Aus dem Institut für Agrar- und Ernährungswissenschaften
(Geschäftsführender Direktor: Prof. Dr. R. Jahn)

der Naturwissenschaftlichen Fakultät III
(Dekan: Prof. Dr. P. Wycisk)

der Martin-Luther-Universität Halle-Wittenberg

Fachgebiet: Pflanzenzüchtung

Genetic and molecular characterisation of resistance factors and candidate genes for scab resistance in apple (*Malus x domestica* Borkh.)

Dissertation

zur Erlangung des akademischen Grades
doctor agriculturarum (Dr. agr.)

von

Diplomagrarwissenschaftler Anastassia Boudichevskaia

Verteidigung am: 02.02.2009

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List of Abbreviations

APS	ammonium persulfate
bp	base pair
°C	Celsius degree
cM	CentiMorgan
CTAB	Cetyltrimethylammonium bromide
ddH_2O	double destilled water
ddNTP	2',3'-dideoxynucleotide triphospates
DNA	deoxyribonucleic acid
dNTP	Desoxy-Nucleotide Triphosfate
EDTA	Ethylene Diamin-N,N,N'N'-tetra acetic acid
Fig.	figure
g	gramm
GMAL	Geneva *Malus* number
h	hour
IPTG	Isopropyl-β-D-thiogalactoside
l	litre
LB	Luria-Bertani medium
LG	Linkage Group
LOD	Logarithm of the odds
LRR	Leucin Reach Repeat
µg	microgram
M	Molar
MAS	Marker Assisted Selection
min	minute
µl	microlitre
µM	micromolar
mM	millimolar
NBS	Nucleotide Binding Site
ng	nanogram
nm	nanometre
no.	number
PCR	Polymerase Chain Reaction
pH	potential of hydrogen
pmol	picomol
PVP	polyvinylpyrrolidone
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
rpm	rotations per minute
SCAR	Sequence Characterized Amplified Region

sec	second
SSR	Simple Sequence Repeat
Tab.	table
TAE	Tris-Acetate-EDTA (buffer)
TE	Tris HCl-EDTA (buffer)
TEMED	N,N,N'N'-Tetramethylethylendiamin
Tris	Tris-hydroxymethyl-aminomethane
U	unit (enzyme activity)
UV	ultraviolet
v.	version
x-GAL	5-Brom, 4-chlor, 3-indoxyl β-D-galactosid
BAZ	Bundesanstalt für Züchtungsforschung an Kulturpflanzen (Federal Centre for Breeding Research on Cultivated Plants)
IOZ	Institut für Obstzüchtung (Institut of Fruit Breeding) Dresden,
JKI	part of BAZ, Julius Kühn Institute

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1. Introduction

1.1 Breeding in apple

The cultivated apple, (*Malus* x *domestica* Borkh.), belongs to the rose family (Rosaceae, subfamily Maloideae or formerly Pomoideae, x=17) along with pear (*Pyrus spp*.), quince (*Cydonia oblonga*), loquat (*Eriobotrya japonica*), and medlar (*Mespilus germanica*). There are about 25-35 species in the genus *Malus* and a number of subspecies of “crabapples” or “crabs”. The taxonomy of this complex genus is still debating (Janick et al. 1996; Way et al. 1990).

The primary centre of origin of cultivars of *Malus* is within the region of Asia Minor, the Caucasus, central Asia, Himalayan India, Pakistan and western China (Watkins, 1995; Zhang et al. 1993), in which at least 25 native species of *Malus* occur (Rehder, 1940). It was suggested that the Old Silk Road from the Black Sea to western China played an important role in the evolution of the cultivated apple (Juniper et al. 1998).

The main ancestor of the domesticated apple (*Malus x domestica* Borkh.) is *M. sieversii* (Lodeb.) M. Roemer, a species with a high degree of diversity (from almost inedible “crabs” to fruits similar to some modern cultivars) found in mountainous areas in southeast Kazakhstan on the border between China, Kazakhstan, Kyrgyzstan and Tajikistan to the edge of the Caspian Sea (Juniper et al. 1998; Hokanson et al. 1998; Harris et al. 2002). Seed and wood collections have been made in the wild apple forests of Kazakhstan (Hokanson et al. 1998) in order to protect these valuable genetic resources, potentially containing interesting diversity for important horticultural traits, from further degradation and to secure them for further development of innovative, market-driven cultivars.

Cultivation of apples has been known for at least 3.000 years in Greece and Persia. The ancient Greeks were familiar with the technique of grafting that was described by Theophrastus in the third century B.C. Romans had knowledge about the techniques of grafting, rootstocks and budding. Pliny the Elder, a Roman statesman (circa 23 CE) described about twenty different varieties of

cultivated apples in his *Historia naturalis*. By the first century CE apples were being cultivated in every region throughout the Rhine Valley. At present there are more than 10.000 different varieties (Morgan and Richardson, 2002) that vary in shape, colour, texture, firmness, crispness, acidity, juiciness, sweetness and harvesting period. However, the commercial apple production is built on only a small number of apple cultivars (e.g. 'Golden Delicious', 'Delicious' and its red sports; Hokanson et al. 1998; Janick et al. 1996). Apple is ranking fourth within the world fruit production after Citrus, banana and grapes. At least 63 million tonnes of apples were grown worldwide in 2006. China is the largest apple producing country, with more than 40% of the world production.The United States, Iran, Poland, Italy and Turkey are other major apple producers (FAO, 2006).

Apples are grown on a wide diversity of soils worldwide, but deep, well-drained, loamy soils with pH 6-7 are the best. Due to relatively late blooming and extreme cold hardiness apples are good adapted to the cool temperate zone from about 35-50° latitude. At the same time apples are adaptable to subtropical climates such as Brazil or South Africa. As a result, apples are produced commercially in 89 countries on about 4.8 million hectares (FAO, 2006). Distribution of apple producing areas in the World political map is shown in Annex (A)1.

Self-incompatibility is common among apples. They need to be planted with another variety since their flowers must be fertilized from the pollen of other apple varieties to set fruit. The ability of apple trees to cross-fertilise one another depends largely on whether they blossom at the same time or not. A few cultivars are pollen-sterile ('Jonagold', 'Winesap', 'Mutsu', 'Gravenstein' and 'Stayman Winesap'). This means that while they need to be pollinated by another variety, their pollen is sterile. It is necessary to plant two other apples with a pollen sterile variety, so that all three will get pollinated. Honey bees are the most effective pollinators. Apples reach maturity about 120-150 days after bloom, with some cultivars maturing in as short as 70 days, and others as long as 180 days. Due to their allogamous nature (cross-fertilization in plants), apple

have a high degree of heterozygosity, and cultivars are therefore propagated vegetatively. The trees are large, requiring much space for field trials.

Apple breeding is a long term and costly project. Generally, it takes as long as 15 years from the first crossing to a commercial apple cultivar. Before crossing, the flowers of the seed parent are usually emasculated at the balloon stage. The pollen of known parent is transferred. In order to prevent contamination with unknown pollen, emasculated flowers are bagged. Obtained fruit is harvested slightly before it ripens. For successful germination seeds are stored in moist conditions at optimal temperatures between 3 and 5°C (so called seed stratification) for period from 6 to 14 weeks, depending partly on the temperature (Janick et al. 1996). Once the seeds have germinated and the first true leaves appeared, the long process of seedling evaluation may begin. A long juvenile phase that lasts 3 to 10 years and a strong self-incompatibility system are probably the two most important limitations to a fast genetic improvement of apple. The juvenile period, when no flowers are produced, depends on the genotype, environmental conditions and the cultural practices. The juvenile phase can be considerably shortened by budding seedlings onto dwarfing rootstocks (Tydeman and Aston, 1965). In the most breeding programmes there are successive stages of testing: seedlings are initially screened for basic agronomic characteristics, and only the most promising lines go on to further testing (Muggleston, 1995). Breeders use several criteria to select the most promising plant material in their program. They screen for disease and pest resistance, tree architecture, high yield, abiotic and biotic stress resistance. At the same time improving fruit quality is a major objective of all fruit breeding programs. Fruit quality refers to all the factors such as colour, flavour, texture, size and shape, which are the main determinants of fruit acceptability, as well as storage and shelf life. Sensory evaluation panels (Hampson et al. 2000) allow more reliable screening of apple breeding selections for dessert quality provided a good preliminary prediction of consumer response.

AppleBreed DataBase (Antofie et al. 2007) developed recently within the framework of the European project ‘High-quality Disease Resistance in Apples

for Sustainable Agriculture' (HiDRAS) (Gianfranceschi and Soglio, 2004) should support apple breeders in their genetic studies and exploration of germplasm collections. Information stored in the database for more than 2.000 apple genotypes (crosses, breeding selections and commercial cultivars) may help in identifying molecular markers associated with important agronomic traits involved in apple fruit quality (ripening, softening, acidity, sweetness, flavour, polyphenols), in allele mining as well as in choosing the best parental cultivars for breeding. The use of innovative biotech tools in modern apple breeding programs should improve selection methods and accelerate the development of novel cultivars. Presently Europe as whole is the world leader in the breeding of new apple varieties followed by North America, Asia and Oceania (Sansavini et al. 2004).

1.2 Major apple diseases

The most important fungal disease is apple scab, which is described in detail further ahead in the text. Besides scab, powdery mildew is the other major disease of apple, caused by the obligate biotrophic ascomycete fungus *Podosphaera leucotricha* (Ell. & Ev.) Salm. Powdery mildew occurs wherever apples are grown. Such factors as climatic conditions, cultivar susceptibility and cultural practices have an impact on economic loss size from mildew. In Western Europe, for example, because of mild winter temperatures and highly favourable environmental conditions during spring, 15 or more fungicide sprays are needed to control the disease (Jones and Aldwinckle, 1990). Powdery mildew attacks leaves, blossoms and stems of apple trees resulting in leaf abscission, shoot stunting, and an overall devitalisation of the tree (Fig. 1a). Powdery mildew may infect the fruit resulting in an unattractive apple covered with a network pattern of cork cells (Fig. 1b). Such apples are frequently used for processing rather than for fresh marketing (Korban and Riemer, 1990).

At present, the majority of apple cultivars of world-wide economic importance is still susceptible (Dunemann et al. 2007). Several sources (both qualitative and quantitative) of resistance to powdery mildew in apple are known. Quantitative

(synonym: partial, horizontal) resistance is little understood but can be expressed as a polygenic trait based on several unlinked genes that usually show equal and additive effects (McDonald and Linde, 2002). A polygenic determinism of resistance to powdery mildew in apple has been identified in certain apple cultivars by Brown (1959) and Misic (1966; 1969). Very recently Calenge and Durel (2006) mapped at least four genes for quantitative resistance (quantitative trait loci, QTLs) to powdery mildew in a F1 apple progeny derived from 'Discovery' x TN10-8 which are both partially resistant to mildew.

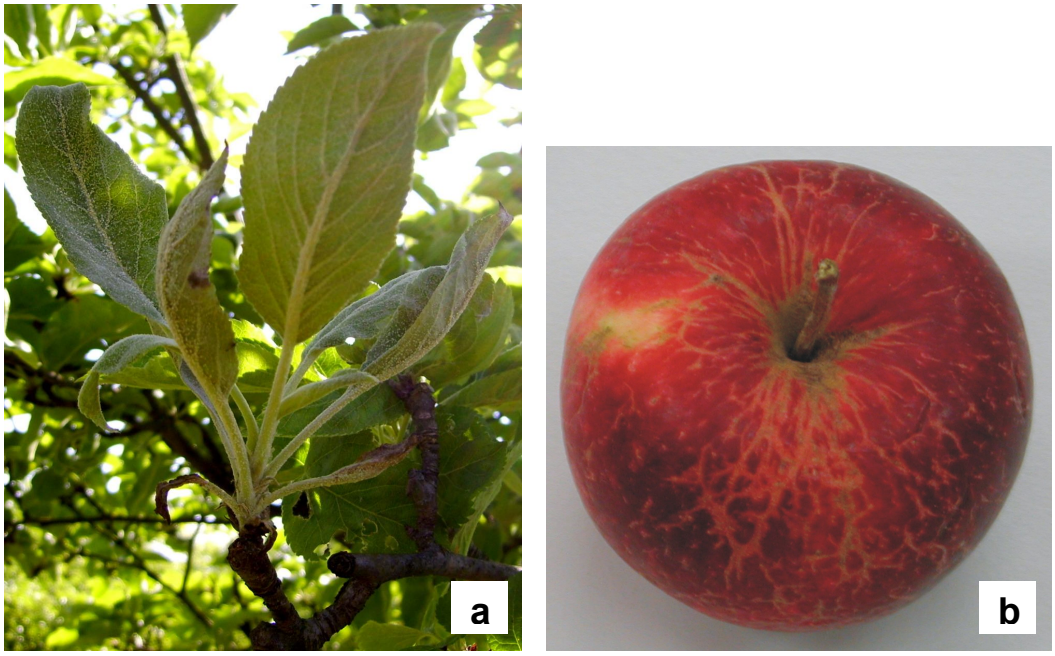


Figure 1: Powdery mildew symptoms: a) whitish, felt like patches of fungal mycelium and spores on leaves; b) fruit covered with a network pattern of cork cells (rasset)

The set of stable QTLs on linkage groups 2, 8 and 13 is of interest for breeding purposes, especially if combined with other major resistance genes (Calenge and Durel, 2006). Monogenic resistance to powdery mildew is generally derived from wild related species or ornamental crab apples. The major genes controlling powdery mildew resistance are listed in Table 1.

Table1: List of powdery mildew resistant sources

Source of resistance	Type of resistance	Gene name	References
Malus robusta	Monogenic	*Pl1*	Knight and Alston, 1968
Malus zumi	Monogenic	*Pl2*	Knight and Alston, 1968
White angel, crab apple	Monogenic	*Plw*	Gallot et al. 1985
D12 clone	Monogenic	*Pld*	Visser and Verhaegh, 1979
Mildew Immune Seedling	Monogenic	*Plmis*	Dayton, 1977
M. baccata jackii	Monogenic	*Plbj*	Dunemann and Schuster, 2007

Molecular markers, which are needed to assist gene selection, are now available for all the known powdery mildew major resistance genes: *Pl1* (Markussen et al. 1995), *Pl2* (Seglias and Gessler, 1997; Dunemann et al. 1999; Gardiner at al. 1999), *Plmis* (Gardiner et al. 2002), *Plw* (Evans and James, 2003), *Pld* (James et al. 2004; James and Evans, 2004) and *Plbj* (Dunemann and Schuster, 2007).

The existence of different physiological races of *Podosphaera leucotricha* (Krieghoff, 1995; Lesemann et al. 2004; Urbanietz and Dunemann, 2005) and the reports on the breakdown of the resistance from *M. robusta* and *M. zumi* (Caffier and Laurens, 2005), 'White Angel' and 'Mildew Immune Selection' (Korban and Dayton, 1983; Lespinasse, 1989) necessitate to concentrate the breeding effort on cumulating ("pyramiding") different monogenic and polygenic resistances in the same cultivar. This approach may allow breeders to create truly durable forms of genetic resistance by offering multiple resistance barriers against powdery mildew.

Another serious disease of apples and most of the *Pomoideae* is fire blight. This destructive bacterial disease caused by the *Erwinia amylovora* (Burrill) Winslow et al. affects blossoms, fruits, shoots, woody tissues, and rootstock crowns. Such factors as the diversity of host tissues susceptible to infection, sporadic nature of fire blight and limited number of tactical options available to manage this disease well have made it difficult to stop or slow down the spread of fire blight. Restricted firstly within the United States of America, fire blight has spread subsequently worldwide. In 2006 it has been reported already in 46 countries (van der Zwet, 2006). Most commercially successful apple cultivars

such as ‘Braeburn’, ‘Fuji’, ‘Gala’, ‘Jonagold’, and ‘Pink Lady’, are very susceptible to fire blight (Steiner, 2000). The most commonly used dwarfing apple rootstocks, Malling (M.) 9 and M.26, are highly susceptible to *E. amylovora* too, and in almost all cases, fire blight infection kills trees by girdling the rootstock (Norelli et al. 2003). Therefore, modern high-density orchards composed of susceptible varieties on susceptible size-controlling rootstocks have increased severity of damage caused by fire blight infection to unprecedented levels. For example, a single fire blight epidemic in southwest Michigan in 2000 resulted in the death of over 220.000 trees and the removal of more than 240 ha of apple orchards, with a total economic loss estimated at 42 million US $ (Norelli et al. 2003). In Dresden-Pillnitz, 1.164 apple trees representing 3.3% of the collections were infected in 2003 (Peil et al. 2004) and had to be consequently discarded from the genebank’s orchards.

Currently, managing fire blight relies on an integrated approach combining several disease control strategies that continually aim at reducing the number and distribution of inoculum sources throughout the orchard, throughout the season every year.

1.3 Apple scab

Apple scab incited by the fungal pathogen *Venturia inaequalis* (Cke.) Wint., is one of the main problems in apple-growing areas worldwide and one of the most costly to control. Scab lesions greatly reduce the fruits overall quality, decrease fruit production and increase susceptibility to invasions by various other pathogens.

Currently, the strategy for apple scab control relies on multiple applications of fungicides, often up to 20 fungicide sprays each growing season. If not controlled, the disease can cause extensive losses (70 percent or greater) where humid, cool weather occurs during the spring months (Jones and Aldwinckle, 1990). The factors influencing the disease severity are: sanitation, topography, cultivar susceptibility and the frequency of infection periods.

1.3.1 Biology

V. inaequalis is placed in the division Ascomycota, order Pleosporales and family Venturiaceae. It overwinters mainly as pseudothecia in infected leaves and fruit that have fallen to the ground. Sacs, called asci, filled with the primary spores, called ascospores, start to develop within the pseudothecia by late winter or early spring. The optimum temperature for ascospore maturation is 16-18°C (Jones and Aldwinckle, 1990). Mature ascospores are discharged into the air during periods of rain. They initiate the primary (ascosporic) infection of new growth. The peak period of ascospore discharge usually occurs between the pink and the full-bloom stages of bud development (Jones and Aldwinckle, 1990). As soon as ascospores deposited on a leaf or fruit surface in a film of moisture, they rapidly germinate. The growth of hyphae between the cuticle and epidermal cell wall over several days results in the development of stroma and, subsequently, in the development of conidiophores and conidia (secondary inoculum) that rupture the cuticle. When this occurs, a scab lesion is clearly visible macroscopically (MacHardy, 1996). During rainfall, the conidia are dispersed to the leaves and fruit by water splash, mainly within the canopy of the infected tree, and cause secondary infections throughout the rest of the season.

V. inaequalis successfully colonizes only species of *Malus*. *Malus* species and cultivars show different levels of resistance to scab, expressed pits, chlorotic flecks, or necrotic lesions that may or may not contain conidia. All commercially important apple cultivars are susceptible to scab in the field (Koch et al. 2000). The leaves and fruits become progressively more resistant to *V. inaequalis* as they age, as shown by many researchers (MacHardy, 1996; Schwabe, 1979; Szkolnik, 1978). However, it has been reported that age-related resistance (“ontogenic resistance”) does not completely prevent conidia from infecting (or penetrating) the under surface of old apple leaves though it significantly reduces the rate of mycelia growth (Li and Xu, 2002). Physiological changes that occur during senescence are thought to increase susceptibility of old leaves to. *V. inaequalis* (Koller et al. 1992; MacHardy, 1996; Olivier and Lespinasse, 1981).

1.3.2 Plant symptoms

Symptoms of scab infection occur on leaf blades, fruit, petioles, sepals, blossoms, young shoots and bud scales. The most common and obvious symptoms occur on leaves and fruit (Fig. 2). Firstly the lower, later both surfaces of leaves can become infected. Young lesions are velvety brown to olive green and have firstly indistinct margins which become clear with time. The tissues adjacent to a lesion thicken, and the leaf surface becomes deformed. The number of lesions per leaf may range from one or two to several hundred. Lesions on young fruit appear similar to those on leaves, but as the infected fruit enlarge, the lesions become brown and corky. Cracks then appear in the skin and flesh, or the fruit may become deformed (Fig. 3).

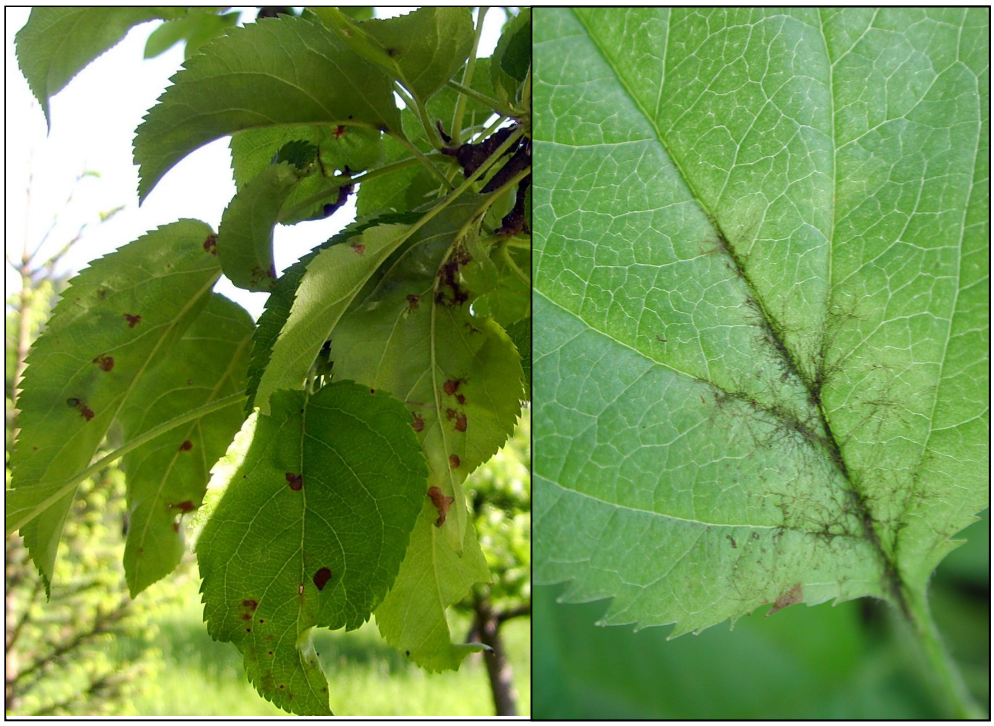


Figure 2: Scab symptoms on leaves

Figure 3: Apple scab fruit symptoms: brown and corky lesions in fruit

1.3.3 Apple scab races

When a pathogen successfully invades the host and causes disease, the pathogen is termed virulent, the host is said to be susceptible and the infection to be compatible (Glazebrook et al. 1997). In the contrary, if a pathogen is unable to establish a compatible (disease) interaction in a host cultivar since it activates defence responses that suppress pathogen colonization, the pathogen is termed avirulent, the host is resistant and the interaction is incompatible.

In 1899, Aderhold had already pointed out that *V. inaequalis* is not a single entity, but can be divided into distinct physiological isolates distinguishable by their different ability to induce sporulating lesions or only flecks on various cultivars (Gessler et al. 2006).

Shay and Williams (1956) identified three physiological races of *V. inaequalis*, among them race 1 is a well sporulating isolate on popular domestic cultivars commonly found in USA and other countries. Race 2 infected *Malus* clones ‘Dolgo’, ‘Geneva’ and certain offspring of R12740-7A (‘Russian Seedling’). Race 3 sporulated on ‘Geneva’. Race 4 was detected in Purdue (USA) on those offspring of R12740-7A that race 2 is not able to sporulate on (Williams and Kuć, 1969). Race 5, detected by Williams and Brown (1968) has the ability to infect plants provoking the pit type of reaction in *M. micromalus* and *M. atrosanguinea*. Race 6 was distinguished from the other five physiological races

by its ability to incite sporulating lesions on hybrids carrying the *Vf* resistance from *M. floribunda* 821. This race was firstly detected in Ahrensburg (Germany), in an experimental orchard (Parisi et al. 1993). Race 7, found in England was able to overcome the *Vf* resistance in original *M. floribunda* 821 and some *Vf* cultivars, while some other *Vf* cultivars were not infected (Bénaouf and Parisi, 2000; Roberts and Crute, 1994). It was discussed in many studies that *Vf* resistance is more complex than the single *Vf* gene and composed of at least two functionally different forms of resistance, *Vf* and *Vfh*. Bénaouf and Parisi (2000) detected that race 7 was unable to infect the cultivar 'Golden Delicious'. This study has led to the postulation of a *Vg* resistance which is overcome by all races except 7. Results from a study about the geographical distribution of scab races 6 and 7 virulent to the *Vf* gene in Europe showed that these races are mainly present in the Northern part of Europe but have spread also to other regions in Europe (Parisi et al. 2004). The latest race to be discovered was race 8 described for a compatible reaction with a *M. sieversii* host genotype carrying a new scab resistance gene named as *Vh8* (Bus et al. 2005b).

Koch et al. (2000) carried out an accurate analysis of eleven commercial apple cultivars which are susceptible in the field to scab to investigate variation in virulence in *V. inaequalis* towards these cultivars. For this purpose 306 single-spore isolates from five different orchard populations were tested for their individual virulence pattern. It was demonstrated that all selected susceptible cultivars were susceptible to some isolates and resistant to others. No cultivar behaved the same way to all isolates. Therefore each tested cultivar carried resistance (so called ephemeral genes) and this resistance was functionally different from the others, analogous to the virulences (Koch et al. 2000). Considering the knowledge mentioned above as well as investigations of Sierotzki et al. (1994), Sierotzki and Gessler (1998), it is plausible that each cultivar has a unique combination of many ephemeral major resistance genes and that there are many races of *V. inaequalis*, as Shay et al. (1962) noted many years ago: "there has been no useful purpose to be served by describing and naming the hundreds of different physiological races that can be differentiated by these hosts".

The high genetic diversity in *V. inaequalis* populations have been determined by using approaches based on DNA markers (Tenzer and Gessler, 1999). The results of this study suggest that the pathogen exhibits an extensive degree of gene/genotype flow that is a process that moves virulent mutant alleles and genotypes among different field or geographically separated populations (McDonald and Linde, 2002). Gessler et al. (2006) discuss that the concept of race as a fixed genetic unit is not valid for an obligatory sexually reproducing organism, and that the term race can not indicate more than the presence of the virulence/avirulence for which the isolate is tested. A race is therefore just a group of genotypes sharing the particular characteristic of being able or not to sporulate on a given set of hosts, or "differential" set (Gessler et al. 2006).

1.3.4 Resistance breeding strategies

MacHardy et al. (2001) put forward a co-evolution hypothesis of *V. inaequalis* and *Malus* which states that over time any scab resistance gene will be overcome by the pathogen's virulence alleles. The sexual stage of *V. inaequalis* enables a yearly adaptation to the host and to environmental conditions (MacHardy, 1996) which suggests a high potential number of scab pathotypes and underpins the need for alternative resistance breeding strategies. In principle, two different strategies in scab resistance breeding are currently followed. One of them is based on polygenic scab resistance sources, preferably under the participation of QTL mapping (Calenge et al. 2004; Durel et al. 2003; Liebhard et al. 2003b). Such quantitative resistance is not based on receptor-elicitor recognition (Chapter 1.5) and thus appears to work equally across all pathogen strains. Therefore, it is often hypothesized that the quantitative resistance is more durable than monogenic resistances. Polygenic resistance, however, is sensitive to environmental conditions and is difficult to handle in practical breeding, and has not yet been widely taken into account in apple breeding. The other strategy favoured presently by apple breeders is to pyramid several distinct major resistance genes into a single cultivar in the hope

that the pathogen will not be able to undergo a sequence of mutations corresponding to each resistance gene (McDonald and Linde, 2002).

A first overview of the position of known major scab resistance genes and scab resistance QTLs was presented by Durel et al. (2004) and was subsequently summarized by Gessler et al. (2006). It was reported that at least 11 chromosomes contain scab resistance factors in different progenies (major genes or QTLs). Some of the major scab resistance genes and QTLs have been mapped to the same regions on the apple genome, suggesting the presence of gene clusters. Typically, resistance gene clusters are known in many crop-disease systems (Michelmore and Meyers, 1998; Young, 2000) and are common in plants infected by (hemi-) biotrophic pathogens (Parlevliet, 2002). Among the major scab resistance genes mapped onto the apple genome there are *Vf* (Maliepaard et al. 1998), *Va*, and *Vb* (Hemmat et al. 2003) mapping to linkage group (LG) 1; *Vbj* (Gygax et al. 2004), *Vh2*, *Vh4* and *Vh8* (Bus et al. 2005a, b), *Vr2* (Patocchi et al. 2004) and *Vx* (Hemmat et al. 2002) mapping on LG 2; *Vb* (Erdin et al. 2006), *Vg* (Calenge et al. 2004) and *Vm* (Patocchi et al. 2005) mapping to LG 12. The sources of the above-mentioned resistance genes are represented in A2. Almost all scab resistant cultivars released from the different apple breeding programmes in the world carry the *Vf* scab resistance gene derived from the small-fruited species *Malus floribunda* clone 821 (Laurens, 1999).

With appearance of scab races 6 and 7 major attention has been focused on other scab resistance factors, in particular the ‘Antonovka’ selections and apple genotype R12740-7A (‘Russian seedling’, RS), which are currently used in several apple breeding programmes.

The scab resistance of the Russian cultivar ‘Antonovka’ is often referred to as polygenic but the evidence is not very clear-cut (Gessler et al. 2006). The reason is that the name ‘Antonovka’ involves different genotypes which show a marked variability and low similarity indicating that the various ‘Antonovka’ accessions are not clones but genetically different (Dunemann unpublished, Hemmat et al. 2003). There are at least several ‘Antonovka’ selections which

are sources of monogenic scab resistance. One of them is Schmidt's 'Antonovka' PI172623. Its resistance gene designated as *Va* induces a hypersensitive pit-type reaction (Dayton and Williams, 1968) and is located at a different locus from that of the *Vm* and *Vf* genes. 'Antonovka' PI 172633 also shows a hypersensitive pit-type reaction, suggesting that it also has the *Va* gene (Lespinasse, 1989). This resistance gene was mapped on the same linkage group as the *Vf* gene, but at a different position (Hemmat et al. 2003). 'Antonovka Monasir' known as a polygenic source of resistance (MacHardy, 1996), in repeated field experiments showed the scab resistance segregation typical for a single dominant gene (Quamme et al. 2003). Other 'Antonovka' derived selections are reviewed by Gessler et al. (2006). The polygenic inheritance of scab resistance in some of them is discussed.

R12740-7A is another Russian source of scab resistance derived from *M. pumila* and identified in the United States from open pollinated apple seeds obtained from Russia in 1935 (Dayton et al. 1953). The genetics of the RS resistance is well but not yet completely understood. At least three major genes, one of them race-nonspecific and the other two specific for races 2 and 4, respectively, are involved in this resistance (MacHardy, 1996). The race-specific genes *Vh2* and *Vh4* (triggering a hypersensitive response, HR) were mapped by Bus et al. (2005a) on the apple linkage group LG 2 using differential segregates of R12740-7A. Bus et al. (2005a) tested the molecular marker S22-SCAR that Hemmat et al. (2002) developed for the R12740-7A scab resistance gene they called *Vx* (also inducing HR). Because the molecular marker was mapped close to *Vh4*, and both genes promote HR and derive from the same selection, Bus et al. (2005a) concluded that *Vh4* and *Vx* are the same gene. The existence of a third (race-unspecific) resistance gene has been postulated for a long time (Shay et al. 1962, Williams and Kuć, 1969), but a genetic and molecular evidence for that gene is still missing.

Over the last 20 years more than 200 scab resistance cultivars were released by apple breeders. Some of these cultivars are with proven fruit quality that appears to be commercially acceptable (e.g., 'Liberty', 'Florina', 'Goldrush').

New promising European apple cultivars resistant to scab and some other diseases are listed in Table 2.

Table 2: Outstanding or promising disease resistance varieties in Europe according to Sansavini et al. (2004)

Variety	Country of the breeding	Parental	Resistance
Ariane	France	Complex hybrid (involving Florina, Prima, Golden Delicious)	Resistance to scab (*Vf*, *Vg*); tolerance to powdery mildew and fire blight
Ariwa	Switzerland	Golden Delicious x A849-5	Resistance to scab, powdery mildew; tolerance to fire blight
Brina	Italy	Sel. PRI2059-101 OP.	Resistance to scab
Choupette	France	Sel. X4598 x Sel. X3174	Resistance to scab
Dalinco	France	Elstar x Sel. X3191	Resistance to scab
Early Free Gold	Poland	Unknown	Resistance to scab
Free Red Star	Poland	Unknown	Resistance to scab
Golden Orange	Italy	Ed Gould Golden x Sel. PRI 1956	Resistance to scab
Harmonie® Delorina	France	Grifer x Florina	Resistance to scab
Initial	France	Gala x Red Free	Resistance to scab
Melfree	Poland	Melrose x Freedom	Resistance to scab
Prime Red	Italy	Prima x Summerred	Resistance to scab
Rebella	Germany	Golden Delicious x Remo	Resistance to scab (*Vf*), powdery mildew, fire blight, bacterial canker
Regine	Germany	Kurzcox x scab resistant clone	Resistance to scab (*Vf*), fire blight, red spider mite
Rubinola	Switzerland	Prima x Rubin	Resistance to scab, powdery mildew
Topaz	Switzerland	Rubin x Vanda	Resistance to scab, moderately tolerance to powdery mildew

Growers interested in organic fruit production should strongly consider planting such varieties. However, to date even in countries like Switzerland and Germany, where integrated and organic production systems are well developed, scab resistant apples account for no more than 5-6% of the market; overall in Italy they stand at less than 1% (reviewed in Sansavini et al. 2004). Yet none of released scab resistant cultivars could compete with standard cultivars on the global market. Sansavini et al. (2004) emphasize three main reasons explaining such situation: 1) scab resistant cultivars are not well known, 2) their diversity has not been appropriately advertised, and, maybe the most important, 3) their sensory qualities generally are neither equal to nor better than the most popular

cultivars. The authors draw an unfavourable conclusion that the organic apple industry continues to rely on 'Golden Delicious' and other susceptible cultivars (which require more than 15 treatments/year) and not on those more suitable to such a market because of their reduced need for disease and pest-control sprays.

1.4 Molecular techniques in plant breeding

In recent years molecular biology has provided valuable tools, suitable for detail analysis of complex genomes. Innovative molecular techniques, in particular the use of molecular markers can considerably accelerate and improve apple breeding. This can be accomplished by looking directly into the plant's DNA sequences at the seedling stage without waiting for the expression of the trait itself (Sansavini et al. 2004). Moreover, the use of molecular markers allows the selection of F1 plants carrying more than one gene for resistance, reducing labour and avoiding time-consuming test crossing (Gianfranceschi et al. 1998). Use of DNA markers is beneficial for traits that are difficult to score (e.g. powdery mildew resistance), for "masked" genes revelation, in the construction of linkage maps as well as for distinguishing closely related biological accessions and accurately cataloguing of germplasm.

DNA (molecular) markers represent genetic differences between individual organisms or species. Generally, they do not represent the target genes themselves but act as "signs". Such markers themselves do not affect the phenotype of the trait of interest because they are only located in close proximity to genes controlling the trait (Collard et al. 2005).

Besides DNA markers, which reveal sites of variation in DNA (Jones et al. 1997), there are two other types of genetic markers, namely morphological ("visible") markers which themselves are phenotypic traits and biochemical markers, which include allelic variants of enzymes called isozymes. Morphological and biochemical markers, which have been extremely useful to plant breeders (Eagles et al. 2001) have, however some disadvantages. They may be limited in number and are influenced by environment or the

developmental stage of the plant (Winter and Kahl, 1995) that is in contrast to DNA markers.

Molecular markers, the most widely used type of genetic markers, arise from different classes of DNA mutations such as substitution mutations (point mutations), rearrangements (insertions or deletions) or errors in replication of tandemly repeated DNA (Paterson, 1996). Collard et al. (2005) have reviewed the application of DNA markers for crop improvement in rice, wheat, maize, barley, tuber crops, pulses, oilseeds, horticultural crop species and pasture species. A lot of markers linked to monogenic traits, mainly resistance to pathogens and pests have been identified in *Malus* too (Gessler et al. 2006; Tartarini and Sansavini, 2002).

In recent years different molecular marker detection methods have been developed. These are Restriction Fragment Length Polymorphisms (RFLPs; Beckmann and Soller, 1986; Botstein et al. 1980), Random Amplified Polymorphic DNAs (RAPDs; Williams et al. 1990), Amplified Fragment Length Polymorphisms (AFLPs; Vos et al. 1995), Simple Sequence Repeats (SSRs; Jacob, 1991), which are called also as microsatellites (Litt and Luty, 1989), Single Nucleotide Polymorphisms (SNPs; Brooks, 1999) and other. The relative advantages and disadvantages of these techniques were summarized by Korzun (2003) and are represented in Table 3.

RFLP method was the first major DNA analysis technique and used initially for human genome mapping (Bostein et al. 1980). RFLPs were further applied in plant genome analysis for genome mapping (Tanksley et al. 1989; Viruel et al. 1995) and estimation of genetic diversity (Iketani et al. 1998; Nybom and Schaal, 1990). RFLPs are based on the hybridization of anonymous probes to genomic DNA digested with specific restriction endonucleases (Botstein et al. 1980). Since the hybridisation-based RFLP method is time-consuming, laborious and expensive as well as results in only one to a few polymorphic fragments per analysis it is not suitable for high-throughput applications.

Table 3: Comparison of the most common used marker systems after Korzun (2003)

Feature	RFLPs	RAPDs	AFLPs	SSRs	SNPs
DNA required (µg)	10	0.02	0.5-1.0	0.05	0.05
DNA quality	high	high	moderate	moderate	high
PCR based	no	yes	yes	yes	yes
Number of polymorphic loci analysed	1-3	1.5-50	20-100	1-3	1
Easy of use	not easy	easy	easy	easy	easy
Amenable to automation	low	moderate	moderate	high	high
Reproducibility	high	unreliable	high	high	high
Development cost	low	low	moderate	high	high
Cost per analysis	high	low	moderate	low	low

As compared to RFLPs, RAPD markers are cheaper, less laborious and much simpler since they do not require sequence data for primer construction as well as use of radioactive isotopes. RAPD markers are DNA fragments amplified by the PCR method (Polymerase Chain Reaction; Mullis et al. 1986) with low stringencies using decamer synthetic primers of random sequence. Such primers can be nearly unlimited in number and the method is amenable to automation. Due to low annealing temperature (35-40°C), the binding is not very specific, which means that primers will bind also to sequences which are not completely complementary. RAPDs easily produce polymorphisms due to variation in the primer annealing sites, but not as frequently as AFLPs. Amplified fragments (from 3-10 genomic sites simultaneously) are separated by gel-electrophoresis. RAPD markers were widely used for creation of genetic maps in a number of woody fruit crops including apple (Conner et al. 1997; Hemmat et al. 1994); blueberry, *Vaccinium darrowi* and *V. elliottii* (Rowland and Levi, 1994); *Citrus* (Cai et al. 1994); grape, *Vitis* (Lodhi et al. 1995); peach, *Prunus persica* (Chaparro et al. 1994) and pear, *Pyrus communis* (Iketani et al. 2001). RAPD markers have proven very useful for cultivar identification and fingerprinting of temperate fruit tree species (Cabrita et al. 2001; Conner and Wood, 2000; Gerlach and Stösser, 1997; Kadkhodaei et al. 2006; Landry et al. 1994; Oliveira et al. 1999; Warburton et al, 1996; Wünsch and Hormaza, 2002).

In *Malus* RAPD method has been also applied to identify markers linked to scab resistance genes such as *Vf* gene (Gianfranceschi et al. 1996; Koller et al. 1994; Tartarini et al. 1999); *Vm* gene (Cheng et al. 1998); *Vx*, *Vr* (Hemmat et al. 2002); *Vb* and *Va* (Hemmat et al. 2003) and *Vbj* (Gygax et al. 2004) as well as for identification of the gene *Sd1* conferring resistance to two rosy leaf curling aphid *(Dysaphis devecta*) biotypes (Roche et al. 1997).

The disadvantage of RAPD markers is their dominant nature where the presence of a particular band is dominant, and its absence is recessive. Such markers cannot discriminate between heterozygotes and homozygotes. Moreover, RAPDs are sensitive to the experimental conditions that lead to poor reliability and reproducibility outside the original laboratory (Karp et al. 1996; Tartarini et al. 1999). The problem of reproducibility may be overcome by the development of SCARs (sequence characterised amplified regions) or STSs (sequence-tagged sites) derived by cloning and sequencing specific RAPD markers (Paran and Michelmore, 1993). For example, RAPD markers M18 and AM19 (Gianfranceschi et al. 1996; Tartarini et al. 1999) tightly linked to the *Vf* resistance gene were converted into robust SCAR markers and used for map-based cloning of *Vf* (Patocchi et al. 1999) resulting in isolation of the *Vf*-region (Vinatzer et al. 2001). RAPD markers transformed subsequently into SCARs were identified also for powdery mildew resistance genes, such as *Pl1* (AT20-SCAR, Markussen et al. 1995) and *Pl2* (N18-SCAR, Seglias and Gessler, 1997). More reliable SCAR markers detect a single locus and may be codominant (Paran and Michelmore, 1993; Tartarini et al. 1999). To date SCAR markers closely linked to scab resistance are still widely used for breeding purposes since they enable the identification of complex genetic combinations and pyramidization of different resistance sources (Gessler et al. 2006).

AFLP method has several advantages that make it one of the most popular research tools in genetic studies. AFLP technique combines the efficiency of PCR based markers such as RAPD with the specificity and reliability of RFLP. A PCR-based AFLP technique allows selective amplification of restriction fragments from digested genomic DNA. The polymorphisms detected are due to modifications of restriction sites, e.g. as a result of point mutation (Vos et al.

1995). The fragments are visualized on denaturing polyacrylamide gels, generally through fluorescence methodologies. In *Malus* AFLP markers were used to access the genetic diversity and verify rootstock identity (Tignon et al. 2000; Tignon et al. 2001; Zhu et al. 2001; Wiedow, 2006), for marker development (James et al. 2004; Ndabambi et al. 2000; Patocchi et al. 2004; Xu and Korban, 2000) as well as for genetic mapping (Kenis and Keulemans, 2005; Liebhard et al. 2003a). AFLP method is widely used for DNA fingerprints and molecular characterization of other fruit tree crops including almond (Sorkheh et al. 2007); apricot (Hagen et al. 2002); cherry (Tavaud et al. 2001) and peach (Xu et al. 2006). The disadvantage of AFLP markers is their dominant nature that results either from restriction site presence or absence, not allowing differentiation between homo- and heterozygous alleles. Since the AFLP technique is complicated, AFLPs may be converted into SCAR markers (Lehmensiek et al. 2001). The use of such converted PCR-based markers is technically simpler, less time-consuming and cheaper (Collard et al. 2005).

Identification of RFLPs, RAPDs and AFLPs linked to useful traits has been often based on bulked segregant analysis (BSA, Michelmore et al. 1991). BSA is based on the creation of separate DNA pools consisting of genotypes exhibiting extreme phenotypes of a trait in a segregating population. The goal is to obtain quickly molecular markers (RFLPs, RAPDs, AFLPs) distinguishing only these two DNA mixes. Such approach reduces the process of genotyping the plants rapidly narrowing down the potential number of primers or primer combinations. Once polymorphic markers have been identified, DNA bulks are separated and individuals are analysed with potential markers for allele frequency. In the work of Michelmore et al. (1991) the number of individuals, comprising each pool varied from 14 to 20 plants. With RAPD markers which usually show dominant polymorphism, only a few individuals are required in each pool. The probability of an unlinked locus being polymorphic between two mixtures of 10 individuals was calculated to be 2×10^{-6} (Michelmore et al. 1991). For RFLPs, which are codominant markers, more individuals would need to be combined to ensure that each allele was represented in the resulting DNA pools at the same frequency as in the population as a whole (Quarrie et al. 1999).

Identification of simple sequence repeat (SSR) molecular markers linked to useful traits has been based on complete linkage maps or the construction of partial maps. SSR markers have been developed more recently for major crop plants. This marker technique is predicted to lead to even more rapid advances in both marker development and implementation in breeding programs (Korzun, 2003). The main advantages of microsatellite markers are good reproducibility, high specificity and unambiguous scorability. Moreover, codominant SSR markers are highly polymorphic, abundant and well distributed throughout the nuclear genomes of eukaryotes. SSR technique is a PCR-based method which can be performed automatically and with a low cost due to use of multiplex reactions, which allow the combining several SSRs in the same reaction. SSRs produce polymorphisms due to variation in the number of repeat units that can be detected by PCR using pair of primers designed from unique sequences bordering the SSR motifs. The main limitation of SSRs is the time and cost required to isolate the flanking sequences to develop species specific primers for SSR polymorphism. SSR markers have proven very useful for diversity study and cultivar identification of temperate fruit tree species such as almond (Amirbakhtiar et al. 2006; Xie et al. 2006); apple (Goulão and Oliveira, 2004; Hokanson et al. 1998; Xuan, 2007); apricot (Hormaza, 2001); *Citrus* (Luro et al. 2001); cherry (Dirlewanger et al. 2002; Struss et al. 2003); peach (Dirlewanger et al. 2002; Sosinski et al. 2000); pear (Kimura et al. 2002); and plum (Qiao et al. 2007).

SSR markers have increased the speed with which genetic maps can be constructed. There are published genetic maps for a number of temperate fruit tree species, such as almond (Sanchez-Pérez et al. 2006); apricot (Dondini et al. 2007) and grape (Adam-Blondon et al. 2004; Riaz et al. 2004). The combination of new microsatellite marker information with previously generated, mostly dominant marker data, allows the construction of the integrated maps (Doligez et al. 2006; N'Diaye et al. 2008; Salava et al. 2007; Verde et al. 2005; Yamamoto et al. 2005). The most common software programmes performing the construction of linkage maps are Mapmaker/EXP (Lander et al. 1987) and JoinMap (van Ooijen, 2006).

More than 300 SSRs were developed for *Malus* (Guilford et al., 1997; Liebhard et al. 2002; Silfverberg-Dilworth et al. 2006). They are represented in HiDRAS SSR Database (http://users.unimi.it/hidras/). The majority of them have been already published (Liebhard et al. 2002; Liebhard et al. 2003a; Maliepaard et al. 1998; Seglias and Gessler, 1997; Silfverberg-Dilworth et al. 2006). The availability of several saturated apple linkage maps sharing a high number of common SSRs (Liebhard et al. 2003a; Silfverberg-Dilworth et al. 2006) has facilitated the placement of resistance genes and QTLs to such diseases as scab (Bus et al. 2005a; Gygax et al. 2004), powdery mildew (Calenge and Durel, 2006; Dunemann et al. 2007; James et al. 2004; James and Evans, 2004), and fire blight (Peil et al. 2007) to a specific linkage group.

Having a very detailed SSR map with highly polymorphic SSRs it is possible to find a linkage between a trait and a SSR without generating a complete genetic map. This strategy was called genome scanning approach (GSA) and was proposed by Patocchi and Gessler (2003). The method consists of testing a reduced number of resistant progeny plants with a few selected and well-spaced SSRs per linkage group (Patocchi et al. 2005). GSA allows detection of a distortion of the expected 1:1 segregation ratio of the SSR alleles of the resistant parent among a small subset of progeny plants (all susceptible or resistant). Such distortion of allele segregation is due to linkage between a SSR and the resistance gene, or to naturally occurring distortions linked to lethal factors (Erdin et al. 2006). GSA method allowed the mapping of apple scab resistance genes such as *Vb*, *Vr2* and *Vm* (Erdin et al. 2006; Patocchi et al. 2004; 2005). SSR markers linked to the scab resistance genes can be incorporated into multiplex PCR reactions and analysed on automatic sequencers with short running times.

An increased amount of available sequence information about functionally characterized target genes as well as genetic maps harbouring genes of known function and QTLs have led to the development of the candidate gene approach in molecular genetics. Candidate gene analysis is based on the hypothesis that known-function genes (the candidate genes) could correspond to loci controlling traits of interest (Pflieger et al. 2001). According to these authors, candidate

genes are either genes with molecular polymorphisms genetically linked to major loci or QTLs (named functional candidate genes), or genes with molecular polymorphisms statistically associated with variation of the studied trait (positional candidate genes). The third type of the candidate genes, named structural candidate genes, can be exemplified with the resistance gene analogs (RGAs). Their isolation is based on conserved motifs of the domains of resistance genes and not on physiological function (see Chapter 1.5).

To select the most promising candidates from a large number of putative candidate genes, gene sequences are tested for linkage to the trait of interest. Population-based fine-mapping experiments allow to precisely locate both candidate genes and the locus (Wei et al. 1999). Another strategy is an association study which allows testing of correlation between phenotypic variation and molecular polymorphisms within candidate genes in a set of genealogically unrelated individuals (e.g. Malosetti et al. 2007). Finally, such candidate genes, for which map co-segregation and/or statistical correlation have been found must be validated. It means that complementary experiments such as genetic transformation, must be conducted to confirm whether the candidate gene is the gene determining the trait variation. A validated candidate gene may be used for identification of varieties as well as for marker-assisted selection. For loci controlling qualitative traits the candidate gene approach provides functional markers of the gene itself. Such functional markers in contrast to the random DNA markers (i.e derived at random from polymorphic sites in the genome) examined above were applied in biodiversity studies, in the context of plant breeding as well as in human genetics (reviewed by Andersen and Lübberstedt, 2003). Successful application of the candidate gene approach in plant genetics and breeding is reviewed in many publications (e.g. Gebhardt et al. 2007; Pflieger et al. 2001). The candidate gene analysis has been utilized in *Malus* too, for example to detect anthocyanin biosynthetic and regulatory genes (Chagné et al. 2007), ethylene regulated aroma biosynthetic genes (Schaffer et al. 2007) and candidate genes involved in ethylene production during apple ripening (Costa et al. 2007).

The candidate gene analysis as a powerful approach for identifying and isolating agronomically important genes controlling qualitative traits became a strategy of the *Malus* study outlined below. Besides, a number of marker assay technologies were applied to identify and characterize resistance factors and candidate genes for scab resistance in apple. The RAPD technology was good established at the molecular laboratory of the Julius Kühn Institute at Dresden-Pillnitz whereas the powerful AFLP and SSR methods were relatively new and not commonly used at the beginning of the research. It is known that introduction of a new technology as well as establishing a protocol takes a long time since multiple replications of each sample are required to ensure that results are accurate. Since the scab resistant cultivar 'Regia' was already exploited at the Julius Kühn Institute in Dresden-Pillnitz to produce a new generation of apple families for breeding purposes, it was the barest necessity to develop molecular markers quickly. Therefore, time requirement was one of the main reasons not to employ SSRs and AFLPs as the detection systems. Moreover, taking into account that the AFLP technique is expensive to set up and it was used rather for discrimination between varieties and species than for marker development RAPD technology became a method of choice for the identification of the scab resistance gene *Vr1*. Subsequent appearance of high-quality SSR-enriched apple molecular maps (Liebhard et al. 2003a; Silfverberg-Dilworth et al. 2006) and sequence information about SSRs made it possible to identify chromosomal locations containing resistance factors and candidate genes for scab resistance in apple.

1.5 Plant resistance genes

Parasites and pathogens of plants are a significant and growing threat to crop production worldwide (Anderson et al. 2004). Development of crops with increased and durable resistance to a spectrum of diseases is one of the major goals of plant breeding. In nature plants are continuously exposed to various enemies such as viruses, bacteria, fungi, parasitic plants, nematodes and insects. The pathogens use various ways to invade plants. It can be direct

penetration of surface layers by using mechanical pressure or enzymatic attack; penetration through natural openings (e.g., stomata or lenticels) or invasion of tissues that have been previously wounded (Hammond-Kosack and Jones, 2000). The invading pathogens deploy one of three main attack strategies to utilize the host plant as a substrate: necrotrophy, in which the plant cells are killed; biotrophy, in which the plant sells remain alive; and hemibiotrophy, in which the pathogen initially keeps the plant cells alive but kills them at later stages of the infection (Hammond-Kosack and Jones, 2000). The strategies utilized by plant pathogens are represented in Table 4.

The process of infection, colonization, and pathogen reproduction is known as pathogenesis (Hammond-Kosack and Jones, 2000). Plants have evolved and developed mechanisms to recognize pathogens and defend themselves against infection. Two basic forms of the recognition process have been proposed: basal or “innate” recognition and specific recognition. The early basal response is mediated by microbe-associated molecular patterns (MAMPs), formerly termed pathogen-associated molecular patterns (PAMPS), which are recognized by the plant as non-self molecules (Chisholm et al. 2006). MAMPs can be conserved vital pathogen structures such as bacterial flagellin protein (Gomez-Gomez and Boller, 2002) or fungal cell-wall polysaccharides (Schulze-Lefert and Panstruga, 2003). This recognition leads to activation of cellular defence responses including generation of reactive oxygen species, a calcium burst, deposition of callose into plant cell walls and induction of pathogen-responsive genes (Chinchilla et al. 2006; Hann and Rathjen, 2007; Zipfel et al. 2004). Typically, the MAMP-triggered basal defence is enough to halt infection before the microbe becomes established (reviewed in Chisholm et al. 2006). However, the pathogens have evolved various strategies to overcome this defence and to be infectious. For example, during infection bacterial pathogens deliver so-called suppressors which are predicted to suppress callose deposition, transcription of defence-related genes and redirect normal host metabolism to facilitate pathogen multiplication and nutrition (Abramovitch and Martin, 2004; Kang et al. 2004; Li et al. 2005; Truman et al. 2006).

Table 4: Strategies utilized by plant pathogens (after Hammond-Kosack and Jones, 2000)

	Necrotrophic	Biotrophic	Hemibiotrophic
Attack strategy	Secreted cell wall degrading-enzymes, host toxins, or both	Intimate intracellular contact with plant cells	Initial biotrophic phase, then necroptophic phase
Specific feature of interaction	Plant tissue killed and then colonized by the pathogen. Extensive tissue maceration	Plant cells remain alive throughhout the infection Minimal plant cell damage	Plant cells alive only in the initial stages of the infection. Extensive plant tissue damage at late stages
Host range	Broad	Narrow; often only a single species of plant is attacked	Intermediate
Examples	Rotting bacteria (e.g., *Erwinia* spp.); rotting fungi (e.g., *Botrytis cinerea*)	Fungal mildews and rusts; viruses and endoparasitic nematodes; *Pseudomonas* spp. bacteria	*Phytophtora infestans; Venturia inaequalis*

Another defence strategy for combating invading pathogens is so called gene-for-gene-mediated defence which is specific to a particular pathogen. The gene-for-gene model has been postulated by Flor working with the flax-flax rust pathosystem as early as in 1940s (Flor, 1942). Flor demonstrated that in the interaction of flax with flax rust *Melampsora lini*, for each dominant resistance (R) gene in the host there was a specific matching dominant avirulence (*Avr*) gene in the pathogen. When corresponding R and *Avr* genes are present, the result is disease resistance. If either is inactive or absent the result is disease (Gurr and Rushton, 2005). Such type of interactions is typical for many pathogens with their hosts. However, there are numerous examples of interactions in which no such relationships have been found (reviewed in Johnson, 1984).

In the last decade, nearly 50 plant R genes and 40 Avr genes have been isolated from different plant pathosystems (Martin et al. 2003; Meyers et al. 2005; Schornack et al. 2006). Generally, the simplest version of the classical gene-for gene model predicts that pathogens produce elicitor molecules (compounds stimulating any type of plant defence) that physically interact with specific receptors in the plant. When a plant cell receptor recognizes a pathogen elicitor, a defence response is activated that often leads to the death of the infected plant cell and inhibition of the pathogen (Keen, 1990; McDonald and Linde, 2002). However, despite the structure and predicted location of R

and Avr proteins a direct physical interaction between Avr and R proteins has been demonstrated in a few cases (Jia et al. 2000; Schornack et al. 2006; Scofield et al. 1996). In most other cases (e.g. Avr9-Cf-9, *Cladosporium fulvum*-tomato, Rowland et al. 2005) no evidence for a direct interaction between the two gene products has been found. Therefore, a more complex basis for recognition was proposed that is formulated in the “guard” hypothesis of van der Biezen and Jones (1998). According to this model, R protein activates resistance when it interacts with another plant protein (a guardee). The latter is recognised, targeted and modified by the pathogen’s Avr product. Resistance is triggered when the R protein detects an attempt to attack its guardee, which might not involve a direct interaction between the R and Avr proteins (McDowell and Woffenden, 2003). Evidence supporting this model for fungal pathosystems is more limited. In case of Avr9/Cf-9 (*Cladosporium fulvum*-tomato) interaction, it was suggested that Cf-9 is probably part of a protein complex (Rivas and Thomas, 2002; van der Hoorn et al. 2003). Although the guard model needs to be proved experimentally, it has gained increasing support from experimental data obtained for most of the intensively studied gene-for-gene pairs (Luderer and Joosten, 2001; van der Hoorn et al. 2002).

Resistance genes cloned and characterized to date confer resistance to bacterial, viral, fungal, oomycete, nematode, or insect attack (Dangle and Jones, 2001; Hammond-Kosack and Kanyuka, 2007). Sequence analysis reveals that resistance genes are highly conserved among plant species. Kruijt et al. (2005) grouped R genes into seven distinct classes according to the protein domains they encode. Class 1 presently consists of only one member, *Pto* from tomato, which has a serine/threonine protein kinase domain (Martin et al. 1993). The second class contain a large number of proteins from diverse plant species that have a leucine-rich repeat (LRR) domain, a putative nucleotide binding site (NBS) domain and an N-terminal putative leucine-zipper (LZ) or coiled-coil (CC) domain (reviewed by Martin et al. 2003). Class 3 is similar to class 2, but instead of an LZ or CC domain, proteins of this class contain a region with high similarity to the Toll and Interleukin 1 receptor protein which are involved in innate immunity in Drosophila and mammals. This region

is referred to as the TIR domain. The R proteins belonging to these three classes are all predicted to reside in the cytoplasm (Kruijt et al. 2005). Schematic diagram illustrating the common structural motifs found in resistance protein classes 1 through 4 is represented in Figure 4. Class 4 of proteins is referred to as receptor-like proteins (RLPs). The first isolated resistance genes of this class are the tomato *Cf* genes grouped in *Hcr2* and *Hcr9* gene families (reviewed by Kruijt et al. 2005). They confer resistance to the fungal pathogen *Cladosporium fulvum*. RLPs have an extracellular LRR domain, a single transmembrane (TM) domain, and a short cytoplasmic domain lacking any obvious signal transduction domain (reviewed by Thomas et al. 1997).

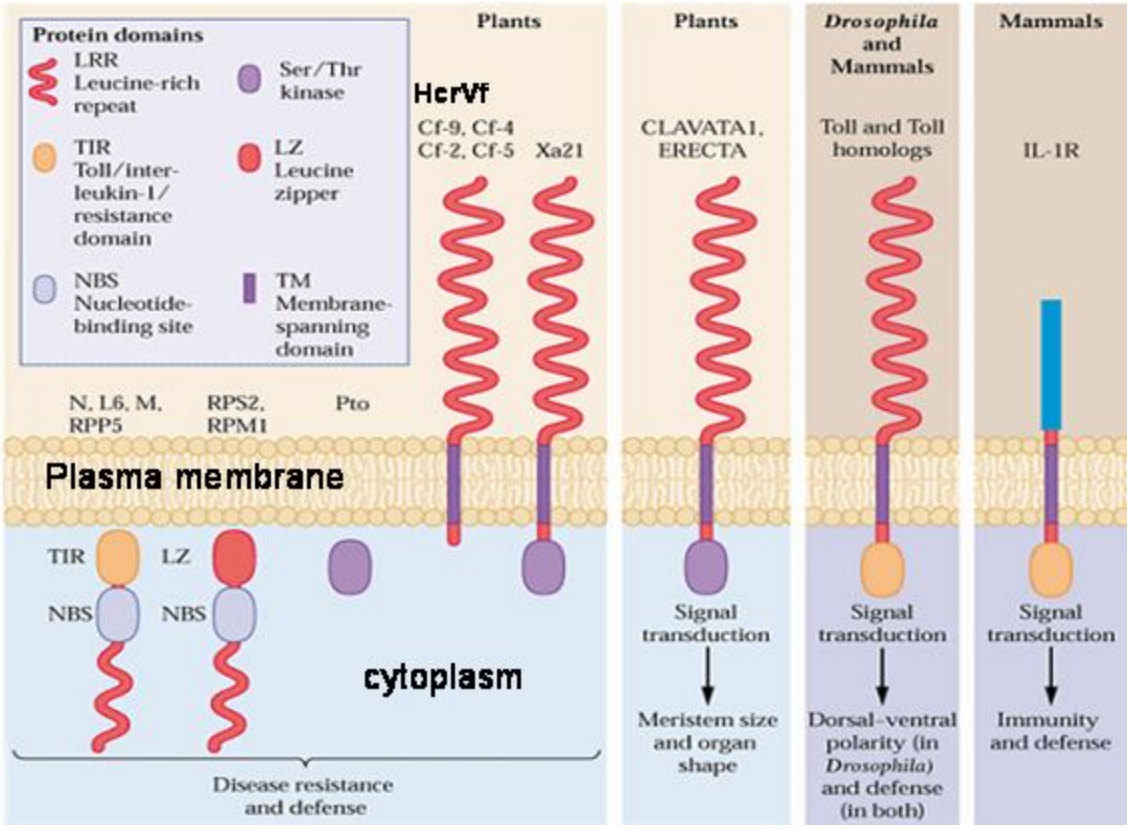


Figure 4: Schematic diagram illustrating 1 to 5 plant resistance protein classes and their cellular location (see text for explanation). For comparison are included structurally related plant proteins that determine floral organ shape and size (CLAVATA1 and ERECTA) as well as other eukaryotic proteins that induce the immune response in animals. Slightly adapted from Hammond-Kosack and Jones (2000): Biochemistry & Molecular Biology of Plants, Chapter 21, p. 1125

These R proteins activate a hypersensitive response upon recognition of their cognate *Avr* proteins. Other genes belonging to this class are *HcrVf* genes from apple (Vinatzer et al. 2001), *Ve1* and *Ve2* from tomato (Kawchuk et al. 2001) and *RPP27* from Arabidopsis (Tör et al. 2004), which are involved in resistance to *Venturia*, *Verticillium* and *Peronospora*, respectively. Class 5 consists of R proteins structurally related to RLPs. In addition to an extracellular LRR domain and a TM domain the proteins of this class possess a cytoplasmic serine/threonine kinase domain (Gomez-Gomez and Boller, 2000). This class of R proteins is known as receptor-like protein kinase (RLKs). It comprises Xa21 protein from rice and the Arabidopsis FLS2 protein. Class 6 is represented by the proteins encoded by two RPW8 genes from Arabidopsis. These cytoplasmatic proteins may be anchored by N-terminally in the plasma membrane and contain a CC domain (Xiao et al. 2001). Class 7 contains the Rpg1 protein from barley (Brueggeman et al. 2002). This cytoplasmic protein is probably not anchored in the plasma membrane and contains two tandem protein kinase domains. There are a few R proteins which can not be categorized into one of these seven classes (Martin et al. 2003).

The largest class of R genes in flowering plants includes genes encoding cytoplasmic proteins with a nucleotide-binding site (NBS) and multiple C-terminal LRR. The conserved motif within the NBS-encoding domain of disease resistance genes has often been used to identify sequences with homologies to plant disease R genes - resistance gene analogs (RGAs) by PCR with degenerate primers. RGAs have been cloned and mapped in many species (e.g. Collins et al. 2001; Di Gaspero et al. 2003; Kanazin et al. 1996; Leister et al. 1996; Mohler et al. 2002; Yu et al. 1996). In *Malus* more than 100 RGAs with NBS-LRR motifs have been identified by PCR based approaches (Baldi et al. 2004; Calenge et al. 2005; Thiermann, 2002). As a result, at least 27 chromosomal regions of the apple genome might contain homologies to RGAs (Baldi et al. 2004). Many NBS markers are organized in clusters close to major genes or quantitative trait loci for resistance to scab and mildew previously identified in different apple progenies (Calenge et al. 2005, Dunemann et al. 2007). However, the first fungal resistance genes isolated from apple,

conferring scab resistance, do not belong to this major resistance gene class but to the receptor-like protein class (Vinatzer et al. 2001; Xu and Korban, 2002). The analysed in detail *Vf* locus has been identified as an array of four paralogs i.e. members of a gene or protein family with similar sequence, structure and function within the same organism. In two independent map-based cloning approaches comparable clusters of RLP genes with homology to the *Cladosporium fulvum* (*Cf*) resistance gene family of tomato were identified (Vinatzer et al. 2001; Xu and Korban, 2002). The first two (*HcrVf1* and *HcrVf2*) of four *HcrVf* genes (Homologue of the *C. fulvum* resistance genes of the *Vf* region) described by Vinatzer et al. (2001) were found to be identical to those of *Vfa1* and *Vfa2*, respectively, isolated later by Xu and Korban (2002). The comparison of the deduced amino acid sequences of the apple *HcrVf* genes with those of the tomato *Cf9* proteins (Jones et al. 1994; van der Hoorn et al. 2005; Fritz-Laylin et al. 2005) shows the presence of extracytoplasmic LRR domains (Vinatzer et al. 2001). Moreover, the *HcrVf* and *Cf* gene clusters share the structure of tandemly repeated genes, which suggests a similar mode of evolution (Kruijt et al. 2005). The C domains of the *HcrVf* genes contain imperfect leucine-rich repeats (LRRs) of different lengths, with an average length of the LRRs of 24 amino acids and a consensus sequence LxxLxxLxxLxL-SxNxLxGxIP (where L = leucine, and x = solvent exposed amino acids; Vinatzer et al. 2001). In the *HcrVf* as well as in the tomato *Cf* resistance gene family, domain C can be divided into two subdomains, C1 and C2, on the basis of degree of conservation, with C1 being more variable than C2 (Fritz-Laylin et al. 2005; Vinatzer et al. 2001). Sequence variation within Cf proteins is generally present in the first 16 LRRs closest to the N terminus (domain C1 in the *HcrVf* gene family).

The evolutionary analysis of the *Vf* gene family (Xu and Korban, 2004) showed a wide range of genetic variation in the *Vf* locus, including a number of point mutations, short duplications or deletions, alterations of LRR units, and a casual insertion of a transposon-like element. The observed by authors highly enhanced ratios of nonsynonymous to synonymous substitutions in putative ligand-binding surfaces within full LRR domains, especially within the first 23

LRRs suggest that the LRR acts as a receptor to recognize pathogen-secreted ligands under diversified selection (Michelmore and Meyers, 1998).

Three *HcrVfs* (*HcrVf1, HcrVf2* and *HcrVf4*) are expressed, also in the absence of the pathogen and co-segregate with the *Vf* resistance phenotype (Vinatzer et al. 2001). By transgenic complementation analysis in the susceptible cultivar 'Gala' it was recently shown that *HcrVf2* controlled by the CaMV35S promotor is a functional resistance gene against *V. inaequalis* (Belfanti et al. 2004). It has been reported that this resistance gene is race-specific and responds to the same fungal race spectrum as known for *Vf* (Silfverberg-Dilworth et al. 2005). Recently, transgenic 'McIntosh' lines carrying the *Vfa1* and *Vfa2* genes driven by their own promoters have been established, which showed increased but not complete scab resistance (Malnoy et al. 2008). Considering the evolutionary analysis of the *Vf* gene family (Xu and Korban, 2004) along with the detail study of transformed apple lines carrying each of *Vfa1*, *Vfa2* or *Vfa4* (Malnoy et al. 2008) it is plausible that only the *Vfa1*/*Vfa2* gene pair is involved in the resistance response to *V. inaequalis*.

1.6 Outline of the thesis

Although fungicide treatments are still a key component in the integrated management of apple scab disease, the use of plant resistance is widely regarded as the most environmentally friendly and cost effective alternative means to the use of fungicides. The first part of the doctoral thesis describes the identification of a major scab resistance gene called *Vr1*, in the apple cultivar 'Regia' derived from the *Malus* scab resistance source R12740-7A ('Russian seedling', RS). The scab resistant cultivar 'Regia' has been developed at the Julius Kühn Institute, Dresden (Germany). Subsequently it has been used to produce a new generation of apple families for breeding purposes. However, molecular markers for a segregating scab resistance gene from 'Regia' have not been available. Therefore, this is one of the main subjects of the study presented in this thesis. The development of DNA markers for a scab resistance gene from the R12740-7A allowing selection of F1 plants, especially

those accumulating different major scab resistance genes in a single genotype is described. During the current study several molecular markers for scab resistance genes from the R12740-7A have been also identified by pathogenicity tests of apple seedlings in the greenhouse by other researchers (Bus et al. 2005a). However, these authors have not reported whether the DNA markers developed are also correlated with the resistance phenotype under natural scab infection conditions. The information about such an "efficient" marker would support the breeder's decision to rely on this resistance source in future crosses. Therefore, the another aim of this study was the verification of the *Vr1*-SCAR within a practical apple breeding programme.

Since the genetics of the R12740-7A resistance is well but not yet completely understood, there was also a high interest to locate *Vr1* on apple linkage maps and to compare this gene with other resistance genes published for the 'Russian Seedling' resistance. Additionally a multiplexing PCR assay based on two codominant SCARs for *Vf* and *Vr1* was adapted and applied in an apple progeny segregating for both genes.

The primary aim of the second part of study presented in this thesis was the identification and molecular analysis of *HcrVf* – type candidate genes in a range of *Malus* cultivars and wild species by using sequence information available from the cloned apple *HcrVf* genes. It was already assumed that different scab resistance genes may share homologous sequences with the *Vf* gene family and that *HcrVf* - like genes are present in multiple loci in the apple genome (Vinatzer et al. 2001; Xu and Korban, 2002). Within the framework of the given research the focus was on distinct from *M. floribunda* 821 apple scab resistance sources, particularly on the studied before R12740-7A and its derivatives. Some susceptible apple cultivars were included into investigation too, since every apple cultivar is assumed to carry a series of scab resistance genes acting against the majority of races of *Venturia inaequalis*, with the exception of those races missing the corresponding avirulence alleles (Boone, 1971; Koch et al. 2000; Sierotzki et al. 1994). Another important objectives were to study the expression of two specific *Vf* homologues, to map them in the *Malus* genome

and to evaluate their usefulness for studying resistance genetics in the *Venturia-Malus* pathosystem as well as for practical apple resistance breeding.

It is thought that the relevant data from the investigations represented and discussed in detail here will provide a valuable guideline for further understanding of the function, chromosomal organization and evolution of *HcrVf* – like genes in *Malus.*

2. Material and Methods

2.1 Plant material

2.1.1 Identification and mapping of a major scab resistance gene from R12740-7A

In this study six apple progenies deriving from the scab resistant cultivar ‘Regia’ (descendant of R12740-7A, 3rd generation) have been included (Tab. 5). Two populations were used for *Vr1* marker identification: 00/213: ‘Regia’ x ‘Pingo’ (192 plants) and 00/214: ‘Regia’ x ‘Pinova’ (186 plants). Three populations were used for marker verification and mapping of SCARs and SSR markers: 02/223: ‘Regia’ x ‘Pingo (147 plants), 03/205: ‘Regia’ x ‘Pinova’ (69 plants) and 03/206 ‘Regia’ x ‘Piflora’ (258 plants). Cultivars ‘Pingo’, ‘Pinova’ and ‘Piflora’ were the scab susceptible crossing parents. The sixth population (00/216) derived from a cross between ‘Regia’ and the *Vf* gene-carrying scab resistant cultivar ‘Rebella’. This progeny was used for marker analyses of the *Vr1*-*Vf*- gene combinations. Crosses were made in 1999 (populations 00/213, 00/214, 00/216), 2001 (population 02/223) and 2002 (03/205, 03/206) at the Federal Research Centre for Cultivated Plants-Julius Kühn Institute (formerly BAZ - Institute of Fruit Breeding) in Dresden-Pillnitz (Germany). The individuals of 03/205 and 03/206 were planted in the field as unselected progenies whereas the other populations were selected for scab resistance and integrated into the apple breeding programme.

The identified markers for scab resistance from R12740-7A were tested on a set of cultivars and scab resistance donors and some additional genotypes representing *Malus* species. The cultivars all originating from the apple breeding programme at Dresden are either without any known scab resistance gene (‘Pingo’, ‘Piflora’, ‘Pinova’, ‘Pirol’), are carrying the *Vf* gene (‘Rewena’, ‘Resi’, ‘Reanda’, ‘Rebella’) or have been obtained from R12740-7A (‘Regia’, ‘Realka’, ‘Remura’). Dihaploid lines DH33/99, DH102/00, DH193/00, DH198/00 and DH235/00 from ‘Remura’ were also included (Höfer et al. 2004).

Table 5: Apple populations investigated in study for scab resistance

Population	♀ parent	♂ parent	Year of cross	Year of test
00/213	Regia	Pingo	1999	2000
00/214	Regia	Pinova	1999	2000
00/216	Regia	Rebella (*Vf*)	1999	2000
02/223	Regia	Pingo	2001	2002
03/205	Regia	Pinova	2002	2003
03/206	Regia	Piflora	2002	2003

The original accession of R12740-7A (existing in German fruit gene bank at Dresden with the accession no. APF1067) was compared with the GMAL 2473, a genotype assumed earlier to be identical with the 'Russian Seedling' and used by a Swiss group to map a resistance gene called *Vr2* (Patocchi et al. 2004). DNA of GMAL 2473 was obtained from A. Patocchi (Zürich, Switzerland). As representatives of *Malus* species three genotypes each of *M. sieversii* (A96/42-29, A96/53-13, A97/42-28), *M. sieboldii* (27, 353, 397), and two accessions of *M. baccata* (419, 420) were analysed. The scab resistance sources *M. floribunda* 821 (*Vf*) and 'Antonovka' selection (*Va*) were also included.

2.1.2 Identification of *HcrVf*-type candidate genes

Forty-one *Malus* accessions were studied with respect to presence of PCR products specific for *Vf* genes. These are: 6 *M. floribunda* accessions, including *M. floribunda* 821; 11 scab resistant cultivars derived from *M. floribunda* 821; 11 accessions carrying different sources of resistance or tolerance to scab (either monogenic, polygenic or to a specific race); and additional 13 cultivars which are known to be susceptible to scab (A3). Four apple populations were used for mapping of *Vf*-type candidate genes: 03/206 ('Regia' x 'Piflora', 214 plants), 04/214 ('Antonovka' x 'Golden Delicious', 81 plants), 05/230 ('Pinova' x *M. sieversii* A96/53-13, 74 plants) and 06/004 ('Golden Delicious' x *M. sieversii* A96/57-4, 106 plants). Apple genotypes A96/53-13 and A96/57-4 were selected from two *M. sieversii* families (GMAL 3667 and GMAL 3688, respectively) available in German fruit gene bank (Dresden-Pillnitz) collection and

representing Kazakh *M. sieversii* germplasm. These apple accessions as well as the cultivar 'Regia' and 'Antonovka' were the scab-resistant crossing parents. Crosses were made in 2002 (population 03/206), 2003 (04/214), 2004 (05/230) and 2005 (06/004) at the JKI in Dresden (Germany). Several scab differential hosts were used for analysis of the *Vf2ARD* candidate gene. These accessions were used under the designations of X4811 and X2250 for host 2, X2045 for host 5, X2249 for host 4 and X2253 for host 3.

2.1.3 Scab assessments

Resistance screening of all progenies in this study was conducted after sowing in a greenhouse. Seedlings were sprayed at the 3-5 leaf stage with a mixed local inoculum of conidia from *V. inaequalis*. The concentration of the conidial suspension was 10^5 conidia/ml. Seedlings of the population 06/004 were sprayed with the monoconidial scab isolate 3650C obtained from a field-grown *M. sieversii* tree. The concentration of such conidial suspension was 4 x 10^5 conidia/ml. The plants were then incubated at 20°C for 48 hours at ~ 100% relative humidity. After 48 h, the seedlings were maintained in a glasshouse cabin at a relative humidity of ~ 90%. After 3 and 6 weeks the seedlings were scored as resistant or susceptible using a scoring scheme from 1 to 6 considering both infection type and sporulation severity (Tab. 6). The resistance scoring classes 1 and 2 were considered to contain the resistant individuals and the classes 3 to 6 the susceptible ones, respectively. A distinct race 7 (strain EU-F02) used for inoculation of the scab susceptible cultivar 'Piflora' was kindly obtained from L. Parisi, INRA Angers, France.

Table 6: Scab assessment scale

Scale	Definition of symptoms
1	No visible symptoms
2	Point necrotic or chlorotic pits
3	Very small sporulating points
4	Sporulating lesions, medium sporulation
5	Obvious and strongly sporulating lesions
6	Very strong sporulation, many lesions with resulting defoliation

Scab assessment data for the family 05/230 were kindly provided by the apple breeder Dr. Peil (JKI, Dresden, Germany).

The populations 03/205 and 03/206 were completely planted in an unsprayed orchard in autumn 2003. Scab assessments were made twice (June and August) during the 2004 vegetation period and one time in July 2005 using the following quantitative 5-step scoring scale: 1 (no scab), 2 (few small scab spots), 3 (scab immediately apparent, with lesions very thinly scattered in the tree), 4 (majority of leaves with at least on lesion), and 5 (heavy infection; multiple lesions and /or large surfaces covered by scab on most leaves).

2.2 Molecular methods

2.2.1 Extraction, purification and quantification of DNA

2.2.1.1. Extraction of genomic DNA from plants

Young leaves were used for DNA extraction. The leaves from all individuals were harvested and immediately frozen and stored at - 80°C before use for the DNA extractions. The isolation CTAB-protocol of Doyle and Doyle (1987) was applied with minor modifications. CTAB is used as detergent disintegrating membranes to separate DNA from proteins and lipids. PVP is for binding of polyphenolics during extraction. The chelating agent EDTA weakens the cells by binding Mg^{++} and Ca^{++} which are needed for membrane stability.

Proteins were extracted with chloroform-isoamylalcohol mixture. RNA was removed by RNase A treatment. Subsequent alcohol precipitation allowed removing residual salts and traces of organic solvents.

1. The leaf material (~ 150 mg) was placed into 2 ml Eppendorf tubes, frozen in liquid nitrogen and grinded to a fine powder for 2.5 min using a vigorous mixer Mill MM300 with metallic beads (Retsch GmbH & Co KG, Haan, Germany).
2. 500 µl of preheated (65°C) CTAB extraction buffer was added to the grinded leaf samples, mixed vigorously and immediately placed for 30 min on a heating block (65°C).

3. An equal volume of chloroform: isoamyl alcohol solution was added to the probe; the tube was vortexed and mixed overhead for 10 min.
4. The sample was centrifuged for 10 min at 10000 rpm, RT (Heraeus Biofuge Pico, Heraeus Instruments GmbH, Hanau, Germany).
5. The obtained upper phase (containing the DNA) was transferred to the new 2 ml tube. A 0.7 volume of 100% isopropanol was added and the tube was several times inverted. The sample was incubated 15 min, RT.
6. The sample was centrifuged for 10 min at 12000 rpm, RT.
7. The supernatant was carefully poured off and the pellet was washed with 1000 µl of 70% ethanol and centrifuged for 5 min at 12000 rpm, RT.
8. The resulting pellet was air dried at RT and dissolved in 100-200 µl of 1 x TE buffer plus 3 µl RNase A (10 mg/ml) per 100 µl TE. DNA was precipitated again with 2 volume of 100% ethanol and 1/10 volume of 3 M Sodium Acetate solution, pH 5.2, incubated 15-30 min, RT and centrifuged for 15 min at 12000 rpm, RT.
9. The pellet was washed with 1000 µl of 70% ethanol, centrifuged for 5 min at 12000 rpm, RT.
10. Finally the resulting pellet was air dried at RT and dissolved in 100-200 µl of 1 x TE.

All buffers and solutions used for the DNA extraction are listed in A4.

2.2.1.2 Extraction of double-stranded PCR products from amplification reactions

The *Vf1RSA* and *Vf2ARD* RT-PCR products (see Chapter 2.2.2.2) were purified with the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. MinElute system is a spin-column technology with the selective binding properties of a silica-gel membrane. DNA is recovered due to special buffers providing also removal of primers, nucleotides, polymerases and salts.

2.2.1.3 Extraction of DNA fragments from agarose gels

PCR fragments of the expected size were excised from gels and purified using the MinElute Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The protocol provides the purification of double-stranded DNA fragments from PCR reactions resulting in high end-concentrations of DNA. Due to spin-column technology DNA fragments adsorb to the silica-membrane in the presence of high salt while contaminants (primers, nucleotides, polymerases) pass through the column. The bound DNA fragment is washed with ethanol containing a buffer and the pure DNA is eluted with Tris buffer (10 mM Tris-Cl, pH 8.5).

2.2.1.4 Agarose gel electrophoresis

DNA fragments were separated in horizontal electrophoresis systems at 3-9 V/cm in 1xTAE buffer (40 mM Tris/Acetat, 0.1 mM EDTA, pH 7.6). The gel concentration varied from 0.8% for genomic DNA to 1.8% for RAPD fragments and those smaller than 300 bp. For visualization of the DNA, ethidium bromide (10 µg/ml) was added into the gels. The data were analysed with the Gel-Doc 2000 documentation system (Bio-Rad Laboratories GmbH, München, Germany).

2.2.1.5 DNA quantification

The concentrations of extracted DNA samples were determined by comparison with DNA mass standards (lambda mass standards, MBI Fermentas, St. Leon-Rot, Germany) on agarose gels. Concentration was estimated with imaging and analysis software Quantity One Version 4.2.2. (Bio-Rad Laboratories GmbH, München, Germany). The sizes of the molecular mass standards are 25, 50, 75, 100, 150 and 200 ng/µl. The use of a low percentage agarose gel (0.8%) in 1% TAE buffer was useful for assessing both the quantity and the quality (non-degradedness) of the genomic DNA. The measurements were documented with the Bio-Rad Gel-Doc 2000 documentation system. All DNA samples were

diluted to obtain equally concentrated working solutions (25 ng/µl). DNA solutions were stored at -20°C as template for the further molecular analyses.

2.2.1.6 Restriction of genomic DNA

Genomic DNA was digested with the *EcoR*I restriction enzyme (Fermentas, St. Leon-Rot, Germany) using approximately 7 µg of DNA in a 50 µl digestion reaction:

Template DNA	7 µg
10 x *EcoR*I buffer	5 µl
*EcoR*I restriction enzyme (20 U/µl)	2 µl
Water to volume of	50 µl

The restriction was performed in the heat block at 37°C overnight. Reaction was stopped by thermal inactivation at 65°C in 20 min. Digested DNA was separated by gel electrophoresis at 100 volts for 4 hours on the 1.0% agarose gel in 1 x TAE buffer.

2.2.2 Total RNA isolation

Total RNA was extracted from 60 mg leaf tissue using the RNeasy Plant Mini Kit (Qiagen). This method designed to isolate total RNA from small quantities of starting material allows extraction up to 100 µg of RNA longer than 200 bp. The technology is based on the selective binding properties of a silica-gel membrane and the speed of microspin technology. Plant material is first lysed and homogenized in the presence of a highly denaturing guanidine isothiocyanate (GITC)-containing buffer, which inactivates RNases to ensure isolation of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy mini column. The total RNA binds to the membrane and contaminants are washed away. High-quality RNA is then eluted in 30 µl of water. Purified RNA was stored at – 80°C.

2.2.2.1 Quantification of RNA

The concentration of RNA was determined by measuring the absorbance at 260 nm (A_{260}) in a RNA/DNA calculator Gene Quant II (Amersham Pharmacia Biotech, Uppsala, Sweden). For determination of RNA concentration RNA sample was diluted in water since the relationship between absorbance and concentration (A_{260} of 1 unit corresponds to 40 µg of RNA per ml) is based on an extinction coefficient calculated for RNA in water. The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provided an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein.

2.2.2.2 cDNA synthesis

Reverse transcriptases are used *in vitro* for first-strand cDNA synthesis with RNA as the starting material. The efficiency of the reaction is highly dependent on the quality and quantity of the starting RNA sample.

Reverse transcription in this study was performed on 1µg RNA using the QuantiTect® Reverse Transcription Kit (Qiagen) according to the manufacturer's instruction. This method takes only 20 min and comprises 2 main steps: elimination of genomic DNA and reverse transcription. The cDNA purity and the success of reverse transcription were verified by PCR using 1 µl cDNA as template in a 25 µl standard PCR reaction (see Chapter 2.2.4.2) with primers specific for an apple housekeeping gene encoding the elongation factor EF1-α. For genomic DNA residues a fragment of 800 bp was expected whereas on cDNA the EF1-α primers amplified only a 700 bp fragment indicating no genomic DNA contamination. The sequences of the primers as well as the PCR amplification conditions are listed in A6 and A7, respectively. The cDNA samples were used in reverse transcription polymerase chain reaction (RT-PCR) and Real-Time PCR (see Chapters 2.2.6 and 2.2.7).

2.2.3 Host and vector systems

As a host the strains of *E. coli* JM109 (Promega, Madison, USA) and TOP10 cells (Invitrogen, Carlsbad, CA, USA) were used. For the cloning of PCR

products the high copy plasmids pGEM-T Easy Vector System (Promega) and pCR® 4-TOPO® (Invitrogen) were utilized. Composition of buffers and solutions is listed in A5.

2.2.3.1 Isolation of plasmid DNA

DNA of the high copy number plasmids pCR®4-TOPO® and pGEM-T Easy Vector System was isolated with Plasmid Isolation Kit QIAprep Spin Miniprep (Qiagen) according to the manufacturer's instructions. The method is based on alkaline lysis of bacteria followed by absorption onto a silica-gel membrane in the presence of high salt. Afterwards, the bound DNA is washed with ethanol containing a buffer and eluted in water or 1 x TE with a yield up to 10 µg.

2.2.3.2 Ligation of PCR products

In the experiments described here, the purified 950 bp PCR fragment amplified with the random decamer primer OPAD 13 was cloned into the plasmid vector pCR® 4-TOPO® (Invitrogen) while all other purified PCR products used in this study were cloned into the pGEM-T Easy Vector System (Promega) following the manufacturer's instructions.

2.2.3.3 DNA transformation

The ligated $OPAD13_{950}$ PCR fragment was transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen). The DNA transformation included following steps:

1. 2 µl of the TOPO® Cloning reaction was added into competent cells and mixed gently.
2. The tubes were incubated on ice for 15 min.
3. Heat-shock of the cells was carried out for 30 sec at 42°C without shaking.
4. The tubes were immediately transferred to ice.
5. 250 µl of room temperature SOC medium was added.

6. The tubes were shaken horizontally (200 rpm) at 37°C for 1 hour.
7. 50 µl from each transformation together with 20 µl of SOC were spread on a pre-warmed selective plate and incubated overnight at 37°C.
8. The TOPO® cloning reaction produced hundreds of colonies. 10 colonies were chosen for analysis.

All other PCR products chosen for cloning were transformed after ligation into *E. coli* JM109 competent cells (Promega). Selection for transformants was made on LB/IPTG/X-Gal plates. DNA transformation procedure involved following steps:

1. 2µl of each ligation reaction was added to a sterile 1.5 ml mictocentrifuge tube on ice.
2. Frozen JM109 competent cells were removed from storage (- 80°C) and placed in an ice bath until they thawed (about 5 min). The cells were mixed gently by flicking the tubes.
3. 50 µl of cells were transferred into each tube prepared in step 1.
4. The tubes were gently flicked and placed on ice for 20 min.
5. Heat-shock of the cells was carried out for 45-50 sec in water bath at exactly 42°C.
6. The tubes were immediately returned to ice for 2 min.
7. 950 ml of room temperature SOC medium was added to the tubes containing cells transformed with ligation reaction.
8. The tubes were incubated for 1.5 hours at 37°C with shaking (about 150 rpm).
9. 100 µl of each transformation culture were spread onto duplicated LB/ampicillin/IPTG/X-Gal plates.
10. The plates were incubated overnight (16-24 hours) at 37°C.

After such transformation blue and white colonies appeared. White colonies generally contain inserts. Storage of plates at 4°C (after 37°C overnight incubation) was used to facilitate blue colour development. Positive clones were screened by PCR using universal primers M13-for (5'-GTAAAACGACGGCC-AGT-3') and M13-rev (5'-GGAAACAGCTATGACCATG-3') with isolated plasmid DNA to check the length of each chosen clone. PCR reaction was performed in

a 25 µl standard PCR reaction (see Chapter 2.2.4.2). The PCR conditions were as follows: one cycle of 3 min at 94°C; 28 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 2 min; final extension of 72°C for 7 min. The right samples are those samples which had the expected size of the cloned fragment. The negative control (blue colony) showed a band at 204 bp, the “recombinants” had amplicons with length equal to original amplicon plus about 204 bp.

2.2.4 Molecular marker techniques

2.2.4.1 RAPD (Random Amlified Polymorphic DNA) markers

The RAPD protocol was performed essentially as described by Williams et al. (1990) with minor modifications in order to optimize the RAPD method to a particular thermal cycler.

Two "resistant" and two "susceptible" bulked DNA samples were prepared each from 10 resistant (resistance classes 1, 2) and 7 clearly susceptible plants (resistance classes 4 to 6) from the progenies 00/213 and 00/214. These pooled DNA samples were used for PCR amplifications to screen 260 decamer primers of arbitrary sequence, as described by Michelmore et al. (1991). Primers (kits: AA, AB, AD, C, F, I, L, O, P, Q, R, U, X) were obtained from Operon Technologies (Alameda, CA, USA). All used in this study 10-base oligonucleotide primers are listed in A8. The primers producing strong, reproducible and not too closely spaced fragments polymorphic between “resistant” and “susceptible” bulks were considered as the most useful for further screening.

Amplification reactions were performed in 25 µl volumes containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM magnesium acetate, 200 µM each dNTPs, 300 nM primer, 1.0 U Taq polymerase (Invitek, Berlin, Germany) and 50 ng genomic DNA. Amplification was performed in Mastercycler gradient (Eppendorf Vertrieb Deutschland GmbH, Köln, Germany). The PCR amplification conditions are listed in A11.

2.2.4.2 SCAR (Sequence Characterized Amplified Region) markers

In this study the AD13-SCAR marker was developed based on the cloned $OPAD13_{950}$ RAPD fragment. Firstly, the 950 bp RAPD PCR fragment amplified with the random decamer primer OPAD13 was cut out from an agarose gel, purified and cloned using the TOPO TA Cloning kit (Invitrogen). Based on the sequence data the following SCAR primers were designed:

AD13-SCAR-for: 5'-GGT TCC TCT GTA AAG CTA G-3' (19 bp)
AD13-SCAR-rev: 5'-GGT TCC TCT GCC CAA CAA-3' (18 bp)

AD13-SCAR was utilized for marker verification in the populations 02/223, 03/205 and 03/206.

The applicability of the S22-SCAR (*Vx* locus) and B18-SCAR (*Vr* locus) identified as first molecular markers for resistance factors of R12740-7A by Hemmat et al. (2002) was evaluated in the populations 'Regia' x 'Pingo' (02/223) and 'Regia' x 'Piflora' (03/206).

PCR amplification of the specific fragments was performed in 25 µl standard PCR reaction using 25 ng of genomic DNA, 1 x *Taq* polymerase buffer with 1.5 mM $MgCl_2$, 100 mM of each dNTPs, 200 nM of each primer and 1 unit of *Taq* polymerase (Invitrogen).

For marker analysis of *Vr1*-*Vf* gene combinations performed in the population 00/216 ('Regia' x 'Rebella'), a multiplex PCR reaction was designed by adding AL07-SCAR primers (Tartarini et al. 1999) and AD13-SCAR primers to the reaction mix at a final concentration of 0.1 µM for AL07 and 0.3 µM for AD13, respectively. Information about sequence of all SCAR markers is in A9. Amplification conditions for all markers are listed in A11. All resulting PCR products (amplification on GeneAmp PE 9700 PCR thermocyclers, Applied BioSystems) were size-separated by electrophoresis on 1.5% agarose gels in 1 x TAE buffer and stained with ethidium bromide.

2.2.4.3 SSR (Simple Sequence Repeats) markers

Microsatellite analysis was carried out using SSR primers end-labelled with the IRDye700 or IRDye800 infrared dyes (MWG Biotech). SSR allele identification was done on a dual-laser automated DNA sequencer (LI-COR, Lincoln, Nebraska, USA). The electrophoresis apparatus was assembled according to the manufacturer's instructions. For microsatellite analysis 25 cm gel plates were chosen. They provide optimum resolution and have adequate run speed. Each gel was loaded up to 3 times. For microsatellite research the genotyping automation software Saga^{GT} developed by LI-COR, was utilized. Saga^{GT} automated electrophoresis and promoted lane finding, location of standards, calibration of band sizes, and allele scoring. The polyacrylamide gel electrophoresis conditions as well as composition of buffers and solutions are listed in A12.

Sample preparation

The respective DNAs, already extracted and quantified, were used as template for the SSR analysis. PCR amplification was performed using 25 ng of genomic DNA in 10 µl volume containing 1 x *Taq* polymerase buffer with 1.5 mM $MgCl_2$, 200 mM of each dNTPs, 5 ng of IRDye-labelled forward frimer, 0.2 µM of reverse primer and 0.5 units of *Tag* polymerase (Invitrogen). PCR amplification reactions were carried out in the GeneAmp PE 9700 PCR thermocycler (Applied BioSystems). After PCR, samples were mixed with formamide-containing loading buffer. Afterwards samples were denatured at 94°C for 3 min, then immediately put on ice and covered to reduce exposure to light. IRDye700- and IRDye800-labelled fragments were visualized on different channels.

Size standards

Size standards were used to allow rapid identification of bands and accurate automated allele sizing over the entire range. Size standards are composed of IRDye-labelled DNA fragments with equal banding intensities in 90% formamide

solution with bromophenol blue. The fragments covered the size range of 50 to 350 bp. The IRDye-labelled DNA standards were stored in the dark at – 20°C. Five size standard lanes per gel were used.

SSR analysis

SSR loci CH02b10, CH02c02a, CH02f06, CH03d10 and CH05e03, all located on Linkage Group LG 2 (Liebhard et al. 2002, 2003a) were analysed in two populations (02/223 and 03/206).

The SSR CH-Vf1, located without recombination to the *Vf1* gene on a BAC clone of the chromosomal region containing the *Vf* locus (Vinatzer et al. 2004) was analysed in the populations 03/206, 04/214, 05/230 and 06/004. SSR loci AG04 and AG11 mapped on LG 1 at approximately 2 and 18 cM from the *Vf* locus (Liebhard et al. 2003a) were analyzed additionally in the population 03/206. These SSRs were kindly provided by A. Patocchi, ETH Zürich, Switzerland and L. Gianfranceschi, University of Milano, Italy The names, sources and sequences of the primers, along with annealing temperatures are represented in A6, A9 and A11.

2.2.5 Identification of *HcrVf*-type candidate genes

2.2.5.1 PCR amplification of *HcrVf*-type candidate genes

Complete genomic sequences of the *Vf* genes *HcrVf1*, *HcrVf2* and *HcrVf3* obtained from GenBank (accession numbers AJ297739, AJ297740 and AJ297741) were used for sequence alignment studies. Based on differences found within the variable domain B and subdomain C1 PCR primers specific to HcrVf1 (Vf1-for, Vf1-rev) and HcrVf2 (Vf2-for and Vf2-rev) were designed. The two sense primers, Vf1-for and Vf2-for were derived from domain B (regions NSDSHWDF and VVCDHVTG) while Vf1-rev and Vf2-rev were based on LRR 22 (DLALSLKS) and LRR 20 (TLNLGGNE), respectively, of subdomain C1. The sequences of the developed primers as well amplification conditions are listed in A6 and A7, respectively. PCR amplification was carried out in a total volume of 25 µl containing 50 ng template DNA, 0.5 U of *Taq* DNA polymerase

(Invitrogen), 1 x *Taq* polymerase buffer, 0.2 µM of each primer, 2.0 mM and 3.0 mM $MgCl_2$ for *HcrVf1* and *HcrVf2,* respectively, and 0.2 mM dNTPs. To enhance amplification of the *Vf1* and *Vf2* fragments in the cultivar 'Regia' the AccuPrime™ *Taq* DNA Polymerase System (Invitrogen) was applied. For such PCR reactions carried out in a total volume of 25 µl, 2.5 µl of 10 x AccuPrime™ PCR buffer II, 0.5 µl of each primer (concentration 10 µM) and 0.5 µl of AccuPrime™ *Taq* DNA Polymerase were utilized. PCR amplification conditions are listed in A5. The amplification products were separated by electrophoresis on a 1.3% agarose gel in 1 x TAE buffer and then visualized by ethidium bromide staining.

2.2.5.2 Development of gene-specific primers

Based on the results of multiple-sequence alignments new gene-specific PCR primers were designed. Based on a unique sequence obtained with the Vf1 primers from the 'Russian Seedling' the primers Vf1RSA-for 5'-GTG GTT TCT TTG- GTC CCC ATT TG-3' and Vf1RSA-rev 5'-CCA ACA AGC ACT GAG ACG AA-3' were developed. These primers were used for amplification of the *Vf1RSA* fragment in 25 µl volume of standard PCR reaction (see Chapter 2.2.4.2) with an annealing temperature of 55 °C for 1 min 30 sec, and an extension time of 7 minutes at 72°C. The second primer pair (Vf2ARD-for 5'-TCT CAA CTT CTT GGA CCT AAG C-3' and Vf2ARD-rev 5'-GTG ATA TTT TGT GAA CTG CCC-3') was designed based on three almost identical sequences amplified with the Vf2 primers in 'Antonovka', 'Realka' and 'Discovery'. The amplification of *Vf2ARD* was performed using the same standard PCR conditions as described in Chapter 2.2.4.2 with an annealing temperature of 52°C. PCR for the specific candidate genes *Vf1RSA* and *Vf2ARD* was performed with *M. floribunda* 821 (*Vf*), some scab resistant cultivars carrying the *Vf* gene ('Florina', 'Prima', 'Rebella'), a scab resistant 'Antonovka' selection (*Va*), Russian Seedling' R12740-7A (*Vh2* and *Vh4/Vx*), its scab resistant derivatives 'Releta', 'Realka', 'Regia' and varieties without any known scab resistance gene ('Pinova', 'Piflora', 'Cox's Orange', 'Idared',

'McIntosh', 'Gala', 'Braeburn'). The polygenic scab resistance source 'Discovery' as well as the cultivar 'Golden Delicious', both carrying the race-specific *Vg* gene, were also analyzed. Additionally the *Vf1RSA* analysis was applied to a set of *M. sieversii* accessions: A97/41-34, A95/12-31, A95/12-50, A96/53-13, A96/48-1, A97/47-23, A96/57-4, A95/08-4, A95/12-43, A96/42-29, A97/40-11, A97/41-20 and A97/42-28, and one accession (no. 419) of *Malus baccata*.

2.2.6 RT-PCR

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) is a sensitive and specific method used for the detection of rare transcripts or for the analysis of samples available in limiting amounts (Carding et al. 1992).

For RT-PCR cDNA was synthesized by reverse transcription (Qiagen). An aliquot of the finished reverse-transcription reaction then used for PCR. The conditions for the *Vf1RSA* and *Vf2ARD* RT-PCR amplification were as described in Chapter 2.2.5.2. Expression of the *Vf1RSA* and the *Vf2ARD* genes was evaluated on the same set of apple genotypes which was analysed on a genomic DNA level.

2.2.7 *Vf2ARD* transcription profiling and Real-Time PCR

Several youngest leaves on each shoot from the accessions 'Prima', 'Florina', 'RS', 'Realka', 'Golden Delicious', 'Antonovka', 'Discovery', 'Pinova', 'Piflora', 'McIntosh', 'Cox's Orange' and 'Idared' were inoculated with a mixed local inoculum of conidia from *V. inaequalis* containing 10^5 conidia/ml. The inoculated shoot tips were wrapped in a piece of wet tissue and placed into plastic bags to ensure 100% humidity (Fig. 5). The plants were kept for 48 h in a growth chamber at 18°C at night and about 20°C during the day with a 16 h light period and a relative humidity of 75%. Leaves were harvested 0 h, 24 h and 48 h post-inoculation and frozen in liquid nitrogen. Total RNA isolation and the reverse transcription were performed as described in Chapter 2.2.2.

Figure 5: Shoot trips Inoculated with a mixed local inoculum from *V. inaequalis*

The basis of Real-Time PCR is a direct positive association between a fluorescent signal with the number of amplicons. The PCR cycle at which a statistically significant increase of the fluorescence signal is first detected is called the ct-value – or threshold cycle. Ct-values form the basis for quantitative comparison of individual real-time PCR reactions. The smaller the ct value is, the bigger the quantity of target DNA in a given Real-Time PCR reaction (www.biolytix.ch). Since the number of target molecules doubles in each PCR cycle (theoretically), a difference in the ct-value of 1 corresponds to a concentration difference of 2.

Quantitative Real-Time PCR described in this study, was performed in a 25 µl PCR reaction using the iQ^{TM} SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and 240 ng first-strand cDNA as template on an iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories). The reactions were performed according the manufacturer's instructions. The PCR reaction was performed by denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for 1 min, 59°C to 63°C for 1 min (depending on the primers) and 72°C for 1 min. The *Vf2ARD* gene specific amplification was evaluated by melt curve analysis for 1 min at 94°C and 1 min at 55°C followed by 80 cycles of 10s at 55°C with an increase of the setpoint temperature after cycle 2 by 0.5°C per cycle. Single melt peaks were obtained for each gene. The *Vf2ARD* PCR products were

subsequently evaluated by agarose gel electrophoresis. To determine the amplification efficiencies and correlation efficiencies of each PCR reaction, 15 ng, 30 ng, 60 ng and 120 ng cDNA of the non-infected sample of 'Antonovka' were analyzed. The PCR efficiencies were used to transform the ct-values into raw data for relative quantification. The determination of PCR efficiency and the calculation of the mRNA transcript levels were done using the Gene Expression Macro™ Version 1.1 (Bio-Rad Laboratories, see Chapter 2.2.11).

The *Vf2ARD* gene transcription of each genotype was quantified using the Vf2ARD-RT primers (Vf2ARD-RT-for 5'-TCT TGG ACC TAA GCA ACA ATG AT-3' and Vf2ARD-RT-rev 5'-AGT TGT CCT GTA AGT TGA TTG GC-3'). These optimized primers were designed based on the same sequence as the previous *Vf2ARD* primers and were utilized in order to overcome some technical difficulties concerning the measurement of the *Vf2ARD* Real-Time PCR specificity. Vf2ARD mRNA expression was normalized by ribulose-1,5-bisphosphate carboxylase/oxygenase activase mRNA, named Rubisco mRNA (acc. no. Z21794) and mRNA of the *M. domestica* cDNA clone Mdfw2033f21.y1 (acc. no.CN579456), which is similar to the RNA polymerase subunit II (RNAPOLII) as internal control samples for each line. The evaluation of the mRNA transcript levels of the reference genes was performed using the PCR primers Rubisco and RNAPOLII (all information about the used primers and amplification conditions is listed in A6). The scaling of the *Vf2ARD* gene expression of the infected leaf tissue samples was performed relative to the mRNA expression level of the non-infected control sample of each genotype set to be one.

2.2.8 Southern hybridisation

In order to determine the presence of other *Vf2ARD* homologous sequences in apple genome, total genomic DNA from the apple accessions R12740-7A, 'Realka', 'Regia', 'Remura'. *M. floribunda* 821, 'Prima', 'Rewena', 'Golden Delicious', 'Antonovka', 'Cox's Orange', 'Discovery', 'Jonathan', 'McIntosh', 'Piflora', 'Pilot', 'Pingo', 'Pinova', 'Elstar' and 'James Grieve' was digested with

*EcoR*I, separated on an agarose gel, blotted onto a nylon filter, and probed with the *Vf2ARD* RT-PCR product from 'Realka'. Composition of solutions is listed in A13.

Preparation of the Southern membrane

1. The ethidium bromide stained gel was photographed and exposed to UV light for 1 min.
2. The gel was depurinated by soaking for 15 minutes in 0.25 N HCl.
3. The gel was rinsed in deionized water, and then soaked in denaturing solution for 15 minutes with gentle rocking.
4. The gel was rinsed in deionized water, and then soaked in neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl, 0,001 M EDTA - pH 7.2) for 15 minutes with gentle rocking.
5. The DNA was transferred to the membrane (Hybond N^+ nylon membrane, Amersham Pharmacia Biotech, Freiburg) by traditional capillary blotting overnight.

Prehybridization of Blot:

1. The Hybond N^+ membrane was rinsed for 5 min in 2 x SSC and fixed for 2 hours at 80°C.
2. The membrane was wetted with 2 x SSC, rolled and transferred into hybridization tube containing 15 ml of Rapid-hyb buffer solution (Amersham Pharmacia Biotech). Then it was incubated at 65°C for 3 hours in a rolling bottle incubator.

Random prime labeling of DNA probes

1. The DNA probe (25 ng) was boiled in 45 µl TE-Buffer for 5 minutes and quickly chilled on ice for 5 min.
2. In specially designated area the denatured probe was mixed with 5 µl [^{32}P]dCTP (50 µCi, Amersham Pharmacia Biotech) and incubated for 10 min at 37°C.

3. Reaction was stopped with 5 µl of 0.2 M EDTA, denatured 5 min and chilled on ice for 5 min.

Southern hybridization

About 500 µl of hybridisation solution Rapid-hyb was removed from the hybridisation rolling tube and mixed with the labelled heat-denatured probe to ensure uniform distribution of the probe. The mixture was added back to the hybridization tube.

1. The membrane was hybridized at 65°C for over night in the rolling bottle incubator.
2. The membrane was washed twice in the hybridisation tube for 15 minutes with 2 x SSC / 0.1% SDS (temperature of buffer 65°C).
3. The membrane was washed twice in the hybridisation tube for 15 minutes at 65°C with 0.1 x SSC / 0.1% SDS, the traces of radioactivity were rechecked.
4. The autoradiograms were taken on the X-Ray film Hyperfilm-MP (Amersham Pharmacia Biotech).

2.2.9 Sequence analysis

Purified PCR products were concentrated by ethanol precipitation, resuspended in water and sent together with specific primers for direct sequencing (MWG-Biotech, Ebersberg, Germany). The concentration of PCR product per cycle sequencing reaction was 40-80 ng/µl. The specific primers for direct sequencing of PCR products were sent in volume of 20 µl (20 pmol/µl). Purified plasmid DNA (2-3 µg) was provided in 20 µl water for sequencing. Standard vector primers M13 Forv (5'-TGTAAAACGACGGCCAGT-3') or M13-Rev (5'-GAGCGGATAACAATT-TCACAC AGG-3') were chosen for sequencing.

2.2.10 Molecular maps construction

The molecular maps of LG 1 and LG 2 were constructed by JoinMap 3.0 (van Ooijen and Voorrips, 2001). A LOD of 3.0 was used to arrange markers into

linkage groups. A LOD value 3.0 between two markers means that the linkage is 1.000 times more probable than no linkage.The Kosambi (1944) mapping function that takes into account interference between crossing-over events was applied to transform recombination frequencies into centiMorgans. The graphical presentation of the maps was generated with the program MapChart 2.1 (Voorrips, 2002). SSR markers were assigned to linkage groups via comparison to the published maps (Liebhard et al. 2002, 2003a).

2.2.10.1 *Vr1* linkage mapping

Genetic maps for the *Vr1*-carrying linkage group LG2 was created for 80 individuals of the population 02/223 (‘Regia’ x ‘Pingo’) and 90 individuals of the population 03/206 (‘Regia’ x ‘Piflora’) selected for marker verification.

2.2.10.2 Mapping of the *HcrVf* like candidate genes

Mapping of the *Vf1RSA* gene was performed using the apple progenies 05/230 and 06/004, whereas linkage analysis of *Vf2ARD* was based on the population 03/206. All these populations were analysed for the SSR CH-Vf1, located without recombination to the *Vf1* gene on a BAC clone of the chromosomal region containing part of the *Vf* locus (Vinatzer et al. 2004). SSR loci AG04 and AG11 mapped on LG 1 at approximately 2 and 18 cM from the *Vf* locus (Liebhard et al. 2003a) were analyzed additionally in the population 03/206. Computer-based linkage analysis was performed with JoinMap 3.0 using the population 03/206 (‘Regia‘ x ‘Piflora‘, 214 individuals).

2.2.11 Computerized data analysis

The sizes of SSR fragments were determined in comparison to a molecular weight standard using the software package Saga^{GT} (LI-COR). Saga^{GT} automated location of standards, calibration of band sizes, and allele scoring.

A nucleic acid homology search was performed using the Blastx program (Altschul et al. 1997). Multiple sequence-alignments were performed utilizing the Lasergene sequence analysis software (DNASTAR, Madison, USA).

In order to estimate quality of the designed primers, the software NetPrimer (PREMIER Biosoft International, USA) was used. This program allowed checking the presence of potential 3' dimers or hairpin formations.

The Gene Expression Macro was used for calculating relative expression values from Real-Time PCR data generated by the iQ Real-Time PCR Detection System (Bio-Rad Laboratories). Bio-Rad Laboratories developed the Gene Expression Macro as a Microsoft Excel workbook containing specialized data analysis functions. The methodology of this macro is based on the algorithms outlined by Vandesompele et al. (2002). Briefly, the Bio-Rad tool calculates a gene expression normalization factor for each sample based on the geometric mean of a user-defined number of housekeeping genes. Such normalization aims to remove all nonspecific differences (such as RNA quantity and quality) in order to identify real gene specific variation. To allow a better normalization between samples within this study, two internal controls were used, namely – Rubisco and Mdfw2033f21.y1 (similar to the RNA polymerase subunit II- RNAPOLII). Then, taking into account the PCR efficiencies for each target mRNA as described in the mathematical model developed by Pfaffl (2001), the algorithm provided values for each sample relative to the sample calibrator.

3. Results

3.1 Development of molecular markers for the scab resistance gene *Vr1* from R12740-7A

3.1.1 Seedling scab assessments

Six apple progenies derived from crosses with the scab resistant cultivar 'Regia' were used to verify the presence and segregation of a single resistance gene by greenhouse scoring (Tab. 7). No clear segregation patterns were observed after greenhouse inoculations when the scab infection data were classified in the six reaction classes described in Table 6. To reduce the number of false positives, only plants without any sporulation symptoms (classes 1 and 2) were considered to contain the scab resistance gene, while those showing different degrees of sporulation (classes 3 to 6) were assigned into the susceptible group (absence of the scab resistance gene). After division of plants in such 2 categories in three (00/213, 00/214 and 03/205) out of five populations, the expected 1:1 segregation ratios were observed (Tab. 7). This segregation fits the assumption that there is a single dominant heterozygous gene present in the resistant parent 'Regia', as is expected from data about genetics of the R12740-7A (Bus et al. 2005a; Dayton and Williams, 1968). The major scab resistance gene was named *Vr1* with the *V* reffering to *V. inaequalis* and the subscript *r1* reffering to the R12740-7A that contains this gene.

During the scab tests of 2002 and 2003, an excess of resistant individuals was observed in two progenies (02/223 and 03/206). Large differences in the intensity of scab infections were found among the two families derived from the same cross parents 'Regia' and 'Pingo'. Family 00/213 showed a clear 1:1 ratio in 2000, whereas in 2002 family 02/223 differed from the expected 1:1 ratio.

The progeny from the cross 'Regia' (*Vr1*) x 'Rebella' (*Vf*) segregating for two resistance genes was used for resistance screening. The segregation data obtained by seedling inoculations is represented in Table 7. Assuming that *Vf* and *Vr1* have the same spectrum of action against the scab inoculum used and

that the parents carry one dominant allele each of the genes *Vf* and *Vr1*, a 3:1 - ratio would be expected in the cross 'Regia' x 'Rebella' (F_1 genotypes: *Vfvf Vr1vr1*, *Vfvf vr1vr1*, *vfvf Vr1vr1*, *vfvf vr1vr1*). However, also in this progeny (00/216), a 1:1 ratio was found (Tab. 7). Although there was inconsistency in segregation patterns for some families, the hypothesis of a single major *Vr1* resistance gene for mapping the scab resistance factor of 'Regia' was maintained.

Table 7: Apple families investigated in study for *Vr1* scab resistance and segregation data obtained by seedling inoculations

					Number of plants			
						Resistant	Susceptible	
Population	♀ parent	♂ parent	Year of cross	Year of test	Total	(classes 1,2)	(classes 3-6)	χ^2 (1:1)
00/213	Regia	Pingo	1999	2000	192	107	85	2.52
00/214	Regia	Pinova	1999	2000	186	88	98	0.54
00/216	Regia	Rebella (*Vf*)	1999	2000	93	47	46	0.01
02/223	Regia	Pingo	2001	2002	147	93	54	10.35*
03/205	Regia	Pinova	2002	2003	69	40	29	1.75
03/206	Regia	Piflora	2002	2003	258	185	73	48.62**

*significant at α = 0.01, **significant at α = 0.001

3.1.2 RAPD marker identification

The RAPD marker technique together with a bulked-segregant analysis as mapping strategy were performed using two apple progenies segregating for a scab resistance gene *Vr1* deriving from 'Regia' in a pseudotestcross situation.

Two hundred sixty RAPD primers were screened on the basis of two "resistant" and two "susceptible" DNA pools and two primers (OPAD13 and OPQ7) appeared to be linked to the *Vr1* gene. These two primers showed polymorphic PCR bands between the resistant and susceptible DNA bulks of the apple populations 00/213 and 00/214 (not shown). The primers OPAD13 and OPQ7 were further analysed using the most resistant and the most susceptible individual seedlings of the segregating progenies from 'Regia' x 'Pingo' (00/213)

and ‘Regia’ x ‘Pinova’ (00/214). Examples of patterns for the primers OPAD13 and OPQ7 are depicted in Figure 6.

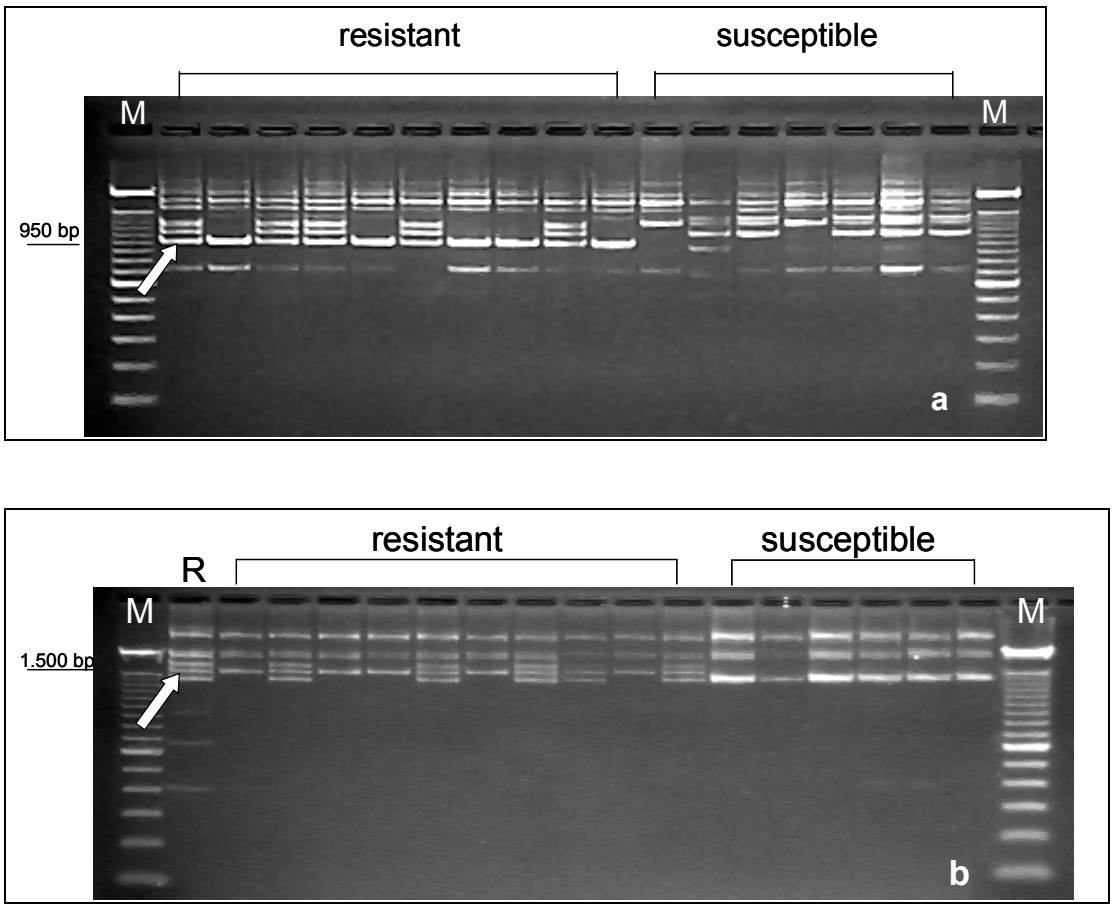


Figure 6: RAPD patterns showing the presence or absence of the $OPAD13_{950}$ (**a**) and $OPQ7_{1500}$ (**b**) in the population from Regia x Pingo (00/213). Scab resistant individuals are marked as resistant. Susceptible individuals are marked as susceptible. R, Regia; M, size marker: 100 bp ladder

Here, the primers produced polymorphic fragments of approximately 950 bp (OPAD13) and 1.500 bp (OPQ7) that well correlated with the segregation of resistance to apple scab in the population from ‘Regia’ x ‘Pingo’. The polymorphic amplification products were designated according to their size (base pairs), $OPAD13_{950}$ and $OPQ7_{1500}$. Since the RAPD primers are known to be sensitive to the experimental conditions, it was decided to estimate the genetic distance between found markers and the *Vr1* resistance gene after the

development of SCARs derived by sequencing of specific $OPAD13_{950}$ and $OPQ7_{1500}$ fragments.

To determine whether RAPD markers $OPAD13_{950}$ and $OPQ7_{1500}$ represent specific DNA sequences introgressed from the resistance donor R12740-7A, a set of cultivars and scab resistance donors was analysed with the found RAPD markers. An example of RAPD patterns obtained with the OPAD13 primer for some apple cultivars without any known scab resistance gene ('Pia', 'Piflora', ''Pirol', 'Pingo', 'Pinova' and 'Delbarestivale'), with the *Vf* scab resistance gene ('Remo', 'Retina', 'Rewena' and 'Rebella') or resistance factors from 'Russian Seedling' ('Realka' and 'Regia') is shown in Figure 7.

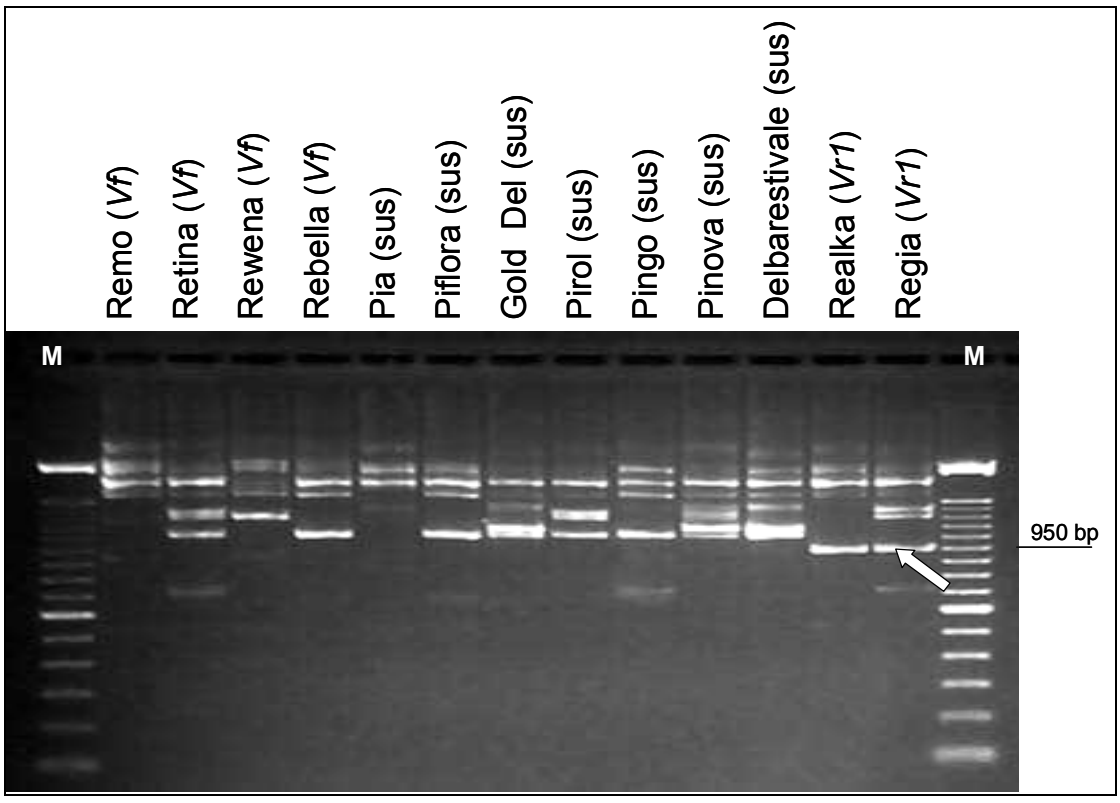


Figure 7: PCR amplification profiles of several apple cultivars carrying the *Vf* or *Vr1* resistance gene, respectively, and some susceptible control cultivars (sus) with the RAPD fragments $OPAD13_{950}$. Gold Del, Golden Delicious; M, size marker:100 bp ladder

In this case, a clear fragment of approximately 950 bp is present only in derivatives from the R12740-7A ('Realka' and 'Regia') but not in any *Vf*-carriers

or susceptible cultivars tested. The presence and absence of the second marker, $OPQ7_{1500}$, is shown in Figure 8. The amplification results were obtained for the following genotypes: the resistance donor R12740-7A, its derivatives ('Remura', 'Regia', 'Releta', 'Realka' and 'Reka'), five available doubled haploids (DH) generated from the cultivar 'Remura' by anther culture (Höfer et al. 2004) and susceptible cultivars ('Golden Delicious', 'Pingo', 'Pinova' and 'Piflora'). The specific RAPD fragment $OPQ7_{1500}$ was absent in any susceptible cultivar tested and present in the R12740-7A as well as in its derivatives ('Realka', 'Regia' and 'Remura'). The specific $OPQ7_{1500}$ was also present in four from five DH-lines (DH33/99; DH193/00; DH198/00 and DH235/00).

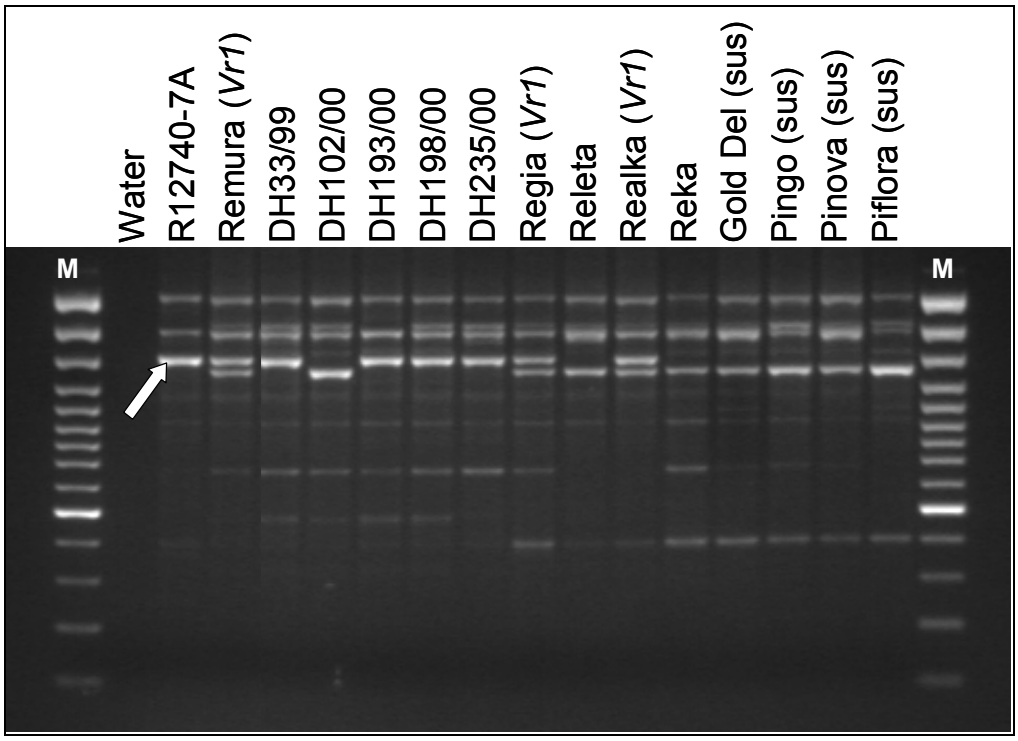


Figure 8: RAPD primer $OPQ7_{1500}$ tested on scab resistant (*Vr1* resistance gene) and susceptible (sus) apple accessions. The fragment 1.500 bp is marked by an arrow. Gold Del, Golden Delicious; DH33/99, DH102/00, DH193/00, DH198/00, DH235/00 are dihaploid lines derived from Remura; Reka and Releta are derivatives from the R12740-7A; M, size marker: 100 bp ladder

Taken together, these results suggest that the RAPD markers $OPAD13_{950}$ and $OPQ7_{1500}$ represent chromosomal regions introgressed from the resistance

donor R12740-7A. At the same time two other cultivars (‘Reka’ and ‘Releta’) derived also from the R12740-7A did not show the specific $OPQ7_{1500}$ (Fig. 8) and $OPAD13_{950}$ (not shown) fragments.

3.1.3 SCAR marker development and analysis

Based on the sequence data of the cloned $OPAD13_{950}$ RAPD fragment several SCAR primers were developed. Finally, it was found that the original OPAD13 RAPD primer prolonged by 8 or 9 bases, respectively, resulted in the most informative fingerprints (A10). The AD13-SCAR can be used as a codominant marker to differentiate between heterozygous and homozygous resistant plants. A total of at least four different alleles were found in the *Malus* genotypes tested for this marker (Fig. 9). In cultivars the most common alleles are *a* (1.300 bp) and *b* (1.100 bp) whereas the "resistance allele" *c* (950 bp) is present in R12740-7A, ‘Regia’, ‘Realka’, ‘Remura’, but also in all three analysed *M. sieversii* genotypes, and in *M. baccata* (419) and *M. sieboldii* (397). In ‘Regia’, ‘Pinova’ and ‘Pirol’ a third fragment with a size of about 1.200 bp was amplified, which cosegrated with the 1.300 bp fragment in population 03/206 (‘Regia’ x ‘Piflora’). The GMAL 2473 genotype also possessed the allele *c* but in addition also the allele *b*, which clearly confirms that GMAL 2473 and R12740-7A are genetically different (Fig. 9). Several cultivars are obviously homozygous regarding the *a* and *b* allele, which is shown by the segregation patterns of the populations. For example, in family 03/206 (‘Regia’ x ‘Piflora’) the cross *ac* x *bb* yielded only descendents with the allele combinations *ab* and *bc* in a 1:1 segregation ratio (Fig. 9). The codominance of the AD13 locus is also demonstrated by the two dihaploid lines generated from ‘Remura’. The fourth allele *d* (750 bp) was only present in one accession of *M. baccata.*

Because the AD13-SCAR marker is not only useful for the molecular description of the allelic situation regarding the *Vr1* marker locus, but can also discriminate cultivars and different accessions of *Malus* species, this marker was designated as a "taxonomic" SCAR marker.

Several attempts to develop a polymorphic SCAR marker derived from the 'Regia' RAPD allele at $OPQ7_{1500}$ have been undertaken. The Q7-SCAR primers amplified the same sized fragment from both parents (not shown). Increasing the annealing temperature did not result in the loss of amplification of the allele from the susceptible parent. The reason for this observation was not investigated deeply. Additional sequencing of the alternate alleles might identify diverged regions for which it may be possible to design allele-specific primers.

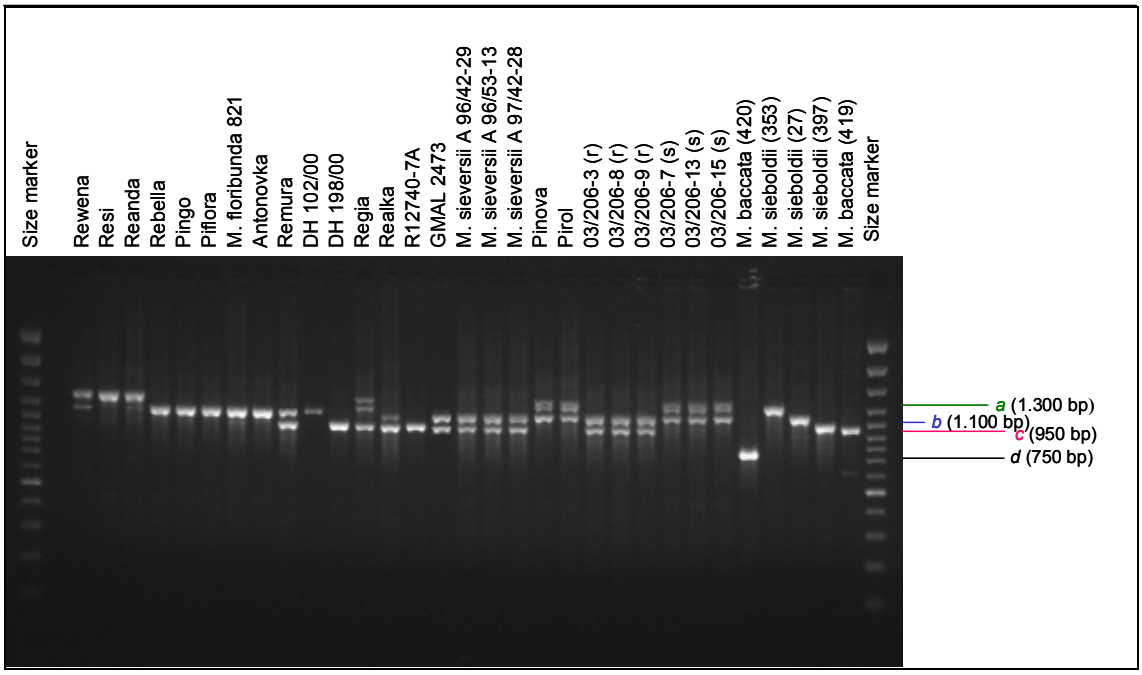


Figure 9: PCR amplification patterns obtained with the AD13-SCAR primers for accessions of *M. sieversii*, *M. sieboldii*, *M. baccata*, and some apple cultivars without any known scab resistance gene (Pingo, Piflora, Pinova, Pirol), the *Vf* scab resistance gene (Rewena, Resi, Reanda, Rebella) or resistance factors from Russian seedling R12740-7A (Regia, Realka, Remura). Six individuals of family 03/206 (Regia x Piflora) are labeled with *r* for resistance and *s* for susceptibility, respectively. DH 102/00 and DH 198/00 are dihaploid lines derived from Remura. Four marker alleles (*a, b, c, d*) are marked by *arrows*. Size marker: 100bp DNA ladder

3.1.4 *Vr1* linkage mapping

Two separate linkage maps were created for the families 02/223 and 03/206 (Fig. 10a, b). Five SSR loci known to map on LG 2 and the AD13-SCAR and OPQ7-RAPD markers were analysed in a subset of 80 (02/223) or 90 progeny

plants (03/206), respectively. A14 represents as example marker data for 90 individuals of the population 03/206 (‘Regia’ x ‘Piflora’) used for construction of genetic map for the *Vr1*-carrying linkage group LG 2. The position and the order of the SSRs mapped in both populations are in good agreement and correspond also with published map information (Liebhard et al. 2002). Using only phenotypes with scab scoring values 1 or 2 for resistance and 4 to 6 for susceptibility, respectively, the recombination frequency between *Vr1* and AD13-SCAR is 11 and 17%, and between *Vr1* and OPQ7-RAPD 3 and 15%, respectively (Fig. 10 a, b). Both markers were located on the same side on the linkage map with respect to the *Vr1* locus.

The *Vr1* locus is also linked to the S22-SCAR and to the SSR CH02c02a as published for *Vx* and *Vh4* by Hemmat et al. (2002) and Bus et al. (2005a), respectively. The utility of the OPB18-SCAR marker was evaluated in the population ‘Regia’ x ‘Piflora’ (03/206). OPB18-SCAR marker amplified a specific fragment in the resistant parent ‘Regia’ and was absent in the susceptible ‘Piflora’. Screening of the most resistant and the most susceptible individuals with OPB18-SCAR showed the presence of a 620 bp fragment in 14 among 28 resistant individuals and in 16 among 26 susceptible individuals (not shown) that demonstrates no correspondence between the 620 bp marker fragment and the resistance phenotypes. SSR loci Ch03d10, CH02b10 and CH05e03 located in the bottom area of LG 2 are linked to *Vr1*, the new *Vr1* markers and to S22-SCAR (Fig. 10). Thus, as it was initially suggested (Boudichevskaia et al. 2004) and as confirmed by Bus et al. (2005a), the *Vh2* gene (CH02b10, CH05e03, CH03d01) and the *Vh4* gene (CH02c02a) map to the same linkage group. Critical observation of the molecular and phenotypic data allowed the identification of so-called "genotype-phenotype-incongruence" (GPI) plants from the mapping population 03/206 following the suggestion of Gygax et al. (2004) and Erdin et al. (2006).

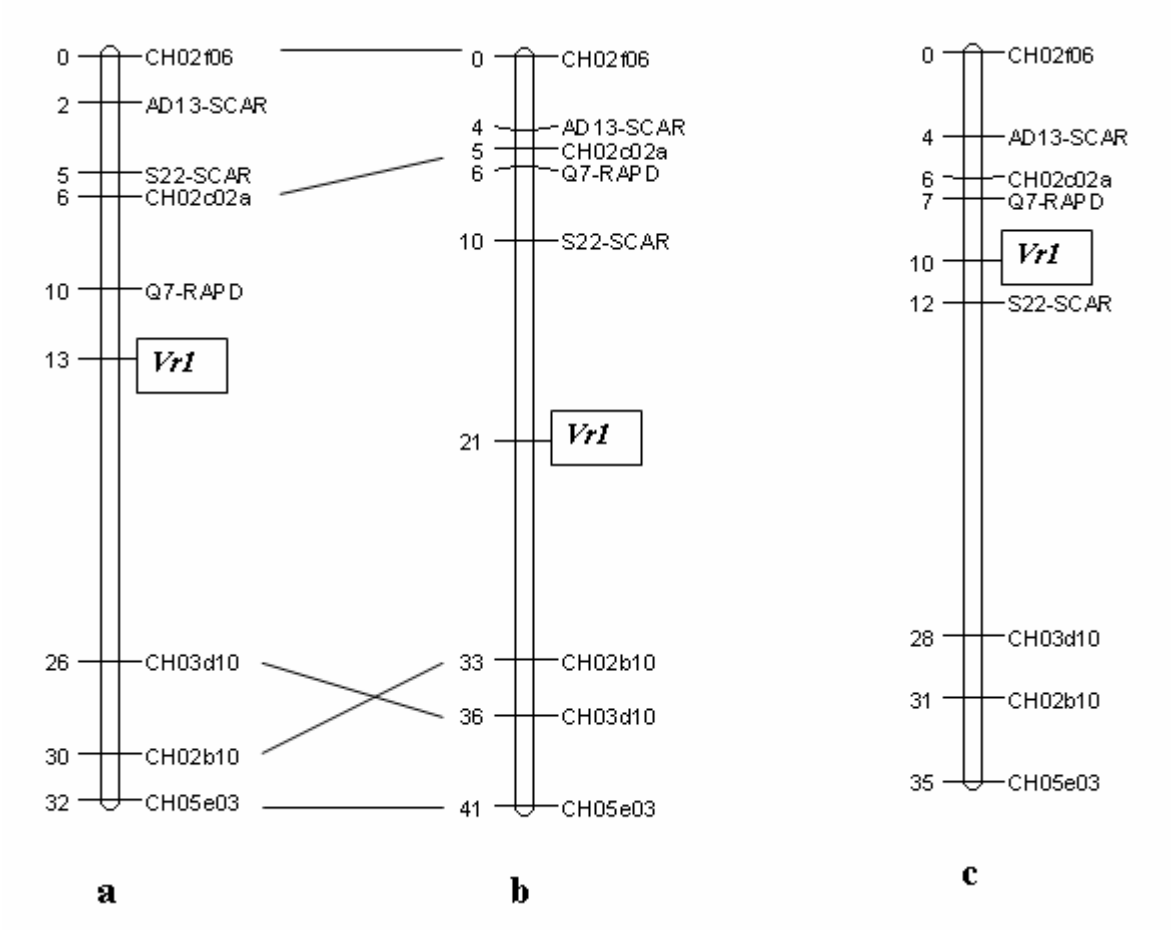


Figure 10: Genetic maps for the *Vr1*-carrying linkage group LG 2 created for **a)** 80 individuals of population 02/223 (Regia x Pingo), **b)** 90 individuals of population 03/206 (Regia x Piflora) selected for marker verification, and **c)** the same 90 individuals selected out of population 03/206 (Regia x Piflora) without 10 genotype-phenotype-incongruence plants (see text for explanation)

Ten individuals (A14) were eliminated from family 03/206 which showed resistance but not any of the 5 markers (Ch02f06, AD13-SCAR, CH02c02a, Q7-RAPD, S22-SCAR) in coupling with *Vr1* or, vice versa, susceptible plant showing all these marker alleles. Linkage mapping of the remaining 80 seedlings resulted in the map shown in Fig. 10c. The position of *Vr1* moved to the proximal end of the chromosome and is more closely located to the *Vx*/*Vr2* locus.

Because *Vr1* mapping was based on scab greenhouse data, it was of interest to verify the assumed linkage between the resistance phenotype and the AD13-

SCAR marker under natural scab infection conditions in the field. Comparison of molecular data concerning presence/absence of the AD13-SCAR, with the scab resistance field data obtained after two years of scoring, revealed only two plants with the AD13-SCAR marker that were clearly susceptible in the field in the population 03/205 (Tab. 8). In the second progeny (03/206) also only two out of 44 marker-carrying individuals showed scab symptoms (Tab. 8).

Table 8: Correspondence between scab resistance field data and *Vr1*-SCAR marker AD13 in the populations 03/205 (Regia x Pinova) and 03/206 (Regia x Piflora); resistance/susceptibility classification based on 2 years of scoring

AD13 marker presence	Number of plants					
	03/205			03/206		
	Resistant	Susceptible	Total	Resistant	Susceptible	Total
AD13 present	28	2	30	42	2	44
AD13 absent	14	25	39	11	35	46
Total	42	27	69	53	37	90

3.1.5 Analysis of *Vr1*-*Vf* gene combinations

A PCR multiplex assay was developed for analysing plants derived from a cross between *Vr1* and *Vf* donors ‘Regia’ and ‘Rebella’ (Fig. 11). The PCR was suited for a simultaneously amplification of two codominant sequence tagged sites (AL-07 and AD-13-SCAR markers). Although ‘Regia’ appeared to be heterozygous regarding the AD13-SCAR locus (Fig. 9), the "susceptible" allele *d* could not be seen in the multiplex PCR of this parent, but appeared in several progeny plants (Fig. 11). Nevertheless, the PCR protocol was suited for a simultaneously amplification of two codominant sequence tagged sites (AL07- and AD13-SCAR markers, alleles *a* and *c* in Fig. 11) in a single PCR reaction. Marker analyses of 93 individuals of progeny 00/216 showed that the 4 expected marker phenotype classes consisted of 25:20:20:28 and didn't deviate from the expected 1:1:1:1 ratio (Tab. 9). However, regarding scab resistance data, there was a difference between the expected (3:1) and the observed (1:1)

segregation ratio (Tab. 9). There was a tendency that plants carrying only the AD13-SCAR (class 3) or this marker in combination with the AL07-SCAR (class 1) had a satisfactory degree of scab resistance. The *Vf*-resistance alone (class 2) was not sufficient to provide a strong resistance phenotype and was comparable with the class 4 containing none of the analysed markers/genes. The reason for this might be in presence of scab races 6 and/or 7 within the naturally occuring scab race spectrum in the orchards at Dresden-Pillnitz that are known to be able to overcome the *Vf* resistance.

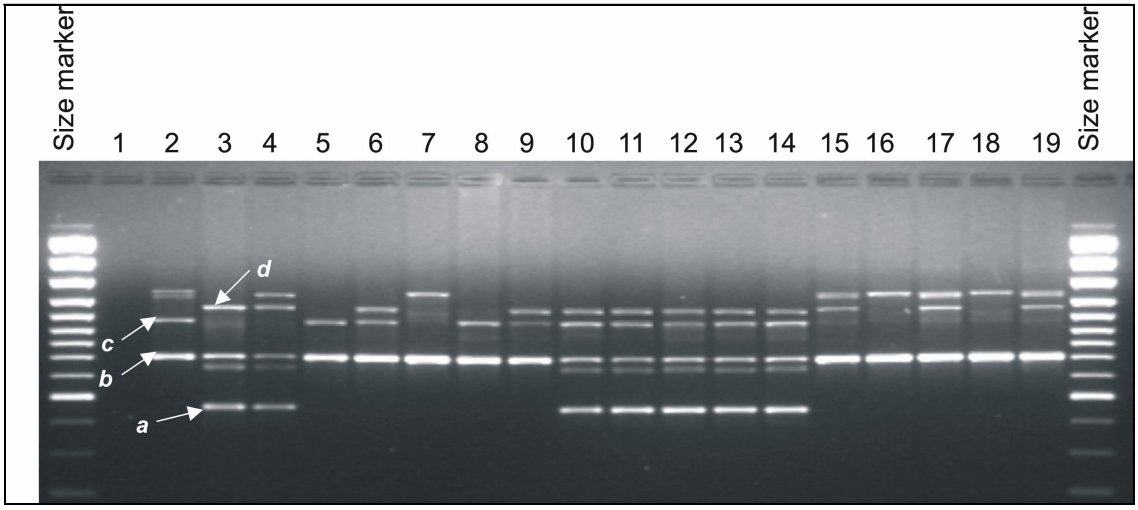


Figure 11: PCR multiplexing for two SCAR markers (AL07-SCAR for the *Vf* gene and AD13-SCAR for the *Vr1* scab resistance gene); arrows *a* (resistant allele) and *b* (susceptible allele) are corresponding to the AL07-SCAR; arrows *c* (resistant allele) and *d* (susceptible allele) to the AD13-SCAR, respectively; lanes from *left* to *right*: *1* water control; *2* Regia (*Vr1*), *3* Rebella (*Vf*), *4* to*19* selected progeny plants of the population Regia x Rebella; *lanes 4* to *9* resistant genotypes with either the *Vr1* marker (*lanes 5*, *6*, *8* and *9*), the *Vf* marker (*lane 4*) or none of the markers (*lane 7*), *lanes 10* to *14*: genotypes assumed to carry both scab resistance genes (marker alleles *a* + *c*); *lanes 15* to *19*: susceptible plants without any marker; Size marker: 100bp DNA ladder

Table 9: Relationship between presence of two SCAR markers AL07 (*Vf*) and AD13 (*Vr1*) and scab resistance in apple population 00/216 (Regia x Rebella)

Scab resistance gene marker combination class	Number of plants					Percent observed/(expected)	
	Resistant			Susceptible	Total**	Resistant	Susceptible
	Class 1	Class 2	Total				
AL07 present, AD13 present	3	20	23	2	25	24.7 (25)	2.2 (0)
AL07 present, AD13 absent	1	1	2	18	20	2.2 (25)	19.4 (0)
AL07 absent, AD13 present	1	13	14	6	20	15.1 (25)	6.5 (0)
AL07 absent, AD13 absent	3	5	8	20	28	8.6 (0)	21.5 (25)
Total*			47	46	93	50.5 (75)	49.5 (25)

Resistance classes 1 (no symptoms) and 2 (point necrotic or chlorotic pits) are specified separately. *χ^2 (1:1)=0.01 (not significant), χ^2 (3:1)=29.68 (significant at α=0.001), ** χ^2 (1:1:1:1)=2.01 (not significant)

3.2 Molecular analysis of *HcrVf*-type candidate genes

3.2.1 Identification of *HcrVf*-type candidate genes

To efficiently identify potential candidate *Vf* genes, a wide range of apple accessions was established to represent different sources of resistance or tolerance to scab as well as cultivars which are known to be susceptible to scab. These genotypes were evaluated with the PCR primers Vf1 and Vf2 designed from the variable subdomain C1 of *HcrVf1* and *HcrVf2*. PCR products matching 711 and 819 bp and corresponding to *Vf1* and *Vf2* homologues, respectively, were found in 11 *Vf* cultivars (‘Prima’, ‘Reanda’, ‘Rebella’, ‘Regine’, ‘Reglindis’, ‘Releika’, ‘Remo’, ‘Renora’, ‘Resi’, ’Retina’, ‘Rewena’) and the six *M. floribunda* accessions (A3). Some cultivars carrying other scab resistance genes (‘Regia’, ‘Releta’ and ‘Discovery’) possessed both fragments, whereas ‘Antonovka kamienna’, ‘Antonovka polutorafuntovaya’ (AP), R12740-7A and ‘Realka’ amplified only one of the two PCR products. Amplicons of other sizes were not detected. None of the cultivars without known scab resistance yielded any PCR products. Figure 12 showes as example, the amplification results obtained with the Vf1 primers for the studied apple accessions. In this case, the 711 bp fragment corresponding to *Vf1* was present in all *Vf*-carriers as well as in apple accessions carrying other scab resistance genes (R12740-7A, ‘Regia’,

'Releta' and 'Discovery') but in none of the scab susceptible cultivars tested in this study.

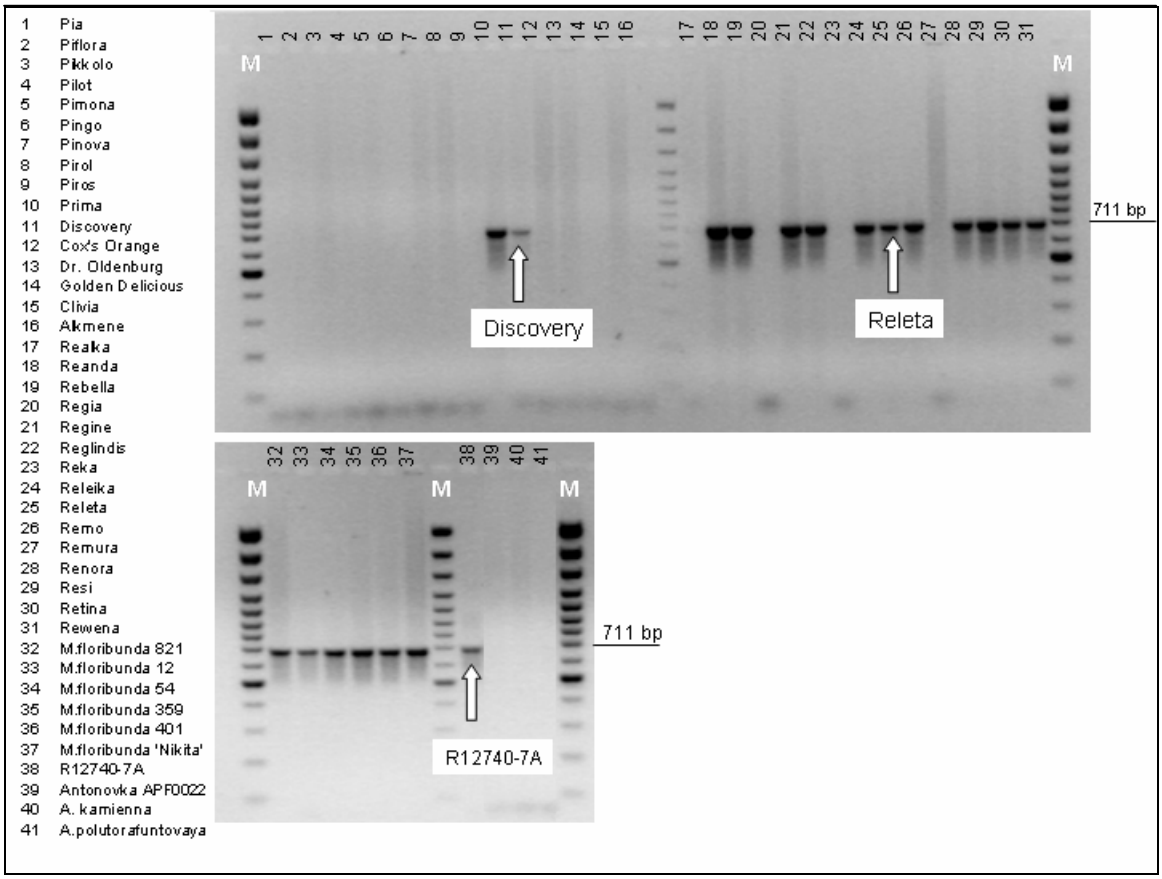


Figure 12: PCR amplification patterns obtained with the Vf1 primers for forty-one apple accessions. Right side, *Vf1* amplification profiles; left side, Table including all tested individuals; M, size marker: 100bp DNA ladder

3.2.2 Sequence analysis of the *HcrVf*-type candidate genes

The *Vf1* PCR products were excised, cloned and sequenced from 'Discovery', 'Prima', 'Russian Seedling', 'Regia' and 'Releta'. Cloning was focused on genotypes that represent different sources of resistance to scab not deriving from *M. floribunda 821*. Analysis of four *Vf1* clones of 'Russian Seedling' yielded two homologous but not identical sequences designated as 'Russian Seedling A' and 'Russian Seedling B'. The homology between the two sequences was 87.4% in amino acid (aa) sequence. The *Vf2* PCR products were sequenced

from ‘AP’, ‘Discovery’, ‘Prima’, ‘Realka’, ‘Regia’ and ‘Releta’. Two homologous sequences having 87.5% identity in amino acid sequence were revealed after analysis of *Vf2* clones of the cultivar ‘Regia’. They have been designated as ‘Regia A’ and ‘Regia B’, respectively. After comparisons with nucleotide sequences present in NCBI GenBank, it appeared that all above-mentioned sequences had a high homology with the original apple HcrVf disease resistance proteins. On the DNA level the identity was 91 - 100%, and for proteins 85 - 100% aa identity was calculated (Tab. 10). The cloned *HcrVf1* homologues of ‘Discovery’ and ‘Releta’ were identical with the *HcrVf1* sequence whereas the ‘Regia B’ sequence matched the *HcrVf2* reference to 100%.

Table 10: Amino acid sequence comparisons of cloned *HcrVf* homologues with *HcrVf1* and *HcrVf2*, respectively (BlastX, NCBI GenBank)

Cloned *HcrVf* homologues	Acc. no.	PCR primers	Length (bp)	Homology	E-value	Identity (%)
Discovery *HcrVf1*	EU635446	Vf1	694	*HcrVf1*	1.4^{E}-95	100.0
Prima *HcrVf1*	EU635447	Vf1	694	*HcrVf1*	8.1^{E}-95	99.6
Regia *HcrVf1*	EU635448	Vf1	685	*HcrVf1*	2.3^{E}-81	87.9
Releta *HcrVf1*	EU635449	Vf1	694	*HcrVf1*	2.5^{E}-95	100.0
Russian Seedling A* *HcrVf1*	EU635450	Vf1	697	*HcrVf1*	2.5^{E}-78	85.4
Russian Seedling B* *HcrVf1*	EU635451	Vf1	694	*HcrVf1*	5.5^{E}-78	84.9
Antonovka polutorafuntovaya *HcrVf2*	EU635452	Vf2	736	*HcrVf2*	4.4^{E}-83	85.4
Discovery *HcrVf2*	EU635453	Vf2	736	*HcrVf2*	2.5^{E}-82	85.8
Prima *HcrVf2*	EU635454	Vf2	748	*HcrVf2*	7.7^{E}-86	88.0
Realka *HcrVf2*	EU635455	Vf2	736	*HcrVf2*	1.9^{E}-83	86.2
Regia A* *HcrVf2*	EU635456	Vf2	748	*HcrVf2*	3.3^{E}-86	88.0
Regia B* *HcrVf2*	EU635457	Vf2	739	*HcrVf2*	2.1^{E}-100	100.0
Releta *HcrVf2*	EU635458	Vf2	739	*HcrVf2*	3.6^{E}-98	98.8

*A and B are different sequences obtained from the same PCR fragment

Quite different picture was observed when other *HcrVf* homologues were compared with the original *HcrVf1* and *HcrVf2* genes. Table 11 represents overall aa alignment data of the shortened (adjusted) *HcrVf* - like sequences and the *HcrVf1* and *HcrVf2* genes. Here, the homology between the sequences ‘Russian Seedling A-HcrVf1’ and ‘Russian Seedling B-HcrVf1’ was higher (86.8%) than between those sequences and the HcrVf1 (84.5 and 85.0%, respectively). The sequences ‘Regia A-HcrVf2’ and ‘Prima-HcrVf2’ shared 100% identity whereas their homology to the HcrVf2 was only (86.8%). The sequences ‘Antonovka-HcrVf2’, ‘Discovery-HcrVf2’ and ‘Realka-HcrVf2’ were most similar to each other (97.7-100%) and less similar to the HcrVf2 (83.6-

84.5%). The sequences of the cloned *HcrVf* homologues were submitted to NCBI GenBank under the accession numbers given in Table 10.

Table 11: Sequence distances between the cloned HcrVf homologues and the HcrVf1/HcrVf2 proteins. Percent similarity is calculated by directly comparing pairs of sequences

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	Prima HcrVf1		99.5	99.5	87.6	84.1	84.5	99.5	85.9	85.9	82.2	83.1	83.1	86.8	85.9	86.8
2	Discovery HcrVf1			100.0	88.0	84.5	85.0	100.0	86.4	86.4	82.6	83.6	83.6	87.3	86.4	87.3
3	Releta HcrVf1				88.0	84.5	85.0	100.0	86.4	86.4	82.6	83.6	83.6	87.3	86.4	87.3
4	Regia HcrVf1					86.2	84.3	88.0	83.4	83.4	82.0	82.5	82.5	85.7	84.8	85.7
5	Russian Seedling A HcrVf1						86.8	84.5	84.2	84.2	80.4	81.3	81.3	85.9	85.0	85.9
6	Russian Seedling B HcrVf1							85.0	80.9	80.9	79.0	79.9	79.9	84.5	83.6	84.5
7	HcrVf1								86.4	86.4	82.6	83.6	83.6	87.3	86.4	87.3
8	Regia A HcrVf2									100.0	81.7	82.2	82.2	86.8	85.9	86.8
9	Prima HcrVf2										81.7	82.2	82.2	86.8	85.9	86.8
10	Antonovka HcrVf2											97.7	97.7	83.6	82.6	83.6
11	Realka HcrVf2												100.0	84.5	83.6	84.5
12	Discovery HcrVf2													84.5	83.6	84.5
13	Regia B HcrVf2														99.1	100.0
14	Releta HcrVf2															99.1
15	HcrVf2															

The alignments of the newly found sequences with those of *HcrVf1* and *HcrVf2* revealed some variation of the solvent-exposed amino acids within the xxLxLxx structural motif of the N-terminal LRRs within the C1 subdomain (Fig 13). Alltogether 47 amino acid substitutions were found in *HcrVf1* alignments (Fig. 13a), and 50 aa changes were detected in *HcrVf2* alignments (Fig. 13b), respectively. Among the nucleotide sequences 88 and 82 SNPs, respectively, were identified in comparison to *HcrVf1* and *HcrVf2* (A18, 19).

Since the aim of the present study was to identify and map candidate genes from others than *Vf* apple scab resistance sources, sequences from the cloned fragments ‘Russian Seedling A-HcrVf1’, ‘Antonovka-HcrVf2’, ‘Realka-HcrVf2’ and ‘Discovery-HcrVf2’ (Tab 10) that appeared to display the lowest homology to the *HcrVf* genes, were used for design the new specific PCR primers.

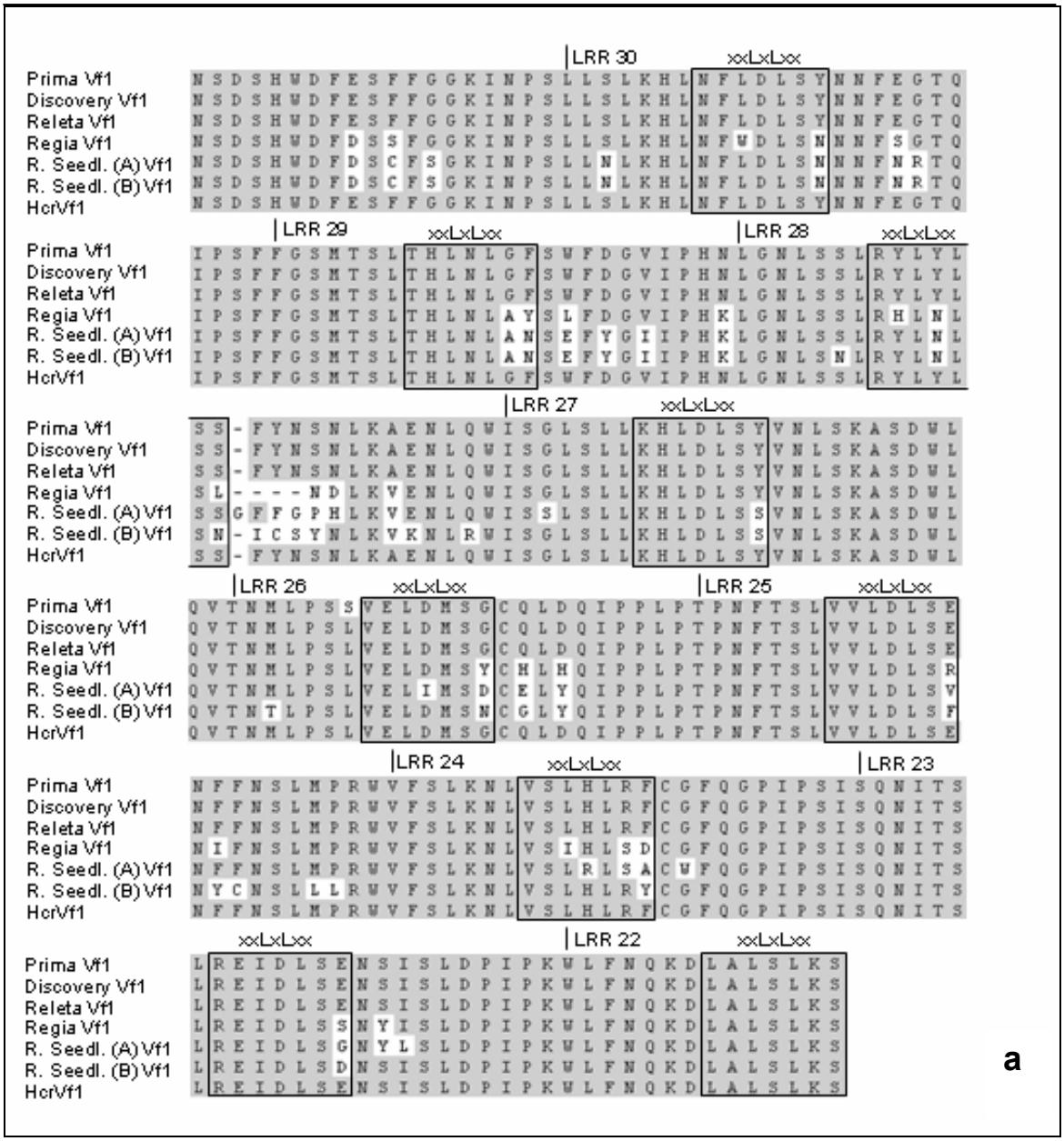
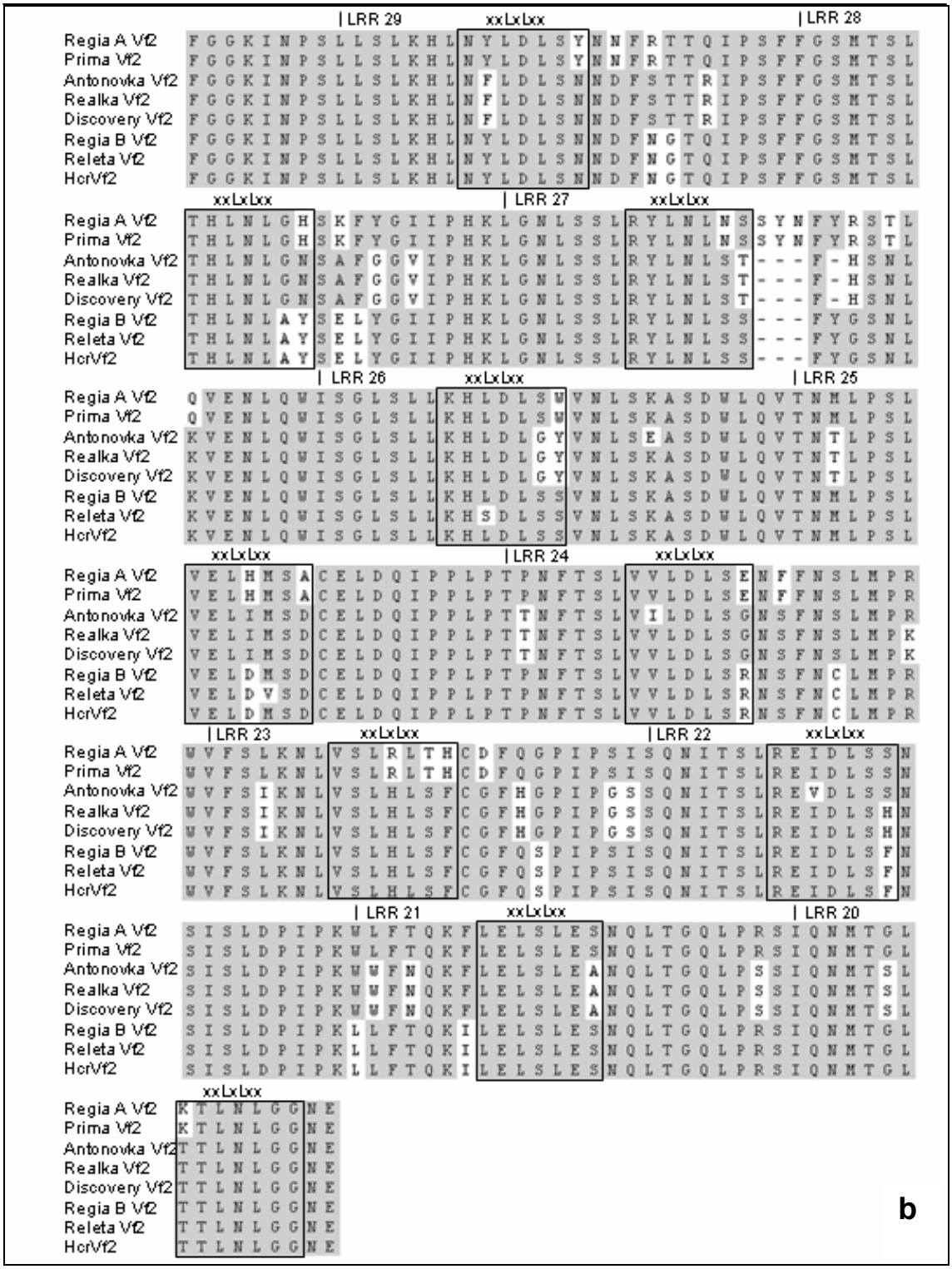


Figure 13: Multiple sequence alignments of **a**) *HcrVf1* (GenBank acc. no. AJ297739) and **b**) *HcrVf2* (GenBank acc. no. AJ297740) and their homologues found in some apple cultivars. Amino acid residues identical to *HcrVf1* and *HcrVf2* are indicated with grey background. Sequence gaps inserted to maintain the alignment are indicated by dashes. LRRs 22-30 (**a**) and LRRs 20-29 (**b**) (C1 subdomain) are indicated above the sequence. Sequences that form part of the putative β-strand / β-turn conserved structural motif in LRR proteins (xxLxLxx, where L is leucine and x is any amino acid) are shown by the dashed box


| LRR 29
xxLxLxx
| LRR 28
Regia A Vf2
Prima Vf2
Antonovka Vf2
Realka Vf2
Discovery Vf2
Regia B Vf2
Releta Vf2
HcrVf2
xxLxLxx
| LRR 27
xxLxLxx
| LRR 26
xxLxLxx
| LRR 25
xxLxLxx
| LRR 24
xxLxLxx
| LRR 23
xxLxLxx
| LRR 22
xxLxLxx
| LRR 21
xxLxLxx
| LRR 20
xxLxLxx
b

3.2.3 Analysis of the HcrVf-type candidate gene *Vf1RSA*

A pair of *Vf1RSA* primers was developed on the basis of the unique sequence of 'Russian Seedling A'. Sequence alignment used for the development of the *Vf1RSA* primers is presented in Figure 14.

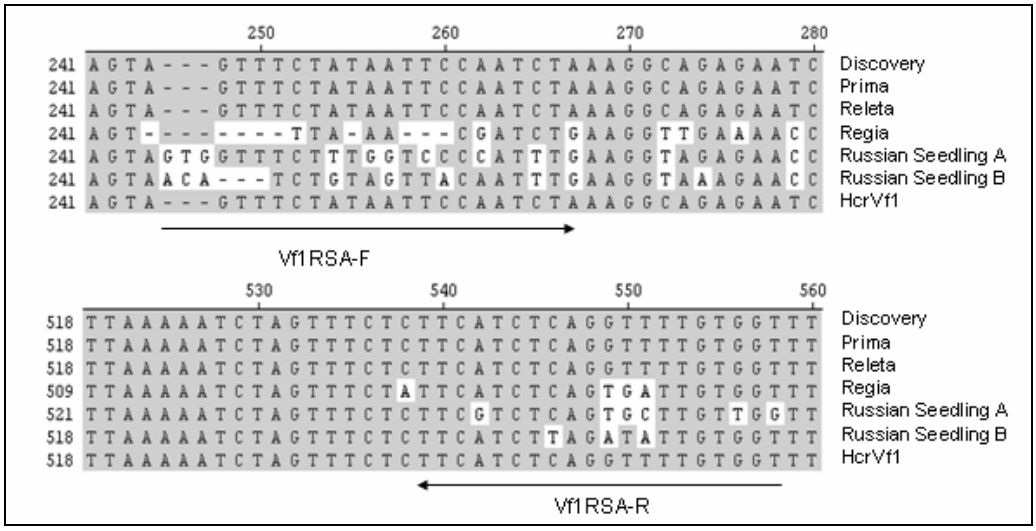


Figure 14: Design of (a) *Vf1RSA*-specific PCR primers based on the multiple nucleotide sequence alignment of *HcrVf1* homologues from different apple accessions. Arrows indicate the position of the forward (F) and reverse (R) primers. Polymorphic nucleotides are marked with white. Dashes correspond to missing nucleotides. (*HcrVf1*- GenBank acc.no. AJ297739)

As a result, specific primers for the 'Russian Seedling A-HcrVf1' amplified a PCR product of the expected size (313 bp) from the 'Russian Seedling' and not from any of the scab susceptible cultivars tested. PCR amplification patterns obtained with the *Vf1RSA* primers from genomic DNA of the studied apple accessions is shown in Figure 15a. Neither the cultivars derived from 'RS' ('Regia', 'Realka', 'Reka' and 'Releta') nor the tested *Vf* cultivars possessed the *Vf1RSA* PCR product. An additional fragment of approximately 520 bp appeared in almost all tested samples.

Since previous taxonomic investigations based on molecular markers have indicated a relationship between *M. sieversii* and 'RS' (Wiedow 2006), 13 *M. sieversii* accessions selected from gene bank collection at Dresden-Pillnitz were tested with the *Vf1RSA* specific primers. The results of this study showed that

three *M. sieversii* genotypes (A96/53-13, A96/57-4, A95/08-4) possessed the 313 bp *Vf1RSA* fragment too (Fig 16).

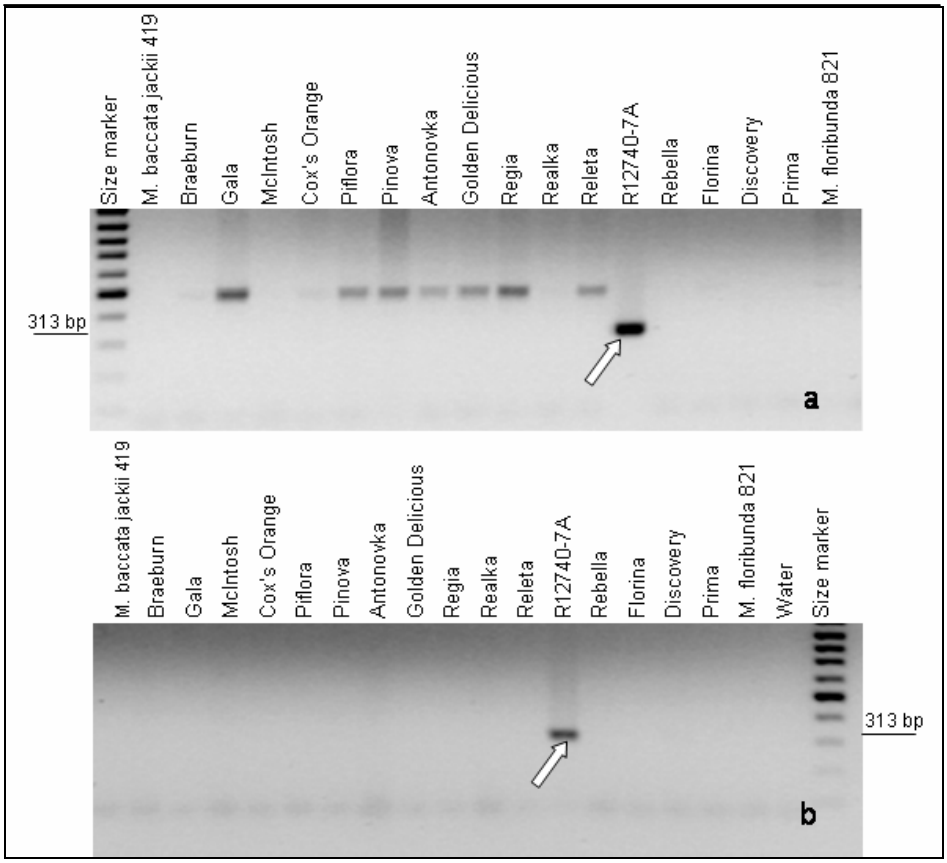


Figure 15: PCR amplification patterns obtained with the *Vf1RSA* specific primers. PCR was performed using either genomic DNA (a) or cDNA (b). The size of *Vf1RSA* PCR product was expected to be 313 bp. Size marker is a 100 bp ladder

To determine whether the *Vf1RSA* fragment might be linked to a scab resistance phenotype inherited by the *M. sieversii* parents A96/53-13 and A96/57-4 to the families 05/230 and 06/004, seedling scab tests were performed in a greenhouse experiment. In the family 05/230 inoculated with a mixture of several races a considerably high number of the resistant plants (25 out of 27) possessed the *Vf1RSA* fragment. In the second population (06/004) inoculated with the newly isolated scab strain 3650C all 54 genotypes with the *Vf1RSA* fragment were scored as resistant whereas half of the plants without the *Vf1RSA* fragment were resistant too (Tab. 12).

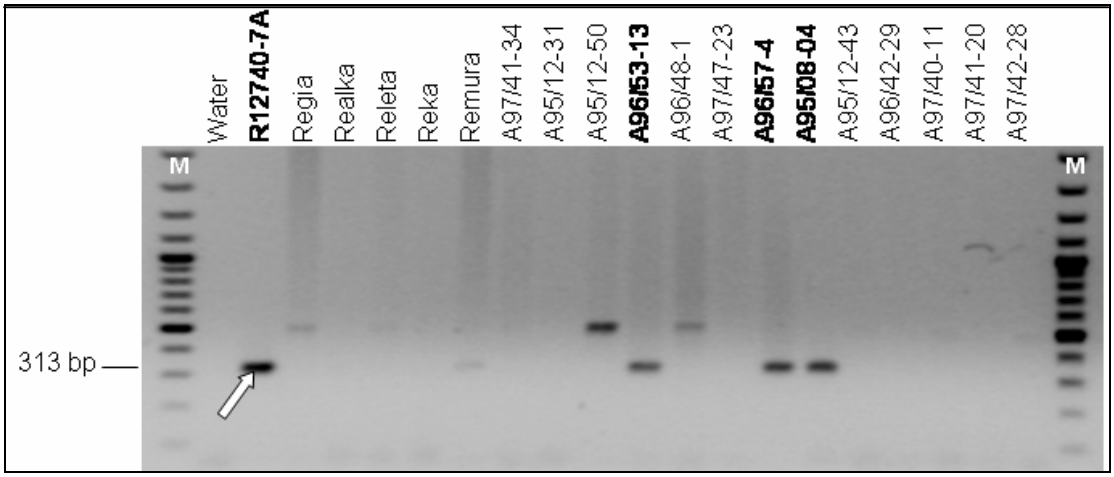


Figure 16: *Vf1RSA* amplification in R12740-7A, its derivative cultivars and thirteen *M. sieversii* accessions selected from gene bank collection at Dresden-Pillnitz. A PCR product of the expected size (313 bp) was amplified from R12740-7A and three *M. sieversii* genotypes (A96/53-13, A96/57-4, A95/08-4) highlighted in bold type. M, size marker:100 bp ladder

Table 12: Correspondence between scab resistance data and presence of the *Vf1RSA* fragment in the populations 05/230 ('Pinova' x *M. sieversii* A96/53-13) and 06/004 ('Golden Delicious' x *M. sieversii* A96/57-4)

Vf1RSA	Number of plants					
313 bp fragment	05/230			06/004		
presence	Resistant	Susceptible	Total	Resistant	Susceptible	Total
fragment present:	25	13	38	54	0	54
fragment absent:	2	34	36	26	26	52
Total	27	47	74	80	26	106

The subsequent cloning and sequencing of *Vf1RSA* fragments from two *M. sieversii* parents (A96/53-13 and A96/57-4) and 'RS' revealed a high protein sequence homology with *HcrVf1* (85 to 88% aa identity, respectively; Tab. 13).

Table 13: Results of the BLAST searches in nucleotide sequences databases (NCBI GenBank) for the *Vf1RSA* specific sequences cloned from R12740-7A and two *M. sieversii* genotypes

Cloned *Vf1RSA* (313 bp)	Length (bp)	Homology	Species	E-value	Identity (%)
R12740-7A	264	*HcrVf1*	*Malus x domestica*	6.5^{E}-23	87.5
M. sieversii A96/53-13	264	*HcrVf1*	*Malus x domestica*	4.0^{E}-22	85.1
M. sieversii A96/57-4	265	*HcrVf1*	*Malus x domestica*	7.9^{E}-23	85.2

3.2.3.1 Transcriptional analysis of the *Vf1RSA* candidate gene

In order to study the expression of the *Vf1RSA* candidate gene, RT-PCR on the RNA extracted three times from young uninfected leaves of 18 accessions including *M. floribunda* 821 was performed. The complete absence of genomic DNA in the cDNA samples was monitored by using primers that amplify an intron-containing gene. This results in a longer PCR product in case of a DNA contamination. The apple housekeeping gene encoding elongation factor EF1-α was chosen as previously reported by Flachowsky et al. (2007). Figure 17 shows as an example the amplification of the EF1-α gene that has been served also to evaluate the sensitivity and efficiency of the RT-PCR reactions. The EF1-α proved to be suited as internal control since a distinct transcript was detected easily in all cDNA samples (Fig. 17). Their fragment size was shorter than one of the genomic DNA controls. Thus, there was no genomic DNA contamination in the cDNAs. The proved cDNA was used in RT-PCR to study the expression of *Vf1RSA* gene in a set of apple accessions. Figure 15b demonstrates that *Vf1RSA* is transcribed in the absence of a pathogen attack. *Vf1RSA*-specific primers resulted in the RT-PCR product with the same size (313 bp) indicating the absence of introns. A distinct band of 313 bp reveals expression of the *Vf1RSA* only in 'RS' and not in any susceptible cultivar or another scab resistance source being tested.

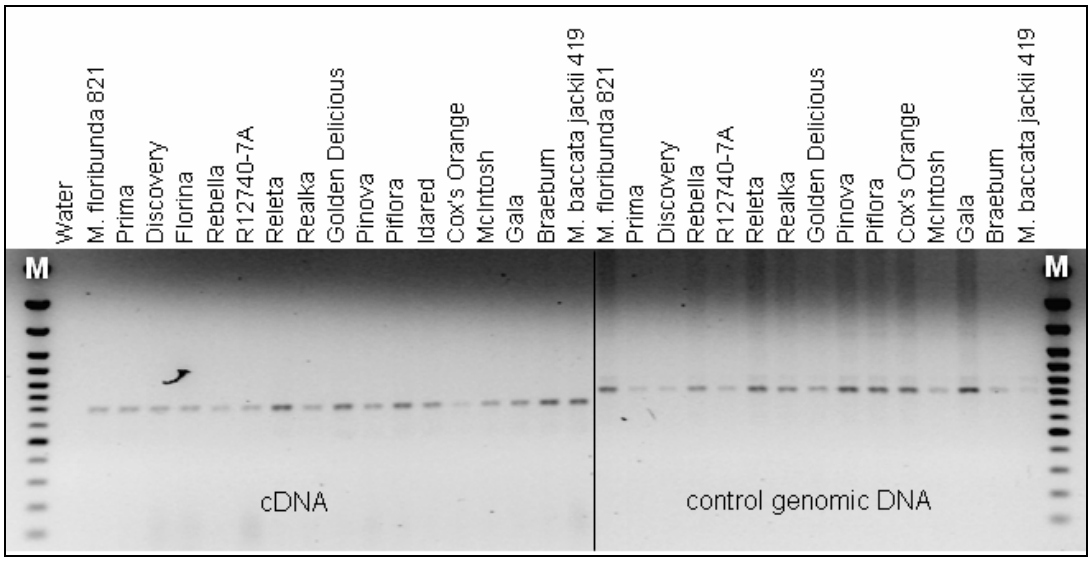


Figure 17: PCR amplification of cDNA isolated from a set of apple accessions using primers EF1-α. The cDNA samples were subsequently applied for RT-PCR and Real-Time PCR analyses. Genomic DNA was used in the EF1-α PCR amplification to control genomic DNA contamination by the fragment size comparison. M, size marker: 100 bp ladder

An additional fragment of approximately 520 bp amplified from genomic DNA of almost all tested samples (Fig. 15a) disappeared when cDNA of the same genotypes was used as a template (Fig. 15b). Interestingly, in three *M. sieversii* genotypes A96/53-13, A96/57-4 and A95/08-4 no *Vf1RSA* transcripts were found. This is exemplified by Figure 18, showing *Vf1RSA* amplification of cDNA isolated from two *M. sieversii* genotypes A96/53-13 and A96/57-4 using the primers Vf1RSA. In this case, a 313 bp band is visible only in genomic DNA samples of two *M. sieversii* genotypes but not in respective cDNAs.

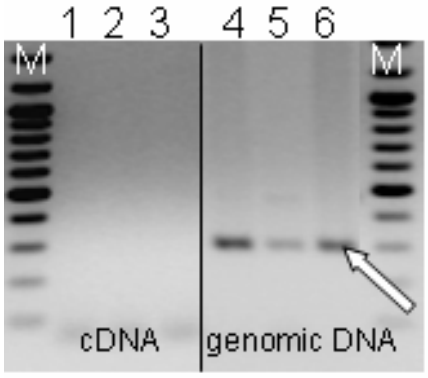


Figure 18: An agarose gel showing amplification of the 313 bp PCR fragment using primers Vf1RSA. Lanes include: 1) cDNA obtained from the RNA extracted from young leaves of *M. sieversii* A96/53-13 and *M. sieversii* A96/57-4, 2-3; 2) genomic DNA extracted from young leaves of R12740-7A, *M. sieversii* A96/53-13 and *M. sieversii* A96/57-4, 4-6. M, size marker: 100 bp ladder. Lane 1 is water control. A 313 bp band marked by an arrow is invisible in the cDNA samples of two *M. sieversii* genotypes

3.2.3.2 Genetic mapping of the *Vf1RSA* gene

Genetic analysis of two apple progenies 05/230 ('Pinova' x *M. sieversii* A96/53-13) and 06/004 ('Golden Delicious' x *M. sieversii* A96/57-4) with specific primers designed for *Vf1RSA* showed 1:1 - segregations for the *Vf1RSA* products (Tab. 14).

Table 14: Segregation of the *Vf1RSA* specific PCR product (313 bp) in two apple progenies 05/230 (Pinova x *M. sieversii* A96/53-13) and 06/004 (Golden Delicious x *M. sieversii* A96/57-4)

Vf1RSA	Number of genotypes	
313 bp fragment	05/230	06/004
Presence	39	54
Absence	35	52
Total	74	106

The same genotypes were then analyzed with the microsatellite marker CH-Vf1. The 159 bp-allele linked in coupling to the scab-resistant *Vf*-allele of *M. floribunda* 821 (Vinatzer et al. 2004) was not detected in any of the genotypes tested. The parent *M. sieversii* A96/57-4 of the cross 06/004 showed three different alleles for CH-Vf1 (137, 139 and 149 bp) whereas A96/53-13 showed two alleles (139 and 143 bp). The 137 bp allele in the population 06/004 as well as the 139 bp allele in the progeny 05/230 co-segregated with *Vf1RSA* without any recombination (A15, 16). Figure 19 shows the amplification results with the *Vf1RSA* (a) and CHVf1 (b) primers pairs as an example for mapping of *Vf1RSA* in the population 06/004 ('Golden Delicious' x *M. sieversii* A96/57-4). In this case, the primers Vf1RSA amplified polymorphic alleles in parents, and it was possible to follow their segregation within the progeny plants. Figure 19a demonstrates also that a distinct 313 bp *Vf1RSA* fragment is present only in scab resistant genotypes. The same genotypes analysed with the CH-Vf1 marker are shown in Figure 19b. The 137 bp allele (coloured with green) derived from the *M. sieversii* A96/57-4 perfectly co-segregates with the 313 bp *Vf1RSA* fragment within the progeny.

In summary, based on marker data obtained for two mapping populations (A14, 15) it was, therefore, assumed that *Vf1RSA* gene should be mapped to the same location as the SSR marker CH-Vf1, i.e. on LG 1 at the known position of *Vf*.

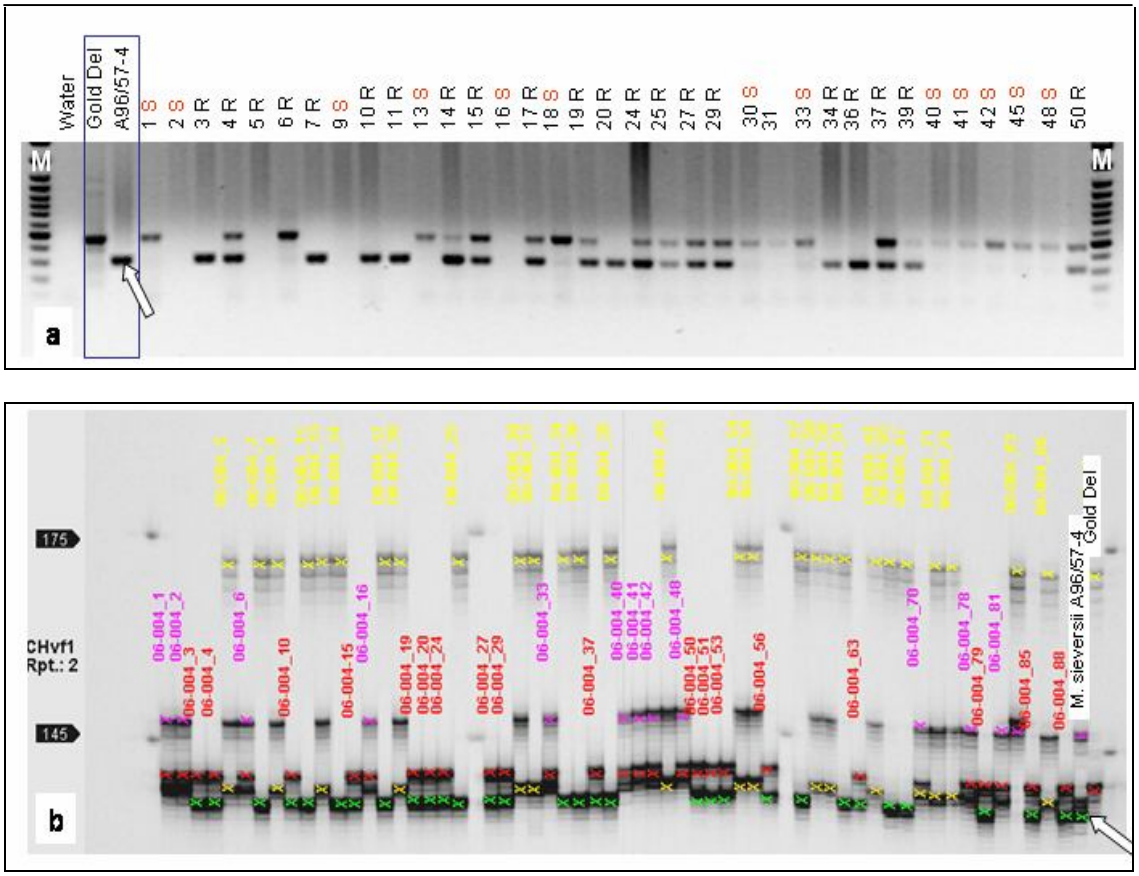


Figure 19: Genetic mapping of the *Vf1RSA* in the population derived from Golden Delicious x *M. sieversii* A96/57-4. The PCR products were amplified from genomic DNA with *Vf1RSA* (**a**) and CHVf1 (**b**) primers and separated by agarose gel electrophoresis and polyacrylamide gel electrophoresis, respectively. (**a**) Segregation for the *Vf1RSA* specific fragment (313 bp) in a subset of population consisting of resistant (R) and susceptible (S) genotypes. The 313 bp fragment is present only in resistant individuals; **(b)** the parent *M. sieversii* A96/57-4 showed three different alleles for CH-Vf1 (137, marked with green colour, 139 and 149 bp). The 137bp-allele in the progeny 06/004 co-segregated with *Vf1RSA*

3.2.4 Analysis of the *HcrVf*-type candidate gene *Vf2ARD*

The *HcrVf2* - homologous sequences of 'Antonovka', 'Realka' and 'Discovery', which are very similar (Figs. 13b, 20), were used to develop the second specific primer pair Vf2ARD (Fig. 20).

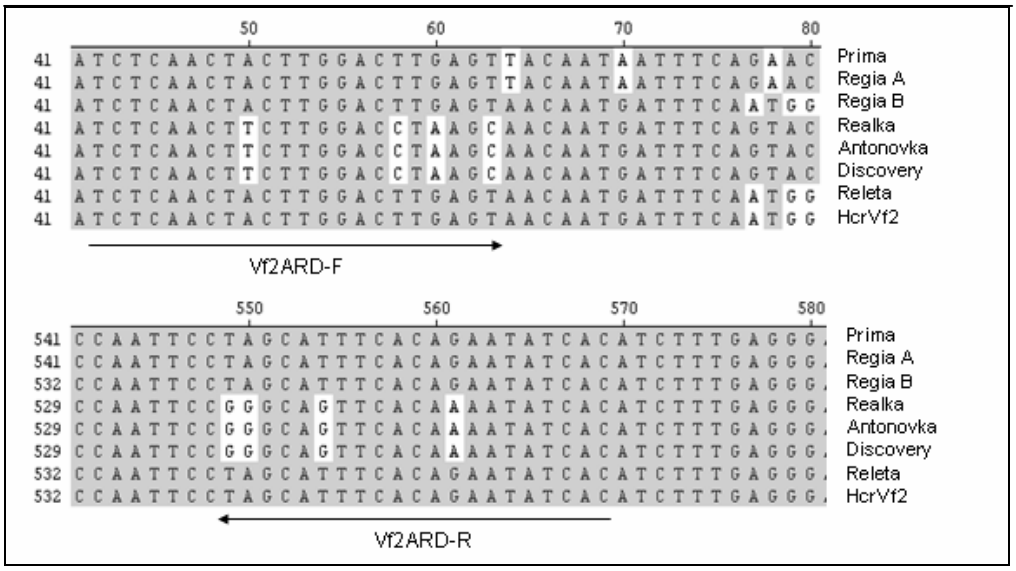


Figure 20: Design of *Vf2ARD*-specific PCR primers based on the multiple nucleotide sequence alignment of *HcrVf2* homologues from different apple accessions. Arrows indicate the position of the forward (F) and reverse (R) primers. Polymorphic nucleotides are marked with white. Dashes correspond to missing nucleotides. (*HcrVf2*- GenBank acc. no. AJ297740).

Vf2ARD PCR analysis of 18 apple genotypes included also in *Vf1RSA* analysis gave the following results (Fig. 21a). The expected 527 bp *Vf2ARD* fragment was present in 'RS' and the cultivars derived from it ('Realka', 'Regia', 'Releta'), the cultivars 'Antonovka' (*Va*), 'Golden Delicious' (*Vg*), 'Discovery' (*Vg*, polygenic), 'Florina' (*Vf*, *Vg*) but also in some cultivars regarded as scab susceptible ('Idared', 'McIntosh', 'Pinova'). However, the *Vf2ARD* PCR product was absent in *M. floribunda* 821, the original source of *Vf* resistance, the *Vf* cultivars 'Prima' and 'Rebella' as well as in the other varieties without any known scab resistance gene ('Piflora', 'Cox's Orange', 'Gala', 'Braeburn'). An additional band of approximately 300 bp appeared in almost all tested samples but was not further found on cDNA of the same genotypes (Fig. 21b). DNA

sequence analysis of this additional fragment revealed no homology with the apple HcrVf disease resistance proteins (not shown). At the same time direct sequencing of the 527 bp *Vf2ARD* PCR fragment, amplified from the mentioned above apple accessions as well as several scab differential hosts and three *M. sieversii* accessions (A96/53-13, A96/57-4 and A95/08-4) confirmed a high sequence homology with the *HcrVf1-HcrVf3* genes.

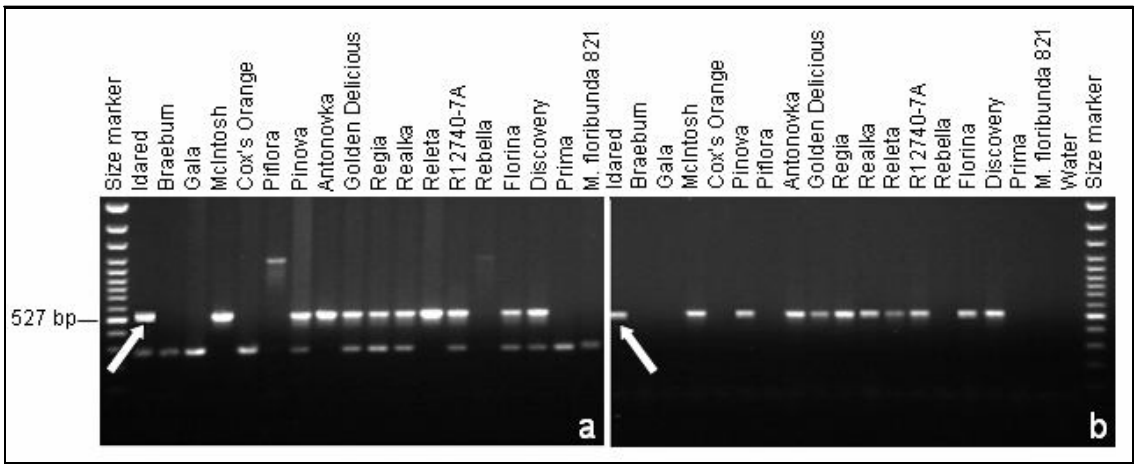


Figure 21: PCR amplification patterns obtained with the *Vf2ARD* specific primers. PCR was performed using either genomic DNA (**a**) or cDNA (**b**).The size of *Vf2ARD* PCR product was expected to be 527 bp. Size marker is a 100 bp ladder

As in case of sequence analysis of other *Vf*-like candidate genes, amino acid polymorphisms within the LRRs have been observed in the alignments of the *Vf2ARD* sequences with those of *HcrVf1-HcrVf3* that is highlighted in A20. A very high sequence homology has been observed among the *Vf2ARD* PCR products. In this respect, of particular interest is a small number of changes that occurred within the putative solvent-exposed residues of the xxLxLxx structural motif of the 23-24 LRRs. Here, the amino acid V (valine) of the 24 LRR is replaced by the equivalent hydrophobic I (isoleucine). Among 24 analysed samples such substitution appears only in 'Antonovka' *Vf2ARD* sequence. Interestingly, the variation at a putative solvent-exposed position of LRR 23 where the conserved S (serine) is replaced by the N (asparagine), is observed only in 'Realka', 'Regia', derived from the 'Russian Seedling' and two scab

differential hosts (X2249, host 4, and X2250, X4811, host 2). Another amino acid polymorphism lies outside of the putative ligand-binding, solvent-exposed residues between LRR23 and LRR 24. Strikingly, the amino acid R (arginine) is replaced by the K (lysine) only in resistant apple accessions such as 'Discovery' and closely related 'Realka', 'Russian Seedling' and *M. sieversii* 96/48-1. Further verification is needed to proof whether such single-amino acid changes within the LRRs of resistance gene products may be attributed to differences in specificities to different races of *V. inaequalis*.

3.2.4.1 Transcriptional analysis of the *Vf2ARD* candidate gene

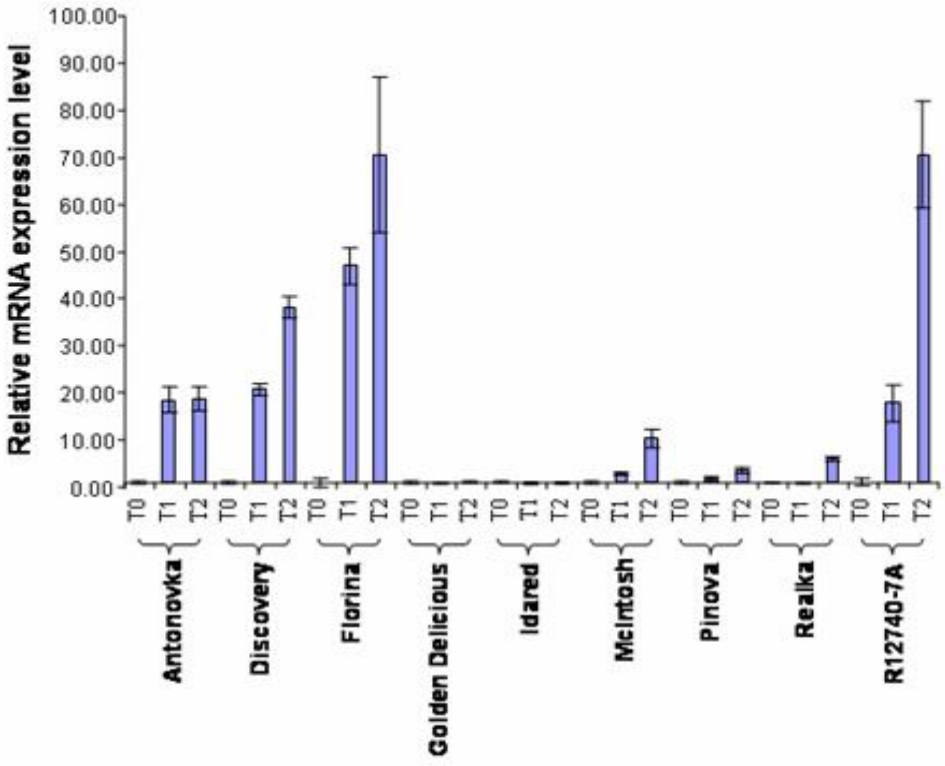
The proved in previous analysis cDNA (Chapter 3.2.3.1) was used in RT-PCR to study the expression of *Vf2ARD* gene in a set of apple accessions. The candidate gene *Vf2ARD* was transcribed under pathogen-free conditions in the same apple accessions for which PCR amplification from genomic DNA occurred (Fig. 21b). The results of the RT-PCR analysis of the expression of the candidate gene *Vf2ARD* is shown in Figure 21b. The expected 527 bp *Vf2ARD* fragment was present in accessions carrying different sources of resistance or tolerance to scab, such as 'RS' and the cultivars derived from it ('Realka', 'Regia', 'Releta'), the cultivars 'Antonovka' (*Va*), 'Golden Delicious' (*Vg*), 'Discovery' (*Vg*, polygenic) and 'Florina' (*Vf*, *Vg*). Interestingly, the same PCR fragment amplified from some cultivars regarded as scab susceptible ('Idared', 'McIntosh', 'Pinova'). However, the *Vf2ARD* PCR product was absent in *M. floribunda* 821, the original source of *Vf* resistance, the *Vf* cultivars 'Prima' and 'Rebella' as well as in the other varieties without any known scab resistance gene ('Piflora', 'Cox's Orange', 'Gala', 'Braeburn').

Vf2ARD-specific primers resulted in the RT-PCR product with the same size (527 bp) indicating the absence of introns. The sequence of *Vf2ARD* RT-PCR product matched the genomic *Vf2ARD* sequence and confirmed transcription of this gene (not shown).

3.2.4.2 Evaluation of *Vf2ARD* transcript levels with and without scab infection

To examine putative changes in defense-associated transcript levels in *Vf2ARD* genotypes after pathogen attack, a Real-Time PCR was applied to RNA which was extracted from leaves harvested at different time points after inoculation with *V. inaequalis*. *Vf2ARD* transcripts were detectable for nine out of twelve genotypes tested that is exemplified in Figure 22. No transcripts were found on cDNA of 'Prima', 'Piflora' and 'Cox's Orange'. This is in accordance with the results of the PCR performed on genomic DNA. *Vf2ARD* transcripts were only detectable in genotypes for which PCR amplification from DNA was found. The relative mRNA expression levels of *Vf2ARD* on non-infected leaf tissue were equal to the expression levels of the reference genes (Fig. 22). No significant differences were found. Although the experiment showed that *Vf2ARD* is expressed in the absence of a pathogen attack, different patterns of gene expression have been detected after scab inoculation. A delayed induction of expression was observed in the resistant cultivar 'Realka' and the susceptible cultivar 'McIntosh'. Only a weak gene induction was found in 'Pinova'. The expression in 'Antonovka' was induced rapidly and strongly, but its induction was sustained and the transcript level was constant for at least 48 hours. *Vf2ARD* was strongly induced in the resistant cultivar 'Discovery'. By contrast, a constant low transcript level of *Vf2ARD* was detected in the cultivar 'Golden Delicious'. Upon infection, the highest induction of expression of the *Vf2ARD* could be observed in R12740-7A and 'Florina' (up-regulated 71-fold in both genotypes, Fig. 22).

These results showed that the *Vf2ARD* gene is scab inducible and that differences in the timing and intensity of gene response to pathogen infection are genotype-dependent.



Genotype	mRNA transcript levels after scab infection T_0	T_1	T_2
Prima	(-)	(-)	(-)
Florina	1.00 ± 0.99	46.90 ± 3.96	70.66 ± 16.55
R12740-7A	1.00 ± 0.79	17.69 ± 3.86	70.65 ± 11.38
Realka	1.20 ± 0.13	1.00 ± 0.21	7.23 ± 0.71
Golden Delicious	1.17 ± 0.24	1.00 ± 0.233	1.32 ± 0.26
Antonovka	1.00 ± 0.24	18.46 ± 2.76	18.86 ± 2.65
Discovery	1.00 ± 0.25	20.71 ± 1.36	38.21 ± 2.14
Pinova	1.00 ± 0.26	1.90 ± 0.50	3.57 ± 0.53
Piflora	(-)	(-)	(-)
McIntosh	1.00 ± 0.28	2.94 ± 0.45	10.42 ± 2.03
Cox's Orange	(-)	(-)	(-)
Idared	1.48 ± 0.21	1.00 ± 0.13	1.08 ± 0.36

Figure 22: Relative mRNA expression levels of *Vf2ARD* depending on the time after scab infection. At the top, diagram showing different patterns of *Vf2ARD* gene expression after scab inoculation. At the bottom, Table summarizing the transcript levels of *Vf2ARD* in relation to the mRNA levels of the reference genes. (-) – no transcripts detectable; T0 – non infected control sample; T1 – 24 h after scab infection; T2 – 48 h after scab infection. The *y*-axis represents the fold difference in gene expression in relation to the mRNA levels of the reference genes. The values given are the mean (±SE) from triplicate samples

3.2.4.3 Presence of the *Vf2ARD* homologues in *Malus* genome

In order to examine the presence of *Vf2ARD* homologues in a range of apple accessions, Southern analysis was performed by using the *Vf2ARD* RT-PCR product from 'Realka' as a probe on *Eco*RI digested genomic DNA (Fig. 23). Some hybridizing fragments appeared to be conserved in all examined genotypes. However, along with similarity of hybridization patterns observed in different apple accessions, variation in the *Vf2ARD* patterns was displayed. In Figure 23 additional hybridizing bands present in scab resistant apple accessions are indicated by *.

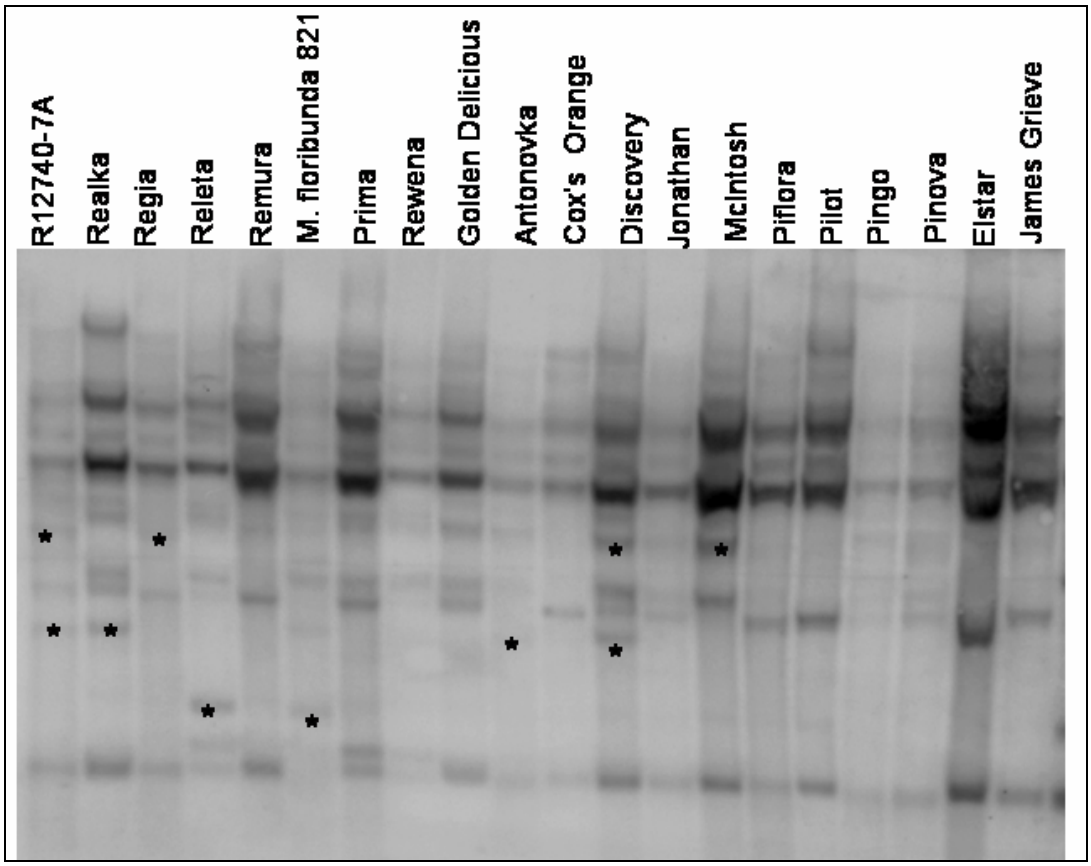


Figure 23: Southern hybridisation autoradiograph of *Eco*RI-digest genomic DNAs probed with the *Vf2ARD* RT-PCR product from Realka. Under investigation there are apple accessions without any known scab resistance gene (Cox's Orange, Jonathan, McIntosh, Piflora, Pilot, Pingo, Pinova, Elstar, James Grieve), the *Vf* scab resistance gene (*M. floribunda* 821, Prima, Rewena) the *Va* resistance (Antonovka), the *Vg* resistance (Discovery, Golden Delicious, Prima), or resistance factors from Russian seedling (R12740-7A, Realka, Regia, Releta, Remura). Additional hybridizing bands present mostly in scab resistant apple accessions are indicated by *

It could be seen that R12740-7A, 'Realka', 'Antonovka' as well as 'Discovery' share the same band that is invisible in other genotypes tested. R12740-7A, 'Regia' and 'Discovery' have another identical band of other size. Interestingly, the same band is present in the susceptible cultivar 'McIntosh' which demonstrated expression of the *Vf2ARD* gene. Another distinct band is present in 'Releta' and *M. floribunda* 821. Susceptible cultivars showed fewer *Vf2ARD*-hybridizing bands (4-5) than resistant accessions (up to 8). The high number of hybridizing bands indicated that multi-copy number of either *Vf2ARD* gene or its homologous sequences is present in the *Vf* region or could be outside the *Vf* locus, in analogy to the presence of various *Cf* clusters in tomato (Kruijt et al. 2005).

3.2.4.4 Genetic mapping of the *Vf2ARD* gene

The candidate gene *Vf2ARD* and the SSR CH-Vf1 were mapped in family 03/206 ('Regia' x 'Piflora') that is exemplified in Figure 24. The *Vf*-specific allele was not present in the resistant parent 'Regia'. CH-Vf1 amplified a 165 bp-band which co-segregated with the *Vf2ARD* gene too. Nevertheless, among 214 tested genotypes four of them showed a recombination between *Vf2ARD* and CH-Vf1. To confirm this finding these individuals were analysed repeatedly and also checked for being putative outcrosses. For fine mapping and placement of the *Vf2ARD* gene in relation to the known *Vf* locus two further SSR markers of LG 1, AG04 and AG11, were analysed in the 03/206 progeny. The linkage map obtained is shown in Figure 25. The positions and order of CH-Vf1, AG04 and AG11 mapped are in good agreement with the published map information (Liebhard et al. 2003b; Silfverberg-Dilworth et al. 2006). A recombination frequency of about 2 cM was calculated for the genetic distance between *Vf2ARD* and CH-Vf1 (Fig. 20). Because the candidate gene *Vf2ARD* appears to be located between CH-Vf1 and AG04, it is suggested that a second genomic region containing *HcrVf* genes of the RLP type has been identified on apple LG 1.

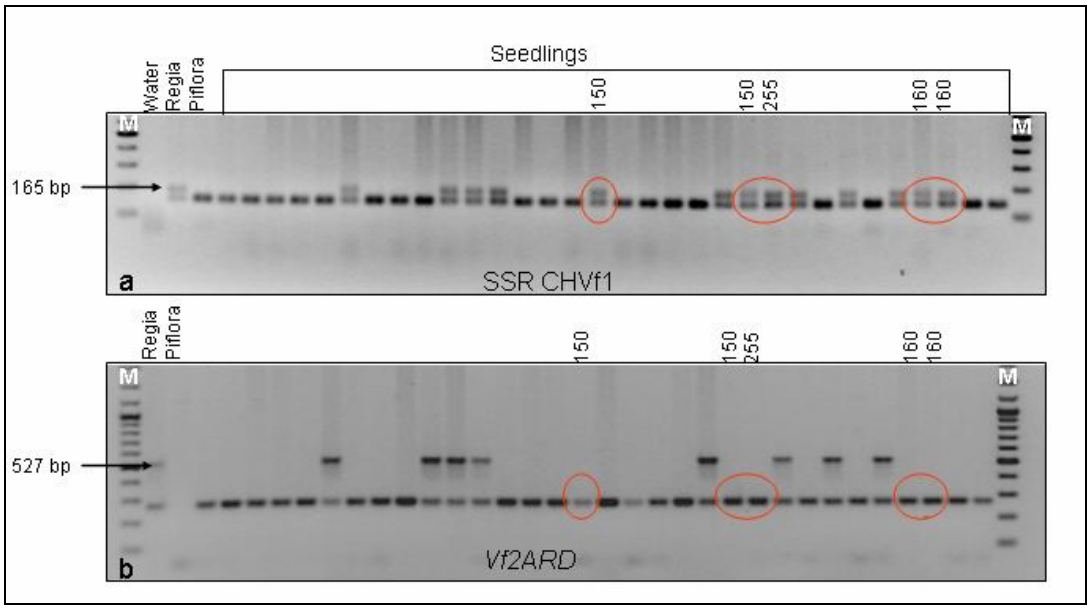


Figure 24: Genetic mapping of the *Vf2ARD* gene in the population derived from Regia x Piflora (03206). Occurence of the recombinant plants allowed separating the *Vf2ARD* from *Vf* genomic region. a) PCR amplification patterns obtained with the SSR CHVf1 for 03206 progeny; the 165 bp band of Regia is cosegregated with the *Vf2ARD*; b) PCR patterns amplified with the Vf2ARD primers. The seedlings under numbers 150 and 160 represented twice on the gels are repeatedly isolated DNA of the corresponding genotypes. The seedlings under the numbers 150, 160 and 255 show a recombination between *Vf2ARD* and CHVf1. M, size marker:100 bp DNA ladder

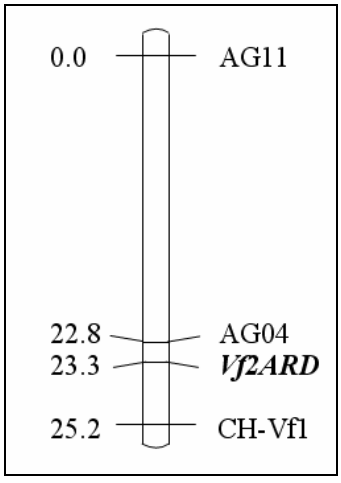


Figure 25: Genetic map of the genomic region adjacent to the *Vf* locus (representing by SSR CH-Vf1) on linkage group LG 1 of the apple cultivar 'Regia'. Markers were mapped in the 03/206 population derived from Regia (resistant) x Piflora (susceptible). Map distances are in centiMorgans

In another mapping population of 'Antonovka' x 'Golden Delicious' (04/214) it was impossible to place the candidate *Vf2ARD* gene toward the *Vf* locus since both parents of this family amplified the same 527 bp *Vf2ARD* PCR fragment

(Fig. 21). Thus, only the SSR CH-Vf1 was analyzed in the subpopulation 04/214. Only reliable genotypes scored during two years as resistant and, respectively, susceptible were chosen for the genetic analysis. Although the cultivar ‘Antonovka’ is often referred to as polygenic resistance source, the progeny of the family ‘Antonovka’ x ‘Golden Delicious’ showed the scab resistance segregation ratio typical for a single dominant gene (A 17). Again the 159 bp-allele linked in coupling to the scab-resistant *Vf*-allele of *M. floribunda* 821 was not detected. Instead the 139 bp allele for the CH-Vf1 originated from ‘Antonovka’ co-segregated with the scab resistance observed during two years in the greenhouse conditions. Analysis of correspondence between scab resistance data and the CH-Vf1 marker revealed two putative recombination events among 81 genotypes that positions the R gene derived from ‘Antonovka’ at about 2 cM from the *Vf* gene, by analogy with *Vf2ARD*. Further verification of the linkage between the resistance phenotype and the CH-Vf1 marker as well as two further SSRs (AG04 and AG11) located on the LG1 by examining accurately genotypes, especially those putative recombinants is required.

4. Discussion

4.1 Scab resistance phenotyping

A major scab resistance gene (called *Vr1*) was identified in R12740-7A - derived apple cultivar 'Regia' and mapped in the close vicinity of scab resistance genes from R12740-7A that were analysed and mapped by other researchers. For greenhouse scab tests of apple seedlings a mixture of several races representing a natural inoculum of the fungus at the location of the apple breeding plots in Dresden-Pillnitz was used. No clear segration patterns were observed because of the considerably high number of individuals in the scoring classes 3 and 4. When only seedlings without any visible symptoms or a hypersensitivity reaction were considered to be scab resistant, an expected 1:1 segregation ratio in three out of the five progenies was found. Although a scoring scheme used in this study is derived from that one developed by Chevalier et al. (1991), the individuals showing slight sporulation were not assigned into the resistance group as done by other researchers (Bénaouf and Parisi 2000, Huaracha et al. 2004) with the purpose to minimize number of false positives for bulked segregant analysis as well as for linkage mapping.

The two other populations showed an excess of resistant plants. One of them ('Regia' x 'Pingo', 02/223) was a replication of the same cross (00/213) tested for scab two years earlier with the result of a 1:1- ratio. The reason for this phenomenon is unknown, but might have been caused by alterations in the climatic conditions in the (different) greenhouse cabins used over the years for the scab tests or by quantitative and/or qualitative differences of the natural scab inoculum used. The variability regarding the inoculum could be minimized when scab inocula with a known composition of defined scab races or even single scab races would be used. Race-specific scab tests have been very informative in studies of the scab resistance genetics (Bénaouf and Parisi 2000, Bus et al. 2005a, b) and the dissection of polygenic resistances (Calenge et al. 2004) but may have the disadvantage that selected scab races must not reflect the real scab situation in the field. In apple breeding programmes it is not practicable to perform several parallel scab tests on the same (cloned)

seedlings. The largest deviation from the expected 1:1-ratio was observed in the 'Regia' x 'Piflora' cross (03/206). The scab susceptible cultivar 'Piflora' inoculated with race 7 (strain EU-F02) unexpectedly showed a very strong hypersensitive reaction (HR) but absolutely no scab symptoms or any sporulating lesions (not shown). Because 'Piflora' is a cultivar derived from a cross between 'Clivia' and 'Golden Delicious' (Fischer and Fischer 1996) it is most probable that the *Vg* resistance gene from 'Golden Delicious' that is effective against race 7 (Bénaouf and Parisi 2000), might have been contributed to the excess of resistant seedlings. *M. floribunda* 821, the original *Vf* donor, is nowadays one of the most heavily scab infected apple genotypes at Dresden location. From this own observations and the results of Parisi et al. (2004) about the geografical distribution of *Vf* virulent scab races it is highly probable that race 7 was present in the local mixed scab inoculum used for seedling scab tests.

In the experiments presented here no stellate necrotic (SN) lesion phenotypes have been found in 'Regia' or in the progenies. 'Regia' displays a typical HR phenotype with small pin-points in the field. During the greenhouse tests of the seedlings not all but a major part of the resistant plants also showed the HR symptoms. Hemmat et al. (2002) postulated two major dominant genes conferring resistance to apple scab. The *Vr* gene produced stellate necrotic (SN) lesions whereas the second gene (*Vx*) caused a pit type HR. These two different resistance phenotypes were confirmed by Bus et al. (2005a) who mapped two genes (named *Vh2* and *Vh4*) by using scab races 2 and 4 and concluded that these genes are the same ones as *Vr* and *Vx*, respectively. Repeated scoring of several common differential hosts including X2250 (host race 2) and X2249 (host race 4) didn't gave any proof of the existence of race 4 in orchards of Dresden-Pillnitz whereas race 2 seemed to be present on the respective hosts. Considering the recent publication about race 2/4 specific genes from R12740-7A (Bus et al. 2005a) and mapping results presented in this study, it is assumed that the analysed and mapped *Vr1* gene confers resistance to race 2, but would cause compatibility with race 4 (named *Vh4* by Bus et al. 2005a).

4.2 DNA markers linked to the scab resistance gene *Vr1* from R12740-7A

Random 10-mer (RAPD) primers together with a bulked-segregant analysis were employed to identify DNA markers linked to the *Vr1* from the scab resistance source R12740-7A. In this study, the supposed resistance gene, *Vr1*, is heterozygous and present only in the resistant parent. As was already noted by Melchinger (1990), the use of heterozygous bulks reduces the probability of a polymorphism to be detected by 50%, since they are informative for the recombination events in one gamete only. In such case only markers in coupling with the resistance gene can be found, perhaps explaining the relatively low number of polymorphisms detected (among 260 primers only markers $OPAD13_{950}$ and $OPQ7_{1500}$). The RAPD markers $OPAD13_{950}$ and $OPQ7_{1500}$ represent chromosomal regions introgressed into the cultivated scab resistant apple from the R12740-7A. Interestingly, two ('Reka', 'Releta') from five derivatives of the 'Russian Seedling' failed to amplify the markers $OPQ7_{1500}$ and $OPAD13_{950}$. Since the genetics of R12740-7A is complex, involving three major genes, the absence of two specific DNA sequences could be attributed to introgression of another, non-*Vr1* resistance factor from the 'Russian Seedling' into the cultivars 'Reka' and 'Releta'. Another possible explanation for the absence of these markers in mentioned derivatives is that the portion of the R12740-7A genome amplified with two RAPDs is not tightly linked to the *Vr1* gene.

Although several attempts have been made to increase the specificity of the RAPD $OPQ7_{1500}$ by converting it into a SCAR marker, the longer primers amplified the same sized fragment from both parents. Further sequencing of the alternate alleles might identify diverged regions for which it may be possible to design allele-specific primers or to apply ribonuclease cleavage. In this study transformation of the second RAPD marker $OPAD13_{950}$ into more reproducible and consistent marker that can be applied directly to apple breeding is performed. In contrast to other SCAR markers published so far for resistance genes from R12740-7A such as the S22- and B18-SCARs (Hemmat et al. 2002) AD13-SCAR is a typical codominant marker. Therefore, heterozygotes can be distinguished from homozygotes which would allow the selection of seedlings

homozygous for the resistance gene provided that a second codominant marker locus located on the other side of the resistance gene is available. In contrast to the *Vf* scab resistance where such a codominant flanking marker system is available (Tartarini et al. 1999), a second codominant *Vr1*-linked SCAR marker or a closely linked SSR is not yet identified. However, with the increasing number of SSRs developed and mapped in *Malus* the gap still existing between SSRs CH02c02a and CH03d10 on LG 2 (Liebhard et al. 2003a) should be filled soon.

The additional alleles detected with the AD13 SCAR primers in a small set of accessions of only a few *Malus* species are highly informative with regard to molecular analyses of relationships between *Malus* genotypes as well as for taxonomic studies. To our knowledge, for this application the AD13 locus seems to be the most informative SCAR published so far for *Malus.* SCARs are usually highly specific for the accessions in which they were identified, but they are often useless in different genetic backgrounds as a result of lack of polymorphism (Vinatzer et al. 2004). AD13-SCAR is nearly as informative as a SSR, but can be analysed on agarose gels. Although in population 03/206 ('Regia' x 'Piflora') three marker alleles are segregating, due to the homozygosity of the *b* allele in 'Piflora' only two marker phenotypes were observed. In an another assumed genetic situation, i.e. in the cross 'Regia' x *M. sieversii* A96/53-13 a more precise linkage mapping would be possible due to the higher statistical information content of this *ac* x *bc*- type marker segregation (Maliepaard et al. 1997). The apple genotype GMAL 2473, which has been described as donor for the *Vr2* scab resistance gene (Patocchi et al. 2004) could be clearly separated from the original R12740-7A. That the two genotypes are differing from each other has been already proven by SSR analysis (Patocchi et al. 2004). However, the common presence of the *c* allele indicate that GMAL 2473 could be an ancestor, a descendent or a related genotype of R12740-7A and support the assumption that *Vr1* and *Vr2* could be the same gene. The three *M. sieversii* accessions selected randomly from a collection of about 1.000 *M. sieversii* trees from the genebank collection at Dresden-Pillnitz showed the same AD13 banding patterns as GMAL 2473. These results match

previously reported finding about existence of a close molecular relationship between *M. sieversii* and R12740-7A (Wiedow, 2006). Recently a new scab resistance gene named *Vh8* from *M. sieversii* was identified which is closely linked (or possibly allelic) with the *Vh2* gene of 'Russian Seedling' (Bus et al. 2005b). In addition, the present studies show amplification of the highly specific *HcrVf*-type candidate gene *Vf1RSA* identified in R12740-7A in a few scab resistant *M. sieversii* accessions. These findings suggest a very close relationship not only with regard to the putative origin of the 'RS' from the *M. sieversii* gene pool but also concerning the existence of the same race specific scab resistance genes in this genetic background.

4.3 *Vr1* linkage mapping

A reliable resistance scoring is essential for precisely mapping a resistance gene. This is especially true for the rating of susceptible plants, where an unsuccessful infection can be due to the accumulation of favourable quantitative trait loci alleles as well as to inappropriate infection conditions. Furthermore, genotypes classified as susceptible might carry the resistance gene, but the background (i.e. modifiers) might reduce its expression how it was demonstrated by Gessler (1989). For these reasons, additional linkage mapping of progeny without critical genotypes, so called "genotype-phenotype-incogruence" (GPI) plants, was performed as previously reported by other researches (Erdin et al. 2006; Gygax et al. 2004; Patocchi et al. 1999).

Linkage mapping around the *Vr1* locus was performed in two different 'Regia'-derived populations and was focused on apple LG 2 because of our earlier suggestion that the two major scab resistance genes *Vr* and *Vx* mapped by Hemmat et al. (2002) are both located on LG 2 (Boudichevskaia et al. 2004). The linkage analyses presented here confirmed that assumption. In this respect the S22-SCAR marker (*Vx*) is located on top of LG 2 and is linked with the *Vr1* phenotype as well as with the AD13-SCAR and the RAPD $OPQ7_{1500}$. In addition, also a linkage of S22-SCAR and AD13-SCAR and the RAPD $OPQ7_{1500}$ markers to two SSR loci mapped on top of LG 2 (Ch02f06,

CH02c02a) was found. These results differ from Hemmat et al. (2002) who mapped *Vr* and *Vx*/S22-SCAR on different linkage groups. However, the *Vr* gene was mapped by these authors on linkage group 11 (Hemmat et al. 2003) which is corresponding to LG 2 in the "European map" (Liebhard et al. 2002). No information was given about the linkage group of *Vx*. Because *Vr1* and its markers seem to be close to both the *Vx* gene and the *Vh4* gene we can follow the argumentation of Bus et al. (2005a), that all these genes are the same. However, the possibility that very tightly linked resistance genes are located in common R gene clusters (top cluster: *Vx*, *Vr1*, *Vr2*, *Vh4*; bottom cluster: *Vr*, *Vh2*, *Vh8*, *Vbj*) can also not be fully excluded.

4.4 Utility of *Vr1* marker AD13 for marker-assisted selection

One of the main goals of this study was the development of molecular markers for a scab resistance gene from R12740-7A that could be used as an alternative or supplement to *Vf* in practical breeding approaches, especially to accumulate different major scab resistance genes in a single apple genotype. After two years of scab scoring of two different families under natural scab infection conditions the utility of the AD13 SCAR marker for this approach was confirmed. The results concerning verification of the ‘Russian Seedling’ scab resistance factor and its molecular marker outside the greenhouse were reported firstly by Boudichevskaia et al. (2006). In this respect, only a minority of two plants in each progeny proved to be suceptible but carried the AD13 resistance marker allele. The relatively high number of plants showing resistance in the field without presence of the marker could be explained by escape plants that have been wrongly classified yet regarding their resistance (Tartarini et al. 2000; Xu and Korban 2000). Additional (minor) genes modifying the expression of resistance that are independent of the major gene may be also responsible for that phenomenon. During the map based cloning of the *Vf* gene Patocchi et al. (1999) found about 9% of resistant individuals that did not carry the marker alleles in coupling with *Vf*. A part of the "field escapes" may

also be explained by an altered scab race spectrum, because during the greenhouse test sporulating scab symptoms were observed on these plants.

As an outlook onto future applications of the AD13-SCAR a marker-based analysis of *Vr1*-*Vf* gene combinations in a cross between 'Regia' (*Vr1)* and 'Rebella' (*Vf*) was performed and somewhat unexpected result was obtained. Only plants carrying the *Vr1* gene either alone or in combination with *Vf* had a satisfactory degree of scab resistance in the seedling test. Hemmat et al. (2002) stated that a two-marker-approach in an advanced population combining *Vr* resistance genes and *Vf* is possible, although these authors also found some inconsistencies between marker and resistance phenotypes. Epistatic gene effects were discussed as a possible explanation. In present experiments the *Vf* gene seemed to have no major effect on the resistance expression. This finding is in agreement with results published by Bénaouf and Parisi (2000) for *Vf* segregating populations. The main reason for this is very probably the presence of scab races 6 and/or 7.

With the availability of highly informative DNA markers for different scab resistance genes located on different apple chromosomes, pyramiding of dominant resistance genes is feasible and would assure better control of the scab fungus. However, further work is required to confirm, whether the scab resistance genes located by different researchers in two resistance gene clusters of apple linkage group 2 are identical loci.

4.5 Molecular analysis of candidate genes homologous to *HcrVf* genes for scab resistance in apple

In the past decade, intensive efforts have resulted in the identification and characterisation of more than 50 R genes from various plant species. In respect of apple, only the *Vf* locus of the crabable *Malus floribunda* 821 that confers resistance to five races of the fungal pathogen *V. inaequalis* has been analysed in detail to date. It has been identified as a complex locus containing four receptor-like *HcrVf* genes (Vinatzer et al. 2001; Xu and Korban, 2002). Additionally, it has been suggested that there are candidate *Vf* genes at other

loci besides the *Vf* region in all apple cultivars (Vinatzer et al. 2001). However, these candidate *HcrVf* –like genes have not been studied at the molecular level. In the present study, identification, partly cloning and molecular analysis for sequence diversity and transcription of a number of *HcrVf*-type candidate genes is reported. These genes exhibit from 85 to 100% amino acid identity with the apple *HcrVf1* and *HcrVf2* disease resistance proteins of the *Vf* locus. The *HcrVf* homologues were found in various apple cultivars, particularly in scab resistant apple accessions known as donors of other scab resistances such as *Vr* ('Russian Seedling' R12740-7A) or *Va* ('Antonovka' selections). For a first PCR-based attempt to identify *Vf* sequences in domesticated apples Afunian et al. (2004) used PCR primers based on conserved regions of the isolated *HcrVf/Vfa* genes (Vinatzer et al. 2001; Xu and Korban 2002) adjacent to a variable portion of the LRR domain. While PCR fragments matching in size *Vfa4(HcrVf3)* were found only in selections of *M. floribunda* and *Vf* cultivars, other PCR products corresponding in size to *Vfa1*(*HcrVf1)* and *Vfa2(HcrVf2)* were present in all *Malus* genotypes. No cloning and sequencing of PCR fragments were performed, but it was suggested that the PCR products might have contained multiple PCR products, potentially representing other *HcrVf*- type resistance gene analogs (Afunian et al. 2004). A key factor of the PCR primer design strategy followed in the study presented here was the use of variable C1-LRR regions themselves. Utilization of specific primers for the *HcrVf1* and *HcrVf2* genes for PCR-based identification of related candidate gene sequences has enabled to clone 13 different *HcrVf*- homologous sequences in apple, in most cases with an amino acid identity between 85 and 90%. Typical for HcrVf proteins, a considerable number of variant amino acids between the found homologues is present in the C1 subdomain. Here, a number of non-conserved amino acids appears to lie outside of the putative ligand-binding, solvent-exposed residues. At the same time, a considerable number of amino-acid substitutions occurs also within the putative solvent-exposed residues of the xxLxLxx structural motif of the LRRs. The predicted solvent exposed residues in LRR regions of many R-gene products are known to be hypervariable (Botella et al. 1998; Dixon et al. 1998; McDowell et al. 1998; Meyers et al. 1998;

Parniske et al. 1997; Thomas et al. 1997). However, whether the multiple amino acid changes observed in the C1 sequences of different apple *HcrVf* homologues could indicate the existence of specific recognition sites comparable to the Cf system in tomato (Van der Hoorn et al. 2005, Fritz-Laylin et al. 2005) is yet unknown. Much more work on the inventory of RLPs in the *Malus* genome would be necessary to establish a set of *HcrVf*–homologous genes that are functionally acting as race-specific scab resistance genes.

4.6 Expression and mapping of two *HcrVf*-like candidate genes: *Vf1RSA* and *Vf2ARD*

This study is focused on the expression and mapping of two *HcrVf*-like candidate genes, a putative highly specific candidate gene derived from 'Russian Seedling' (called *Vf1RSA*), and a more frequently in apple existing *HcrVf* homologue called *Vf2ARD.*

Transcripts from the *Vf1RSA* gene were detected in leaves prior to fungal infection only in 'Russian Seedling' and not in any susceptible cultivar or another scab resistance source being tested. As it was already mentioned, R12740-7A is highly resistant to apple scab. While its *Vh2* and *Vr1/Vh4/Vx* race-specific genes were mapped on LG 2 (Boudichevskaia et al. 2006; Bus et al. 2005b; Hemmat et al. 2002), a third broad-spectrum gene postulated earlier (Dayton and Williams, 1968) remains still to be detected and mapped. To our knowledge, this is the first report demonstrating the presence of an expressed *HcrVf*- homologous sequence in 'Russian Seedling' R12740-7A, which is located on LG 1 at the known position of *Vf*. Although *Vf1RSA* was not expressed in the *M. sieversii* accessions showing the *Vf1RSA* fragment on a genomic DNA level, a linkage between the *Vf1RSA* "marker fragment" and a scab resistance phenotype was evident in two progenies derived from the *M. sieversii* parents. This finding could be explained by the co-segregation of a different allele of the *Vf* locus not deriving from *M. floribunda* 821. However, since the number of the individuals screened in the two *M. sieversii*-derived mapping populations may not have been sufficient to detect the recombinant

genotypes, we cannot exclude completely that *Vf1RSA* represents another genomic region tightly linked to the *Vf* locus.

The PCR primer pair for the second candidate gene *Vf2ARD* was designed from homologous sequences of three distinct apple cultivars (‘Antonovka’, ‘Realka’ and ‘Discovery’). However, the band of predicted size was found in the majority of the resistant genotypes analysed. Expression analyses for *Vf2ARD* by RT-PCR indicated that this gene is constitutively expressed in the same genotypes. A DNA fragment of the same size was amplified in genomic DNA as well as in cDNA, that was expected since the *Vf* genes are known to be intronless genes (Xu and Korban, 2004). Interestingly, the *Vf2ARD* RT-PCR product was absent in *M. floribunda* 821 and the *Vf* cultivars ‘Prima’ and ‘Rebella’, but present in ‘Golden Delicious’ and some scab susceptible cultivars such as ‘Idared’ or ‘McIntosh’. Direct sequencing revealed a very high sequence homology among the *Vf2ARD* PCR products indicating that this gene might be relatively frequent in *Malus*. Although only partial sequence of *Vf2ARD* candidate gene was available for analysis (LRR22-LRR28), a small number of amino acid changes has been observed within the 23-24 LRRs. Notably, such substitutions appear in *Vf2ARD* sequences of others than *Vf* apple scab resistance sources. The question arises whether such minor sequence variation observed within the LRRs of *Vf2ARD* sequences is essential in generation of novel recognition specificities. Further analysis of the full extent of *Vf2ARD* sequences derived as from resistant as from scab susceptible genotypes would provide novel insights into the molecular basis of recognition specificity in *HcrVf*-type genes. In this respect, *Vf2ARD* observed in some cultivars that do not confer resistance to *V. inaequalis* may, by analogy with *Hcr9*s, represent a reservoir of variation that facilitates the generation of novel *HcrVf*-like genes (van der Hoorn et al. 2001).

The appearance of *Vf2ARD* PCR fragment not only in resistant genotypes but in some susceptible ones prompted us to investigate whether differential expression of the *Vf2ARD* gene could be detected after scab inoculation of the genotypes. Whereas Real-Time PCR analysis revealed no significant differences in the induction of *Vf2ARD* genes in susceptible and resistant apple

accessions before inoculation, different levels and speeds of responses after infection with *V. inaequalis* occurred. An early gene response in apple genotypes is obviously established upon fungal infection. Similar results have been shown by analyzing changes in gene expression of resistant and susceptible *Arabidopsis* plants (Tao et al. 2003) and resistant and susceptible olive cultivars (Benitez et al. 2005) to the pathogens *Pseudomonas syringae* and *Spilocaea oleagina*, respectively. In these studies plant responses in compatible and incompatible interactions were qualitatively similar but quantitatively different, with a delayed induction of genes in the compatible interaction. A limitation of present approach for evaluation of *Vf2ARD* transcript levels is that a mixture of several races representing a natural inoculum of the fungus was used. Race-specific scab tests would be essential to proof the resistance spectrum of the identified *HcrVf* homologues. On the other hand, selected races must not reflect the real scab situation in the field since there may be hundreds of different physiological races that can be differentiated by the various cultivars of *M. x domestica* (Shay and Williams, 1956). The candidate *Vf2ARD* genes identified in the susceptible cultivars such as 'McIntosh' and 'Idared' could correspond to ephemeral resistance genes which have been previously postulated in apple (Sierotzki et al. 1994; Koch et al. 2000).

4.7 Existence of a further *Vf* - like locus located closely to *Vf* on apple linkage group LG 1

The first isolated resistance genes of the RLP class are the tomato *Cf* genes which are presently the most intensively studied RLP resistance genes. They are usually found in clusters of several homologues in particular chromosomal regions (Kruijt et al. 2005). A number of the different *Cf* resistance genes have been cloned, which were shown to group into two large gene families, namely: *Hcr2* (Homologues of the *C. fulvum* resistance gene *Cf-2*) and *Hcr9* (Homologues of the *C. fulvum* resistance gene *Cf-9*) gene families (Kruijt et al. 2005). Whereas *Hcr2*s are closely linked and map on chromosome 6, the *Hcr9*

genes are found at least at five different loci on the short arm of chromosome 1 (*Nothern Lights*, *Milky Way*, *Aurora*, *Orion*, *Southern Cross*) (Rivas and Thomas, 2002; Kruijt et al. 2005; de Kock et al. 2005). In relation to apple, from the map-based cloning approaches towards the *Vf* gene isolation there are some indications for the existence of *HcrVf* homologues that were apart from the *Vf* locus (Vinatzer et al. 2001; Xu and Korban 2002). At least four genomic regions containing *HcrVf*-like genes were identified and three of them were mapped on LG 1 at approximately 2, 3 and 18 cM from *Vf* (Gianfranceschi et al. unpublished data, cited in Gessler et al. 2006). In the present study, mapping analysis for the 214 genotypes derived from the mapping population of 'Regia' x 'Piflora' resulted in localization of *Vf2ARD* separately from the known *HcrVf* gene cluster with a genetic distance of about 2 cM indicating the presence of a second *Vf*-like locus on apple LG1 (Boudichevskaia et al. 2008). In another mapping population of 'Antonovka' x 'Golden Delicious' (04/214) it was impossible to place the candidate *Vf2ARD* gene toward the *Vf* locus since both parents of this family amplified the same 527 bp *Vf2ARD* PCR fragment. The interesting aspect is that occurrence of two putative recombination events between scab resistance data and the CH-Vf1 SSR closely linked to the *Vf* locus, positions the R gene derived from 'Antonovka' at about 2 cM from the *Vf* gene, by analogy with *Vf2ARD*. It would be essential to further verify the linkage between the resistance phenotype and the CH-Vf1 marker examining accurately genotypes, especially those putative recombinants. Increasing the number of the individuals screened in the 04/214 population may lead to detection of further recombinant genotypes. There are already indications that different 'Antonovka' selections might have the resistant gene(s) located on the same chromosome carrying the *Vf* gene, however at a different position (Hemmat et al. 2003, reviewed by Gessler et al. 2006). In summary, additional studies to elucidate whether *Vf2ARD* candidate gene highly expressed in 'Antonovka' corresponds to the R gene mapped in the population 04/214 are required.

In conclusion, PCR-based cloning and molecular characterization of further *HcrVf* homologues derived from different resistant and susceptible *Malus*

genotypes as well as full-length cloning and additional transriptional analyses of the *HcrVf*-like genes *Vf1RSA* and *Vf2ARD* would enhance the restricted knowledge about structure and chromosomal organization of *HcrVf* genes in *Malus.* To date, detailed evolutionary studies of RLPs have mainly focussed on the extensively studied tomato *Cf* genes. Thus, functional analysis of apple *HcrVf* – like genes would elucidate whether they have evolved by similar mechanisms as *Cf* genes. The identification of functions of *HcrVf* –type genes will allow better understanding of biological mechanisms determining a partial and durable disease resistance. Furthermore, as molecular characterization of resistance genes proceeds, it will become easier to manipulate genetic resistance and transfer resistance genes within and among species. Genetic engineering can have an advantage over traditional breeding methods since novel pyramids of major resistance alleles (represented as a cassette of linked genes) can be simultaneously introduced into semi-elite and elite germplasm through a single transformation step. Co-evolution observed in *Malus*-*Venturia* pathosystem suggests that it is unlikely to eliminate the pathogen by combining new receptors and putting them into apple cultivars. Pathogens will continue to evolve. But genetic engineering approach could be used to create quickly multilines by inserting different resistance alleles into superior agronomic genotypes (McDonald and Linde, 2002). This will slow pathogen evolution and allow to stay a few steps ahead of the scab pathogen.

5. Summary/Zusammenfassung

5.1 Summary

In the first part of the underlying doctoral thesis, an identification of a major scab resistance gene called *Vr1* in the apple cultivar 'Regia' derived from the *Malus* scab resistance source R12740-7A (‘Russian seedling’, RS) was described. The developed codominant AD13-SCAR marker, suitable for the molecular description of the allelic situation regarding the *Vr1* marker locus, allowed also discriminating cultivars and different accessions of *Malus* species. Additional alleles of the AD13-SCAR marker locus proved to be informative for the analysis of genetic relationships within *Malus* including putative relatives of R12740-7A.

The *Vr1* resistance gene was located on apple linkage group (LG) 2 and compared with other resistance genes published for RS resistance. Separate linkage maps were created for two families derived from crosses with 'Regia'. Based on phenotypic data from the greenhouse scab tests, the recombination frequency between *Vr1* and AD13-SCAR was calculated between 6 and 17%. The *Vr1* locus appeared to be closely linked to the *Vx* (Hemmat et al. 2002), *Vr2* (Patocchi et al. 2004) and the *Vh4* gene (Bus et al. 2005a). Present linkage analysis of molecular markers identified by Hemmat et al. (2002) for two scab resistance factors from RS (*Vr* and *Vx*) indicates that both genes are separated by a large distance on apple LG 2 (Boudichevskaia et al. 2004). This is in agreement with the results of Bus et al. (2005a) who concluded that 1) the RS-derived *Vh2* is identical to *Vr*; 2) the RS-derived *Vh4* is identical to *Vr1*; 3) *Vh2/Vr* and *Vr1/Vh4/Vx* map on opposite sides of LG 2. However, further work is required to confirm, whether the scab resistance genes located by different researchers in two resistance gene clusters of apple linkage group 2 are identical loci.

The utility of the AD13-SCAR within a practical apple breeding programme was verified during two years under natural scab infection conditions in two families investigated. In this respect, this is the first report about the confirmation of a molecular marker for a RS resistance factor in a 2 - years field experiment.

Finally, a multiplexing PCR assay based on two codominant SCARs for *Vf* and *Vr1* was tested in an apple progeny segregating for both genes. The result of the two-marker-approach was discussed with respect to scab races, which are able to overcome the *Vf* resistance gene.

The focus of the second part of research was on the identification and characterisation of candidate *HcrVf*-type genes in a wide set of apple cultivars. Based on published sequences for *HcrVf1* and *HcrVf2* PCR primers were designed from the domain B and the variable leucine-rich repeat (LRR) C1 subdomain. PCR products with high amino acid identity (85 to 100%) to *HcrVf1* and *HcrVf2* were obtained not only from *M. floribunda* 821 and *Vf* cultivars but also from other apple scab resistance sources, such as R12740-7A (*Vr*, *Vr1/Vh4/Vx*, *Vh2* resistance) or 'Antonovka polutorafuntovaya' (VA resistance). A series of thirteen *HcrVf* candidate genes have been partly cloned from the PCR fragments spanning N-terminal LRRs 20 – 30 and the sequences were submitted to NCBI GenBank. A considerable number of amino acid exchanges within the solvent-exposed xxLxLxx structural motives were detected among the homologous sequences. However, further investigation is required in order to clarify whether the multiple amino acid changes observed in the C1 sequences of different apple *HcrVf* homologues can generate novel recognition specificities.

This work focused further on expression analyses and mapping of two *HcrVf*-like candidate genes, a putative highly specific candidate gene derived from 'Russian Seedling' (called *Vf1RSA*), and a more frequently distributed in apple *HcrVf* homologue called *Vf2ARD.* RT-PCR experiments showed that *Vf1RSA* as well as the *Vf2ARD* are expressed under pathogen-free conditions. However, the results of a quantitative PCR-based transcription profiling performed for *Vf2ARD* suggest that this gene is scab-inducible in some resistant cultivars. Generally, different levels and speeds of responses after infection with *V. inaequalis* were detected among tested apple cultivars. *Vf1RSA* has been mapped so far without any recombination to the known *Vf* cluster whereas *Vf2ARD* was separated from the *Vf* locus with a genetic distance of about 2 cM. The latter might be a member of a second *Vf* - like locus on apple LG 1

supporting the existence of several *Vf* - like loci on apple linkage group 1. Additional studies are needed to evaluate further *HcrVf* candidates genes derived from different resistant and susceptible *Malus* genotypes.

Full-length cloning and additional transriptional analyses of the *HcrVf*-like genes *Vf1RSA* and *Vf2ARD* are suggested to enhance the knowledge about structure and chromosomal organization of *HcrVf* genes in *Malus.*

5.2 Zusammenfassung

Der Apfelschorf (*Venturia inaequalis)* ist der wirtschaftlich wichtigste pilzliche Schaderreger im Apfelanbau. Züchterische Ansätze haben sich in den letzten etwa 50 Jahren schwerpunktmäßig auf das *Vf*-Resistenzgen aus *Malus floribunda* konzentriert, welches jedoch inzwischen durch neu aufgetretene Erregerrassen durchbrochen wurde. Vor diesem Hintergrund ist eine Erweiterung der genetischen Basis der Resistenz eine wichtige Grundlage für zukünftige Resistenzzüchtungsstrategien.

Im ersten Teil der hier vorliegenden Dissertation wird die Identifizierung eines neuen Schorfresistenzgens beschrieben. Das Resistenzgen stammt ursprünglich aus dem sogenannten “Russischen Sämling" (R12740-7A). Die genetische Charakterisierung der Resistenz erfolgte in Kreuzungsnachkommenschaften, die auf die hochgradig schorfresistente Apfelsorte ’Regia’, einem Abkömmling des “Russischen Sämlings“, zurückgehen. Der nach Bulked-Segregant-Analyse auf der Basis von drei verschiedenen Apfelnachkommenschaften identifizierte RAPD-Marker AD13 mit einer Rekombinationsfrequenz zum Zielgen von etwa 10 cM wurde in einen codominanten SCAR-Marker umgewandelt. Die weitere genetische Untersuchung und SSR-markergestützte Kartierungen zeigten, daß es sich bei dem kartierten Resistenzgen um ein neues Resistenzgen (genannt *Vr1*) handelt. Das *Vr1*-Gen wurde im oberen Bereich der *Malus*-Kopplungsgruppe LG 2 kartiert, und zwar in einem Genomabschnitt, in dem in einem parallelen Forschungsvorhaben in Neuseeland das ebenfalls aus dem R12740-7A stammende rassenspezifische Resistenzgen *Vh4* lokalisiert wurde. Aufgrund

seiner Eigenschaft als "multi-allelischer" Marker erwies sich der AD13-SCAR als dafür geeignet, Sorten und verschiedene Akzessionen von *Malus* - Arten zu differenzieren und mögliche verwandschaftliche Beziehungen zu analysieren.

Um gleichzeitig eine Markeranalyse für die zwei züchterisch wichtigen Schorfresistenzen *Vf* und *Vr1* durchführen zu können, wurde ein PCR-Multiplex-Assay für die Frühselektion der beide Resistenzgene tragenden Sämlinge entwickelt. Es konnte in dieser Arbeit auch erstmals für ein Resistenzgen des "Russischen Sämlings" demonstriert werden, daß die molekularen Markerinformationen mit dem Resistenzverhalten der Bäume unter natürlichen Befallsbedingungen im Versuchsfeld weitgehend übereinstimmten.

Der zweite Teil dieser Dissertation umfasste die molekulare und genetische Charakterisierung von Schorfresistenz-Kandidatengenen unter Verwendung der bei der Isolierung des *Vf*-Locus vor einigen Jahren publizierten Sequenzinformationen über die beteiligten *HcrVf*-Gene, die zur Resistenzgenklasse der "receptor-like proteins" (RLP) gehören. Es sollte im Rahmen dieser Arbeit untersucht werden, ob außer in *M. floribunda* und den daraus abgeleiteten *Vf*-Sorten *HcrVf*-Gene auch in anderen *Malus*-Genotypen vorkommen. Insbesondere sollte geklärt werden, ob auch alternative, d. h. "Nicht-*Vf*"-Schorf-resistenzdonoren wie der "Russische Sämling" (*Vr*-Resistenz) oder 'Antonovka' (*Va*-Resistenz) funktionell aktive *HcrVf*-Gene besitzen und ob diese möglicherweise in kausalem Zusammenhang mit ihrer Schorfresistenz stehen. Ausgehend von den publizierten Sequenzen der klonierten *HcrVf*-Gene wurden mittels einer 2-stufigen PCR-basierten Klonierungsstrategie *HcrVf*-Kandidatengene in verschiedenen *Malus*-Genotypen identifiziert, partiell kloniert und im Fall von zwei ausgewählten Kandidatengenen auch auf ihre Expression überprüft. Den Abschluß der Untersuchung bildete die Kartierung der zwei speziellen Kandidatengene und der Vergleich ihrer Kartenpositionen mit dem bekannten *Vf*-Locus.

Die Expression der als *Vf1RSA* und *Vf2ARD* bezeichneten Kandidatengene wurde mittels RT-PCR und im Fall von *Vf2ARD* auch unter Einbeziehung einer quantitativen PCR überprüft und nachgewiesen. Beim Kandidatengen *Vf2ARD*

wurde bei einigen Sorten eine Expressionszunahme nach Schorfinokulation festgestellt. Den vorläufigen Abschluß der Untersuchung bildete die Kartierung von *Vf1RSA* und *Vf2ARD* für den Vergleich ihrer Kartenpositionen mit dem bekannten *Vf*-Locus. Die Kartierungsversuche mit dem aus dem "Russischen Sämling" klonierten *HcrVf*-Kandidatengen *Vf1RSA* ergaben bislang in beiden untersuchten Nachkommenschaften keine Rekombination zum bekannten *Vf*-Locus auf Kopplungsgruppe LG 1. Das aus 'Antonovka' stammende Gen *Vf2ARD* liegt dagegen etwa 2 cM vom bekannten *Vf*-Locus entfernt und gehört daher sehr wahrscheinlich zu einem zweiten, neu identifizierten *Vf*-Locus auf Kopplungsgruppe LG 1. Die strukturellen Vergleiche der Aminosäuresequenzen deckten eine erhebliche allelische Variabilität zwischen den *HcrVf*-Varianten der verschiedenen *Malus*-Herkünfte auf, auch innerhalb der vermutlich für die Rassenspezifität bedeutsamen Genabschnitte.

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Annex 7.

A1: World apple production according to FAO data (2006). The leading apple producing countries are marked with red, other countries growing apple are marked with green. Source of the political map of the World: www.nationsonline.org

A2: List of scab resistant sources after Gessler et al. (2006)

Source of resistance	Type of resistance*	Gene name
Malus atrosanguinea 804		
"three-type"	M	Allelic to *Vf*
"pit-type"	M	*Vm*
Malus micromalus 245-38		
"three -type"	M	Allelic to *Vf*
"pit-type"	M	*Vm*
Hansen's baccata # 2	M	*Vb*
Malus baccata jackii	M	*Vbj*
Malus floribunda 821	M	*Vf*
Malus pumila R12740-7A	Complex	
from GMAL 1462	M	*Vr*
race 2 differential	M	*Vh2*
race 4 differential	M	*Vr1/Vh4/Vx*
GMAL 2473	M	*Vr2*
"Antonovka type"		
PI 172612	M	-
PI 172623 ("pit-type")	M	*Va*
PI 172633)"pit-type")	M	*(Va)*
PI172632 ("three-type"; known as Schmidt's Antonovka) and its derivative TN10-8	P	-
Antonovka $1^1/_2$ lb and other Antonovka selections	P	VA
Freedom	-	-
Malus prunifolia 19651	M	Allelic to *Vf*
Malus prunifolia microcarpa 782-26 and Hansen's baccata # 1	M	Allelic to *Vf*
Malus prunifolia xanthocarpa 691-25, M.A. 4, M.A. 8, M.A. 16 and M.A. 1255	M	Allelic to *Vf*
Jonsib crab	M	-
Cathay crab	M	-
Malus sargenti 843, *Malus sieboldii* 2972-22, *Malus toringo* 852, *Malus ringo* 840	P	-
Malus zumi calocarpa	P	-
Discovery	P	-
Golden Delicious	M (race 7)	*Vg*
Durello di Forlì	P M (race 6)	*Vd*
Dülmener Rosenapfel, Colapius, Rubin, Z190, Lombarts Calville, Alkmene, Firiki and President Roulin	P	-

*M = Monogenic; P = Polygenic

A3: Amplification of *HcrVf* gene homologues in a set of apple cultivars and scab resistance sources with PCR primers Vf1 and Vf2

Variety or accession	Resistance*	*HcrVf* homologues	
		Vf1	*Vf2*
Alkmene	-	-	-
Antonovka acc. APF0022	VA	-	-
Antonovka kamienna	VA	-	+
Antonovka polutorafuntovaya	VA	-	+
Clivia	-	-	-
Cox's Orange	-	-	-
Discovery	*Vg*, polygenic	+	+
Dr Oldenburg	-	-	-
Golden Delicious	*Vg*	-	-
M. floribunda 12	*Vf*	+	+
M. floribunda 54	*Vf*	+	+
M. floribunda 359	*Vf*	+	+
M. floribunda 401	*Vf*	+	+
M. floribunda 821	*Vf*	+	+
M. floribunda Nikita	*Vf*	+	+
Pia	-	-	-
Piflora	-	-	-
Pikkolo	-	-	-
Pilot	-	-	-
Pimona	-	-	-
Pingo	-	-	-
Pinova	-	-	-
Pirol	-	-	-
Piros	-	-	-
Prima	*Vf, Vg*	+	+
R12740-7A	*Vh2, Vr1(Vh4/Vx), Vr*	+	-
Realka	*Vr1* (*Vh4/Vx*)	-	+
Reanda	*Vf*	+	+
Rebella	*Vf*	+	+
Regia	*Vr1* (*Vh4/Vx*)	+	+
Regine	*Vf*	+	+
Reglindis	*Vf*	+	+
Reka	unknown from R12740-7A	-	-
Releika	*Vf*	+	+
Releta	unknown from R12740-7A	+	+
Remo	*Vf*	+	+
Remura	*Vr1* (*Vh4/Vx*)	-	-
Renora	*Vf*	+	+
Resi	*Vf*	+	+
Retina	*Vf*	+	+
Rewena	*Vf*	+	+

According to the literature and own molecular marker analyses

A4: Buffers and solutions for DNA extraction (adapted from Doyle and Doyle, 1987)

Extraction buffer

	% concentration	500 ml	1000 ml	stock solution
NaCl	1.4 M	140 ml	280 ml	5M
Tris HCl	0.1 M	25 ml	50 ml	2M, pH 8.0
EDTA	20 mM	20 ml	40 ml	0.5 M, pH 8.0
CTAB	2%	10 g	20 g	
PVP-40	2%	10 g	20 g	
β Mercaptoethanol	1%	5 ml	10 ml	p.a.

Chloroform:Isoamylalcohol (24:1) solution

	% concentration	250 ml	500 ml	stock solution
Chloroform	96%	240 ml	480 ml	p.a.
Isoamyl alcohol	4%	10 ml	20 ml	p.a.

Sodium acetate solution

	% concentration	100 ml	250 ml	stock solution
Sodium acetate	3 M	40.8 g	102.0 g	
ddH_2O to		100 ml	250 ml	

TE buffer

	% concentration	100 ml	250 ml	stock solution
Tris HCl	10 mM	1.0 ml	2.5 ml	1 M, pH 8.0
EDTA	1 mM	0.2 ml	0.5 ml	0.5 M, pH 8.0

RNA solution (Qiagen)

Stock solution: 100 mg RNase A/ml
From 1:10 diluted stock solution (10 mg/ml) 3 µl RNase solution per 100 µl DNA solution

A5: Composition of buffers and solutions for DNA transformation

LB liquid medium	Bacto-Trypton	10 g
(per litre medium)	Yeast extract	5 g
	NaCl	5 g

The solution was adjusted to pH 7.0 with NaOH

LB-Agar with ampicillin	LB liquid media	
	Bacto-Agar	15 g

The solution was autoclaved and cooled to 50°C before adding ampicillin to a final concentration of 100 µg/ml.

Indicator plates	LB-Agar +	
	IPTG	0.5 mM
	X-Gal	0.004%

SOC medium (100 ml)	Bacto-Trypton	2.0 g
	Bacto-Yeast extract	0.5 g
	1M NaCl	1 ml
	1M KCl	0.25 ml
	2M Mg^{++} stock*	1 ml
	2M Glucose*	1 ml

* filter-sterilized

Bacto-Trypton, Bacto-Yeast extract, NaCl and KCl were added to 97 ml distilled water and dissolved in it. The solution was autoclaved and cooled to RT. 2M Mg^{++} stock and 2M glucose were added, each to a final concentration of 20 mM. Sterile, distilled water was added to get 100 ml. The final pH was adjusted to 7.0.

2M Mg^{++} stock	20.33 g $MgCl_2 \cdot 6\ H_2O$
	24.65 g $MgSO_4 \cdot 7\ H_2O$

Distilled water was added to 100 ml. The solution was filter-sterilized.

X-Gal

100 mg 5 bromo-4 chloro-3indolyl-β-D-galactoside was dissolved in 2 ml N-N-dimethylformamide, covered with aluminum foil and stored at – 20°C.

IPTG

Distilled water was added to 1.2 g IPTG to 50 ml final volume, filter-sterilized and stored at 4°C.

A6: Molecular primers used for the amplification and mapping of the *Vf* candidate genes; sequences and annealing temperatures of the primers

Primers/sequence	Annealing temperature (°C)
Vf1 Forv: 5'-AATTCCGACTCTCATTGGGATTTCG-3' Rev: 5'-TGGATTTTAGACTCAAGGCAAGGTC-3'	Ta 62
Vf2 Forv: 5'-GTTGTTTGTGATCATGTAACCGGCC-3' Rev: 5'-ATTCGTTCCCCCCGAGATTAAGAGT-3'	Ta 66
Vf1RSA Forv: 5'-GTGGTTTCTTTGGTCCCCATT TG-3' Rev: 5'-CCAACAAGCACTGAGACGAA-3'	Ta 55
Vf2ARD Forv: 5'-TCTCAACTTCTTGGACCTAAG-3' Rev: 5'-GTGATATTTTGTGAACTGCCC-3'	Ta 52
Vf2ARD-RT Forv: 5'-TCTTGGACCTAAGCAACAATGAT-3' Rev: 5'-AGTTGTCCTGTAAGTTGATTGGC-3'	Ta 55
EF1-α Forv: 5'-ATTGTGGTCATTGGYCAYGT-3' Rev: 5'-CCAATCTTGTAVACATCCTG-3'	Ta 56
Rubisco Forv: 5'-GCTTGTCCAAGAGCAAGAGAAT-3' Rev: 5'-CTCCCTCCCCTCAATTATAACC-3'	Ta 60
RNAPOLII Forv: 5'-ATATGCCACCCCGTTCTCTACT-3' Rev: 5'-CACGTTCCATTTGTCCAAACTT-3'	Ta 58
CH-Vf1-SSR Forv: 5'-ATCACCACCAGCAGCAAAG-3' Rev: 5'-CATACAAATCAAAGCACAACC-3'	Ta 60

A 7: PCR profiles for molecular primers used for identification, characterization and mapping of the *Vf* candidate genes

Vf1

	temperature	time
1 cycle	94°C	5 min
5 cycles	94°C	1 min
annealing	62°C	2 min
elongation	72°C	2 min
33 cycles	94°C	1 min
annealing	62°C	1 min
elongation	72°C	1 min
extension	72°C	7 min
hold	4°C	

Vf2

	temperature	time
1 cycle	94°C	2 min
30 cycles	94°C	1 min
annealing	66°C	3 min
elongation	72°C	2.5 min
extension	72°C	10 min
hold	4°C	

Vf1 for the cultivar 'Regia'

	temperature	time
1 cycle	94°C	2 min
35 cycles	94°C	30 sec
annealing	55°C	30 sec
elongation	68°C	1 min
hold	4°C	

Vf2 for the cultivar 'Regia'

	temperature	time
1 cycle	94°C	2 min
35 cycles	94°C	30 sec
annealing	64°C	30 sec
elongation	68°C	1 min
hold	4°C	

A 7 (continued): PCR profiles for molecular primers used for identification, characterization and mapping of the *Vf* candidate genes

Vf1RSA

	temperature	time
1 cycle	94°C	5 min
35 cycles	94°C	30 sec
annealing	55°C	1.5 min
elongation	72°C	1.5 min
extension	72°C	7 min
hold	4°C	

Vf2ARD

	temperature	time
1 cycle	94°C	5 min
35 cycles	94°C	30 sec
annealing	52°C	1 min
elongation	72°C	1.5 min
extension	72°C	8 min
hold	4°C	

CH-Vf1-SSR

	temperature	time
1 cycle	94°C	5 min
35 cycles	94°C	30 sec
annealing	60°C	1 min
elongation	72°C	1.5 min
extension	72°C	8 min
hold	4°C	

EF1-α

	temperature	time
1 cycle	94°C	2 min
30 cycles	94°C	30 sec
annealing	56°C	1 min
elongation	72°C	1.5 min
extension	72°C	10 min
hold	4°C	

A8: Decamer primers of arbitrary sequence from Operon Technologies (Alameda, CA, USA) used in this study

KIT NAME	PRIMER NAME	SEQUENCE
KIT AA	OPAA-01	AGACGGCTCC
	OPAA-02	GAGACCAGAC
	OPAA-03	TTAGCGCCCC
	OPAA-04	AGGACTGCTC
	OPAA-05	GGCTTTAGCC
	OPAA-06	GTGGGTGCCA
	OPAA-07	CTACGCTCAC
	OPAA-08	TCCGCAGTAG
	OPAA-09	AGATGGGCAG
	OPAA-10	TGGTCGGGTG
	OPAA-11	ACCCGACCTG
	OPAA-12	GGACCTCTTG
	OPAA-13	GAGCGTCGCT
	OPAA-14	AACGGGCCAA
	OPAA-15	ACGGAAGCCC
	OPAA-16	GGAACCCACA
	OPAA-17	GAGCCCGACT
	OPAA-18	TGGTCCAGCC
	OPAA-19	TGAGGCGTGT
	OPAA-20	TTGCCTTCGG
KIT AB	OPAB-01	CCGTCGGTAG
	OPAB-02	GGAAACCCCT
	OPAB-03	TGGCGCACAC
	OPAB-04	GGCACGCGTT
	OPAB-05	CCCGAAGCGA
	OPAB-06	GTGGCTTGGA
	OPAB-07	GTAAACCGCC
	OPAB-08	GTTACGGACC
	OPAB-09	GGGCGACTAC
	OPAB-10	TTCCCTCCCA
	OPAB-11	GTGCGCAATG
	OPAB-12	CCTGTACCGA
	OPAB-13	CCTACCGTGG
	OPAB-14	AAGTGCGACC
	OPAB-15	CCTCCTTCTC
	OPAB-16	CCCGGATGGT
	OPAB-17	TCGCATCCAG
	OPAB-18	CTGGCGTGTC
	OPAB-19	ACACCGATGG
	OPAB-20	CTTCTCGGAC
KIT AD	OPAD-01	CAAAGGGCGG
	OPAD-02	CTGAACCGCT
	OPAD-03	TCTCGCCTAC
	OPAD-04	GTAGGCCTCA
	OPAD-05	ACCGCATGGG
	OPAD-06	AAGTGCACGG
	OPAD-07	CCCTACTGGT
	OPAD-08	GGCAGGCAAG

KIT NAME	PRIMER NAME	SEQUENCE
KIT AD	OPAD-09	TCGCTTCTCC
	OPAD-10	AAGAGGCCAG
	OPAD-11	CAATCGGGTC
	OPAD-12	AAGAGGGCGT
	OPAD-13	GGTTCCTCTG
	OPAD-14	GAACGAGGGT
	OPAD-15	TTTGCCCCGT
	OPAD-16	AACGGGCGTC
	OPAD-17	GGCAAACCCT
	OPAD-18	ACGAGAGGCA
	OPAD-19	CTTGGCACGA
	OPAD-20	TCTTCGGAGG
KIT C	OPC-01	TTCGAGCCAG
	OPC-02	GTGAGGCGTC
	OPC-03	GGGGGTCTTT
	OPC-04	CCGCATCTAC
	OPC-05	GATGACCGCC
	OPC-06	GAACGGACTC
	OPC-07	GTCCCGACGA
	OPC-08	TGGACCGGTG
	OPC-09	CTCACCGTCC
	OPC-10	TGTCTGGGTG
	OPC-11	AAAGCTGCGG
	OPC-12	TGTCATCCCC
	OPC-13	AAGCCTCGTC
	OPC-14	TGCGTGCTTG
	OPC-15	GACGGATCAG
	OPC-16	CACACTCCAG
	OPC-17	TTCCCCCCAG
	OPC-18	TGAGTGGGTG
	OPC-19	GTTGCCAGCC
	OPC-20	ACTTCGCCAC
KIT F	OPF-01	ACGGATCCTG
	OPF-02	GAGGATCCCT
	OPF-03	CCTGATCACC
	OPF-04	GGTGATCAGG
	OPF-05	CCGAATTCCC
	OPF-06	GGGAATTCGG
	OPF-07	CCGATATCCC
	OPF-08	GGGATATCGG
	OPF-09	CCAAGCTTCC
	OPF-10	GGAAGCTTGG
	OPF-11	TTGGTACCCC
	OPF-12	ACGGTACCAG
	OPF-13	GGCTGCAGAA
	OPF-14	TGCTGCAGGT
	OPF-15	CCAGTACTCC
	OPF-16	GGAGTACTGG

A8 (continued): Decamer primers of arbitrary sequence from Operon Technologies (Alameda, CA, USA) used in this study

KIT F	OPF-17	AACCCGGGAA
	OPF-18	TTCCCGGGTT
	OPF-19	CCTCTAGACC
	OPF-20	GGTCTAGAGG
KIT I	OPI-01	ACCTGGACAC
	OPI-02	GGAGGAGAGG
	OPI-03	CAGAAGCCCA
	OPI-04	CCGCCTAGTC
	OPI-05	TGTTCCACGG
	OPI-06	AAGGCGGCAG
	OPI-07	CAGCGACAAG
	OPI-08	TTTGCCCGGT
	OPI-09	TGGAGAGCAG
	OPI-10	ACAACGCGAG
	OPI-11	ACATGCCGTG
	OPI-12	AGAGGGCACA
	OPI-13	CTGGGGCTGA
	OPI-14	TGACGGCGGT
	OPI-15	TCATCCGAGG
	OPI-16	TCTCCGCCCT
	OPI-17	GGTGGTGATG
	OPI-18	TGCCCAGCCT
	OPI-19	AATGCGGGAG
	OPI-20	AAAGTGCGGG
KIT L	OPL-01	GGCATGACCT
	OPL-02	TGGGCGTCAA
	OPL-03	CCAGCAGCTT
	OPL-04	GACTGCACAC
	OPL-05	ACGCAGGCAC
	OPL-06	GAGGGAAGAG
	OPL-07	AGGCGGGAAC
	OPL-08	AGCAGGTGGA
	OPL-09	TGCGAGAGTC
	OPL-10	TGGGAGATGG
	OPL-11	ACGATGAGCC
	OPL-12	GGGCGGTACT
	OPL-13	ACCGCCTGCT
	OPL-14	GTGACAGGCT
	OPL-15	AAGAGAGGGG
	OPL-16	AGGTTGCAGG
	OPL-17	AGCCTGAGCC
	OPL-18	ACCACCCACC
	OPL-19	GAGTGGTGAC
	OPL-20	TGGTGGACCA
KIT O	OPO-01	GGCACGTAAG
	OPO-02	ACGTAGCGTC
	OPO-03	CTGTTGCTAC
	OPO-04	AAGTCCGCTC

KIT O	OPO-05	CCCAGTCACT
	OPO-06	CCACGGGAAG
	OPO-07	CAGCACTGAC
	OPO-08	CCTCCAGTGT
	OPO-09	TCCCACGCAA
	OPO-10	TCAGAGCGCC
	OPO-11	GACAGGAGGT
	OPO-12	CAGTGCTGTG
	OPO-13	GTCAGAGTCC
	OPO-14	AGCATGGCTC
	OPO-15	TGGCGTCCTT
	OPO-16	TCGGCGGTTC
	OPO-17	GGCTTATGCC
	OPO-18	CTCGCTATCC
	OPO-19	GGTGCACGTT
	OPO-20	ACACACGCTG
KIT P	OPP-01	GTAGCACTCC
	OPP-02	TCGGCACGCA
	OPP-03	CTGATACGCC
	OPP-04	GTGTCTCAGG
	OPP-05	CCCCGGTAAC
	OPP-06	GTGGGCTGAC
	OPP-07	GTCCATGCCA
	OPP-08	ACATCGCCCA
	OPP-09	GTGGTCCGCA
	OPP-10	TCCCGCCTAC
	OPP-11	AACGCGTCGG
	OPP-12	AAGGGCGAGT
	OPP-13	GGAGTGCCTC
	OPP-14	CCAGCCGAAC
	OPP-15	GGAAGCCAAC
	OPP-16	CCAAGCTGCC
	OPP-17	TGACCCGCCT
	OPP-18	GGCTTGGCCT
	OPP-19	GGGAAGGACA
	OPP-20	GACCCTAGTC
KIT Q	OPQ-01	GGGACGATGG
	OPQ-02	TCTGTCGGTC
	OPQ-03	GGTCACCTCA
	OPQ-04	AGTGCGCTGA
	OPQ-05	CCGCGTCTTG
	OPQ-06	GAGCGCCTTG
	OPQ-07	CCCCGATGGT
	OPQ-08	CTCCAGCGGA
	OPQ-09	GGCTAACCGA
	OPQ-10	TGTGCCCGAA
	OPQ-11	TCTCCGCAAC
	OPQ-12	AGTAGGGCAC
	OPQ-13	GGAGTGGACA

A8 (continued): Decamer primers of arbitrary sequence from Operon Technologies (Alameda, CA, USA) used in this study

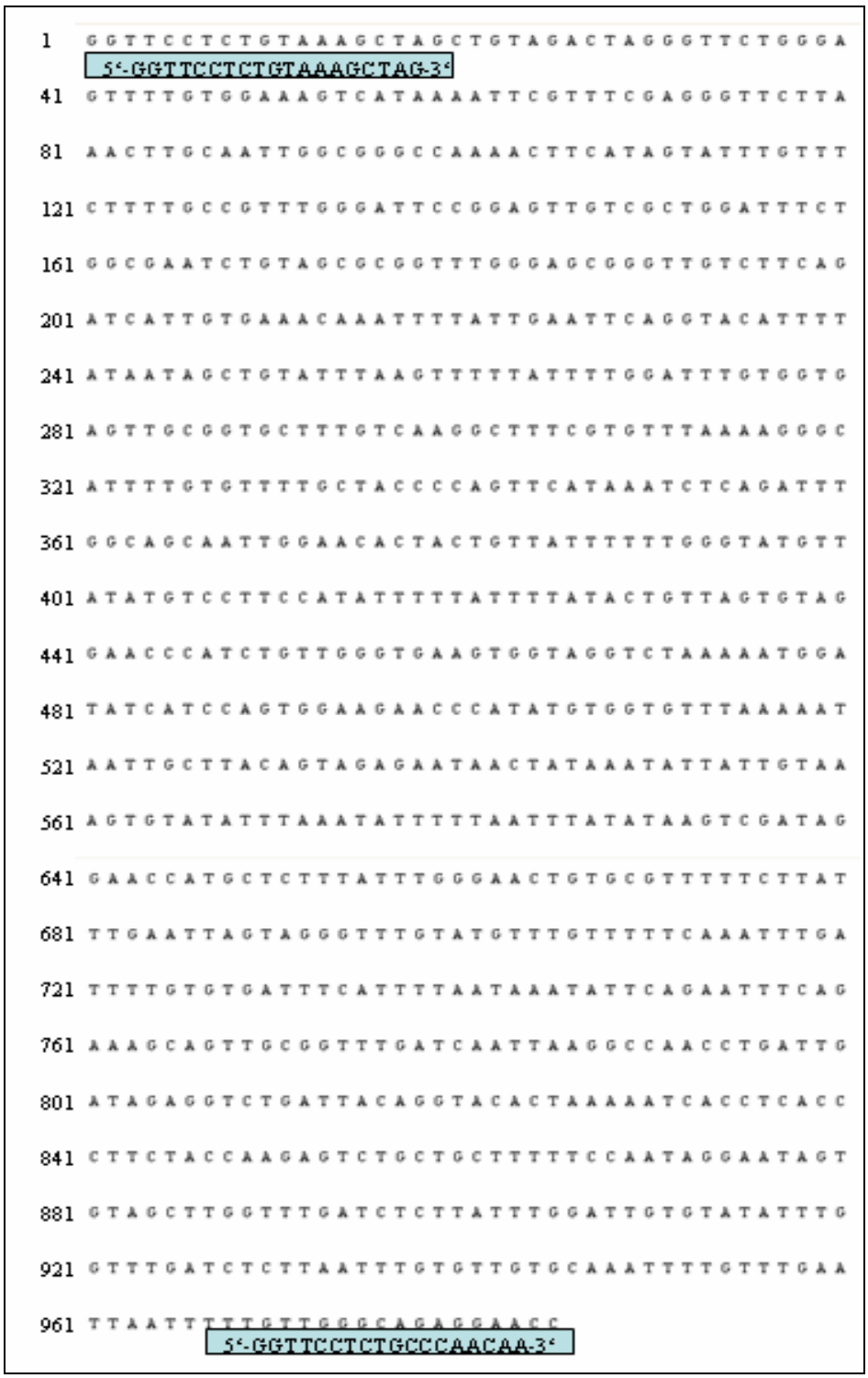
KIT Q	OPQ-14	GGACGCTTCA
	OPQ-15	GGGTAACGTG
	OPQ-16	AGTGCAGCCA
	OPQ-17	GAAGCCCTTG
	OPQ-18	AGGCTGGGTG
	OPQ-19	CCCCCTATCA
	OPQ-20	TCGCCCAGTC
KIT R	OPR-01	TGCGGGTCCT
	OPR-02	CACAGCTGCC
	OPR-03	ACACAGAGGG
	OPR-04	CCCGTAGCAC
	OPR-05	GACCTAGTGG
	OPR-06	GTCTACGGCA
	OPR-07	ACTGGCCTGA
	OPR-08	CCCGTTGCCT
	OPR-09	TGAGCACGAG
	OPR-10	CCATTCCCCA
	OPR-11	GTAGCCGTCT
	OPR-12	ACAGGTGCGT
	OPR-13	GGACGACAAG
	OPR-14	CAGGATTCCC
	OPR-15	GGACAACGAG
	OPR-16	CTCTGCGCGT
	OPR-17	CCGTACGTAG
	OPR-18	GGCTTTGCCA
	OPR-19	CCTCCTCATC
	OPR-20	ACGGCAAGGA
KIT U	OPU-01	ACGGACGTCA
	OPU-02	CTGAGGTCTC
	OPU-03	CTATGCCGAC
	OPU-04	ACCTTCGGAC
	OPU-05	TTGGCGGCCT
	OPU-06	ACCTTTGCGG

KIT U	OPU-07	CCTGCTCATC
	OPU-08	GGCGAAGGTT
	OPU-09	CCACATCGGT
	OPU-10	ACCTCGGCAC
	OPU-11	AGACCCAGAG
	OPU-12	TCACCAGCCA
	OPU-13	GGCTGGTTCC
	OPU-14	TGGGTCCCTC
	OPU-15	ACGGGCCAGT
	OPU-16	CTGCGCTGGA
	OPU-17	ACCTGGGGAG
	OPU-18	GAGGTCCACA
	OPU-19	GTCAGTGCGG
	OPU-20	ACAGCCCCCA
KIT X	OPX-01	CTGGGCACGA
	OPX-02	TTCCGCCACC
	OPX-03	TGGCGCAGTG
	OPX-04	CCGCTACCGA
	OPX-05	CCTTTCCCTC
	OPX-06	ACGCCAGAGG
	OPX-07	GAGCGAGGCT
	OPX-08	CAGGGGTGGA
	OPX-09	GGTCTGGTTG
	OPX-10	CCCTAGACTG
	OPX-11	GGAGCCTCAG
	OPX-12	TCGCCAGCCA
	OPX-13	ACGGGAGCAA
	OPX-14	ACAGGTGCTG
	OPX-15	CAGACAAGCC
	OPX-16	CTCTGTTCGG
	OPX-17	GACACGGACC
	OPX-18	GACTAGGTGG
	OPX-19	TGGCAAGGCA
	OPX-20	CCCAGCTAGA

A9: Molecular markers used for *Vr1* linkage mapping and molecular resistance tests; their sources, sequences and annealing temperatures

Scab resistance locus	Marker/sequence	Annealing temp (°C)	Product size	Marker references
Vr1/Vh4/Vx	**AD13-SCAR** Forv: 5'-GGTTCCTCTGTAAAGCTAG-3' Rev: 5'-GGTTCCTCTGCCCAACAA-3'	Ta 58	950 bp	Boudichevskaia et al. 2006
Vf	**AL07-SCAR** Forv: 5'-TTCTTACTGAGGAAACCAG-3' Rev: 5'-CAAGGGAACTGATCTTTCGTTG-3'	Ta 60	466 bp, 724 bp	Tartarini et al. 1999
Vr1/Vh4/Vx	**OPQ07-RAPD** 5'-CCCCGATGGT-3'	Ta 36	1500 bp	Operon Technologies (USA)
Vr/Vh2/Vh8/Vbj	**OPB18-RAPD** Forv: 5'-CCACAGCAGT-3'	Ta 36	620 bp	Hemmat et al. 2002
Vr1/Vh4/Vx	**S22-SCAR** Forv: 5'-GTCGTGGAAGAGGACCGA-3' Rev: 5'-GTCGTGGAAATCCTCGTGAG-3'	Ta 62	1300 bp	Hemmat et al. 2002
Vr/Vh2/Vh8/Vbj	**CH02b10-SSR** Forv: 5'-CAAGGAAATCATCAAAGATTCAAG-3' Rev: 5'-CAAGTGGCTTCGGATAGTTG-3'	Ta 58	122 bp	Hemmat et al. 2002 Liebhard et al. 2002
Vr1/Vh4/Vx	**CH02c02a-SSR** Forv: 5'-CTTCAAGTTCAGCATCAAGACAA-3' Rev: 5'-TAGGGCACACTTGCTGGTC-3'	Ta 60		Liebhard et al. 2002
	CH02f06-SSR Forv: 5'-CCCTCTTCAGACCTGCATATG-3' Rev: 5'-ACTGTTTCCAAGCGATCAGG-3'	Ta 60	145 bp	Liebhard et al. 2002
	CH03d10-SSR Forv: 5'-CTCCCTTACCAAAAACACCAAA-3' Rev: 5'-GTGATTAAGAGAGTGATCGGGG-3'	Ta 60	178 bp	Liebhard et al. 2002
	CH05e03-SSR Forv: 5'-CGAATATTTTCACTCTGACTGGG-3' Rev: 5'-CAAGTTGTTGTACTGCTCCGAC-3'	Ta 58	166 bp	Liebhard et al. 2002

A10: Sequence of the cloned OPAD13$_{950}$ RAPD fragment. Boxed sequences are two AD13-SCAR primers



A 11: PCR profiles for molecular markers used for the *Vr1* linkage mapping and molecular resistance tests

AD13-SCAR, SCAR multiplexing (AD13-SCAR and AL07-SCAR)

	temperature	**time**
1 cycle	94°C	2 min
30 cycles	94°C	1 min
annealing	58°C	3 min
elongation	72°C	1.5 min
1 cycle	94°C	1 min
annealing	58°C	3 min
elongation	72°C	10 min
hold	4°C	

AL07-SCAR

	temperature	**time**
1 cycle	94°C	2.5 min
annealing	60°C	1 min
elongation	72°C	1.5 min
35 cycles	94°C	30 sec
annealing	60°C	1 min
elongation	72°C	1.5 min
extension	72°C	10 min
hold	4°C	

RAPD-primers (after Koller et al. 1994)

	temperature	**time**
1 cycle	94°C	1 sec
2 cycles	94°C	30 sec
annealing	36°C	30 sec
elongation	72°C	2 min
20 cycles	94°c	20 sec
annealing	36°C	15 sec
annealing	45°C	45 sec
elongation	72°C	2 min
19 cycles	94°C	20 sec*
annealing	36°C	15 sec
annealing	45°C	15 sec
elongation	72°C	2 min**
extension	72°C	10 min
hold	4°C	

* increased 1 sec/cycle

** increased 3 sec/cycle

A 11 (continued): PCR profiles for molecular markers used for the *Vr1* linkage mapping and molecular resistance tests

OPB18-SCAR, CH02b10-SSR, CH05e03-SSR

	temperature	**time**
1 cycle	94°C	5 min
annealing	58°C	1 min
elongation	72°C	1.5 min
38 cycles	94°C	30 sec
annealing	58°C	1 min
elongation	72°C	1.5 min
1 cycle	94°C	30 sec
annealing	58°C	1 min
elongation	72°C	6 min
extension	72°C	1 min
hold	4°C	

S22-SCAR

	temperature	**time**
1 cycle	94°C	5 min
annealing	62°C	2 min
elongation	72°C	2 min
38 cycles	94°C	1 min
annealing	62°C	1 min
elongation	72°C	1 min
extension	72°C	8 min
hold	4°C	

CH02c02a-SSR, CH02f06-SSR, CH03d10-SSR

	temperature	**time**
1 cycle	94°C	5 min
annealing	60°C	1 min
elongation	72°C	1.5 min
38 cycles	94°C	30 sec
annealing	60°C	1 min
elongation	72°C	1.5 min
1 cycle	94°C	30 sec
annealing	60°C	1 min
elongation	72°C	6 min
extension	72°C	1 min
hold	4°C	

A12: Polyacrylamide gel electrophoresis

Loading buffer

95% Formamide

0.01 NaOH

0.05% Xylencyanole

0.05% Bromophenol blue

Parameters for run using 25 cm gel

Parameter	
Plate length	25 cm
Spacer thickness	0.25 mm
Gel composition	50% Long Ranger TM
Run time (for 350 bp)	1.5 hours

Preparing buffer

Gel and running solutions were prepared from a standard 10 x TBE buffer. Gel and running buffer contained 1 x TBE. For preparing 10 x TBE the following components were added to a 1000 ml beaker:

Component	Amount	Molarity
Tris Base	107.8 g	0.89 M
Boric acid	550 g	0.89 M
EDTA	7.4 g	0.02 M
Distilled water	950 ml	
Total volume	1000 ml	

The solution was adjusted to pH 8.3. This solution was stored at RT. For 1 x runnig TBE buffer 100 ml of prepared as above 10 x TBE was mixed well with 900 ml of distilled water.

Gel preparation

Acrylamide

The components for mixing of gel solution are listed below. Measured urea was put into the beaker. Then the acrylamide solution and 10 x TBE buffer were added. Water was added to get volume of 20 ml gel solution. Solution was mixed well at RT. 150 µl of 10 % APS was added to the gel solution and swirled

gently. Just before pouring, 15 µl TEMED was added. The time to pour the gel before its polymerization was 3-5 min.

Components	25 cm
	0.25 mm 6.5%
Urea (7 M)	8.4 g
50% Long Ranger ™ acrylamide	2.6 ml
10 x TBE	2.0 ml
ddH_2O	to 20 ml

Preparing Ammonium Persulfate Solution

APS provides a source of free radicals needed for polymerization of the gel (Sambrook, 1989). A 10 % APS solution was made by adding 0.1 g APS to 1.0 ml deionised water in a small tube.

A13: Composition of solutions for Southern blot analysis

Solutions

Solution for DNA transfer		
to membranes (20 x SSC)	NaCl	3 M
pH 7.0	sodium citrate	0.3 M
Denaturation solution	NaCl	1.5 M
	NaOH	0.5 M
Neutralising solution	NaCl	1.5 M
pH 7.2	Tris-HCl	0.5 M
	EDTA	0.001 M

A14: Representation of marker data for 90 individuals of the population 03/206 (Regia x Piflora) used for construction of genetic map for the *Vr1*-carrying linkage group LG 2

03/206	Genotype	*Vr1*	CH02f06	AD13-SCAR	CH02c02a	Q7-RAPD	S22-SCAR	CH02b10	CH03d10	CH05e03
Nr		<lmxll>*	<efxeg>*	<lmxll>*	<lmxll>*	<lmxll>*	<lmxll>*	<abxcd>*	<abxcd>*	<abxcd>*
1	**1**	lm	ef	lm	ll	lm	ll	bc	ad	bc
2	**3**	lm	fg	lm	ll	lm	lm	bd	ac	bd
3	**4**	lm	ef	lm	ll	--	lm	ac	bd	ac
4	**6**	ll	ef	ll	lm	ll	ll	ac	ad	ac
5	**7**	ll	eg	ll	lm	ll	ll	ad	bc	bd
6	**8**	lm	fg	lm	ll	lm	lm	bd	ac	bd
7	**9**	lm	fg	lm	ll	lm	lm	bc	ad	bc
8	**10****	ll	fg	lm	ll	lm	lm	bd	ac	bd
9	**11**	ll	ee	ll	lm	ll	ll	bc	ad	bc
10	**13**	ll	eg	ll	lm	ll	ll	ad	bc	ad
11	**14**	ll	ee	ll	lm	ll	ll	ac	ad	ac
12	**15**	ll	eg	ll	lm	ll	ll	ac	bd	ac
13	**16**	lm	fg	lm	ll	lm	ll	bd	ac	bd
14	**17**	lm	ef	lm	ll	lm	lm	bd	ac	bd
15	**21**	lm	fg	lm	ll	lm	lm	bc	ad	bc
16	**25**	lm	ef	lm	ll	lm	lm	bd	ac	bd
17	**29**	lm	fg	lm	ll	lm	lm	bd	ac	bd
18	**32**	lm	ef	lm	ll	lm	lm	bd	ad	bd
19	**33**	ll	eg	ll	lm	ll	ll	ac	bd	ac
20	**35**	lm	fg	lm	ll	lm	lm	bc	ad	bc
21	**36**	lm	ef	lm	ll	lm	lm	bd	ac	bd
22	**37**	lm	eg	ll	lm	ll	ll	bc	ad	bc
23	**40**	lm	fg	lm	ll	--	lm	bd	ac	bd
24	**41**	ll	ee	ll	lm	ll	ll	ac	bd	ac
25	**42**	ll	ef	lm	ll	lm	lm	bc	ac	bd
26	**43**	lm	ef	lm	ll	lm	lm	bc	ad	bc
27	**45**	ll	fg	lm	lm	ll	ll	ac	bd	ac
28	**46**	ll	eg	ll	lm	ll	ll	ad	bc	ad
29	**47**	ll	eg	ll	lm	ll	ll	ad	bc	ad
30	**48**	ll	eg	ll	lm	ll	ll	ac	bd	ac
31	**49**	lm	fg	lm	lm	lm	lm	ad	--	bd
32	**52**	lm	fg	lm	ll	lm	lm	bc	ac	bd
33	**54**	lm	fg	lm	ll	lm	lm	bc	ad	ac
34	**55**	ll	ee	ll	lm	ll	ll	ad	bc	ac
35	**58**	ll	eg	ll	lm	--	ll	ad	bc	ad
36	**61**	lm	ef	lm	ll	lm	lm	bd	ac	bd
37	**63**	ll	ef	lm	ll	lm	lm	bc	ad	bd
38	**67**	lm	fg	lm	ll	lm	lm	ad	bc	ad
39	**69**	ll	fg	lm	ll	lm	ll	ad	bc	ad
40	**70**	ll	ee	ll	lm	ll	ll	ad	bc	ac
41	**71**	lm	ef	lm	ll	lm	lm	ac	bd	ac
42	**72**	ll	eg	ll	lm	ll	ll	ad	bc	ad
43	**73**	lm	ee	ll	lm	ll	ll	bc	ad	bd
44	**74**	ll	ee	ll	lm	--	ll	ad	bc	ad
45	**75**	ll	eg	ll	lm	ll	ll	ac	bd	ac
46	**78**	ll	ee	ll	lm	--	ll	ad	bc	ad
47	**80**	lm	ee	ll	lm	ll	ll	bc	ad	bc
48	**81**	lm	ef	lm	ll	lm	lm	ac	--	ac
49	**82**	lm	ef	lm	ll	lm	lm	ad	--	ad
50	**83**	lm	ef	lm	ll	lm	lm	ac	bd	ac
51	**89**	ll	ee	ll	lm	ll	ll	ac	bd	ac
52	**90**	lm	fg	lm	ll	lm	lm	ac	bd	ac

A14 (continued): Representation of marker data for 90 individuals of the population 03/206 (Regia x Piflora) used for construction of genetic map for the *Vr1*-carrying linkage group LG 2

03/206	Genotype	*Vr1*	CH02f06	AD13-SCAR	CH02c02a	Q7-RAPD	S22-SCAR	CH02b10	CH03d10	CH05e03
Nr		<lmxll>*	<efxeg>*	<lmxll>*	<lmxll>*	<lmxll>*	<lmxll>*	<abxcd>*	<abxcd>*	<abxcd>*
53	**91**	ll	ee	ll	lm	--	ll	ad	bc	ad
54	**92**	lm	ee	ll	lm	lm	ll	ac	bd	ac
55	**94**	ll	eg	ll	lm	ll	ll	ad	bc	ad
56	**96**	lm	ef	lm	ll	--	lm	bc	ad	bc
57	**98**	lm	fg	lm	ll	--	lm	bd	ac	bd
58	**99**	ll	eg	ll	lm	ll	ll	ac	bd	ac
59	**101**	lm	fg	lm	ll	lm	lm	bc	ad	bc
60	**105**	lm	fg	lm	ll	--	lm	ac	bd	ac
61	**106**	lm	fg	lm	ll	lm	lm	bc	ac	bd
62	**107**	lm	ee	ll	lm	ll	lm	bc	ad	bc
63	**108**	lm	eg	ll	lm	ll	ll	ac	bd	ac
64	**110**	lm	ef	lm	ll	lm	lm	bc	ad	bc
65	**115**	ll	ef	lm	ll	lm	lm	bd	ad	bc
66	**117**	ll	ee	ll	lm	ll	ll	ac	bd	ac
67	**118**	ll	ee	ll	lm	ll	ll	bc	ad	bc
68	**120**	lm	fg	lm	ll	lm	lm	ad	bc	ad
69	**122**	ll	ef	lm	ll	lm	lm	ac	bd	ac
70	**124**	ll	eg	ll	lm	ll	ll	ad	bc	ad
71	**126**	ll	ee	ll	lm	ll	ll	ad	bc	ad
72	**129**	ll	ee	ll	lm	--	ll	ad	bc	ad
73	**132**	ll	ef	ll	lm	ll	ll	ad	bc	ad
74	**133**	lm	ee	ll	lm	--	ll	ad	bc	ad
75	**138**	lm	ef	lm	ll	lm	lm	bd	ac	bd
76	**153**	ll	ee	ll	lm	ll	ll	ac	bd	ac
77	**157**	ll	ef	ll	lm	ll	ll	ac	bd	ad
78	**159**	lm	ef	lm	ll	lm	lm	bd	ac	bd
79	**160**	lm	ee	ll	lm	--	ll	bc	ad	bc
80	**161**	lm	ef	lm	ll	lm	lm	bc	ad	bc
81	**162**	lm	ef	lm	ll	lm	lm	bd	ac	bd
82	**165**	lm	ef	lm	--	lm	lm	--	--	bc
83	**166**	ll	eg	ll	lm	ll	ll	ad	bc	ad
84	**172**	ll	eg	ll	lm	ll	ll	ac	bd	ac
85	**176**	ll	ee	ll	lm	ll	ll	ac	bd	ac
86	**215**	ll	eg	ll	lm	ll	ll	bd	ac	bd
87	**222**	ll	eg	ll	lm	--	ll	ad	bc	ad
88	**225**	ll	eg	ll	lm	ll	ll	ad	bc	ad
89	**235**	ll	ee	ll	lm	ll	ll	bd	ac	bd
90	**247**	ll	eg	ll	--	ll	ll	--	--	ad

* Segregation type of a locus. The two characters left of the ‘x’ represent the alleles of the first parent, the two characters on the right represent those of the second. Hence, below the possible progeny genotypes are presented. There are three essentially distinct segregation types of a locus providing recombination information: 1) two alleles, one parent heterozygous (lm x ll), 2) two alleles, both parents heterozygous (ef x eg), 3) four alleles (ab x cd).

** Marked with grey colour genotypes are ”genotype-phenotype-incogruence” (GPI) plants from the mapping population 03/206

A 15: Seedling scab assessments in family 05/230 after greenhouse inoculation and its genetic analysis with the *Vf1RSA* and CH-Vf1 primers

Genotype	Greenhouse data		CH-Vf1	Vf1RSA	Genotype	Greenhouse data		CH-Vf1	Vf1RSA
	inoculation 01.02.05	inoculation 01.02.05	139 bp	313 bp		inoculation 01.02.05	inoculation 01.02.05	139 bp	313 bp
	assessment: 01/02.03.05	assessment: 15/16.03.05				assessment: 01/02.03.05	assessment: 15/16.03.05		
	(Dr.Peil)	(Dr.Peil)				(Dr.Peil)	(Dr.Peil)		
1	3	3	0	0	42	3	3	0	0
2	1	1	1	1	43	3	5	0	0
3	2	1	1	1	44	1	1	1	1
4	3	3	0	0	45	5	5	--	--
5	2	1	1	1	46	3	5	0	0
6	1	1	1	1	47	1	1	0	0
7	3	3	1	1	48	1	1	1	1
8	2	3	1	1	50	3	3	1	1
9	3	3	0	0	51	1	1	1	1
10	3	5	1	1	52	1	1	1	1
11	3	1	1	1	53	1	1	1	1
12	1	1	1	1	54	1	1	1	1
13	3	5	0	0	55	1	1	1	1
14	3	3	0	0	56	3	5	0	0
15	3	1	1	1	57	3	5	0	0
16	3	3	0	0	58	3	5	0	0
17	3	1	1	1	59	1	1	1	1
18	3	3	0	0	60	3	3	1	1
20	1	3	0	0	61	3	5	0	0
21	1	1	1	1	62	3	3	0	0
22	1	1	1	1	63	1	1	0	0
23	3	5	0	0	64	3	3	0	0
24	3	5	0	0	65	1	3	0	0
25	2	1	1	1	66	3	5	0	0
26	3	5	1	1	67	2	1	1	1
27	1	1	1	1	68	3	5	0	0
28	3	3	0	0	69	3	5	0	0
29	3	5	1	1	70	1	1	1	1
30	3	3	1	1	71	5	5	0	0
31	3	3	1	1	72	1	3	1	1
32	3	3	0	0	73	3	3	--	--
33	3	5	0	0	74	1	1	1	1
34	1	3	0	0	75	3	3	1	1
35	1	1	1	1	76	3	3	0	0
36	1	1	1	1	77	3	5	--	--
38	1	1	1	1	78	3	3	0	0
39	3	3	0	0	79	3	3	0	0
40	3	3	0	0	80	3	5	0	0
41	1	1	1	1	81	3	3	1	1

A 16: Seedling scab assessments in family 06/004 after greenhouse inoculation and its genetic analysis with the Vf1RSA and SSR primers

Genotypes	**Scab resistance*[1]**	**Vf1RSA**	**CH-Vf1** 137 bp			**AG04**	**AG11**
Golden Delicious		0	141	173		0	0
M. sieversii A97/57-4		1	137	147	139	1	1
1	3	0	141	147	139	0	0
2	4	0	141	147	139	0	1
3	1	1	137	141		1	1
4	1	1	137	141		1	1
5	1	0	147	173	139	0	1
6	1	0	141	147	139	0	1
7	1	1	137	173		1	1
9	3	0	147	173	139	0	0
10	1	1	137	173		1	1
11	1	1	137	173		1	1
13	3--4	0	147	173	139	0	0
14	1	1	137	173		1	1
15	1	1	137	141		1	1
16	2--3	0	147	141	139	0	0
17	1	1	137	173		1	1
18	3	0	147	173	139	0	0
19	1	1	137	141		1	1
20	1	1	137	141		1	0
22	1	0	147	141	139	0	0
24	1	1	137	141		1	1
25	1	1	137	173		1	1
26	1	1	137	173		1	1
27	1	1	137	141		1	1
29	1	1	137	141		1	1
30	3	0	147	173	139	0	1
33	3--4	0	141	147	139	0	0
34	1	1	137	173		1	0
36	1	1	137	173		1	1
37	1	1	137	141		1	1
38	1	1	137	141		1	1
39	2	1	137	173		1	1
40	3	0	141	147	139	0	1
41	3--4	0	141	147	139	0	0
42	3	0	141	147	139	0	1
45	3	0	147	173	139	0	1
48	3	0	141	147	139	0	0
50	1	1	137	141		1	1
51	2	1	137	141		1	1

A 16 (continued): Seedling scab assessments in family 06/004 after greenhouse inoculation and its genetic analysis with the Vf1RSA and SSR primers

Genotypes	Scab resistance*[1]	Vf1RSA	CH-Vf1 137 bp			AG04	AG11
Golden Delicious		0	141	173		0	0
M. sieversii A97/57-4		1	137	147	139	1	1
52	3--4	0	147	173	139	0	0
53	1	1	137	141		1	1
54	3	0	147	173	139	*	0
55	1	0	147	173	139	0	0
56	1	1	137	141		1	1
57	2	0	147	173	139	1	1
58	1	0	147	173	139	0	0
61	1	1	137	173		1	1
63	1	1	137	141		1	1
64	2	0	147	173	139	0	0
65	1	1	137	173		1	1
67	1	1	137	173		1	1
69	2	0	147	173	139	1	0
71	1	0	147	173	139	0	0
74	3	0	147	173	139	0	1
76	1	1	137	173		1	0
78	1	0	141	147	139	0	0
79	1	1	137	141		1	0
81	1	0	141	147	139	0	0
83	1	0	147	173		1	0
85	1	1	137	141		1	1
86	1	0	147	173	139	0	0
88	1	1	137	141		1	1
89	1	0	139	141		0	0
90	1	0	139	141		0	1
91	2	0	147	173	139	0	0
92	3	0	147	173	139	0	0
93	1	0	141	147	139	0	0
94	1	1	137	173		1	1
99	2--3	0	139	141		0	0
100	1	1	137	141		1	1
102	1	0	139	141		0	0
104	3	0	147	173	139	0	0
108	1	0	147	173	139	0	0
109	3	0	141	147	139	0	0
110	1	1	137	173		1	1
111	1	0	139	141	173	0	0
113	1	1	137	173		1	1
115	1	1	137	173		1	1

A 16 (continued): Seedling scab assessments in family 06/004 after greenhouse inoculation and its genetic analysis with the Vf1RSA and SSR primers

Genotypes	**Scab resistance*[1]**	**Vf1RSA**	**CH-Vf1** 137 bp			**AG04**	**AG11**
Golden Delicious		0	141	173		0	0
M. sieversii A97/57-4		1	137	147	139	1	1
118	1	0	147	173	139	0	1
119	1	0	141	147	139	0	0
120	1	1	137	141		1	1
122	1	1	137	141		1	1
124	1	1	137	173		1	1
125	1	1	137	173		1	1
126	1	1	137	173		1	0
127	3	0	141	147	139	0	0
131	1	1	137	173		1	0
132	1	0	147	173	139	0	0
134	3	0	147	141	139	0	1
135	1	1	137	141		1	1
136	1	1	137	141		1	1
137	1	1	137	141		1	0
138	1	1	137	173		1	0
139	4	0	141	147	139	0	0
140	4	0	141	147	139	0	0
142	2--3	0	141	147	139	0	0
143	1	1	137	141		1	0
144	1	1	137	141		1	1
146	1	1	137	141		1	1
148	1	0	141	147	139	0	0
150	1	0	141	147	139	0	1
151	1	1	137	141		1	0
157	1	1	137	173		1	1
158	1	1	137	173		1	1
159	1	0	139	141		0	1
161	4--5	0	139	141		0	0
162	1	1	137	173		1	1

*Scab race MS36

[1] See definition of scab symptoms in Chapter 2.1.3

A 17: Seedling scab assessments in family 04/214 after greenhouse inoculation during two years and its genetic analysis with the CH-Vf1 SSR marker

	Scab 2005[1]	Scab 2006[1]	CH-Vf1 (SSR)	
Antonovka			139	173
Golden Delicious			141	173
10	1	1	139	141
15	1	1	139	141
17	1	1	139	141
18	1	1	139	141
22	3	3	173	173
24	3	3	173	173
26	1	1	139	141
27	1	1	139	173
32	1	1	139	146
41	1	1	139	173
53	1	1	139	141
61	3	3-4	141	173
65	2-3	3	141	173
77	3	2-3	173	173
79	1	1	139	173
81	3	2-3	173	173
83	3	3	173	173
86	1	1	139	141
90	1	1	139	173
95	1	1	139	173
98	3	3	173	173
99	1	1	139	141
100	3	2-3	141	173
101	3-4	3	141	173
103	3	2-3	173	173
119	1	1	139	141
127	3	2-3	141	173
132	1	1	139	173
155	1	1	139	141
174	1	1	139	141
183	1	1	139	173
186	3	3	141	173
194*	1	1	141	173
195*	1	1	173	173
196	1	1	139	141
203	1	1	139	173
204	3	2-3	141	173
206	1	1	139	173
207	1	1	139	173
210	1	1	139	141
211	3	2-3	141	173
212	1	1	139	141
215	1	1	139	141
217	3	3	141	173
222	2-3	3	173	173
230	1	1	139	173
231	4	2-3	141	173

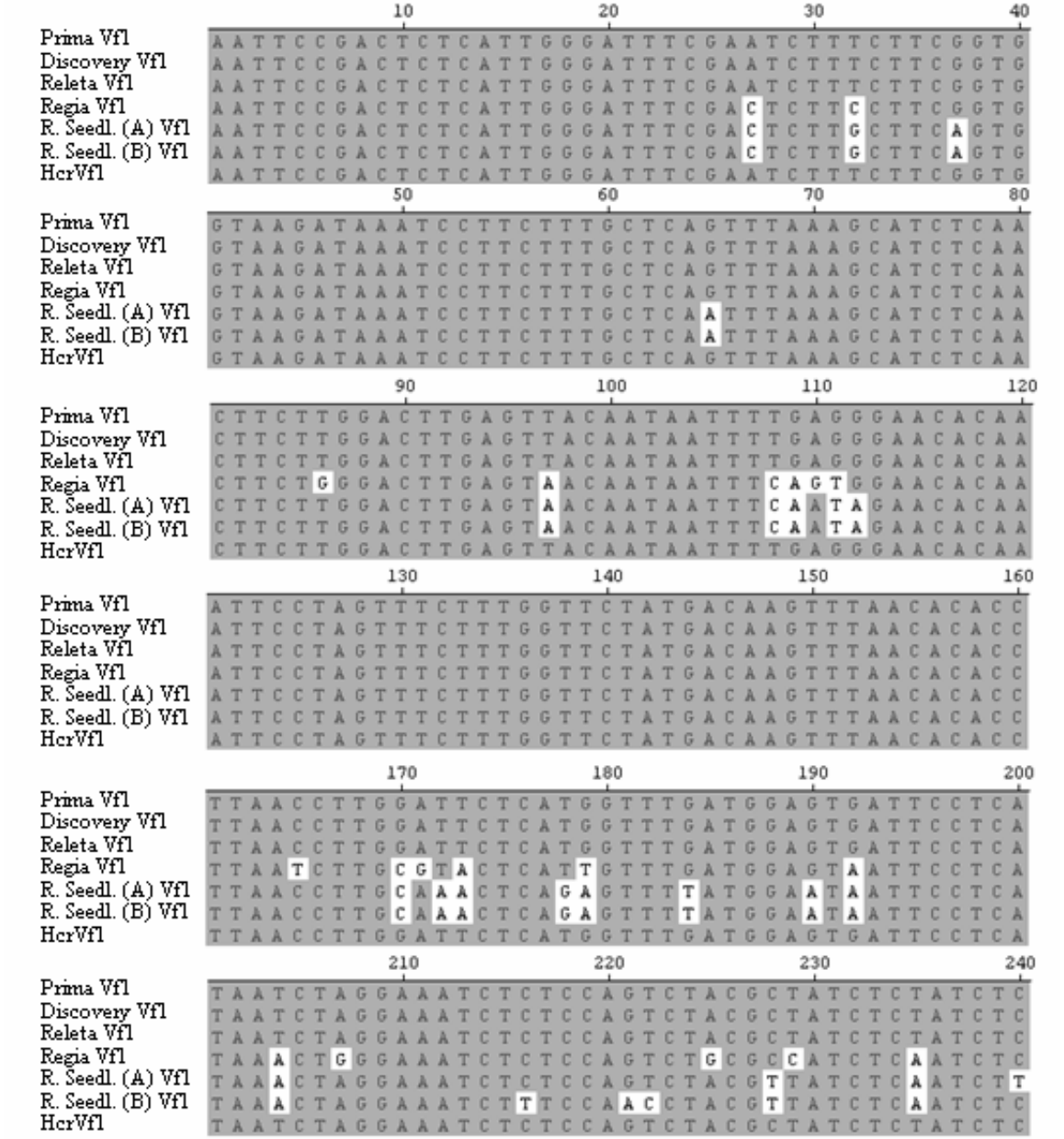
A 17 (continued): Seedling scab assessments in family 04/214 after greenhouse inoculation during two years and its genetic analysis with the CH-Vf1 SSR marker

	Scab 2005[1]	Scab 2006[1]	CH-Vf1 (SSR)	
Antonovka			139	173
Golden Delicious			141	173
235	1	1	139	173
236	1	1	139	141
240	1	1	139	173
243	2	3-4	173	173
249	1	1	139	173
251	3-4	2	141	173
252	3-4	2-3	173	173
259	1	1	139	173
260	1	1	139	173
261	1	1	139	141
265	3	2-3	141	173
266	1	1	139	173
272	1	1	139	173
280	1	1	139	173
283	1	1	139	173
287	1	1	139	173
293	3	3	141	173
294	3	3	141	173
295	3	3	173	173
313	3	3	141	173
317	2-3	2-3	173	173
326	3	2-3	141	173
349	3	2-3	141	173
364	3	2-3	141	173
379	1	1	139	141
381	3	3	173	173
388	3	2-3	173	173
391	3	3	141	173
392	3	3	173	173
400	3	3	141	173
402	3	2-3	141	173
403	3	2-3	141	173
405	3	2-3	173	173
406	2-3	3	141	173

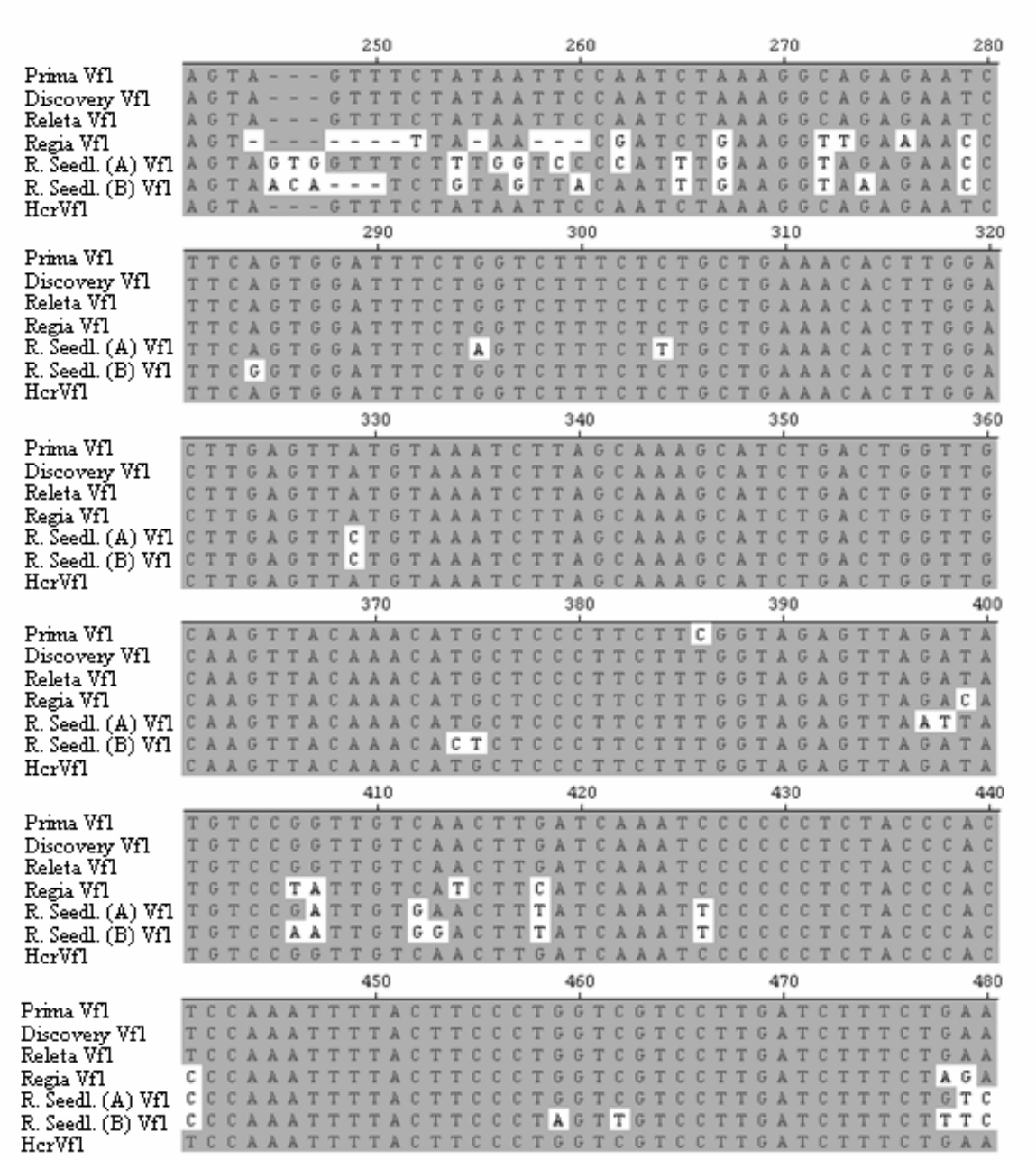
* Genotype showing putative recombination event between scab resistance data and the CH-Vf1 SSR marker

[1] See definition of scab symptoms in Chapter 2.1.3

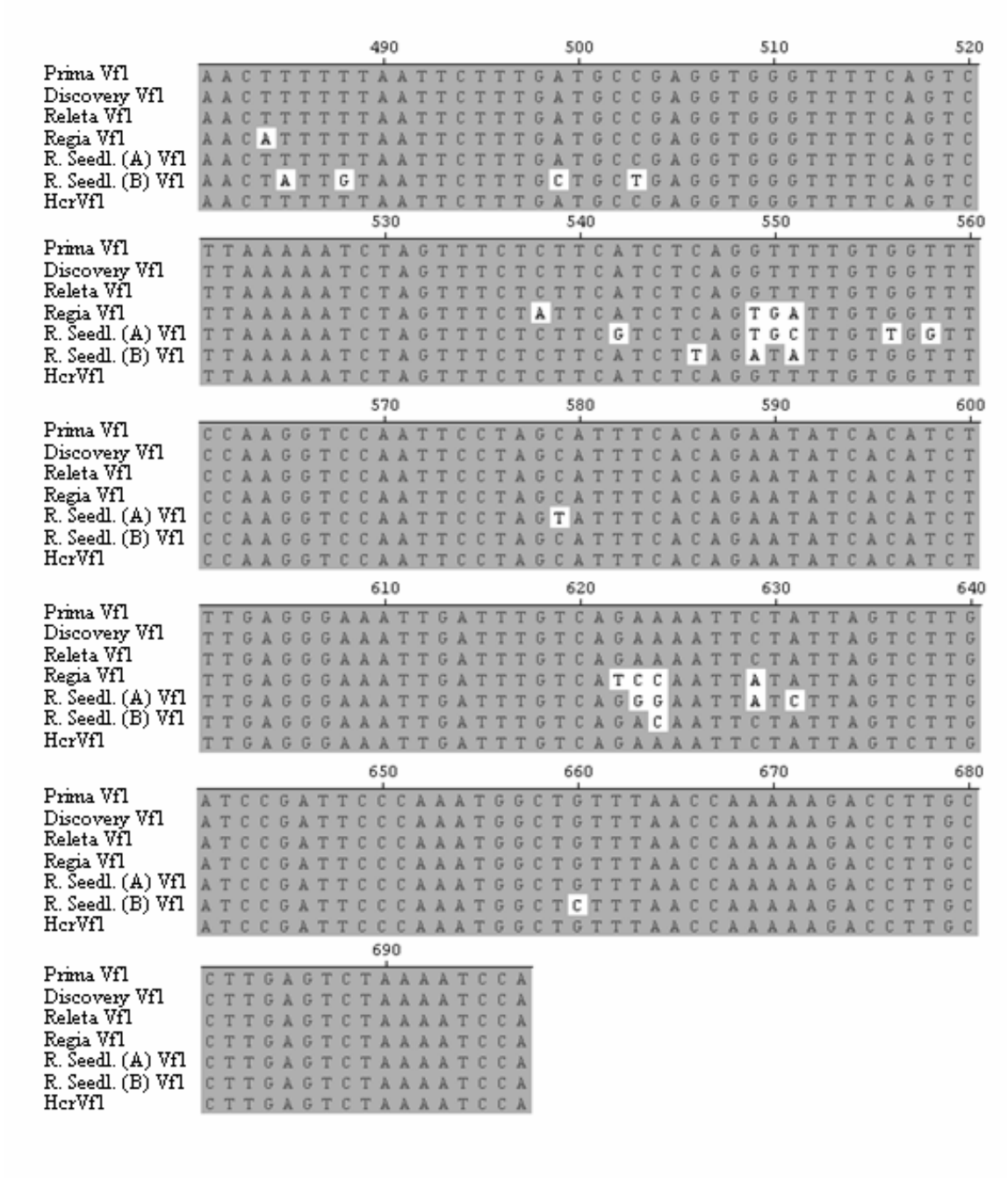
A 18: Multiple nucleotide sequence alignments of *HcrVf1* (GenBank acc. no. AJ297739) and their homologues found in some apple cultivars. Nucleotides identical to *HcrVf1* are indicated with grey background. Sequence gaps inserted to maintain the alignment are indicated by dashes



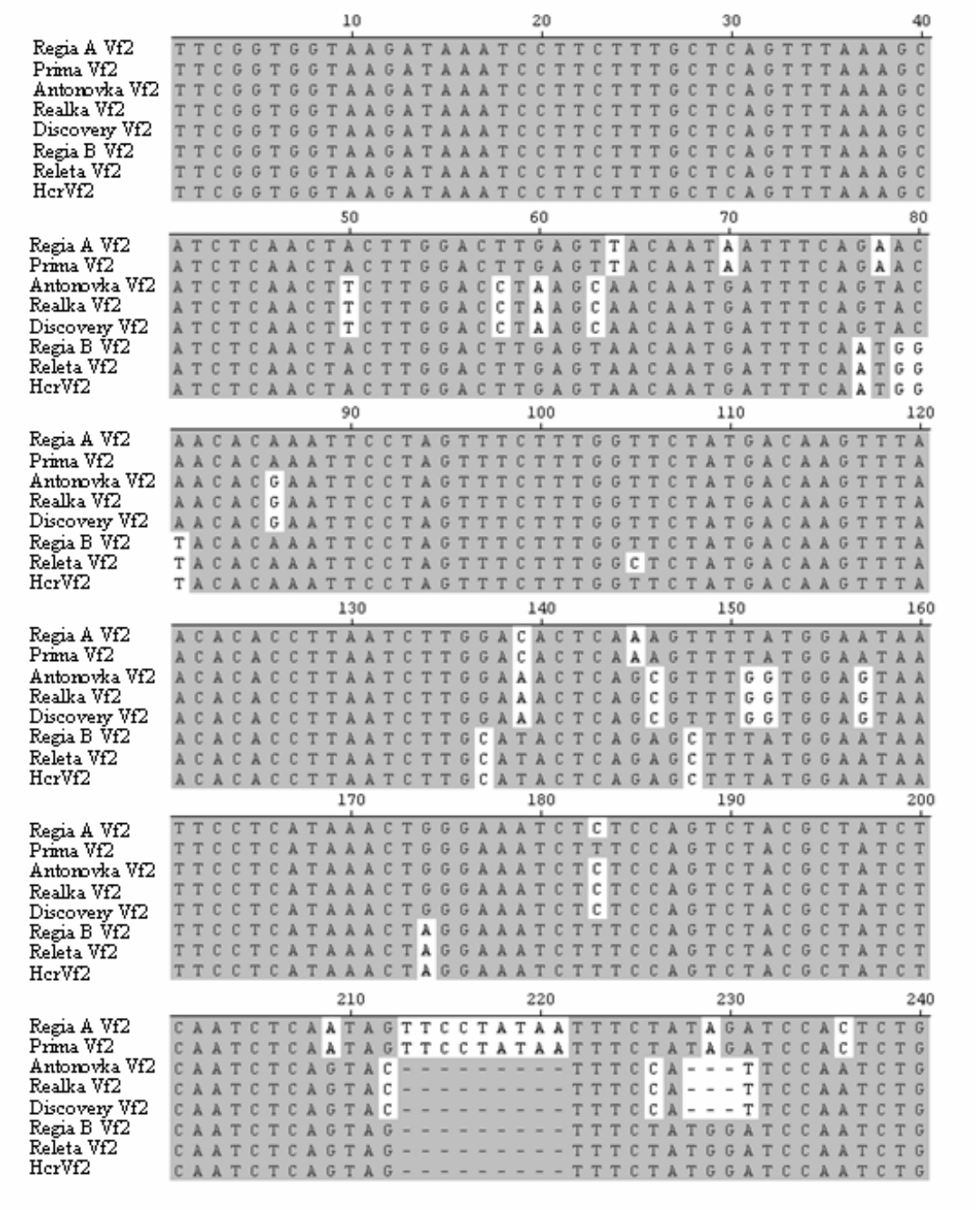
A 18 (continued): Multiple nucleotide sequence alignments of *HcrVf1* (GenBank acc. no. AJ297739) and their homologues found in some apple cultivars. Nucleotides identical to *HcrVf1* are indicated with grey background. Sequence gaps inserted to maintain the alignment are indicated by dashes



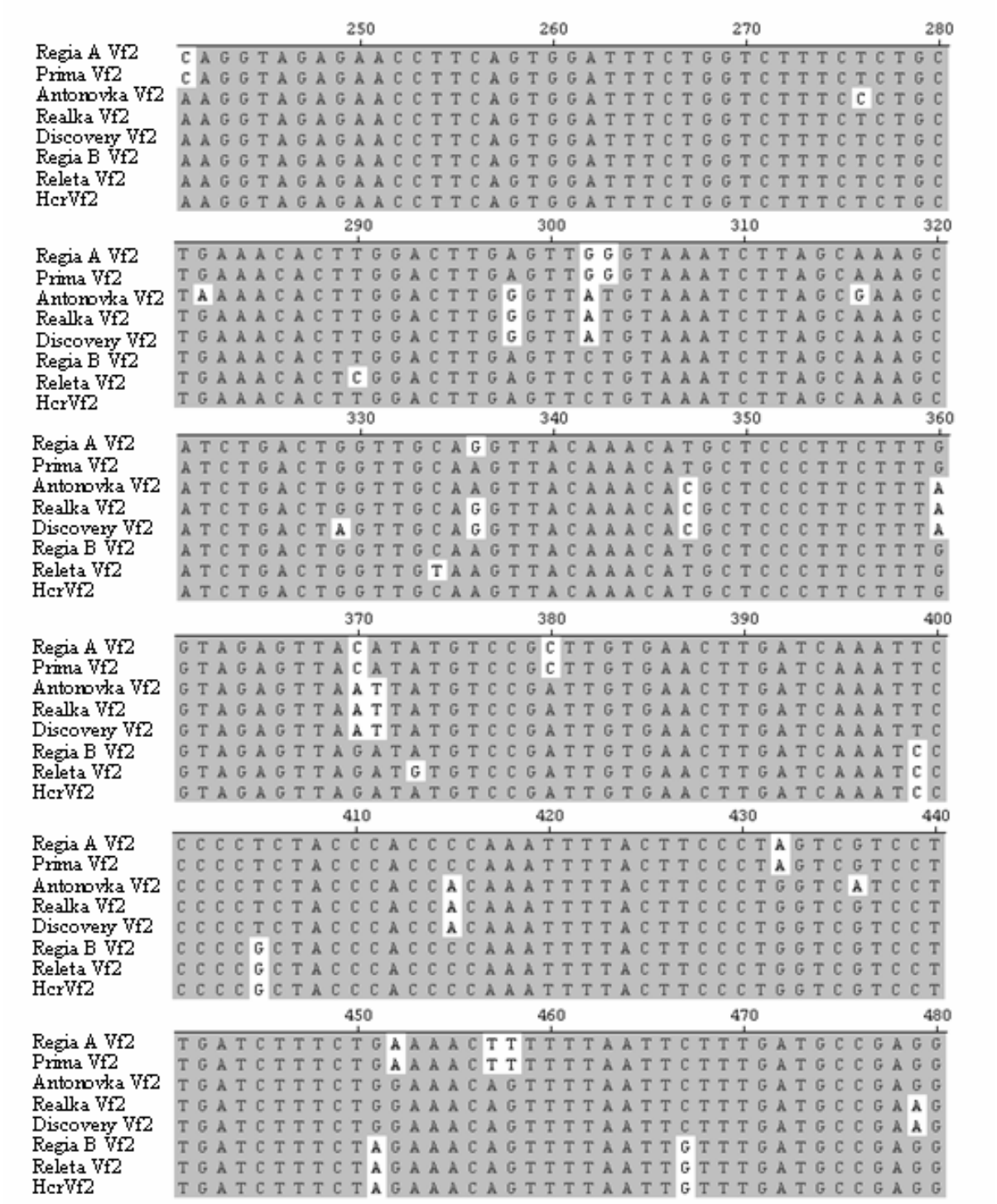
A 18 (continued): Multiple nucleotide sequence alignments of *HcrVf1* (GenBank acc. no. AJ297739) and their homologues found in some apple cultivars. Nucleotides identical to *HcrVf1* are indicated with grey background. Sequence gaps inserted to maintain the alignment are indicated by dashes



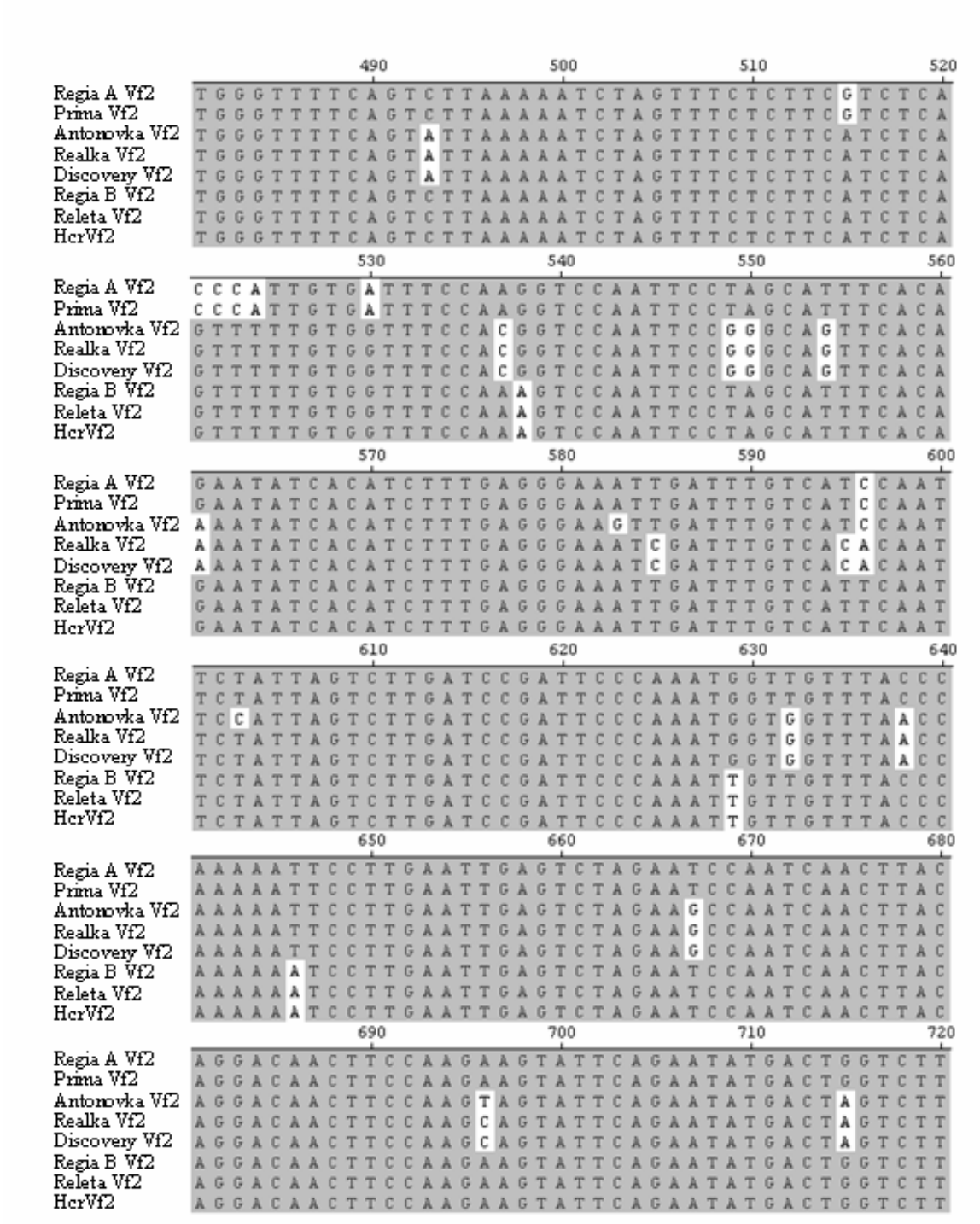
A19: Multiple nucleotide sequence alignments of *HcrVf2* (GenBank acc. no. AJ297740) and their homologues found in some apple cultivars. Nucleotides identical to *HcrVf2* are indicated with grey background. Sequence gaps inserted to maintain the alignment are indicated by dashes



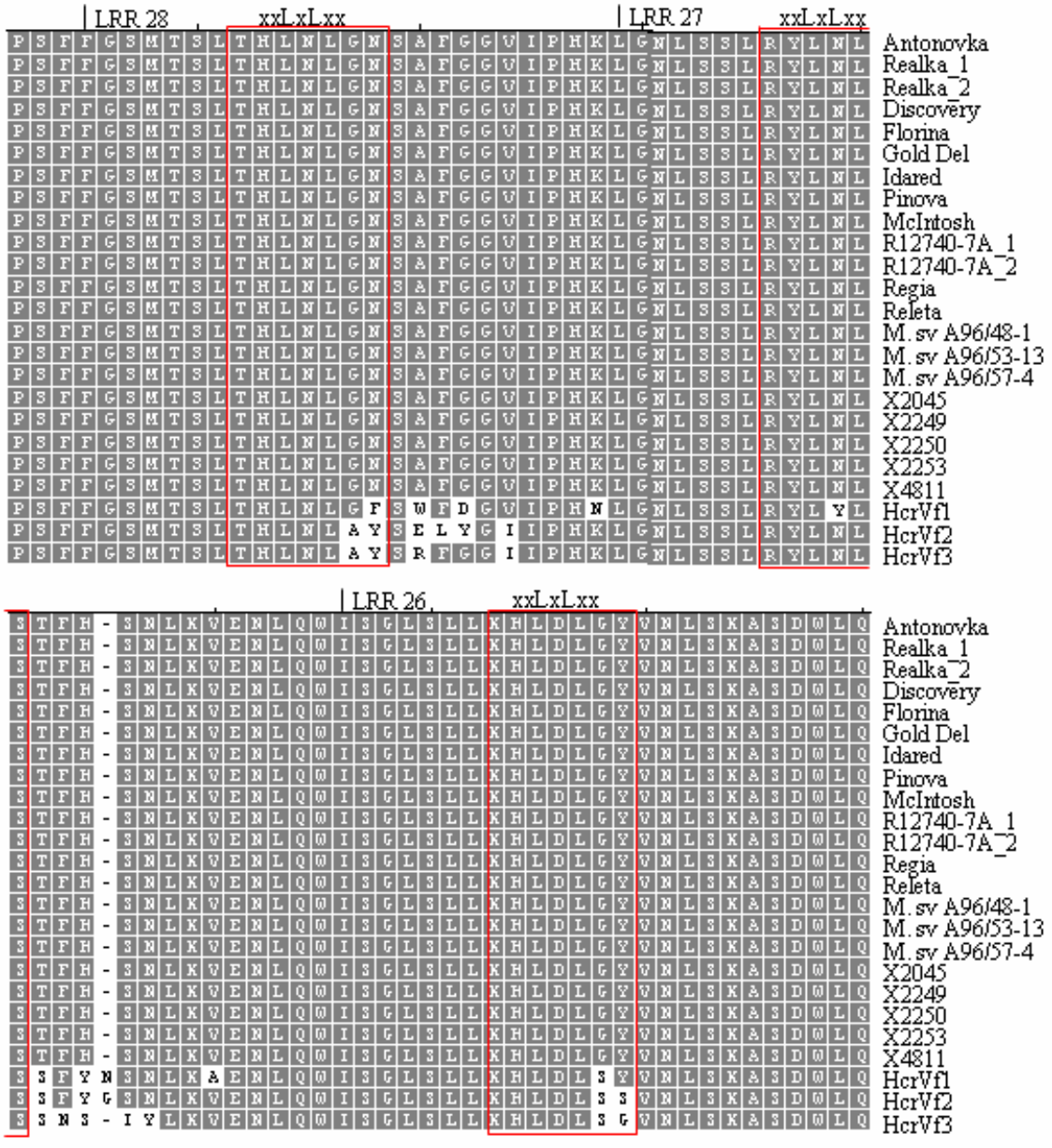
A19 (continued): Multiple nucleotide sequence alignments of *HcrVf2* (GenBank acc. no. AJ297740) and their homologues found in some apple cultivars. Nucleotides identical to *HcrVf2* are indicated with grey background. Sequence gaps inserted to maintain the alignment are indicated by dashes



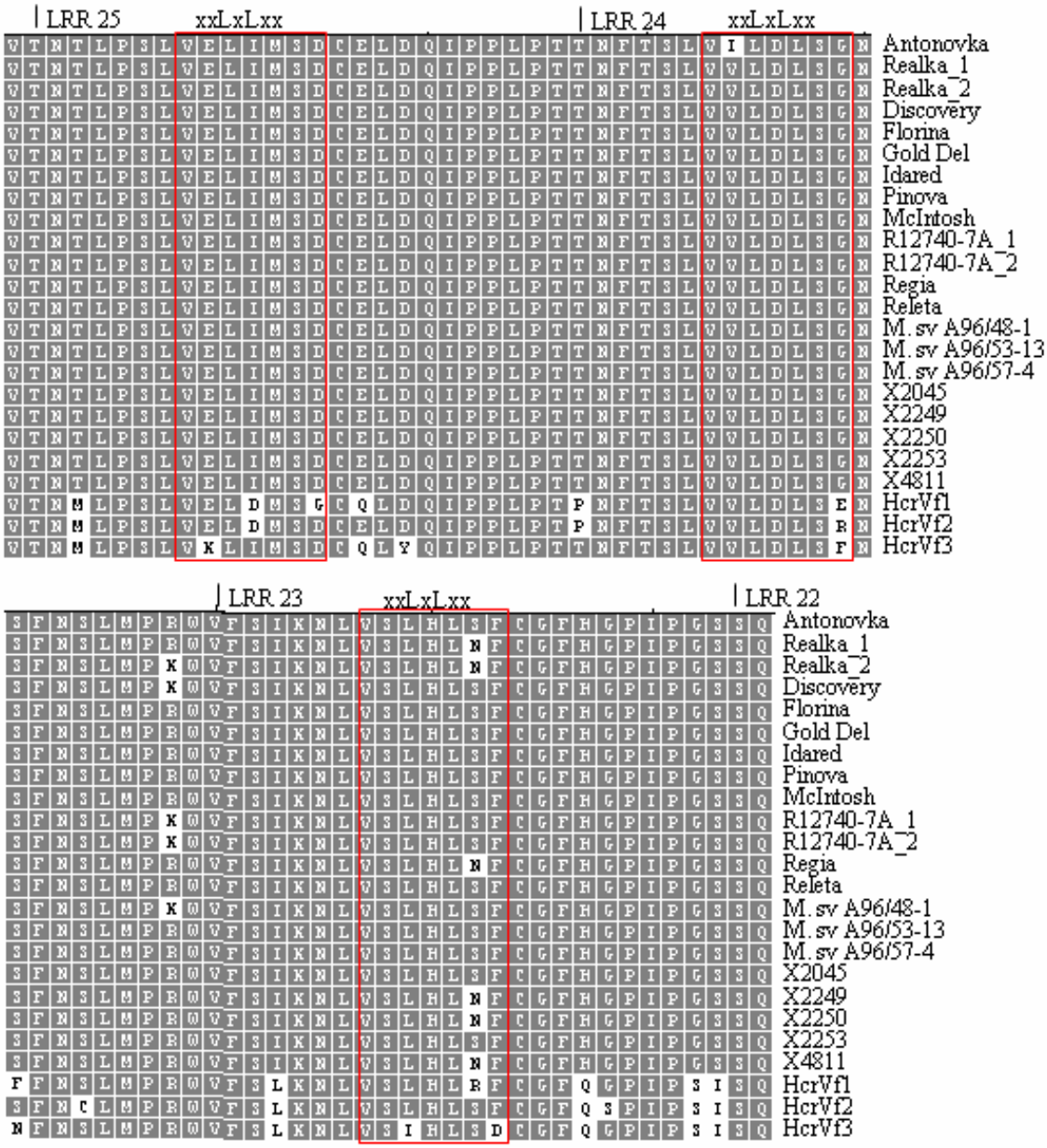
A19 (continued): Multiple nucleotide sequence alignments of *HcrVf2* (GenBank acc. no. AJ297740) and their homologues found in some apple cultivars. Nucleotides identical to *HcrVf2* are indicated with grey background. Sequence gaps inserted to maintain the alignment are indicated by dashes



A20: Multiple sequence alignments of *HcrVf1* (GenBank acc. no. AJ297739), *HcrVf2* (GenBank acc. no. AJ297740), *HcrVf3* (GenBank acc. no. AJ297741) and their *Vf2ARD* homologous sequences found in some apple cultivars. Identical amino acid residues are indicated with grey background. Sequence gaps inserted to maintain the alignment are indicated by dashes. LRRs 22-28 (C1 subdomain) are indicated above the sequence. Sequences that form part of the putative β-strand / β-turn conserved structural motif in LRR proteins (xxLxLxx, where L is leucine and x is any amino acid) are shown by the red dashed box



A20 (continued): Multiple sequence alignments of *HcrVf1* (GenBank acc. no. AJ297739), *HcrVf2* (GenBank acc. no. AJ297740), *HcrVf3* (GenBank acc. no. AJ297741) and their *Vf2ARD* homologous sequences found in some apple cultivars. Identical amino acid residues are indicated with grey background. Sequence gaps inserted to maintain the alignment are indicated by dashes. LRRs 22-28 (C1 subdomain) are indicated above the sequence. Sequences that form part of the putative β-strand / β-turn conserved structural motif in LRR proteins (xxLxLxx, where L is leucine and x is any amino acid) are shown by the red dashed box



ERKLÄRUNG

Hiermit erkläre ich, dass mit dieser wissenschaftlichen Arbeit noch keine vergeblichen Promotionsversuche unternommen wurden.

Desweiteren erkläre ich, daß keine Strafverfahren gegen mich anhängig sind.

Halle/Saale, den

Publikationen

Boudichevskaia, A., Flachowsky, H., Fischer, C., Hanke, V., Dunemann, F. (2004): Development of molecular markers for *Vr1*, a scab resistance factor from R12740-7A apple. In: F. Laurens, K. Evans (eds) XIth Eucarpia Symposium on Fruit Breeding and Genetics. Acta Hortic, 663: 171-175.

Boudichevskaja, A., Flachowsky, H., Dunemann, F. (2007): Identification and molecular characterisation of *Vf* candidate genes in cultivated apples and selections from *M. sieversii*. XII Eucarpia Symposium on Fruit Breeding and Genetics. 16 - 20.09. 2007; Zaragoza, Spain, 96.

Boudichevskaia, A., Flachowsky, H., Dunemann, F. (2008): Identification and molecular analysis of candidate genes homologous to *HcrVf* genes for scab resistance in apple. Plant Breeding, 127 (4), online.

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Dunemann, F., Boudichevskaja, A., Lesemann, S. (2005): Molekulare Forschung für die Züchtung von krankheitsresistenten Sorten mit hoher Fruchtqualität beim Apfel. Vorträge für Pflanzenzüchtung, Heft 67: 127-138.

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Kouassi, A. B., Laurens, F., Costa, F., Tartarini, S., Evans, K., Fernandez, F. Govan, C., Boudichevskaja, A., Dunemann, F., Antofie, A., Lateur, M., Stankiewicz-Kosyl, M., Soska, A., Tomala, K., Lewandowski, M., Rutkovski, K., Zueawicz, E., Guerra, W., Barbaro, E., Mott, D., Durel, C. E. (2007): Estimation of genetic parameters of apple fruit quality traits using pedigreed plant material from eight European countries. XII Eucarpia Symposium on Fruit Breeding and Genetics. 16-20.09 2007, Zaragoza, Spain: 53-54.

Patocchi, A., Fernández-Fernández, F., Evans, K., Gobbin, D., Rezzonico, F., Boudichevskaia, A., Dunemann, F., Stankiewicz-Kosyl, M., Mathis-Jeanneteau, F., Durel, C. E., Gianfranceschi, L., F. Costa, F., Toller, C., Cova, V., Mott, D., Komjanc, M., Barbaro, E., Kodde, L., E. Rikkerink, E., Gessler, C., van de Weg, W. E. (2008): Development and test of 21 multiplex PCRs composed of SSRs spanning most of the apple genome. Tree Genetics & Genomes, online First ™ 13.

Patocchi, A., Fernandez, F., Evans, K., Silfverberg-Dilworth, E., Mataschi, C. L., Gobbin, D., Rezzonico, F., Boudichevskaja, A., Dunemann, F., Stankiewicz-Kosyl, M., Matisse, F., Soglio, V., Gianfranceschi, L., Durel, C. E., Toller, C., Cova, V., Mott, D., Komjanc, M., Barbaro, E., Costa, F., Voorrips, R., Yamamoto, T., Gessler, C., van de Weg, E. W. (2007): Development of a set of apple SSRs markers spanning the apple genome, genotyping of HIDRAS plant material and validation of genotypic data. XII Eucarpia Symposium on Fruit Breeding and Genetics. September 16-20, 2007, Zaragoza, Spain: 55-56.

Soufflet-Freslon, V., Kouassi, A. B., Laurens, L., Mathis, F., Gobbin, D., Patocchi, A., Rezzonico, F., Evans, K., Fernandez, F., Boudichevskaja, A., Dunemann, , Stankiewicz-Kosyl, M., Komjanc, M., Mott, D., Gianfranceschi, L., van de Weg, E., Bink, M., Durel, C. E. (2007): Pedigree-based mapping of scrab resistence QTL in apple. XII Eucarpia Symposium on Fruit Breeding and Genetics. September 16-20, 2007, Zaragoza, Spain: 94-95.

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Poster und Vorträge

First German Meeting on Woody Plant Genomics
Vortrag: Development of DNA-markers for *Vr* conferring resistance to apple scab. Grosshansdorf/Ahrensburg, Germany, 06-09.10.2002.

Congress “In the wake of the double helix. From the green revolution to the gene revolution”
Boudichevskaia, A., Fischer, C., Flachowsky, H., Dunemann, F.
Poster: Development of Molecular Markers for a *Vr* Scab Resistance Factor from R12740-7A Apple. University, Bologna, Italy, 27-31.05.2003.

11th Molecular Markers Symposium of the GPZ
Boudichevskaia, A., Fischer, C., Flachowsky, H., Dunemann, F.
Poster: Development of Molecular Markers for a *Vr* Scab Resistance Factor from R12740-7A Apple. IPK Gatersleben, Germany, 16-17.09.2003.

3rd International Rosaceae Genomics Conference
Dunemann, F., Boudichevskaja, A., Lesemann, S. Poster:
Molecular research for breeding disease resistant apple cultivars with high fruit quality. Napier, New Zealand, 19-22.03.2006.

Plant Genetics Conference
Boudichevskaia, A., Peil, A., Dunemann, F. Poster: Cloning and characterization of NBS – LRR and receptor – like protein (RLP) – type disease resistance genes in apple. Kiel, Germany, 20-23.09.2006.

First International Symposium on Biotechnology of Fruit Species
Dunemann, F., Boudichevskaia, A., Grafe, C., Ulrich, D. Poster:
QTL and candidate gene mapping for aroma compounds in the apple progeny 'Discovery' x 'Prima'. Dresden, Germany, 01-05.09.2008.

Lebenslauf

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