) were followed by decontamination (200 mg·liter⁻¹ cefotaxime) and selection (50 mg·liter⁻¹ kanamycin) on the two media. Regenerating shoots were proliferated on A17 (21) and rooted on half-strength MS medium supplemented with 2 mg·liter⁻¹ indolebutyric acid (P. Negri, personal communication). Transformed lines were named Ga2,

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Abbreviation: cv., cultivar.

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for Gala transformed with *HcrVf2*, and numbered consecutively as they regenerated. As controls for greenhouse apple scab inoculation, *in vitro*-grown shoot tip cultures of Gala, Florina, and Enterprise were rooted, and the plants were acclimated to the greenhouse.

Evaluation of Apple Scab Resistance. Apple scab resistance of the five transgenic lines Ga2-2, Ga2-5, Ga2-7, Ga2-8, and Ga2-21 was tested by greenhouse inoculation. Young leaves of actively growing plants were sprayed with a suspension of *V. inaequalis* conidia (at least 200,000 conidia per ml) derived from cvs. Gala, Red Chief, and Golden Delicious growing in an untreated orchard. Conidia vitality was verified after 24 h by determining the fraction of germinated conidia ($\approx 80\%$). Inoculum ability to infect Gala was tested by including, in all experiments, cv. Gala and/or a regenerated Gala line lacking the *HcrVf2* gene (Ga2-7); the varieties Florina and Enterprise served as scab-resistant controls. A completely randomized scheme was adopted for plant location in the greenhouse. The environmental conditions were set as follows: $19 \pm 5^\circ\text{C}$ and 100% humidity for 48 h postinoculation and 70–80% thereafter. Three independent infection cycles were carried out in an attempt to test all transformed plants and controls twice.

Macroscopic symptoms and infection severity were evaluated on each leaf 10, 15, and 21 days postinoculation according to Chevalier (22) and Croxall (3, 23) scales. The maximum level of symptoms recorded 21 days postinoculation and their median and confidence limits were computed to describe the overall response of each line.

The presence of germinated *V. inaequalis* conidia on the leaf surface of inoculated plants was assessed at the end of the second inoculation round by observing by optical microscopy one inoculated leaf per plant. At 21 days postinoculation, leaves were prepared for microscopy by following the methods of Silfverberg-Dilworth *et al.* (24).

DNA Extraction and Detection of T-DNA Integration. T-DNA [portion of the Ti (tumor-inducing) plasmid that is transferred to plant cells] integration was confirmed by Southern blot analysis. *Hind*III-digested genomic DNA from greenhouse-acclimated plants was transferred to nylon membranes (Hybond-N+, Amersham Pharmacia Biosciences) and hybridized with probes produced by radiolabeling PCR-generated fragments of the entire *HcrVf2* ORF and a section of the neomycin phosphotransferase II (*nptII*) gene (Fig. 1).

RNA Extraction and RT-PCR. For transgenic and control plants, the presence or absence of the *HcrVf2* and *nptII* mRNAs was determined 21 days postinoculation. Total RNA was extracted from 30 mg of young leaf tissue and quantified. cDNAs were synthesized from 0.2 μg of total RNA by using the ImProm-II Reverse Transcription system (Promega) with an oligo(dT) primer. PCR was performed by using *HcrVf2*-specific primers (RT1 forward and RT2 reverse) (11) and *nptII*-specific primers (25). PCR using total RNA as template was performed to detect any DNA contamination.

Results

Transfer of *HcrVf2* Gene to the Scab-Susceptible Apple cv. Gala. *Agrobacterium*-mediated transformation of cv. Gala with *HcrVf2* was successful and yielded several independent transformed lines. Of these, five proliferating well in culture *in vitro* were acclimated to the greenhouse and tested for scab resistance. Integration of the T-DNA (Fig. 1a) and uniformity among regenerated plants of the same line were confirmed by Southern hybridization of DNA extracted from single plants of the lines Ga2-2, Ga2-5, Ga2-7, Ga2-8, and Ga2-21. Two probes, one from *HcrVf2* and the other from the selectable marker *nptII*, which

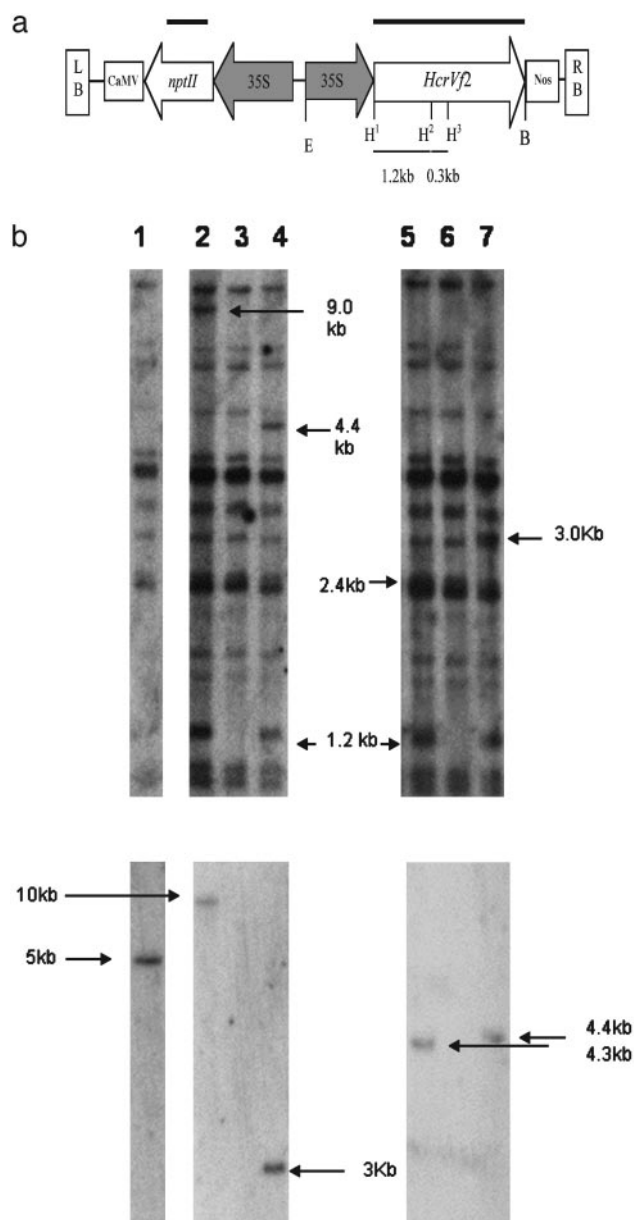


Fig. 1. (a) Schematic representation of T-DNA from pCORF2 used in the transformation experiment. *nptII* and the putative scab resistance gene *HcrVf2* are regulated by the cauliflower mosaic virus 35S promoters (filled arrows). E, *Eco*RI; B, *Bst*EII; H, *Hind*III; LB, left border; RB, right border; CaMV, cauliflower mosaic virus 35S poly(A) signal; Nos, Nos poly(A) signal. H¹, H², and H³ represent restriction sites 1, 2, and 3, respectively, and thin bars represent the distance between *Hind*III restriction sites. Thick bars represent Southern blot probes. (b) T-DNA integration and copy number. Southern blot hybridization of *Hind*III-digested genomic DNA. Lanes: 1, Ga2-7; 2, Ga2-2; 3, Gala; 4, Ga2-5; 5, Ga2-21; 6, Gala; 7, Ga2-8. (Upper) T-DNA was hybridized with *HcrVf2* probe, showing, in addition to the 1.2-kb specific fragment of the *HcrVf2* gene, a second fragment for the estimation of the copy number. [The 300-bp fragment produced by *Hind*III sites 2 and 3 (H² and H³ in a) was too small and was not transferred to the membrane.] Ga2-2, Ga2-5, Ga2-8, and Ga2-21 each contain a single copy of the pCORF2 T-DNA. Ga2-7 contains no *HcrVf2* gene. T-DNA integration is shown by fragments of 9.0, 4.4, 3.0, and 2.4 kb hybridizing to this probe for the lines Ga2-2, Ga2-5, Ga2-8, and Ga2-21, respectively. (Lower) *nptII* probe. All transgenic lines show one single hybridization band of specific size demonstrating the presence of and integration to the *nptII* gene.

confers resistance to kanamycin, were used. Twelve fragments hybridizing to the *HcrVf2* probe were present in untransformed Gala DNA. Additional hybridization signals supported trans-

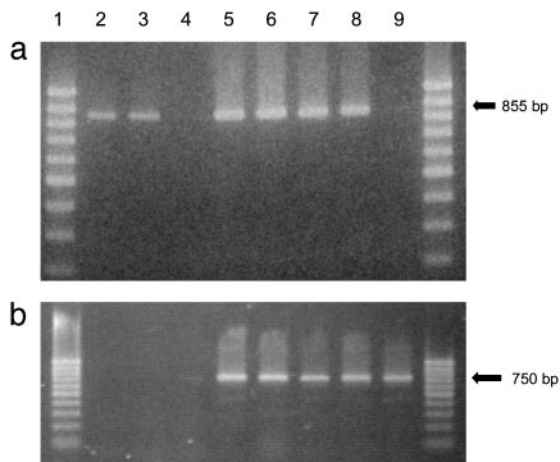


Fig. 2. Transcription evaluated by RT-PCR. Lanes: 1, 100-bp ladder; 2, Enterprise; 3, Florina; 4, Gala; 5, Ga2-2; 6, Ga2-5; 7, Ga2-8; 8, Ga2-21; 9, Ga2-7. (a) *HcrVf2* primers show that the gene is expressed in *Vf* cvs. and in all transformed lines except Ga2-7. (b) *nptII* primers show that the gene is expressed only in transformed lines.

gene integration in transformed regenerated plants (Fig. 1b). Four transgenic lines (not Ga2-7) showed two additional bands. One gene fragment (1.2 kb) was produced by *Hind*III sites within the T-DNA and another, of at least 1.5 kb, was produced by a *Hind*III site in the T-DNA and an additional site within the apple genome. This latter fragment, the same size for all individual plants of each line, was 9.0, 4.4, 3.0, and 2.4 kb for the lines Ga2-2, Ga2-5, Ga2-8, and Ga2-21, respectively. Southern analysis based on the *nptII* probe showed a single band with a specific size for each transgenic line, including Ga2-7 (Fig. 1b).

The hybridization results support the presence of one complete copy of the T-DNA in the transgenic lines Ga2-2, Ga2-5, Ga2-8, and Ga2-21. Plants of the line Ga2-7 most likely contain a partial integration of the T-DNA, because only fragments also hybridizing with Gala DNA hybridized to the *HcrVf2* probe, whereas one clear hybridization signal was evident for the *nptII* probe. Moreover, PCR amplification of 35S::*HcrVf2* from this line was negative and that from the *nptII* positive, thus support-

ing the conclusion of a partial integration of the transformation construct in line Ga2-7 (data not shown).

Transcription of *HcrVf2* is demonstrated by RT-PCR, because the expected fragment (855 bp) was amplified from the *Vf*-resistant cvs. Enterprise and Florina, as well as all plants of lines Ga2-2, Ga2-5, Ga2-8, and Ga2-21. No amplification was noted from Gala or Ga2-7 plants (Fig. 2a). The *nptII* gene transcription was confirmed in all transgenic lines but not in control cvs. (Fig. 2b). PCR carried out directly with RNA showed that no DNA contamination was present in any of the samples (data not shown).

Scab Resistance Evaluation. To assess scab resistance in transgenic lines, plants were evaluated 21 days postinoculation with *V. inaequalis* conidia. The untransformed cv. Gala and the line Ga2-7 showed clear symptoms of susceptibility, whereas untransformed *Vf*-resistant varieties and *HcrVf2* transgenic plants proved resistant (Table 1). Resistant plants were inoculated twice, and the results were consistently similar, as indicated by the median value and confidence limits (Table 1). Gala showed high scab susceptibility to the inoculum, with sporulation observed as early as 10 days postinoculation. Symptom severity peaked after 21 days, with heavy sporulation observed on all inoculated young leaves; in addition, Gala showed no resistance reaction (Fig. 3). Similar symptoms were observed in plants from the line Ga2-7, which contains a functional copy of the *nptII*, but not the *HcrVf2*, gene. Additional inoculations of Ga2-7 and Gala plants proved impossible because of the extreme damage suffered after the first inoculation round.

On leaves of the *Vf*-carrying cv. Enterprise, small chlorotic lesions appeared after 10 days; ~25% of the leaf surface of one to two leaves per plant showed chlorotic lesions after 21 days. During the second inoculation round, necrosis and a very limited level of sporulation were observed on two Enterprise leaves. Leaves of the other *Vf*-carrying cv., Florina, were inoculated only once and showed chlorotic lesions after 21 days.

The highest level of resistance was observed in transgenic lines Ga2-5, Ga2-8, and Ga2-21, which had either no symptoms or rare, pinpoint pits as resistance reactions (Fig. 3). The transgenic line Ga2-2 showed no symptoms on approximately half of the young inoculated leaves and small chlorotic lesions after 10 days on the other leaves; in a few cases, after 21 days the chlorosis

Table 1. Pooled results of the three greenhouse scab inoculation rounds 21 days postinoculation

Line or cv.	<i>HcrVf2</i> gene presence	No. of inoculated plants	Symptom*				Severity†			
			Confidence limit				Confidence limit			
			Med.	L	U	Max.	Med.	L	U	Max.
Ga2-2	+	6	0	0	2	3b	0	0	2	3
Ga2-5	+	4	0	0	0	1	0	0	0	3
Ga2-8	+	4	0	0	0	1	0	0	0	3
Ga2-21	+	3	0	0	0	2	0	0	0	1
Ga2-7	-	4	4	4	4	4	6	4	7	7
Gala	-	7	4	4	4	4	6	4	7	7
Enterprise	+	2	2	0	3b	3b	3	0	4	4

Median values of symptoms and their confidence limits were calculated by using the data from the three youngest leaves present at the time of each plant's inoculation. Med., median; Max., maximum; L, lower; U, upper. *Symptoms were assessed after Chevalier *et al.* (22) with 0 = no macroscopically visible symptom, 1 = hypersensitive pinpoints, 2 = chlorotic areas with no sporulation, 3a = chlorotic and necrotic areas with no sporulation, 3b = chlorotic and necrotic areas with sparse sporulation, and 4 = sporulation covering entire leaf. These values represent degrees of resistance, with 4 representing susceptibility. Confidence limit $\alpha = 0.01$.

†Infection severity values were assessed on a modified Croxall scale (3, 23): 1 indicates a percentage of leaf area with sporulating symptoms from 0% to 1%; 2, from 1% to 5%; 3, from 5% to 10%; 4, from 10% to 25%; 5, from 25% to 50%; 6, from 50% to 75%; and 7, from 75% to 100%. Confidence limit $\alpha = 0.01$.

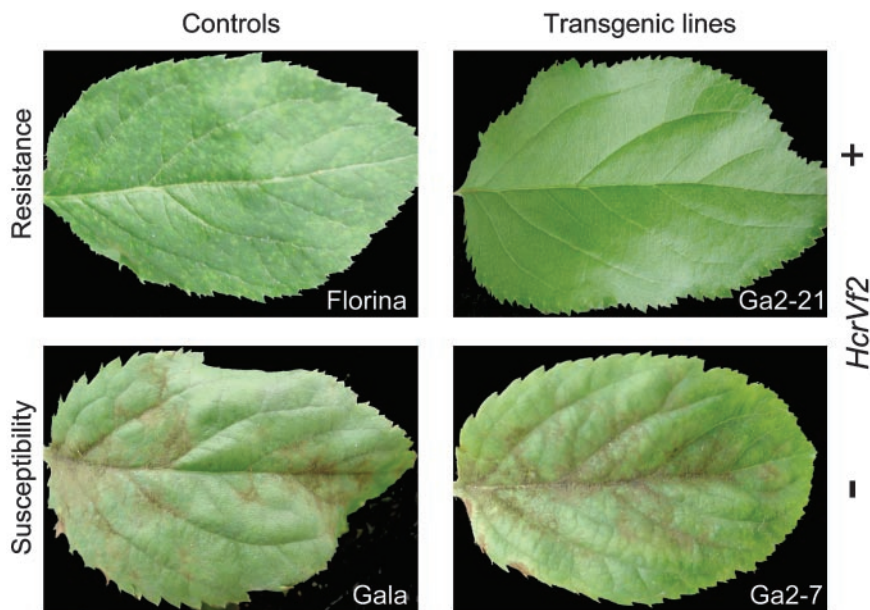


Fig. 3. Leaf symptoms 21 days after inoculation with *V. inaequalis* conidia. Transgenic line Ga2-21 (Upper Right), containing *HcrVf2*, represents typical symptoms observed on all *HcrVf2* transformed lines. Transgenic line Ga2-7 (Lower Right) contains only the selective *nptII* gene and is thus a transgenic control. The susceptible cv. Gala (Lower Left) and resistant cv. Florina (Upper Left) are included for the comparison of scab symptoms.

became more severe, and small necrotic lesions with restricted sporulation were observed on one leaf of one plant. Although Ga2-2 occasionally showed some restricted fungal development, the line still can be considered more resistant than the *Vf*-carrying cv. Enterprise, as indicated by the median (Table 1).

The presence of germinated *V. inaequalis* conidia was confirmed on the leaves of plants showing either a resistance reaction or no symptoms from lines Ga2-2, Ga2-5, Ga2-8, and Ga2-21 (Table 2). In most cases (97% of conidia observed), no fungal development beyond that of appressoria and penetration peg formation was observed (class γ). In a few rare cases, subcuticular primary stroma formed, but expansion did not exceed 40 μm (class β). On leaves from two plants of the line Ga2-2 that had small chlorotic lesions, small isolated areas of stroma with sporulating conidiophores were found (class α). However, an equivalent fungal development was seen on a leaf of the *Vf*-resistant cv. Enterprise.

Table 2. Presence of germinated *V. inaequalis* conidia on leaf surface of resistant plants

Line or cv.	No. of leaf samples*	No. of conidia		Fungal development†		
		Average per leaf sample	Total	α	β	γ
Ga2-2	6	97	582	55	8	519
Ga2-5	4	137	548	0	1	547
Ga2-8	4	100	401	0	0	401
Ga2-21	3	80	240	0	0	240
Enterprise	1	54	54	10	33	11

This table does not contain the data from susceptible plants because on Gala and transgenic Ga2-7 leaf samples the new stroma and conidiophores were too dense, making conidia counting impossible.

*For each plant, one leaf section ($\approx 1.5 \times 1 \text{ cm}$) was examined.

†Fungal development for each conidium was defined according to the following classes: α , isolated areas of stroma with sporulating conidiophores; β , primary stroma expanding no more than $\approx 40 \mu\text{m}$; and γ , no fungal development beyond that of appressoria and penetration peg.

Discussion

The reported data show that the *HcrVf2* gene can confer resistance to apple scab when transformed in the scab-susceptible cv. Gala. Of the five transgenic lines, the Ga2-5, Ga2-8, and Ga2-21 showed a very high level of resistance. Line Ga2-2 showed a level of resistance at least comparable with that of *Vf* cvs., although the peak symptoms observed in these plants were more severe than those seen in plants of the other transgenic lines. Because all of the transgenic resistant lines contain only a single copy of the transgene, the differences between Ga2-2 and the other scab resistance transformants are most likely attributable to the integration site of T-DNA, which may modify transgene expression (26, 27) and, consequently, influence the level of resistance. The slight differences observed between inoculations were probably due to environmental variation, because they were observed in both transformed and control plants. The fact that transgenic line Ga2-7, containing the *nptII*, but not the *HcrVf2*, gene, is scab-susceptible proves that neither the method used to regenerate plants nor the *nptII* gene affects plant resistance to *V. inaequalis*.

The interaction of *V. inaequalis* with *Malus* genotypes follows the gene-for-gene rule (28, 29). Our data, in fact, clearly show that, when transformed with the *HcrVf2* gene, the scab-susceptible cv. Gala can develop an incompatible interaction with a mixed-strain inoculum of *V. inaequalis*.

HcrVf2 is one of at least three resistance genes mapping at the *Vf* locus (11). The influence of the *HcrVf1* and *HcrVf4* genes on scab resistance must be investigated to determine whether *HcrVf2* is the only gene able to trigger a resistance response. Of the >30 plant resistance genes characterized so far, at least half are members of gene clusters (30). In tomato, the *Cf* genes confer resistance to *C. fulvum*, and different members of the *Cf* gene family recognize different *avr* genes (31, 32). The two resistance genes *Cf-4* and *Cf-9* each are found within a cluster of five ORFs, and within each cluster two ORFs confer resistance to *C. fulvum* (*Cf-4* and *Hcr9-4E*, *Cf-9* and *Hcr9-9A* or *Hcr9-9B*, respectively). Interestingly, *Hcr9-9A* and *Hcr9-9B* are active only in adult plants, whereas *Cf-9* and *Cf-4* are active in seedlings, too, showing a differentiated expression pattern between the mem-

bers of a cluster (33, 34). The resistance gene *Xa21* in rice, which confers resistance to *Xanthomonas oryzae* pv. *oryzae*, encodes a receptor-like kinase with an extracellular leucine-rich repeat and a serine/threonine kinase in the putative intracellular domain. Of seven ORFs studied within the *Xa21* gene family, only two were found to contain the complete sequence encoding for receptor kinase-like proteins (35), and only *Xa21* has been shown to confer resistance (36). The *Pto* resistance gene in tomato mediates resistance to strains of *Pseudomonas syringae* pv. *tomato* expressing *avrPto*. *Pto* is the only gene of a cluster of seven found to interact with the AvrPto protein and, hence, to confer resistance, although several genes of the cluster have been shown to be transcribed. Note, however, that only one *avr* ligand has been identified from pv. *tomato* and that the other ORF found at the *Pto* locus may interact with other strains of the pathogen (37). The examples cited show that, although *HcrVf2* clearly can confer resistance to apple scab, it will be important to clarify the function of all genes within the *Vf* cluster.

The cloning of an apple scab resistance gene represents the basis for further investigation of the resistance mechanism. It also represents a step toward a gene therapy (restoring resistance where lost) of the scab-susceptible cvs. that currently dominate

the apple industry. This strategy will allow the transfer of resistance from a wild apple species to any commercial apple genotype while maintaining the horticultural and fruit-quality traits growers and consumers prize most. It may also be possible to achieve greater resistance durability by the simultaneous transfer of several resistance genes from wild apple species. Going one step further, it may be possible to use apple promoters and novel techniques that, by eliminating selective marker genes (38, 39), generate transgenic varieties without any foreign genes and, hence, may make genetically modified plants more acceptable to growers and consumers alike.

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