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REVIEW

Vf scab resistance of *Malus*

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Abstract The apple production in temperate regions with spring rains, the Scab caused by the fungus *Venturia inaequalis* is the most important constraint. To produce spotless apples and avoid damage that develops during storage, growers apply fungicide on a regular or weather-determined basis. All major apple cultivars are highly susceptible to this disease. To limit the need for fungicide applications, apple breeders are currently introgressing disease resistance from wild *Malus* accessions into commercial lines. The first attempts to do this were made 100 years ago. As apples are self-incompatible, pseudo-backcrossing is used to eliminate unwanted traits from wild *Malus* and select new cultivars that are attractive to both producers and consumers. This process, from the first cross of a commercial cultivar with a wild, disease-resistant *Malus*, is extremely long due to apple's long juvenile phase, the need for more than seven backcross steps and the high heterozygosity of this genus. Therefore, most of today's scab-resistant cultivars rely on a single introduction of scab resistance from *Malus floribunda* 821, referred to as Vf. In this paper, we trace the history of Vf from its initial identification through its use in breeding and commercial production. We sum up the literature describing how and where Vf resistance has been overcome by new pathotypes of *V. inaequalis*. Finally, we describe the current knowledge of the genes behind Vf resistance, its mode of action and the use of *Vf* genes in gene technology.

Keywords Apple · Biotechnology · Resistance breeding · *Venturia inaequalis*

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Introduction

Apple is one of the major fruit crops of temperate regions. Early on, humans learned to graft wood or buds from a particular apple tree onto a rootstock, to maintain the desirable traits that are overwhelmingly lost when apple is propagated through seeds. This approach eventually led to the high genetic uniformity (few cultivars) of today's apple orchards. The causal agent of apple scab (*Venturia inaequalis*) was favored by this system and apple scab became a disease requiring intense chemical control programs (MacHardy et al. 2001), which are costly, cumbersome and pollute the environment. Scientists recognized the importance of breeding for resistance early on and some apple breeders recognized the potential benefit of introgressing resistance from wild *Malus* species (Hough et al. 1953; Dayton and Mowry 1970; Lespinasse et al. 1976; Kellerhals and Furrer 1994). However, they also were, and are aware that it takes many double pseudo-backcrosses, each involving a different cultivar as the donor of high-quality traits, to eliminate undesirable "wild" traits. In addition, for each pseudo-backcrossing step, a large number of descendants have to be analyzed. The result was that each breeder continued the work of his predecessor using the most advanced selections for his own crosses, with the final result that a single source of resistance was used almost exclusively. In the breeding of scab resistance into apple populations, the Vf resistance that originated from a single

tree referred to as *Malus floribunda* 821 was used in this way. This review traces the story of Vf.[1] Many details have been presented in previous review articles on apple scab (Boone 1971; MacHardy 1996), as Vf resistance has always played a major role in any applied or fundamental research on *V. inaequalis* (Gessler et al. 2006). However, this review honors all researchers who dedicated their effort in using and understanding this resistance, fundamental for the current apple breeding and apple production with reduced fungicide input either organic or conventional. About 400 research papers deal with the Vf resistance of which three-fourth have focused on the breeding of resistant cultivars (CAB data base 1910 until Feb 2011). We will cite publications which we judge to contribute to our purpose, and leave out the breeding and testing of Vf cultivars. We request all authors to excuse us for whose work we do not acknowledge appropriately.

Identification of Vf resistance and its use in breeding

At the University of Illinois, Crandall (1926) crossed a high number of crab apples with commercial cultivars. In 1914 and 1915 he used the apple cultivar "Rome Beauty" and *Malus floribunda* accession No. 821 and produced around 450 seedlings which were planted and further crossed. Some of this material survived and 20 years later two siblings ($F_2$26829-2-2 and $F_2$26830-2) recognized to be scab resistant (Hough 1944) were used for further crosses and the inheritance of scab resistance analyzed (Hough et al. 1953), and named Vf [*Venturia floribunda*; (Williams et al. 1966). These two scab-resistant plants were the starting material for the PRI Cooperation Program (Purdue University (IN), Rutgers University (NJ) and the University of Illinois]. $F_2$26829-2-2 was crossed with Golden Delicious or Jonathan and some of the resistant offspring were then crossed with other commercial cultivars. "Prima" was the first of a long series of PRI cultivars with an acceptable fruit size and quality (Dayton and Mowry 1970). All are heterozygous for the dominant genetic factor Vf. Some have additional disease resistance, so Prima under field conditions, it is also resistant to *Erwinia amylovora* and *Phyllosticta solitaria*. Most of the PRI cultivars ripen early; Prima ripens about 1 month before "Red Delicious" and has a short shelf-life (Williams et al. 1972a, b).

By analyzing the segregation of scab resistance derived from selected genotypes of *M. prunifolia*, *M. atrosanguinea*, *M. baccata*, *M. prunifolia microcarpa* and some *Malus* accessions (MA4, MA8, MA16 and MA 1255), Williams et al. (1966) concluded that the genes involved in this resistance to *V. inaequalis* are the same or allelic to those involved in the Vf resistance of *M. floribunda* 821 (see review in Gessler et al. 2006). During 1970 s and 1980 s, there were intensive efforts to breed scab resistance around the world. Most of these programs involved the advanced selections of the PRI program (Janick 2002). In some cases, breeders attempted to diversify the sources of resistance and accumulate various functionally different resistances against scab (Boldyzheva et al. 1985; Braniste, 1981; Denardi et al. 1988; Fomina 1980; Korban et al. 1988, 1990; Krasova 1981; Lespinasse et al. 1976; Mehlenbacher et al. 1988; Sedov and Zhdanov 1981; Zhdanov and Fomina 1981).

In 1992, Crosby et al. (1992a, 1992b) reported that over 17 breeding programs were using mainly Vf resistance and that those efforts had yielded 48 named varieties. Currently, we estimate that the number of released and named cultivars is above 80. In some cases (i.e. "Nova Easygro"), even if the breeder attempted to use a different resistance gene (Vr) (Lespinasse and Olivier 1981), the use of molecular markers later revealed that the introduced resistance was actually the same classic Vf resistance (Gianfranceschi et al. 1996). The major obstacle to stacking different resistance genes has been the lack of a fast and cheap system for recognizing individual daughter plants that have inherited both parental resistance genes, since different types of resistance can usually not be phenotypically distinguished. Therefore, only an analysis of the segregation pattern among the F_2 progeny of the resistant (heterozygous) and a susceptible selection (Williams et al. 1966; Ishchenko et al. 1981) will reveal the presence of two resistant genes. A 3:1 (resistant:susceptible) segregation ratio indicates the presence of two types of resistance; whereas a 1:1 ratio indicates the presence of only one in a heterozygous (r/s) allelic state. There may also be special cases in which races that specifically overcome a single type of resistance might be used and/or specific resistance reaction symptoms might be induced (e.g. rapidly forming pin-point pits, such as those induced by the presence of Vm resistance) (Williams and Kuc 1969).

Currently, in almost all scab-resistant cultivars, the resistance is solely due to *Vf*-genes. All major Western breeding programs currently view the selection of disease-resistant cultivars as a priority. Scab resistance still primarily relies on Vf, but efforts to combine it with resistances from other sources are underway (Kellerhals et al. 2006; Kozlovskaya et al. 2000; Lefrancq et al. 2004; Sansavini et al. 2002; Soufflet-Freslon et al. 2008). The

[1] As the naming of resistances after their origin and the various pathotype of *V. inaequalis* able to overcome these resistances with numbers may lead to confusions Bus et al. (2009) renamed all resistances and corresponding pathotypes, so that in future the Vf resistance will change to Rvi6, all pathotypes of *V. inaequalis* unable to infect a Rvi6, will be named race AvrRvi6 and those able to infect a Rvi6, virRvi6.

development of DNA-based markers linked to other resistances than Vf was the key for Marker-assisted selection of individuals carrying several heterozygous resistances (Baniulis et al. 2008; Kellerhals et al. 2009; Sansavini et al. 2005). While several high-quality and competitive scab-resistant cultivars have been released in recent years, their market share has been negligible. In organic production, however, scab-resistant cultivars find acceptance. For example, scab-resistant cultivars account for 40% of organic apple production in Switzerland. Meanwhile, scab-resistant cultivars account for only a minor share of the 12 million tons of apples produced in the conventional or integrated apple production systems of Europe each year. In the statistics, scab-resistant cultivars accounts for about 0.5% of Europe's (EU27, 2007) annual apple production surface (Topaz 0.24%, Prima 0.1%), as compared to "Golden Delicious" (13%), Jonagold (6.6%) or "Gala" (6%). (http://epp.eurostat.ec.europa.eu/portal/page/portal/agriculture/data/database)

Vf plant reaction against scab

Vf resistance does not give uniform reaction to scab inoculation. *M. floribunda* 821 and some of its resistant descendants react to scab inoculation by forming pin-point pits. The reactions of selections $F_2$26829-2-2 and $F_2$26830-2 (obtained from sibling F_1') and all of the resistant cultivars derived from them range from no macroscopic visible reaction to the formation of chlorotic and necrotic lesions up to the formation of a few restricted necrotic lesions with sparse sporulation (class 3; (Lespinasse 1989; Parisi and Lespinasse 1996). Williams and Kuc (1969) suggested that "the original Vf resistance was not due to a single qualitative gene, but either to a group of rather closely linked quantitative genes or to a class 3 reaction qualitative gene,[2] closely linked with one or more quantitative genes."

However, Williams and Kuc (1969) could not explain the absence of the pin-point pit reaction in certain descendants of *M. floribunda* 821. With the identification of pathotype 6, which overcomes the resistance of cultivars possessing Vf resistance (without any pin-point pit reaction) and not that of the original *M. floribunda* 821, and pathotype 7, which overcomes the original *M. floribunda* 821 resistance it became possible to undertake a genetic analysis of host-pathogen interactions. Parisi and Lespinasse (1999); Bénaouf and Parisi (2000) demonstrated the existence of a second dominant gene in *M. floribunda* 821 which was inherited to F_2 26830-2. This gene, which is different from *Vf,* but linked[3] to it, was named *Vfh* because it seemed to induce a hypersensitive reaction. Vfh resistance was not inherited to F_2 26829-2-2 so all descendent from this siblings can not carry *Vfh*. Still, the fact that Vf resistance, as found in advanced selections and varieties, did not yield a uniform reaction required some explanation. Rousselle et al. (1974) suggested that the cumulative effect of the minor genes inherited from both the susceptible and the resistant parent modify the level of resistance conferred by Vf. As different numbers of such minor genes will be inherited by each individual daughter plant, the degree of resistance will also vary. Some of these minor genes are lost in subsequent backcrosses (Rousselle et al. 1974), which explains why a cultivar exhibiting a class 3b reaction can still be classified as resistant and carrying the Vf gene (Tartarini et al. 2000).

The results of an analysis of older segregation data (Gessler 1989) and a microscopic analysis point in the same direction (Gessler 1992). Loci of postulated genes that modify Vf resistance were later identified by mapping in a A679-2 (Vf carrier) × "Iduna" cross and an analysis of quantitative resistance loci (background resistance) was performed based on field data (Seglias-Hodel 1997). Seglias defined loci that contribute quantitatively to the resistance only in the Vf carriers as "modifier loci" and loci that always contribute to resistance or contribute only in the non-carriers of Vf as "background resistance loci". The Vf resistance on one hand, corresponds to the definition of a qualitative resistance (yes/no) governed by a single gene (allele) in a gene for gene reaction (see below). On the other hand, the Vf resistance reaction not being uniform, but depending on the genetic environment in which it is embedded, has attributes of a quantitative resistance (more/less). Moreover, homozygote Vf individuals appear to express a stronger resistance than the heterozygous in populations derived from a cross of two heterozygous Vf

[2] Reaction to *V. inaequalis* inoculation or natural infection was scored in classes as follows: 0, signifying no macroscopic evidence of infection; 1, pin-point pits and no sporulation; 2, irregular chlorotic or necrotic lesions and no sporulation; 3, a few restricted, sporulating lesions; and 4, extensive, abundantly sporulating lesions. Since the original reaction classes were described, another (class M) has been added. This class is described as a mixture of necrotic, non-sporulating and sparsely sporulating lesions. This same method of scoring plants is used in the PRI program, in which only class 4 is considered to be field-susceptible. The class 1 or pin-point reaction is considered to be a hypersensitive response. The host epidermal cells below the infection peg collapse within 40–72 h and, shortly thereafter, the fungus is killed. In contrast, the other reaction types are not expressed until 3–12 days after inoculation and the fungus remains viable for as long as 21 days. This scheme of classification was later adopted and described microscopically by Chevalier et al. (1991), however class 3 was named class 3a and class M, class 3b.

[3] We have to assume that Vf and Vfh are linked and in a heterozygous state r/s as the segregation of resistance in progenies from the F_2 26830.2 is 1:1. If the two resistances would be independent Hough et al. (1953) would have noted a segregation of 3:1 instead of 1:1.

parents (Gessler et al. 1997; Gianfranceschi et al. 1999; Tartarini et al. 2000).

The breakdown of Vf resistance

Several breeders have warned that the widespread use of Vf may increase the risk of selecting for pathogen genotypes that are able to overcome this resistance (Lespinasse 1989; Lespinasse and Olivier 1981). In the collection of scab-resistant *Malus* in Ahrensburg, Germany, scab symptoms have been observed on "Prima" seedlings in the field since 1984. In 1988, small, sporulating scab lesions were found on some other Vf selections in the same collection. Detailed inoculation studies carried out at INRA Angers in France (Parisi et al. 1993) showed that the inoculum from Ahrensburg was able to infect and sporulate on all the tested Vf cultivars or selections, while *M. floribunda* 821 itself and the ornamental crabapple (*Malus* × *Perpetu*) "Evereste" were still resistant. The progeny from a cross between a resistant (Vf) cv (Prima) and a susceptible cv (Fiesta) were completely susceptible to the inoculum from Ahrensburg (Durel et al. 2000; Durel et al. 2003). However, in response to inoculation with local inoculum, the resistance of this same set of progeny segregated at a resistant:susceptible ratio of 1:1. Another relevant fact was the clear distinction between the resistance of *M. floribunda* 821 and that of the Vf cultivars and selections.[4] The Ahrensburg *V. inaequalis* pathotype was named "race 6". Further studies confirmed that *M. floribunda* 821 has an additional resistance, which was lost during the breeding process (Bénaouf et al. 1997; Parisi and Lespinasse 1996). Roberts and Crute (1994) reported that a spore mixture derived from the naturally infested leaves of a *M. floribunda* tree in a local garden near East Malling GB elicited the formation of sporulating lesions on the original *M. floribunda* 821, as well as the Vf cultivars "Jonafree", "Liberty", "Macfree", "Redfree", "Novamac" and "Priscilla", but not on "Florina", "Priam", "Prima" or "Sir Prize". This mixture was also able to sporulate on "Golden Delicious" and "Nova Easygro". Single-spore isolates were used to assess the host spectrum of this pathotype, but did not confirm completely the results obtained with mass-spore inoculum by Roberts and Crute (1994); the pathotype named "race 7" after single-spore tests was avirulent to cv Golden Delicious (Bénaouf and Parisi 1997).

The susceptibility of "Nova Easygro" (Crosby et al. 1992a; Lespinasse and Olivier 1981) is not surprising, as we know that it owes its resistance to the Vf system and not to Vr [resistance originating a Russian apple seedling from the Caucasus Mountains (R12740-7A)] (Gianfranceschi et al. 1996; Parisi and Lespinasse 1996). However, the resistance of "Florina", "Priam", "Prima" and "Sir Prize" to pathotype[5] 7 can be only explained by the assumption that they inherited an unknown ephemeral resistance (MacHardy et al. 2001) from one of their scab-susceptible ancestors that is still functioning against that particular *V. inaequalis* population. Since Golden Delicious posses the identified ephemeral resistance gene Vg (Bénaouf and Parisi 2000), this gene explains the resistance to race 7 of this cv, and also the resistance of some Vf cultivars to race 7; the cv Golden Delicious has been widely used in breeding programs and is present in the pedigree of many Vf resistant cultivars. It is the case for example for Florina (Bénaouf and Parisi 2000). However, we can also assume that some Vf selections possess other resistance factors not overcome by the original pathotype 7.

The identification of pathotypes 6 and 7 demonstrated the vulnerability of all Vf orchards to *V. inaequalis*. We can draw some conclusions from the fact that pathotype 6 was found in a *Malus* collection and pathotype 7 on an ornamental tree near an institution that is home to such a collection, and not in a "trap orchard" that includes cultivars and selections with specific resistances generally used to determine pathotypes (Lespinasse 1989). Trap orchards cannot be regarded as a reliable early-warning device for detecting new pathotypes, as the distribution and frequency of pathotypes breaking a functioning resistance may be too localized and low-level to be noticeably present at any specific site. The sites for the first sightings of the Vf-virulent pathotypes suggest an introduction with a specific host plant; mutation from *avir* to *virVf* appears to be extremely rare or nonexistent as the first appearance of this phenomenon was not in one of the abundant Vf orchards. In the case of Ahrensburg, we may hypothesize that pathotype 6 was introduced with breeding material. This hypothesis is supported by the fact that the original strain lacked most of the genes that would enable it to grow on commercial cultivars and only through several steps of

[4] We consider a cultivar (= cultivated variety) to be any apple genotype to which a name has been given. Cultivars have usually been described and officially released for marketing from a breeding program (e.g. 'Florina', 'Gala') or may be older genotypes that have been named and described (e.g. 'Golden Delicious', 'Elstar'). A selection (breeder's selection) is any apple genotype which is used in a breeder's program for further crosses or is already being tested for future marketing. We define as an accession any apple genotype present in a collection that is neither a cultivar nor a selection. Accessions and selections are usually identified by specific codes.

[5] Pathotype is used here to identify all genotypes with the ability to infect the corresponding host; race is often used as synonym. However, a race is defined by the resistance genes of the host which it can overcome. As *V. inaequalis* recombines its virulences each winter, a particular pathotype may be found to be composed of many different races if the analysis is extended to more differential hosts (Gessler et al. 2006).

recombination (years) did it acquire a level of fitness that allowed it to spread to commercial cultivars.

The data furnished by Bengtsson et al. (2000) can be interpreted similarly. In that case, the first vir-Vf pathotype was found in the researchers' own collection orchard. They reported that, in August 1998, *M. floribunda* 821 had an average leaf scab incidence of 94% and an average severity of 7.8 on a scale from 1 to 9 (similar to "Golden Delicious"); whereas no scab was observed on "Florina" and the incidence of leaf scab on "Prima" was 5.9%, with a severity of 1.7. However, in that study, the true identity of *M. floribunda* 821 was not confirmed. In later years, scab developed on additional cultivars present at that location with increasing severity.

In several commercial orchards, farmers have experimented with Vf-cultivars and there have been anecdotal reports of scab lesions on "Prima". However, these observations have not been consistent over the years. We may assume that, under conditions that are extremely favorable for the development of disease, the resistance of "Prima" is insufficient to prevent the development of scab and not that pathotype 6 was present.

In 1994, for the first time, a Vf scab-resistant cultivar in a commercial orchard was found to be infected with a "Vf-breaking" pathotype (The Netherlands; Schouten and Schenk 1997). In Normandy in 1995, the cider apple "Judeline" was heavily infected while its half-sib "Judaine" remained scab-free. Both cultivars carry Vf inherited from the scab-resistant parent "Priam". Since "Judaine" remained uninfected while "Judeline" did not, we can assume that "Judaine" must carry some sort of additional resistance. In a study involving various pathotypes, Parisi et al. (2000) were able to determine that Vf was overcome in this case. The resistance observed in Judaine was attributed to the ephemeral resistance Vg, which is present in "Golden Delicious" and "Priam" and is assumed to have been passed down from "Priam" to "Judaine", but not to "Judeline". This pathotype was identified as race 7.

This observation was followed, in 1997, by a similar observation of race 7 on "Judeline" in Aubel, Belgium, followed by a similar observation in 2000 in a nearby trap orchard of fully scabbed *M. floribunda* 821 (Lateur et al. 2002). In 1997, scab was also found on a *M. floribunda* 821 tree near Wagenigen in the Netherlands and at 150 km in an unsprayed experimental orchard at the Wilheminadorp NL research station. In this orchard, heavy scab infections were detected on the Vf cv "Otava" and low to moderate levels of scab were observed on "Santana" and "Ecolette". The rapid scab build-up is astonishing; as of 1996, no scab had been seen on any of these cultivars. An inoculation test with conidia collected from lesions on "Otava" and vigorously sporulating lesions on *M. floribunda* 821 revealed the presence of pathotype 7 (Schouten and Schenk 1997).

In 2004, the geographical distribution of Vf-virulent strains was assessed as part of the EU-DARE project (Parisi et al. 2004). This study found that pathotypes 6 and 7 are present in northern Europe (mainly in northern France, Belgium, Holland, Denmark, southern Sweden and northern Germany), but no quantitative information can be obtained from these data. Pathotype 7 was once found as clone on a single *M. floribunda* 821 tree in Switzerland, but it was eradicated and no other evidence of pathotypes 6 or 7 is currently present in that country (A. Patocchi, personal communication). Recently, the Czech Republic was added to the list of regions with extensive Vf breakthrough (Vavra and Bocek 2009).

In the USA, there have not been any reports of Vf resistance being overcome in a commercial orchard or on any Vf apple cultivar. At the Secrest Arboretum, Ohio state University a large number of crab apples were observed for scab symptoms since 1972. Among the scab free accession was also a *M. floribunda* tree until 1997, when the first signs of weak scab infection were noted on this tree. In 2003 and 2005, Beckerman et al. (2009) detected heavy scab infection on this *M. floribunda* tree, suggesting the presence of pathotype 7 in North America for the first time. However, as the *M. floribunda* tree in question was not unequivocally identified as being the accession 821 (Beckerman et al. 2009), further confirmation is necessary. In light of the above data, we may ask about the present and future role of crabapple-collections in introducing pathotype 7 into a geographical region, as well as the origin of pathotype 7 (e.g. imported from the Far East or the result of a spontaneous mutation at the site of discovery).

Even in regions in which Vf resistance has been largely overcome, the phenomenon is restricted to particular organic orchards. Vf cultivars are mostly planted in organic orchards, often without any fungicide application. Complete loss of resistance appears to be associated with the absence of any control measures. In the Netherlands, orchards planted with the Vf cultivars "Collina", "Santana" and "Topaz" have been affected by this problem (Trapman 2005). In Vf orchards in which some basic rules are followed [absence of susceptible cultivars or a minimal distance to these cultivars, spraying when there is a high risk of scab infection[6] (Jamar et al. 2010a;

[6] Mills (1947), Mills and LaPlante (1951) determined that the risk of scab infection is a function of the duration of leaf wetness and the temperature during this period. Gadoury and MacHardy (1986), MacHardy and Gadoury (1985) added the amount of ascospores present (potential ascospore dose, PAD) and the amount of mature ascospores to be released with the onset of rain during darkness to these physical parameters. The current, most advanced and most widely used scab prognosis and warning program RIMPro (Vittone et al. 2007) integrates all of these factors.

MacHardy 2000; Triloff 2006), as indicated by percentage of ascospore maturation, temperature and leaf wetness duration and presence of young leaves; and sanitation measures to eliminate the ascosporic inoculum found on the leaves of susceptible cultivars], the Vf cultivars remain free of scab (Trapman 2005) or with a low disease level (Caffier et al. 2010). This demonstrates that, with appropriate management, the current Vf resistance can still be effective. Based on this experience, some advisory services recommend treating scab-resistant cultivars with a minimal program of two–three fungicide applications at the peak of ascospore maturation, when there is a high risk of scab infection (Höhn et al. 2010; Jamar et al. 2010b).

In conclusion, while Vf resistance has been overcome, these events appears to be rare and are probably due to particular *V. inaequalis* genotypes that originated outside of Europe or North America and have bred into local scab populations. The risk of the ability to overcome Vf resistance spreading in the local *V. inaequalis* population under the selection pressure of Vf cultivars is most acute in organic and cider-apple orchards.

Mechanism of action of Vf resistance

The mechanism of action of Vf resistance is still unknown. The resistance present in *M. floribunda* 821 was originally dissected into two types of symptomatology: Vfh and Vf. Vfh is characterized by the formation of "pin-point pits", due to a rapid (develops within 40 h) classical hypersensitive response (Williams and Kuc 1969) that is characterized by the rapid death of the cells around the penetration peg (Chevalier and Lespinasse 1989; Chevalier et al. 1991). Vf is characterized by either the absence of symptoms or by visible chlorosis and/or necrosis, sometime with sparse sporulation depending on the genetic background in which this resistance is embedded. Separating the reactions due to Vfh from those due to Vf, Chevalier and colleagues described extensive histocytological modification of the upper epidermal layer with destruction in the necrotic zones, which may extend to the palisade parenchyma. In contrast, in a susceptible tissue where the fungus expands and latter sporulates, the cells are mostly similar to those of non-infected tissue (Chevalier 1988; Chevalier and Lespinasse 1989; Chevalier et al. 1991).

The cuticle is the first potential obstacle to infection by *V. inaequalis*. It appears that, even in the Vf cultivars, the fungus is able to penetrate the cuticle and form the primary stroma on the youngest leaf, similar as on older ontogenic resistant, however, still expanding leaves of susceptible cultivars. However, the difference between a Vf cultivar and susceptible cultivars lies in the number of appressoria under which such primary stroma can be observed and the extent to which this phenomenon is observed (Valsangiacomo and Gessler 1988). This phenomenon may be somehow analogous to the ontogenic resistance (increased resistance to scab with the age of the leaf) present in all cultivars (Gessler and Stumm 1984)

In a study of the interaction between *V. inaequalis* and apple, Maeda (1970) interpreted the ultra-structural changes in the plant's cell wall below the fungal stroma as the degradation of pectic substances by the fungus. However, in an experiment involving the ^{14}C-labelled cell walls of apple leaves, Valsangiacomo et al. (1992) were able to exclude any correlation between Vf resistance and the absence of cell wall degradation. Similarly, inhibitors of fungal cellulase and polygaracturonase that were extracted from "Florina" (Vf) or "Golden Delicious" inhibited the corresponding two enzymes of *V. inaequalis* to the same extent (Koller et al. 1992).

Treutter and Feucht (1990) found that the Vf cultivars had higher quantities of flavan-3-ols in their leaf tissue and fruit skins than susceptible cultivars; levels were 6.5-fold higher in leaves and 3-fold higher in fruit skins. In addition, the resistant group had approximately twice the number of different flavan-3-ols in its leaves and fruit skin than the susceptible group had. These flavan-3-ols accumulated after inoculation. However, the constitutive quantitative differences in the flavan-3-ol content of the Vf cultivars play no role in the observed resistance. In fact, in an analysis of two sets of progeny that both had "Florina" (Vf) as a parent, no correlation was detected between the level of scab resistance and the amount of flavan-3-ol in the leaves (Sierotzki and Gessler 1993).

Treatment with L-alpha-aminooxy-beta-phenylpropionic acid (AOPP), an inhibitor of phenylalanine ammonia-lyase, broke the resistance of "Sir Prize", a Vf scab-resistant cultivar. In this experiment, the fungus sporulated and the defense reaction involving the synthesis of flavonols was not activated, leading to the hypothesis that the genes that confer resistance to apple scab also regulate phenol synthesis (Michalek et al. 1999). Vf cultivars generally have higher total phenol contents, as well as greater amounts of particular phenolic molecules, as compared with susceptible cultivars, even as these levels vary over the course of the season (Petkovsek et al. 2009) and are influenced by cultural practices (Petkovsek et al. 2010). We conclude that such quantitative differences between the phenolic contents of Vf-derived cultivars and the conventional non-Vf cv "Areto" should be attributed to genetic drag derived from the wild ancestors, rather than any direct association with Vf resistance.

The development of molecular markers associated with Vf resistance

The first molecular marker reported to be associated with the Vf resistance trait was *Pgm-1*, the gene responsible for variation in the most anodal isoenzyme of phosphoglucomutase in apple, which is located about 8 cM from Vf (Manganaris et al. 1994). In parallel to work involving isoenzymes, new types of markers based on PCR-amplified DNA segments were developed for use in apple. The first of these were random amplified polymorphic DNA (RAPD) markers (Koller et al. 1993). In the highly heterozygous apple genome, the RAPD technique yields an extremely high number of polymorphisms with a dominant presence of a particular band on gels. By constructing bulk lots of individual susceptible and resistant offspring (bulk segregant analysis, BSA), we can ensure that only polymorphisms linked to the segregation trait are present or absent. BSA was used in combination with the RAPD technique to generate the first markers for Vf resistance.

Two RAPD markers were found with recombination frequencies[7] of 10.6 and 19.7% relative to the Vf locus (Koller et al. 1994). Yang and Kruger (1994) identified a RAPD marker with a 20–25% recombination frequency. Tartarini (1996) identified five Vf-linked RAPD markers. A map of the Vf region was constructed that contained eight genetic markers spread over approximately 28 cM (Gardiner et al. 1996). With these studies, it was immediately clear that genetic markers are a faster, easier and more reliable alternative to classical selection methods based on phenotype and the tool of the future for the analysis of segregating progeny for the identification of the various types of resistance (Virscek-Marn et al. 1996; Yang and Korban 1996).

A further step was the evolution from RAPDs to sequence characterized amplified region SCARs (Tartarini et al. 1999; Yang and Korban 1996), which are easy to score as a single amplification product that is either present or absent. With the development of whole-genome maps (Maliepaard et al. 1999), maps of the Vf region became more and more saturated (Hemmat et al. 1998; King et al. 1999; Xu and Korban 2000). New types of markers were used, such as cleaved amplified polymorphic regions (CAP) and simple sequence repeats (SSRs); (Gianfranceschi et al. 1998; Vinatzer et al. 2004). The latters were an important step forward as, unlike all the others, they are highly polymorphic, codominant, multi-allelic and sufficiently reliable to be used across different labs.

However, as more and closer markers were added to the map of the Vf genome region, there was a major reshuffling of the map containing the Vf phenotype data. During the fine mapping of the *Vf* gene, Patocchi et al. (1999a) classified approximately 9% of the plants they surveyed as resistant individuals, but showed in the flanking markers the alleles in repulsion to Vf. The exclusion of the phenotype data collected from these plants was necessary for the correct mapping of the gene, as was later demonstrated with the map-based cloning of the *Vf* gene (Patocchi et al. 1999b). Incongruency between genotype and phenotype of particular individuals appears to be a major source of erroneous localization of resistance genes, especially when using single gene mapping procedures (Gygax et al. 2004).

The identification and characterization of the *Vf* gene

In efforts to identify the gene(s) responsible for Vf, Patocchi et al. (1999a, b) used a map-based cloning approach. He determined that the physical distance between two closest flanking markers was 870 kb (Patocchi et al. 1999a) and that, therefore, a chromosome walk could be performed using a bacterial artificial chromosome (BAC) library constructed for the Vf cv "Florina" (Vinatzer et al. 1998). With this technique a BAC clone contig covering the genomic region between the Vf resistance flanking markers was obtained (Patocchi et al. 1999b). This same approach, involving a BAC library of *M. floribunda* 821, was used to identify three BAC contigs in the Vf region (Xu et al. 2001). In 2001 firstly Vinatzer et al. (2001) reported the identification of a cluster of four receptor-like genes with homology to the family of tomato genes associated with resistance to *Cladosporium fulvum* (*Fulvia fulva*). The deduced structure of the amino acid sequences coded by these genes contained an extracellular leucine-rich repeat (LLR) domain and a transmembrane domain with a high level of dissimilarity in the LLR region. They named these four genes *HcrVf1, HcrVf2, HcrVf3 and HcrVf4*. The transcription of three members of the cluster is constitutive and complete, while the *HcrVf3* is not functional being its sequence truncated. These same genes have also been identified in the resistance region of *M. floribunda* 821 (Xu and Korban 2002), where they are referred to as *Vf1* through *Vf4*.

Differential expression profiles were observed among the four paralogs during leaf development. *Vfa1*, *Vfa2* and *Vfa3* were active in immature leaves, but were expressed at very low levels in mature leaves. In contrast, *Vfa4* was active in immature leaves and was highly expressed in mature leaves. Using Agrobacterium-based transformation techniques and the binary vector pCAMBIA2301, *HcrVf2* was introduced into the scab-susceptible cv 'Gala' under the control of the CaMV35S promoter (Sansavini et al. 2003). *V. inaequalis* was unable to form extensive stroma

[7] Recombination frequency is a measure of genetic distance given in centiMorgans cM.

or sporulate on these regenerated transgenic plantlets (Barbieri et al. 2003; Belfanti et al. 2004a). Under greenhouse conditions, no scab symptoms were present on transgenic plants inoculated with *V. inaequalis* (Belfanti et al. 2004b). The *HcrVf2* gene regulatory sequences were identified (Silfverberg-Dilworth et al. 2005) and used (Joshi 2010; Szankowski et al. 2009) in transformation experiments involving "Gala" and "Elstar". In most cases, the transformants were resistant to scab and the quantitative expression of the target gene was clearly more similar to the expression observed in classical Vf-resistant cultivars ("Florina", "Santana") than that observed in plants transformed using the 35S (up to 100-fold higher) or Rubisco promoters (50- to 150-fold higher).

Similar transformation experiments were performed by Malnoy et al. (2008) using *Vfa1*, *Vfa2* and *Vfa4*. Both *Vfa1* and *Vfa2* incited resistance against scab once inserted into "Galaxy"[8] and "McIntosh", decreasing susceptibility to apple scab by 50 and 38%, respectively (measured as leaf area covered by sporulating lesions). However, contrary to the earlier data showing constitutive expression of *Vfa1*, *Vfa2*, and *Vfa3* (Xu and Korban 2002) and data from *HcrVf2*, the relative expression of the *Vfa1* and *Vfa2* transgenes was identical to that observed in "Galaxy" respectively "McIntosh" prior to inoculation with *V. inaequalis*. As both "Galaxy" and "McIntosh" possess neither the *Vfa1* nor the *Vfa2* gene, we must assume that neither of these genes were expressed earlier in uninfected plants and the reference expression (Galaxy, respectively, McIntosh) is actually the background level. However, 24 h after inoculation, expression in the transgenic plants was 2- to 30-fold higher than that observed in the trans not inoculated controls (e.g. the levels observed in "Galaxy" and "McIntosh") (Malnoy et al. 2008). The role of *HcrVf1* (which corresponds to *Vfa1*) in resistance is, however, questionable, as Joshi (2010); Joshi et al. (2011), in a very detailed analysis, was not able to identify any change in susceptibility, even in transformants expressing the gene at levels several hundred-fold higher than the Vf control ("Santana").

'In summary, it is clear that of the four originally identified *HcrVf* genes, *HcrVf3* is not functional, *HcrVf1* and 4 play no role in scab resistance while in all published reports *HcrVf2* has been shown to provide a variable degree of resistance, and this resistance is generally similar to the resistance symptom spectrum found in a set of progeny (ranging from no symptoms to necrosis and slight sporulation). *HcrVf2* is also expressed constitutively in transgenic plants under the control of its own promoter and terminator sequences, similarly to the classical bred Vf cultivars. *HcrVf2*-transformed plants recognize all scab genotypes, except pathotypes 7 (Silfverberg 2004) and 6 (Joshi 2010). Strains of these two pathotypes[9] sporulate on the *HcrVf2* transformants just as they do on the original "Gala" (Silfverberg et al. 2005). We can, therefore, conclude that *HcrVf2* functions in the transformants just as it does in the classically bred Vf cultivars and that the defense cascade induced by the mechanism of Vf resistance present in the Vf cultivars is also present, intact and functional in the susceptible cultivars. For the time being, the mechanism of Vf resistance is still not understood, as no element of the defense pathway has yet been identified nor have we any explanation of the genes that modify Vf resistance.

Recognition of the pathogen by Vf and the defense mechanism

'The sequences and functional domains of the *HcrVf*s proteins are similar that of the *Cf-9* proteins of tomato, which protects tomato against *Cladosporium fulvum* (Vinatzer et al. 2001). The predicted protein includes a signal peptide (domain A), the NH_2 terminus of the mature protein (B), a leucine-rich repeat region (C), a domain with an unknown function (D), an acidic domain (E) and a hydrophobic transmembrane domain (F) with a basic C-terminus (G) (Vinatzer et al. 2001). The structural similarities between apple and tomato for the *HcrVf* and *Cf-9* genes suggest the presence of similar resistance mechanisms between the two systems. The *Cf-9* gene product recognizes the pathogen *Avr9* gene product and induces a rapid hypersensitive cell-death response in tomato and in transgenic tobacco and potato plants carrying the *Cf-9* gene (Hammond-Kosack et al. 1998). In transgenic Cf-9 tobacco, two mitogen-activated protein (MAP) kinases are transiently activated after injection of the avir9 protein. These kinases have been identified as WIPK (wounding-induced protein kinase) and SIPK (salicylic-acid induced kinase) (Romeis et al. 2000). Different types of kinases were found to be differentially expressed in plants carrying the *Vf* gene after pathogen challenge (Paris et al. 2009). Cova et al. (2010) described four putative leucine-rich repeat receptor-like protein kinases (LRPKm) in "Golden Delicious", "Gala" and "Florina". Two appear to be transiently up-regulated 24 h after *V. inaequalis* inoculation in 'Florina' and in the transgenic Vf "Gala", but not in the untransformed, susceptible "Gala" or "Golden Delicious". At the cytological level, the LRPKm proteins were localized in the plasma membranes of epidermal cells in

[8] 'Galaxy' is one of the many sports (selected mutants) of the original 'Gala'.

[9] Using the new nomenclature (Bus et al. 2009) we would refer to the two pathotypes as race virRvi6.

resistant genotypes following pathogen challenge. These genes have been mapped on linkage group (LG) 5 and 10 and are not associated with Vf, which maps on LG 1 in 'Florina' (Cova et al. 2010).

It can be speculated that the two LRPKinases may play roles in the signal transduction pathways after pathogen recognition by the HcrVf2 protein. However, we currently have no clue as to the defense mechanism, except that, in apple, we did not observe any rapid (within 48 h) hypersensitive response, as has been observed in the tomato system. The recognition of an unknown avirVf product of the pathogen by the HcrVf2-protein induces a host reaction which in some cases is not macroscopically visible but stops highly efficaciously the pathogen, in others it leads to the formation of macroscopically visible chlorosis and necroses. But, this process takes at least 1 week (see also above) in transgenic *HcrVf2* "Gala"/"Elstar". The expression of the *HcrVf2* gene is similar to the classical bred Vf cv Florina only when an upstream sequence of the HcrVf2 of 115 bp is used as promoter. In all the other cases (288 and 779 bp, 35S, rubisco) the level of transcription is much higher than in Vf cv Florina. (Szankowski et al. 2009; Joshi 2010). The range and type of reaction in those transgenic "Gala" plants is identical to that observed in the classically bred Vf genotypes. The difference between a Vf scab resistant individual and a susceptible is the lack of the *HcrVf2* gene or rather the correct allele and its product, the recognition protein. This product should recognize a pathogen molecule (avrRvi6) that is still unknown, and then activate the defense cascade. Paris et al. (2009) used a PCR-based suppression subtractive hybridization between cDNA from challenged leaves of *HcrVf2*-resistant transgenic Gala and susceptible cv. Gala plants to identify apple genes that are differentially expressed after *V. inaequalis* inoculation. They identified 523 differentially expressed unigenes and characterized them by assigning a putative function via comparison with public databases. This set of pathogen-modulated apple genes includes many defense-related genes. Degenhardt et al. (2005) compared the Vf cv. Remo to the susceptible cv Elstar and describe the upregulation of transcripts encoding a number of proteins related to plant defense (such as beta -1,3-glucanase, ribonuclease-like PR10, cysteine protease inhibitor, endochitinase, ferrochelatase, and ADP-ribosylation factor) or detoxification of reactive oxygen species (such as superoxide dismutase). A large number of EST clones derived from mRNAs for metallothioneins of type 3 (91 out of 262) were found in Remo. The corresponding transcripts were only present in small amounts in young uninfected leaves of the cv. Elstar, but were up-regulated in the susceptible cultivar after inoculation with *V. inaequalis*. The authors indicate that constitutively high-level expression of PR proteins may protect cv. Remo from infection by different plant pathogens. However, which of those genes finally are responsible for inhibiting the pathogen growth still escapes our knowledge. On the pathogen side attempts to identify the avirulence gene are currently undertaken (Broggini et al. 2007; Broggini et al. 2009a; Broggini et al. 2011).

The *HcrVf2* gene is not unique in the apple genome. As discussed above, two other similar genes are present in the Vf-resistance genomic region (*HcrVf1* and *HcrVf4*) and six constitutively expressed genes with structures similar to that of *HcrVf2* (paralogs) have been identified and mapped in "Florina", but they did not map near any genomic site known to carry functional resistance genes (Broggini et al. 2009b). It is possible that they might be scab resistance-recognition genes that are overcome by the overwhelming majority of *V. inaequalis* genotypes present in the world-wide population (ephemeral resistance genes (MacHardy et al. 2001)).

Vf and recombinant DNA technology

The use of recombinant DNA technology to transform a scab-susceptible cultivar into a scab-resistant cultivar is an appealing idea, as all of a cultivar's characteristics remain preserved in this type of genetic manipulation, unlike the case in classical breeding. Foreign genes of very diverse origins have been used to transform and introduce resistance into popular susceptible cultivars (review in Gessler and Patocchi 2007) and some of these efforts have even reached the stage of field trials (Borejsza-Wysocka et al. 2010). Since the use of apple's own genes (as opposed to genes of other species)[10] might be more acceptable to producers and consumers (Gaskell et al. 2010; http://ec.europa.eu/public_opinion/archives/ebs/ebs_341_winds_en.pdf), there is significant interest in using *HcrVf2* to introduce resistance into susceptible cultivars; *id est*. as such cg Gala lines can be used to demonstrate feasibility and benefit, however, for commercial use additional *Malus* scab resistance genes need to be added so to render the resistance durable. True cisgenic have been created using as promoter a relatively short upstream sequence of 242 bp lines (Vanblaere et al. 2011). Trees of selected cg-Gala lines grown in glasshouses have *HcrVf2* expression several fold below theat of Florina. They show a strong chlorotic reaction with leaf deformation up to necroses upon inoculation with *V. inaequalis* conidia. In some cases also sporulation is evident (Vanblaere 2011).

[10] Genetic modified plants containing only genes and regulatory sequences derived from a crossable donor are defined as cisgenic (Schouten et al. 2006).

Outlook

The interaction *Malus* and *V. inaequalis* is under natural conditions regulated by many specific resistances of which an unknown number has been overcome by specific pathotypes present to a variable proportion in the local *V. inaequalis* population. This proportion is in relation to the frequency of the resistance in the apple population (MacHardy et al. 2001). The Vf resistance was introduced relatively recently and to a small proportion, still race 6 and/or 7 currently present in many areas where Vf cvs are grown, probably spread over longer distance by man activity and then bred into the local *V. inaequalis* population favored by monoculture. Appropriate preventive *V. inaequalis* control measures can retard this development. We have to assume that any resistance governed by a single gene will follow the same fate. The sole use of Vf in scab-resistance breeding programs and in GM-plants is not advisable. Probably only a high diversity of resistances as present in natural conditions (MacHardy et al. 2001) in and between orchards can reduce *V. inaequalis* populations permanently. Pyramiding various resistances into commercial cultivars comes closest to such a functional diversity. Currently several excellent cultivars and breeders' selections with Vf are available, so Vf can serve as a first source of resistance (still effective in many areas), to which others should be added to gain momentarily in this arm race between the pathogen and host. As breeders are receiving more and better tools new cultivars with several effective resistances can be released in shorter intervals. Currently sufficient resistances with closely associated molecular markers are available, so to pyramid resistances with "marker assisted selection" (Kellerhals et al. 2009; Patocchi et al. 2009). Faster generation cycles will also help, recently inserting a gene from silver birch into Pinova, Flachowsky et al. (2011) were able to reduce the juvenility phase drastically to a generation turn over of 1 year. Genetic engineering may contribute rendering a popular cv disease resistant by creating functional resistance diversity in an otherwise homogeneous genetic cultivar. Once better technologies are available, such as replacing non-functional resistance alleles with still effective resistance alleles such GM-cvs may become more acceptable. The main constraint is currently the lack of cloned resistance genes, being *HcrVf2* cloned in 2004 (Belfanti et al. 2004b) the only one available. In a longer time span new approaches could derive from the identification and sequencing of avirulence genes of *V. inaequalis*, for example, the product of the avir Vf which is recognized by *HcrVf2*, as well as the mutated gene whose product is no longer recognized, rendering the host susceptible once again. Once we understand the binding patterns and changes that lead to non-recognition, it may be possible to create artificial resistance alleles by making small changes in the recognition zone (LRR) and selecting artificial mutations of the *HcrVf2* so that the pathogen product may once again be recognized.

For the current orchards planted with Vf cvs the advice to growers is to protect Vf cvs against scab during periods of high risk of scab infection and that Vf scab resistant trees should never be kept in the vicinity of scab susceptible and infected trees.

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