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TESI DI DOTTORATO DI RICERCA

Isolation and characterisation of the *Rvi5 (Vm)* apple scab resistance gene in *Malus ×domestica.*

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Summary

Apple scab, caused by the fungal pathogen *Venturia inaequalis* (Cke.) Wint.is the major disease affecting apples grown in temperate climates around the world. The disease can cause a significant economic impact as diseased fruit are not marketable. Complete crop loss is possible if appropriate control measures are not taken. Control of the disease is achieved through good cultural practices, such as leaf litter reduction to prevent primary inoculum production, but prevention is primarily achieved through frequent applications of fungicides. Whilst the application of chemical fungicides is effective at controlling the spread of the disease, they are not environmentally friendly, and *V. inaequalis* has successfully developed resistance against some fungicides. Thus, there is a need for more sustainable methods to control the disease, and as a result, research into identifying and exploiting natural resistance to scab from *Malus* germplasm is well established.

Breeding for apple scab resistance primarily involves the introgression of resistance genes from the varieties of cultivated and wild apple species, and to date, a total of 17 scab resistance loci have been characterised. The apple scab resistance gene *Rvi6* (Vf) derived from the Asiatic wild apple accession *Malus floribunda* 821 is the most studied and mostly utilized scab resistance gene in resistance breeding programs. However, identification of *V. inaequalis* isolates that can overcome natural *Rvi6*-based resistance demonstrates the importance of the use of various sources of resistance in apple varietal development and highlights the need to develop durable resistance through pyramiding of resistant genes from various sources.

The apple scab resistance gene *Rvi5*, mapped on the distal end of LG 17 believed to be inherited from *Malus micromalus 245-38* and *Malus atrosanguinea 804*, described as a pit type gene induces hypersensitive response for the pathogen of apple scab after 3-5 days of inoculation. *V.inaequalis* race 5 has overcome the gene, which is first discovered in England. However, recent surveys conducted under the frame of VINQUEST initiative,

suggested that this virulence is not widely widespread. The well-studied apple scab resistance gene *Rvi15* also induces the hypersensitive response for the pathogen, but *Rvi15* elicits slow response compared to the *Rvi5*, which requires 15 days to elicit the resistance symptoms after the inoculation. The time differences required to elicit the symptoms in *Rvi15* and *Rvi5* may be due to the different genes responsible for the resistance reaction. The limited spread of *V.inaequalis* race 5 and the differences of time required to elicit the resistance reaction with respect to *Rvi15*, makes this gene interesting to study aiming resistance gene pyramiding. The *Rvi5* gene was found to segregate for a single dominant locus and a co-segregating molecular marker linked with the resistance has been reported.

For the purpose of isolation and characterisation of the *Rvi5* (Vm) apple scab resistance gene, we first fine mapped the region surrounding the *Rvi5* locus using 1243 progeny plants of 'Golden Delicious' × 'Murray' and 'Galaxy' × 'Murray', which permitted to delimits the locus controlling the resistance to a 1 cM in 'Murray' genome and developed three co-segregating markers for the resistance gene (Vm_ SCAR1, Vm_SNP5, FMACH_Vm3). The locus controlling the resistance was flanked by newly designed two molecular markers, SSR FMACH_Vm2 and SSR FMACH_Vm4. The Bacterial Artificial chromosome (BAC) library of 'Murray' was first screened for the resistance allele of SSR FMACH_Vm2 and continued by chromosome walking until found a positive clone for the SSR FMACH_Vm4 to isolate the *Rvi5* resistance locus. Three bacterial artificial chromosome (BAC) clones spanning the resistance locus were identified, completely sequenced and assembled, which allowed identifying the putative *Rvi5* locus in a region of 154kb in size. The open reading frame prediction and functional annotation of the identified region revealed the presence of one putative gene homologous to TMV resistance protein of *Malus* × *domestica*, characterized by a Toll and mammalian interleukin-1 receptor protein nucleotide-binding site leucine-rich repeat structure.

The identified candidate gene has been transformed to the scab susceptible cultivar ‘Gala’ with the help of gateway directional cloning technology and *Agrobacterium* mediated transformation. Plant DNA extracted from obtained transgenic plants was used to confirm the insertion of the gene of interest. Five confirmed transgenic ‘Gala’ plants with inserted *Rvi5* candidate gene were obtained as first transgenic plants inserted with *Rvi5* gene. Before conducting the scab inoculation experiments, transgenic plants are needed in order to undertake multiplication and regeneration to obtain sufficient plant materials for inoculation experiments. Once a sufficient amount of plant material has been obtained from transgenic plants, the shoots will be micro-grafted onto ‘Golden Delicious’ rootstocks. When the micro-grafted shoots reach the ten leaf stage, scab inoculation experiments will be continued with monoconidial *V. inaequalis* isolates, with differential interaction with *Rvi5*.

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1. INTRODUCTION

1.1 The apple: origin, distribution and economic importance

The cultivated apple, *Malus* × *domestica* Borkh, is one of the most widely cultivated fruit tree species in the world (MacHardy 1996). The apple belongs to the *Malus* genus, in the Maloideae subfamily of the Rosaceae family. The main temperate fruit crops, such as the peach (*Prunus persica*), cherry (*Prunus avium* and *Prunus cerasus*), plum (*Prunus domestica* and *Prunus salicina*), apricot (*Prunus armeniaca*), pear (*Prunus communis*) and strawberry (*Fragaria* × *ananassa*) are also members of the Rosaceae family. Apples are cultivated throughout the temperate region. They can adapt to conditions ranging from the extremely cold regions of northern China to the much warmer environments of Colombia and Indonesia.

It is widely accepted that the domesticated apple originated in the mountain ranges of Central Asia (Harris et al. 2002). In early Neolithic times, this region was crossed by famous trade routes that ran from Rome (Italy) to Luoyang (China), through Samarkand in Uzbekistan. It is believed that travellers or their domesticated animals, especially horses, carried the seeds of the Asian wild apple to the West, either in saddle bags or in horse gut. Although it is difficult to determine exactly when the apple was first domesticated, there is archaeological evidence showing that the Greeks and Romans cultivated apples at least 2,500 years ago. The Romans introduced and spread the apple across the European and Mediterranean areas and it was taken to the new world by European settlers during the sixteenth century. In terms of apple domestication, the *Malus* genus is the most important group of species.

Most of the *Malus* species are diploids (2n=34), but there are a few triploids and tetraploids (Way et al. 1989). The *Malus* genus belongs to the Maloideae subfamily (Challice 1974). This subfamily is considered to be an allopolyploid that evolved due to

hybridization between speroideae (x=9) and Prunoideae (x=8), leading to the basic haploid number of x=17 (Lepinasse et al. 1999). Apples are largely self-incompatible and some are apomictic. They are usually propagated in a vegetative manner, with separate root stocks and scions.

The domesticated apple has been referred to as *M.domestica Borkh*, but more recently it was argued that the correct nomenclature is *Malus pumila* Mill (Korban and Skirvin 1984) and the species should include the wild apple identified as *M.sylvestris* (L) (Mabberley et al. 2001). When describing the centres of origin of cultivated plants Vavilov (1951) also referred to the wild apple as *M.pumila*, which is in direct conflict with Ponomarenko, a well-known Russian botanist, who denied the existence of this species (Way et al. 1989). However, the relatedness of wild and domesticated apples is demonstrated by the small degree of morphological, biochemical, and molecular variation between the two species (Harris et al. 2002). Together with *M.sieversii*, the European wild crab apple *M.sylvestris* also belongs to *M.pumila* Mill (Way et al.1989) and may have been the result of separate introduction of the wild apple into Europe.

Apples are the second largest fruit crop in the world and today global apple production exceeds 80 million metric tons (FAOSAT, 2013). China is the largest producer and produces almost half of the total. The United States is the second largest producer, with more than 6% of world production. Turkey is third, followed by Italy, India and Poland. However, most major producers do not export their products, as they have large internal markets, and a large amount of fruit is probably processed. Being an extreme versatile crop, apples can be eaten directly from the tree or can be stored for up to a year in a controlled atmosphere. They can be transformed into juice, sauce and slices. There are more than 7,500 known cultivars of apples, with a range of desired traits. Different

cultivars are bred for various tastes and uses, including cooking, eating raw and cider production.

1.2 Diseases of the apple

Apple trees are susceptible to several pests and diseases including bacteria, fungi, virus and phytoplasma (Way et al. 1991). Of these, few cause a severe loss of yield or the death of plants. Fire blight (*Erwinia amylovora)* is the most devastating bacterial disease, affecting all parts of the tree and leading to its death. Apple scab (*Venturia inaequalis*) and powdery mildew (*Podosphaera leucotricha*), are the main fungal pathogens that lead to severe losses. Of these pathogens, *Venturia inaequalis* (Cooke) G.Wint., is one of the most devastating diseases for apples, particularly in temperate zones with humid growing seasons. Both leaves and fruit can be affected by this fungal pathogen. If uncontrolled, the fungus may defoliate trees and blemish fruits to a point where they are unmarketable, with significant production losses.

1.2.1 Apple scab

Apple scab is the most devastating fungal disease, caused by the ascomycete fungus *Venturia inaequalis* (Cke) Wint. The pathogen has a negative economic impact, due to yield losses, and has been reported to have wide geographical range, so it has been found in almost all areas in which apples are cultivated commercially. The severity of the pathogen is high in temperate countries with cool humid climates in the early spring (Machardy et al. 1996). Apple scab attacks leaves, petioles, blossoms, sepals, fruits, pedicels and less frequently, young shoots and bud scales. Slightly bulging black-olive brown lesions on the upper leaf surface and deformed corky fruits are the most common symptoms that can generate economic loss. Leaf infections can cause premature foliar dissemination and the ultimate result is yield reduction, due to the slow growth of plants. If the infection takes place on fruits it affects the shape and size of the fruits and causes a

loss in the commercial value of fruit. Apple scab can lead to yield losses of up to 70% if it is not treated. (Jha et al. 2009)

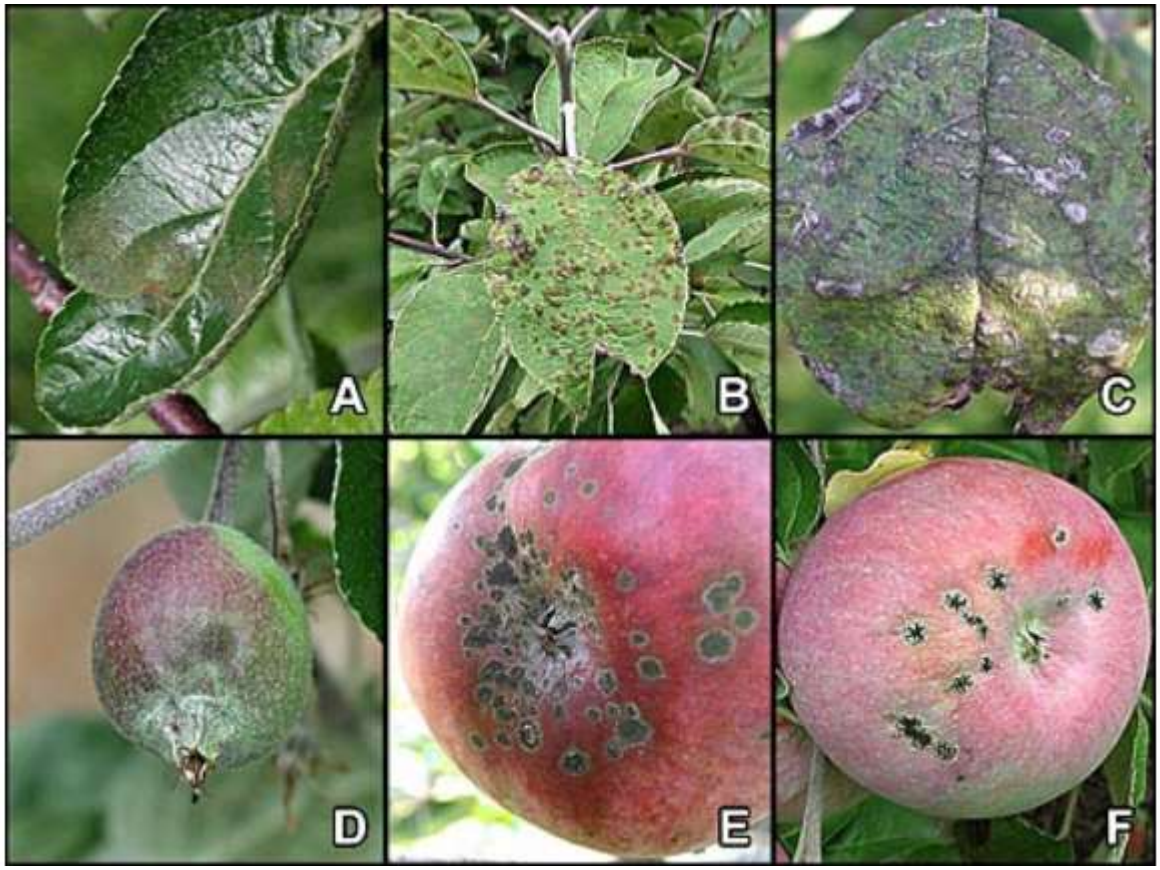

Figure 1: Apple scab symptoms on different apple tissue parts
Symptoms on (A) a cluster leaf (B) a terminal leaf (C) old or exhausted apple scab lesions on a terminal leaf (D) young lesion on a developing fruit. (E) and (F). well-developed lesions (E) on a mature fruit with characteristic cracking (Turechek and Koller 2004)

1.2.2 Taxonomy of the pathogen: Scientific classification

Venturia inaequalis is a heterothallic ascomycetes fungus with seven haploid chromosomes, that reproduces both asexually and sexually (Day et al. 1956). Debate over the taxonomic classification of many fungal species is also affected by the taxonomy of *V.inaequalis*. Until very recently, *V. inaequalis* (Cooke) Wint. was classified under the *Venturia genus,* Venturiaceae family, Pleosporales order, Dothideomycetes class, Pezizomycotina subdivision, Ascomycota division in the Fungi kingdom (Eriksson and Hawksworth 1998; Godwin 2004). In a very recent study conducted on members of

Dothideomycetes using comprehensive molecular phylogenetic data, the venturiaceae family was placed outside the Pleosporales order, without assigning a subclass or order. (Kruys et al. 2006; Schoch et al. 2009). There is also some controversy regarding classification related to the anamorph stage of fungi. Schubert et al. (2003) proposed that the anamorph of *V.inaequlis* should be classified as *Fusicladium pomi* (Fr.) Lind, replacing the old classification of *Spilocaea pomi* (Fr.), although this has not been universally accepted and researchers in the USA still use the old name of S.pomi. (Bini et al. 2008; Jha et al. 2009).

1.2.3 Life cycle

V. inaequalis is a hemibiotrophic fungus that passes the winter forming pseudothecia (sexual fruiting bodies) inside infected fallen leaf tissues. Pseudothecia are formed only from heterothallic mating, requiring two different mating types (Keitt et al. 1938). Each Pseodothecium contain many asci, each containing four pairs of ascospores resulting from meiotic division, followed by mitotic division. Due to rain and high humidity in spring or early summer, pseudothecia releases ascospores which have ability to germinate and penetrate through the cuticle. This penetration can happen either directly or via an approssorium and the development of stromata. Stromatas are presumed to help obtain nutrients from the sub cuticular space. Conidia (secondary inoculum) develop from the upper surface of the stromata and are produced throughout the growing season, initiating multiple rounds of infection.

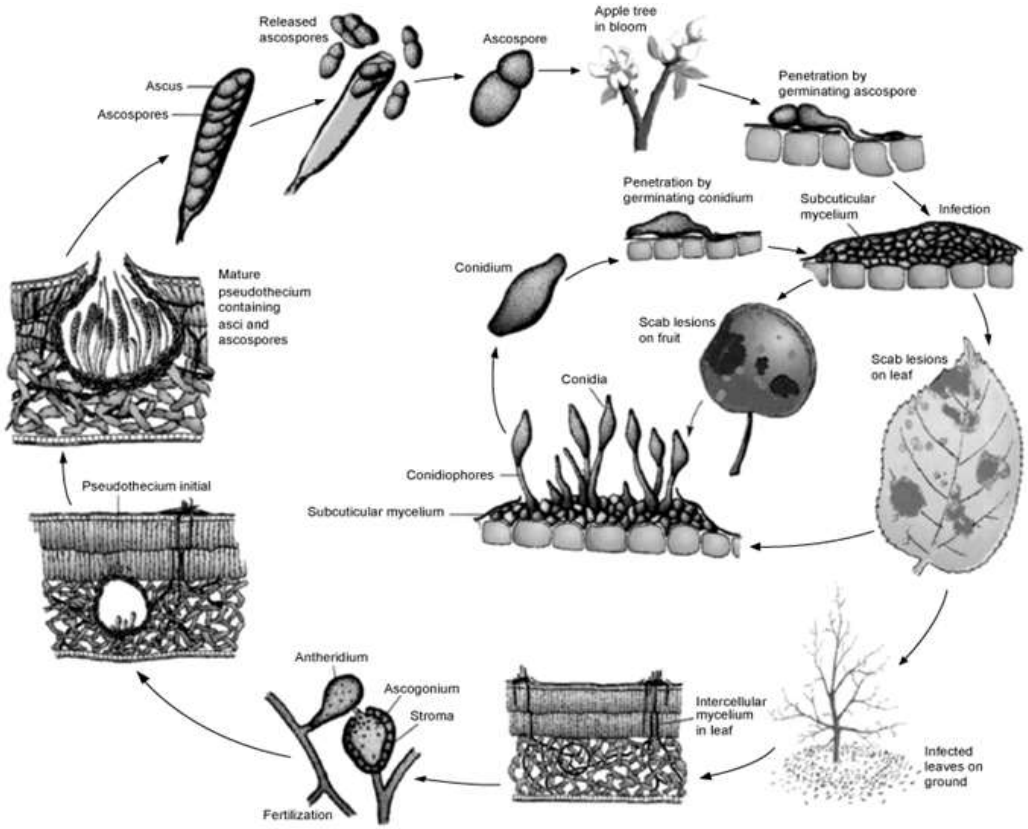

Figure 2: The life cycle of *Venturia inaequalis*

1.2.4 Plant Symptoms

Signs associated with apple scab are evident to most plant pathologists and apple farmers; however these signs are only significant in the case of fully compatible interaction. The characteristic circular black, olive–brown clearly-defined lesions, often slightly bulging out from the upper leaf surface and corky spots with or without deformed fruit shape are symptoms which lead to a loss in commercial value. The variability of symptoms in terms of intensity and type can be due to ontogenic resistance or interaction between particular cultivars or genotypes and different pathogen genotypes.

Ontogenic resistance is observable in all apple cultivars and never appears to be overcome by the pathogen. It provides protection against scab on mature tissues by reducing the frequency and extent of stroma formation and sporulation with the increasing age of host

tissue (Gessler and Stumm 1984). The basis of ontogenic resistance is still unknown but it is clearly evident. Ontogenic resistance ceases at the end of the growing season when the leaves start to senesce and dormant lesions start their development even in mature leaves (Kollar 1996). However, senescence may compromise the ability of the plant to induce the resistance response, although the appearance of small black spots mainly on the underside of the leaf may be the result of earlier infection during the season that was blocked due to ontogenic resistance.

Particular symptoms are correlated to the presence of a particular type of resistance in the host plants. The hypersensitive response or appearance of small pin point pits and tissue browning on foliar tissues 72 hours after pathogen infection is historically associated with *Rvi5* resistance (Dayton and Williams 1968). However, more recently hypersensitivity has been reported with various other forms of resistance.

The host pathogen interaction of the *Malus* genus with *V.inaequalis* was described and classified by Hough (1944) using a quantitative 5 class system, with 0 representing no visible symptoms, 1 the hypersensitive response or pin point pits observed in *Rvi5* resistance, 2 flecks or necrotic lesions with no sporulation, 3 a few restricted lesions with sporulation and 4 susceptible interaction with abundantly sporulated lesions. This classification was further refined by introducing classes 3a and 3b Chevalier et al. (1991), by considering the microscopic observations of the progeny reaction of *Rvi6* (*Vf*) resistance. In Chevalier's classification system, class 1 is the availability of the typical hypersensitive response, with extended and rapid cell death including changes in palisade tissue in the cell in contact with the pathogen; the reactions associated with classes 2,3a and 3b are described by the presence of necrotic sub-cuticular stroma of fungus and significant modification of epidermal and palisade cells. The differences between classes 2, 3a and 3b are quantitative and lead to class 4. The symptom variability associated with

Rvi6 (*Vf)* resistance spread over classes 0, 2, 3a and 3b with a few individuals in class 4 (Gianfranceschi et al. 1996), but other scab resistance genes elicited more specific symptoms, for example *Rvi2* (*Vh2)* led to stellate necrosis 4-6 days after inoculation (Bus et al. 2005a), *Rvi5* (*Vm)* and *Rvi4* (*Vh4)* elicited the classic hypersensitive response, while *Rvi12* (*Vb)* and *Rvi11* (*Vbj)* elicited flecks from yellow to necrosis and necrosis with sparse sporulation similar to *Rvi6* (*Vf)* resistance.

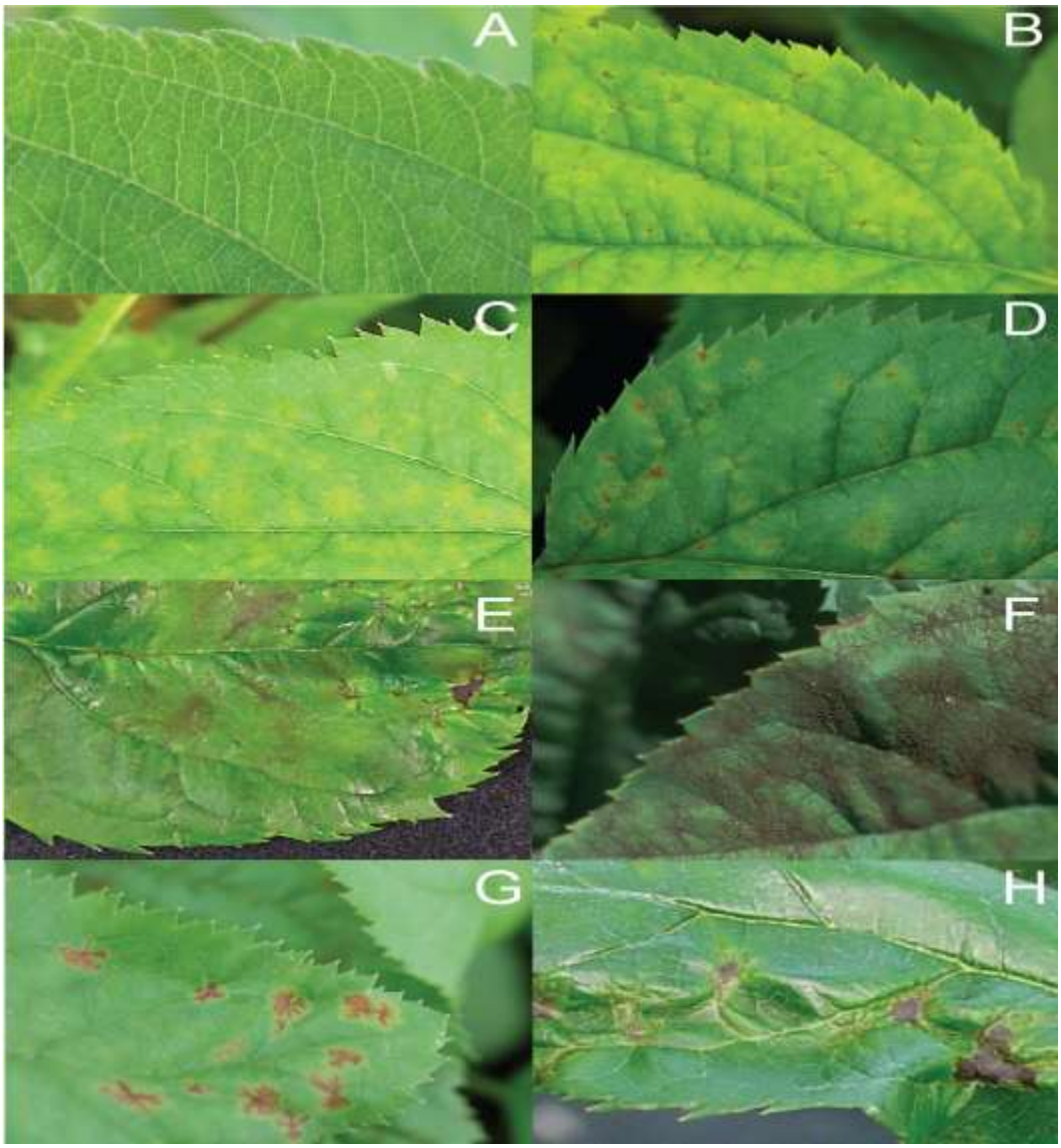

Figure 3: Characteristic scab resistance reactions on apple leaves.
A- No symptoms (Class 0), B-Hypersensitive response (Class 1), C–Chlorosis (Class 2), D- Chlorosis with necrotic lesions (class 3a), E- Light sporulation (Class 3b) F-Complete susceptibility (Class 4),G- Stellate necrosis, H- Necrosis with wrinkled leaves (Vg)

1.2.5 Pathogen Races

V.inaequalis populations are hyper-variable and exhibit differential pathogenicity on differential hosts. The annual sexual phase followed by asexual multiplication during the growing season allows opportunities for the adaptive selection of new strains. The old *V.inaequalis* pathogenicity race nomenclature was a simple system based on differences between pathogens and had eight physiological races. The term physiological race of *V.inaequalis* is used for a particular pathogen population that has the ability to induce a common range of virulence/avirulence on a particular genotype/differential host.

Table 1 : Pathogenicity of different *Venturia inaequalis* races on the range of differential host cultivars (Gessler et al.2006)

		Venturia inaequalis races[b]							
Differential cultivars	R gene	1	2	3	4	5	6	7	8
Royal Gala (h1)	None	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
Dolgo (h2)	None	R	**S**	R	R	R	R	R	R
TSR34T132 (h2)[a]	*Vh2*	R	**S**	**S**	R	R	R	R	R
Geneva (h3)	Not named	R	**S**	**S**	R	R	R	R	R
TSR33T239 (h4)	*Vh4*	R	R	R	**S**	R	R	R	R
9-AR2T196 (h5)	*Vm*	R	R	R	R	**S**	R	R	R
Florina (h6)[a]	*Vf* and *Vg*	R	R	R	R	R	**S**	R	n.a.
M. floribunda 821 (h7a)	*Vf*	R	R	R	R	R	R	**S**	R
Golden Delicious (h7b)	*Vg*	**S**	**S**	**S**	**S**	**S**	**S**	R	**S**
M. sieversii W193B (h8)	*Vh8*	R	**S**	n.a.	n.a.	n.a.	n.a.	n.a.	**S**

[a]The differential hosts for race 2 and 6 in Bus *et al.* (2005b) were TSR34T15 and Prima.
[b]n.a. = data not available.

Race 1: well sporulating on popular domestic cultivars and elicits flecks or necrotic lesions without sporulation on the 'Dolgo', R12740-7A and 'Geneva' Malus clones . (Shay and Williams 1956)

Race 2: sporulates on 'Dolgo', 'Geneva' and some progenies of R12740-7A.

Race 3: sporulating lesions on 'Geneva', non sporulating lesions on 'Dolgo' and R12740-7A.

Race 4: non sporulating lesions on ‘Dolgo’, ‘Geneva’ and sporulating lesions on the progenies of R12740-7A, on which race 2 cannot sporulate.

Race 5: sporulation lesions on Vm (*Rvi5)* resistance gene containing cultivars.

Race 6: Sporulating lesions on *Rvi6* (*Vf)* hybrids but cannot infect *Malus floribunda* 821 containing the *Rvi7* (*Vfh)* resistance gene.

Race 7: Cannot infect cultivars with the *Rvi6* (*Vf)* and *Rvi7* (*Vfh)* resistance gene and cannot infect ‘Golden Delicious’, which contains Rvi1 (*Vg)* gene.

Race 8: Infects ‘Golden Delicious’, ‘Royal Gala’ and cultivars containing the *Rvi8* (*Vh8)* resistance gene.

However, some of the *Venturia Inaequalis* races are able to infect more than one differential host, in which case they are difficult to assign to a particular race using the available simple numeric nomenclature. To improve the nomenclature of *Venturia inaequalis* races, Bus et al. 2011, proposed a new nomenclature system. They replaced differential hosts with more than one resistance gene in the previous nomenclature system with new accessions/selections that carry only one resistance gene. Furthermore, the new nomenclature system can be updated with new differential hosts when new resistance genes are discovered. According to the new nomenclature system, race determination is based on the combination of resistance genes and pathotypes that have the ability to overcome the particular resistance gene, hence the number of races is equal to the number of major resistance genes identified.

Table 2: Nomenclature for the gene-for-gene relationship between *V.inaequalis* and *Malus*.
The races are defined by the avirulence genes they are lacking, resulting in the susceptibility of the complementary host.

Malus						Venturia inaequalis		
Differential host			Resistance locus			Avirulence locus		
Number	Accession	Phenotype	Historical	LG[a]	New	New	Old	Race
h(0)	Royal Gala	susceptibility			–	–		(0)
h(1)	Golden Delicious	necrosis	*Vg*	12	*Rvi1*	*AvrRvi1*		(1)
h(2)	TSR34T15	stellate necrosis	*Vh2*	02	*Rvi2*	*AvrRvi2*	p-9	(2)
h(3)	Geneva[b]	stellate necrosis	*Vh3*	04	*Rvi3*	*AvrRvi3*[b]	p-10	(3)
h(4)	TSR33T239	hypersensitive response	*Vh4* = *Vr* = *Vrf*	02	*Rvi4*	*AvrRvi4*[b]		(4)
h(5)	9-AR2T196	hypersensitive response	*Vm*	17	*Rvi5*	*AvrRvi5*		(5)
h(6)	Priscilla	chlorosis	*Vf*	01	*Rvi6*	*AvrRvi6*		(6)
h(7)	*Malus x floribunda* 821[c]	hypersensitive response	*Vfh*	08	*Rvi7*	*AvrRvi7*		(7)
h(8)	B45	stellate necrosis	*Vh8*	02	*Rvi8*	*AvrRvi8*		(8)
h(9)	K2	stellate necrosis	*Vdg*	02	*Rvi9*	*AvrRvi9*	p-8	(9)
h(10)	A723–6[c]	hypersensitive response	*Va*	01[c]	*Rvi10*	*AvrRvi10*[b]		(10)
h(11)	A722–7	stellate necrosis/chlorosis	*Vbj*	02	*Rvi11*	*AvrRvi11*[b]		(11)
h(12)	Hansen's baccata #2[b]	chlorosis	*Vb*	12	*Rvi12*	*AvrRvi12*[b]		(12)
h(13)	Durello di Forlì	stellate necrosis	*Vd*	10	*Rvi13*	*AvrRvi13*[b]		(13)
h(14)	Dülmener Rosenapfel[b]	chlorosis	*Vdr1*	06	*Rvi14*	*AvrRvi14*[b]		(14)
h(15)	GMAL2473	hypersensitive response	*Vr2*	02	*Rvi15*	*AvrRvi15*[b]		(15)
h(16)	MIS op 93.051 G07–098[b]	hypersensitive response	*Vmis*	03	*Rvi16*	*AvrRvi16*[b]		(16)
h(17)	Antonovka APF22[b]	chlorosis	*Va1*	01	*Rvi17*	*AvrRvi17*[b]		(17)

[a]LG = linkage group of apple.
[b]Temporary differential host until the host has been confirmed as being monogenic, or a monogenic progeny from this polygenic host has been selected.
[c]Provisional placement based on the assumption that the resistance in sources PI 172623 and PI 172633 are identical.
[d]Gene-for-gene relationship not confirmed to date.

1.2.6 Differences between the old and new nomenclature systems for *Venturia inaequalis* races

The common race, avirulent to all hosts and defined as race 1 in the old classification system, was renamed as race 0 in the new nomenclature system. This addition will provide a theoretical definition, because there are no reference candidates exhibiting a completely avirulent pattern for any available hosts. Race 1 induces necrosis on 'Golden Delicious', hence it carries the AvrRvi1 avirulence locus for the *Rvi1(Vg)* resistance gene. Likewise, there are 17 different races defined by Bus et al. 2011 carrying 17 different avirulent genes that have the ability to overcome the 17 major resistance genes identified. However, there are still races that are difficult to classify using the nomenclature system above, especially races able to overcome two resistance genes for such races. Further research is required in order to recognise differential hosts. (Bus et al. 2011)

1.3 Apple scab resistance genes

Non *Malus* plants are not affected by *Venturia inaequalis*, however not all *Malus* genotypes are susceptible to the pathogen. Even those that are infected by *V.inaequalis* have a different level of susceptibility. Two types of resistance can be observed in *Malus* – *Venturia* interactions.

1.3.1 Qualitative resistance

Qualitative resistance is also called vertical resistance. This type of resistance is specific to the pathogen race, which means the resistance mechanism is particularly effective against a specific race of pathogen and is controlled by one or a few genes. The durability of this type of resistance is low, because of the pathogen's high selection pressure. Genotypes that elicit this type of resistance show a high level of resistance characteristics and these are inherited according to Mendel's laws. However, as mentioned above, resistance is controlled by a single locus or a very limited number of loci, so it is easy to identify the gene responsible for resistance, or at least the chromosome region carrying the resistance genes, by evaluating the cross population created by crossing resistant and susceptible cultivars. To date, more than 18 major apple scab resistance genes have been identified in different *Malus* species and mapped on the apple genome. Molecular markers closely linked to the resistance genes identified have been identified for the use of marker assisted breeding (MAS). Specifically, the *Rvi6, Rvi10* and *Rvi17* genes were mapped on three different regions of linkage group (LG) 1, the *Rvi12, Rvi4, Rvi8, Rvi9, Rvi11* and *Rvi15* genes on different positions of LG 2, *Rvi1* and *Rvi12* on different regions of LG 12, the *Rvi16, Rvi3, Rvi1, Rvi7, Rvi13* and *Rvi5* genes on the LGs 3, 4, 6, 8, 13 and 17 respectively (Bus et al. 2011). In addition to these genes, the *Rvi18* apple scab resistance gene was recently mapped on LG 11 (Soriano et al. 2014). To date, only three R genes have been cloned in the apple (Vinatzer et al. 2001; Belfanti et al. 2004; Joshi et al. 2011):

Rvi6 deriving from *Malus floribunda 821* (Crandall 1926), *Rvi15* from the GMAL 2473 accession (Galli et al. 2010a; Schouten et al. 2014) and *Rvi1* discovered in 'Golden Delicious' (Benaouf and Parisi 2000; Gessler at el. 2006; Cova et al. 2015). It is also relatively easy to identify markers for major resistance to apple scab in comparison with other resistance. This is because of the relative reliability of phenotypes obtained from glasshouse screening of very young seedlings from mapping populations in response to infection by *Venturia inaequalis*. Different types of molecular markers are available for the identified apple scab resistance genes, for which there are RAPD (Rapid Amplified Polymorphic DNA) markers (Koller et al. 1994;Yang and Kruger 1994; Hemmat et al. 1995;Gardiner et al. 1996;Tartarini et al. 1996; Yang et al. 1997a,b), SCAR (Sequence Characterised Amplified Regions) or SSR markers (Simple Sequence Repeat or Microsatellites), CAPS (Cleaved Amplified Polymorphic Sequences) (Gianfranceschi et al. 1996;Yang and Korbon 1996;Yang et al. 1997(a,b) ;Tartarini et al. 1999;Liebhart et al. 2003b,) and SNP markers (Single Nucleotide Polymorphism) (Padmarasu et al. 2015; Jänash et al. 2015).

The *Rvi6* (*Vf)* apple scab resistance gene from *Malus floribunda* 821 (Janick et al. 1996) has been extensively studied and is the most widely used resistance gene in breeding programs. The *Rvi6* gene conferring resistance to five out of seven known races of *V. inaequalis* held up quite well in orchards for over 80 years until 1993, when two new races of the pathogen (races 6 in Germany and 7 in England) were able to produce scab lesions on some apple cultivars carrying *Rvi6* (Parisi et al, 1993; Roberts and Crute 1994; Benaouf and Parisi 2000). *Rvi6* was mapped on LG 1 of the Prima cultivar by Maliepaard et al. 1998. King et al. 1998 and Patocchi et al. 1999 then carried out fine mapping of the region around *Rvi6,* locating the resistance in a short interval between the OPM18 and OPAL7 markers. The first physical map of *Rvi6* was constructed by Vinatzer et al. 2001 in

a region of 350kb. Identification of CH-Vf1 and CH-Vf2 microsatellite markers closely linked to the *Rvi6* gene on LG 1 of the apple was carried out using Bacterial Artificial Chromosome (BAC) clones of the genomic *Rvi6* region by Vinatzer et al. 2004. Utilisation of different molecular markers developed for *Rvi6* resistance made it possible to recognise four coding sequences for receptor-like proteins, these genes showing a higher sequence similarity with members of a cluster of the *Cladisporium fulvum (Cf)* resistance gene family in the tomato. Because of this similarity, the genes were called *HcrVfs* (Homologous to *C.fulvum* resistance genes of the *Vf*-region (Vinatzer et al. 2001). The same set of genes was identified and named *Vfa1* (synonym for *HcrVf-1*), *Vfa2* (synonym for *HcrVf-2*), *Vfa3* (synonym for *HcrVf-3*) and *Vfa4* (synonym for *HcrVf-4*) by Xu and Korban (2002), using a slightly different approach. Barbieri et al. (2003) published the first evidence of *HcrVf2* inducing apple scab resistance under the control of the constitutive promoter CaMV35s. This was introduced into the 'Gala' apple scab susceptible cultivar, and in vitro scab evaluation assay was developed to perform early evaluation of plantlets. The results obtained by the transgenic 'McIntosh' line carrying *HcrVf-1* and *HcrVf-2* genes driven by their own promoter demonstrated increased resistance, but the level of protection was not complete for the pathogen (Malnoy et al. 2006). The *HcrVf-4* gene does not seem to be involved in the scab resistance induction mechanism, because all the transgenic lines showed a high level of sporulation after inoculation (Malnoy et al .2008).

Apple scab resistance *Va* (*Rvi10)* was identified in the PI172623 apple accession deriving from the Russian apple cultivar Antonovka, which has ability to induce hypersensitive reaction (Dayton and Williams 1968). The gene was mapped on linkage group 1, in the same linkage group where the *Rvi6* gene was mapped, but in a different position (Hemmat et al. 2003) about 25cM away from the *Rvi6* gene (Zini et al. 2005).

The *Vb (Rvi12)* gene from *Malus baccata #2* was initially positioned by Hemmat et al. 2003 on linkage group 1, approximately 25 cM from the *Rvi6* locus. However, using a cross created with 'Golden Delicious' × 'Hansen's baccata' *#2* Erdin et al. 2006 positioned the *Rvi12* locus on linkage group 12, between the SSR markers Hi02d05 and Hi07f01. The *Rvi12* locus was fine mapped by Padmarasu et al. 2015 and narrowed down to a region of 985kb by developing closely linked SNP markers. Plants carrying *Rvi12* resistance show the same range of symptoms as *Rvi6,* and in the USA there is evidence of the possible breakdown of *Rvi12* resistance.

The *Vbj* (*Rvi11)* gene from *Malus baccata jackii* has been mapped on the distal part of linkage group 2. The closest molecular marker associated with *Rvi11* resistance is SSR Ch05e03, which was mapped at 0.6 cM from the locus (Gygax et al. 2004).The resistance symptoms associated with this gene vary from the absence of symptoms to chlorotic lesions and chlorotic and necrotic lesions with weak sporulation. Plants carrying abundant sporulation are considered to be susceptible to *Rvi11*. To date there are no reports available about the breakdown of the scab resistance conferred by the *Rvi11* gene.

The *Vg* (*Rvi1)* apple scab resistance gene identified in the 'Golden Delicious' cultivar (Bénaouf et al. 1997,2000) shows race-specific resistance to *V.inaequalis* race 7 and was mapped on LG 12 (Durel et al. 2000). Resistant plants carrying the *Rvi1* gene showed large necrotic lesions with wrinkled leaves 10 days after *V.inaequlais* race 7 inoculation. Fine mapping of the region surrounding *Rvi1* has been reported by Cova et al. 2015, with prediction of possible candidate genes.

When Bus et al. (2005) reviewed the genes deriving from the GMAL 1462 accession (*Vr, Vh2 (Rvi2), Vh4 (Rvi4)* and *Vr1 (Rvi4)* for the new nomenclature with differential hosts revealed the problems associated with them. Dayton and Williams (1968) assigned the *Vr* gene as a non-race-specific gene present in R12740-7A, but they did not describe the symptoms associated with the *Vr* gene. However, Aldwinckle et al. (1976) described it as

being associated with R12740-7A, promoting stellate necrosis. Bus et al. (2005a) found this association to be incorrect. When studying the resistance genes of *Rvi2* (*Vh2)* and *Rvi4 (Vh4)* from R12740-7A derived differential hosts TSR34T15 and TSR33T239 respectively, they suggested that the *Vr* gene promoting stellate necrosis was identical to the *Vh2* gene, which also promotes stellate necrosis and has been mapped in the same region as *Vr*-stellate. For this reason, the molecular markers developed for *Vr*-stellate by Hemmat et al. (2002) are for *Vh2,* and the original *Vr* described as non-race- specific has remained without markers and unmapped. Bus et al. (2005a) mapped *Vh2* on the distal end of LG 2, between SSR CH05e03 and CH02c02a. Hemmat et al. (2002) described another resistance gene from R12740-7A inducing hypersensitive reaction as *Rvi4* (*Vx)* and developed a S22-SCAR marker for it. Bus et al. (2005a) confirmed that the *Vx* gene is identical to the *Vh4* resistance gene deriving from the same selection. The *Vr1* gene described by Boudichevskaia et al. 2004, 2006 from R12740-7A was also mapped on the proximal part of LG2, like *Vh4*, and was found to be associated with the S22-SCAR marker, and Boudichevskaia et al. 2006 concluded that *Vr1* may be identical to the *Vh4* (and *Vx*) gene.

The *Vr2 (Rvi15)* apple scab resistance gene deriving from the GMAL 2473 accession has been mapped on LG 2, very close to the *Vh4* gene; the closest marker for *Vr2* was CH02c02a, mapped approximately 2 cM from *Vh4* (Patocchi et al. 2004). Later Galli et al. (2010) described this gene as a gene inducing slow hypersensitive resistance and developed two flanking SSR markers ARGH17 and GmTNL1, mapped on both sides of the resistance locus at 0.3cM and 0.2cM respectively. This resistance gene was then physically isolated and functionally characterised by Schouten et al. (2014). It was the second cloned apple scab resistance gene after the *Rvi6* gene.

The *Rvi8 (Vh8)* gene deriving from *M.sieversii* W193B was described as an ephemeral resistance gene (Bus et al. 2005a). After inoculation with a mixed inoculum, half of the

cross population created by crossing ‘Royal Gala’ × *M.sieversii* W193B showed heavy sporulation, while the other half of the seedlings showed stellate necrosis with sporulation. Research has confirmed that the mixed inoculum used contained *V.inaequalis* race 8 and that it is incompatible with the newly discovered apple scab resistance gene. The *Vh8* locus is located on LG 2, linked or possibly allelic to *Vh2*.

The *Vfh (Rvi7*) apple scab resistance gene deriving from *M.floribunda* 821 was identified in progeny plants of the ‘Golden Delicious’ × *M.floribunda* 821 cross, with two race 1 single spore isolates (Bénaouf and Parisi 2000). The results of inoculation elicited two key findings: 1) most of the resistant progeny had the hypersensitive response, 2) a 2:1:1 ratio was observed by grouping different resistant reactions (Classes 0 and 1; 2 and 3a; 3b and 4). This ratio corresponds to the segregation ratio of two independent resistance genes, with the gene inducing hypersensitive response being epistatic to the gene inducing the class 2 and 3a reactions. It was suggested that the second gene was *Vf (Rvi6),* while the gene inducing the hypersensitive response was a new gene called *Vfh (Rvi7).*

The *Vd (Rvi13)* scab resistance gene deriving from the old ‘Durello di Forli’ Italian apple cultivar has been described as conferring both high field tolerance to apple scab (3b type reaction) and stellate necrotic reaction in glasshouse-grown seedlings exposed to the EU-D-42 race 6 reference strain of *V. inaequalis.* QTL analysis of scab resistance in a progeny deriving from the ‘Durello di Forli’ × ‘Fiesta’ cross made it possible to identify three main QTLs on LGs 10, 11 and 17. Although scab resistance from ‘Durello di Forli’ is considered to be quantitative (Sansavini et al, 2000), only a strong QTL on LG10 deriving from this Italian cultivar was identified. The other QTLs derived from the ‘Fiesta’ parent. Inoculation of the same progeny with a mono-conidial strain of race 6 (EU-D-42) made it possible to identify a previously unknown major gene for scab resistance. This gene was mapped on LG10 and was called *Rvi13* (*Vd*; Tartarini et al. 2004). The OPAF07/880

(RAPD) and G63Tru91a (CAPS) markers flanking *Rvi13* span about 10 cM at the top of LG10 (Tartarini et al. 2004).

The *Vdr1 (Rvi14*) apple scab resistance gene was identified in the 'Dülmener Rosenapfel' apple cultivar, demonstrating broad spectrum resistance to apple scab (Laurens et al. 2004). The resistance gene induced chlorosis after inoculation. This is the first scab resistance gene to have been reported under the new nomenclature system presented by Bus et al. (2011) and is also the first *R* gene mapped on LG6, towards the top near the HB09 (128) SSR marker. Resistance is overcome by the 301, EU-D42, and EU-B04 *V. inaequalis* isolates, all of which are characterised by their suitability as reference isolates.

Most of the disease resistance genes characterised to date in the apple are single dominant genes. Such genes commonly confer resistance to the pathogen in a gene-for-gene manner and are therefore, in theory, easily overcome by the pathogen's ability to mutate to virulence (Crute and Pink 1996). The main problem with the resistance breakdown of perennial crops is that unlike an annual species, a new variety cannot be planted and become profitable the following year; it can take at least 10 years to recover the cost of planting an orchard. This is because an apple orchard remains in the ground for a production period of around 15 years, and it takes several years for the trees to come into commercial production. It is therefore imperative that the resistance of new commercial varieties is durable. The combination of functionally different resistance genes in the same genotype, also known as "pyramiding" was proposed as a promising strategy for obtaining durable scab resistance over time (Gessler and Blaise.1994; Laurens and Lespinasse. 1994); Fischer 1995; Evans 1997). It is likely that durable resistance to apple pathogens will be established through the pyramiding of different resistance genes with different resistance specificities in a single cultivar.

The *Vmis (Rvi16)* gene was identified in the open pollinated mildew immune selection (MIS) progeny 93.051 G07-098 (Bus et al. 2010). The gene conditioned a range of

resistance reactions, from predominantly non-visible symptoms with a single J222 spore isolate, to chlorosis reactions with a mixture of *V. inaequalis* isolates. The resistance mapped at the lower end of LG3, near the SSR marker AU223657.

Recently, two genes were mapped in an Antonovka APF22 progeny, one of which is *Va2* and possibly the same as *Rvi10*, whereas the other is *Va1*, *Rvi17* (Dunemann et al. 2010). The gene was identified in a progeny screened in the field and confirmed on a subset of plants inoculated in the glasshouse. *Rvi17* maps within 1 cM of *Rvi6* on LG1, but is a different gene from *Rvi6* since it is not overcome by race (6) isolates and has a specific CH-Vf1 marker allele of 138 bp linked to it.

The *Vm (Rvi5)* apple scab resistance genes deriving from *Malus micromalus* 245-38 and *Malus* × *atrosanguinea* 804 were identified early in the PRI breeding program (Hough 1944) as highly resistant sources conditioning hypersensitive response, visible within three days of inoculation (Shay and Hough 1952) The HR-conditioning gene (*Rvi5*) in *M. micromalus* 245-38 segregate as a single gene in progeny of the F1 accession PRI 76-27 (Shay et al. 1953) and was later confirmed in related progenies. A race of *V. inaequalis* was identified among isolates collected from *M. micromalus* trees in England, and in France in 1968 certain *Rvi5* progeny of *M. micromalus* 245-38 became infected (Lespinasse et al. 1979). Genetic markers for *Rvi5* were identified in *M.* x *atrosanguinea* 804 derivatives NY748828-12 and OR45T132 (Cheng et al. 1998) and *Rvi5 (Vm)* was mapped on the distal end of LG17 (Patocchi et al. 2005). The designed HI07h02 SSR marker (Patocchi et al. 2005) was considered as a co-segregating marker for *Rvi5* resistance.

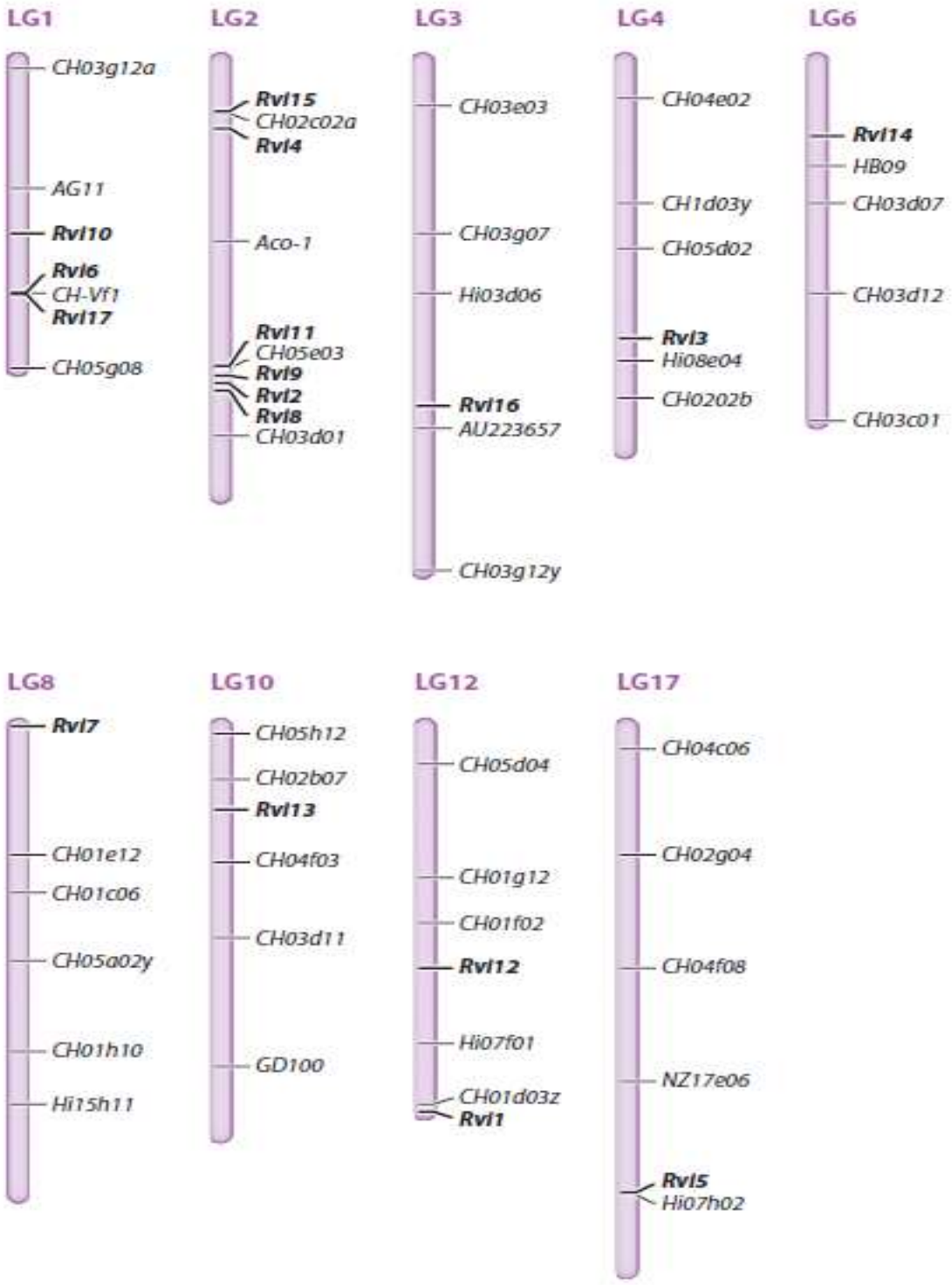

Figure 4: Global positions of the 17 *Rvi* scab resistance genes named to date on the apple genome

The skeleton genetic map is based on the integrated consensus map by N'Diaye et al. 2008.

1.3.2 Quantitative resistance

This type of resistance is not race-specific and is also called polygenic resistance/ horizontal resistance or partial resistance to apple scab. The resistance is characterised by a reduced level of sporulation on leaves or fruits (Mac Hardy 1996). Sporulation is only observable in wet conditions, but not every year and this is not followed by progressive invasion of the leaf or fruit tissues of susceptible cultivars. Consequently, sporulation appears to be restricted to small areas of the leaf and/or fruit skin. When partially resistant cultivars are crossed with highly susceptible cultivars, this resistance can be traced back in a very small proportion of the resulting progenies, thus it is generally believed that this type of resistance is inherited on a polygenic basis, but no molecular evidence is available to prove this polygenic control. However, the use of quantitative resistance in breeding programmes is less common than the use of qualitative resistance. Due to low selection pressure on the pathogen, this type of resistance is more durable than the resistance conferred by the major gene.

The pathogen resistance conferred by QTLs would be a valuable addition to the breeding portfolios of the major resistance genes, as incorporating QTLs into a single cultivar is likely to be more effective than combining the major genes alone (Parlevliet and Zadoks 1977). QTLs were identified for apple scab resistance using the reference genetic maps constructed for the following populations; 'Prima' × 'Fiesta' (Durel et al.2003), 'Fiesta' × 'Discovery' (Liebhard et al. 2003) and 'Discovery' × TN10-8 (Calenge et al. 2004). Durel et al. (2003) used two monoconidial strains of race 6 to identify QTLs controlling resistance in both 'Fiesta' and 'Prima'. Detailed QTL analysis using both MCQTL (Jourjon et al. 2000) and Map QTL (Van Ooijen 2004) software identified four genomic regions that were significantly involved in partial resistance, characterised by a reduction in sporulation. One of these regions was located close to the original *Rvi6* gene and it is

possible that the partial resistance observed was due to a closely linked gene, or the result of a residual effect of the overcome *Rvi6* gene (Durel et al. 2003). The remaining three additional regions identified on LG 15, 11 and 17 were new locations for association with scab resistance. Liebhard et al. (2003) carried out extensive assessment of field resistance to apple scab over a three year period involving three different geographical sites. Using MapQTL, eight QTLs were identified that contributed to apple scab resistance; six for leaf scab and two for fruit scab. Interestingly, the 'Discovery' cultivar demonstrated a greater degree of resistance; most of the identified QTLs were attributed to 'Fiesta', the more susceptible parent, indicating a high degree of homozygosity at the resistance gene loci in 'Discovery' that prevented their detection in the progeny because of the lack of segregation. The high levels of resistance observed in individuals during the study confirmed that 'Discovery' was a strongly resistant parent for breeding (Liebhard et al. 2003). The strongest scab resistant QTL from 'Prima' × 'Fiesta' mapped at LG 17 (Liebhard et al. 2003), coinciding with a scab resistant QTL that Durel et al.(2003) identified, and similarly LG 11 was identified in both studies as having a region of interest. One of the QTLs detected by Liebhard et al. (2003), which accounted for 4% of phenotypic variability, was located on LG 12, in a position comparable to *Rvi1* (*Vg;* Van de Weg, unpublished data). Calenge et al. (2004) used a panel of eight monoconidial isolates to inoculate replicated progeny from a 'Discovery' × TN10-8 cross, resulting in the identification of numerous QTLs across seven linkage groups (with MapQTL), depending on the isolate used. Combining QTLs with overlapping confidence intervals and close likelihood peaks revealed three major QTLs on LG 1, LG 2 and LG 17. The region identified on LG 1 corresponds to the region around *Rvi6* that Durel et al. (2003) identified as contributing to between 16% and 17.8% of phenotypic variation, and the QTL identified on LG 17 (Calenge et al. 2004) is also in agreement with a QTL mapped in both 'Fiesta' and 'Discovery' that explained 23% of the observed phenotypic variability.

Calenge et al. (2004) also detected additional QTLs on LGs 5, 13 and 15 in only one or two isolates and a QTL on LG 2 that appeared to control more broad-spectrum resistance to apple scab. This QTL spans a region around the major *Rvi11, Rvi2 and Rvi8* scab resistance (Calenge et al. 2004; Durel et al. 2004). Identification of isolate-specific QTLs seems to indicate that some QTL for partial resistance could be involved in a pathogen-mediated recognition response, in a similar way to major genes.

1.4 Plant resistance genes

Plant pathogen interactions are well studied mechanisms which involve the activation of signals that can occasionally induce rapid defence responses against plant pathogens. These defence mechanisms protect the host plants from further infection by the disease. Induction of plant defence signals involves the recognition of specific pathogen effectors by the products of specialist host genes called resistance (R) genes (Belkhadir et al. 2004). Significant efforts by several laboratories in the past 5–10 years have resulted in the identification of many R genes from model and crop species (Bent 1996; Ellis and jones 1998 and Jones 2001). The isolated functional R genes encode resistance to bacterial, viral, fungal, nematode and insect pathogens. Despite this wide range of pathogen taxa and their presumed pathogenicity effector molecules, plant resistance genes can be broadly divided into eight groups based on their amino acid motif organisation and their membrane spanning domains (Gururani et al. 2012).

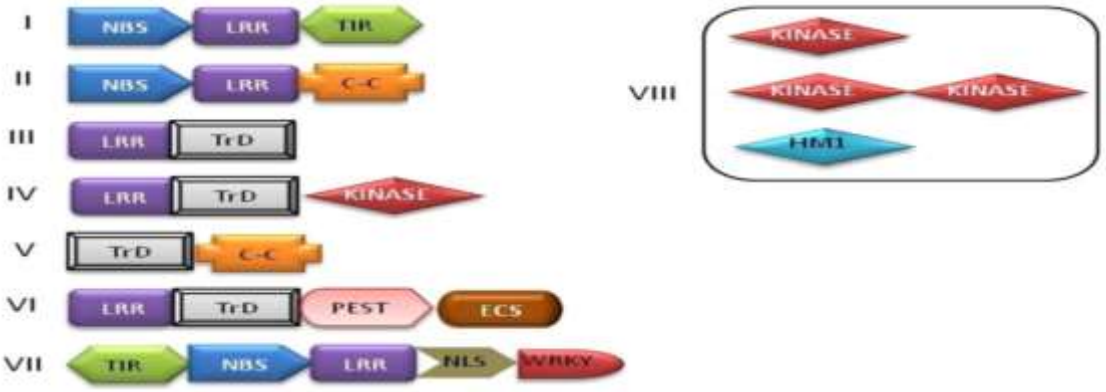

Figure 5: Major classes of plant resistance genes based on the arrangement of the functional domains
LRR - Leucine rich repeats; NBS - Nucleotide-binding site; TIR Toll/Interleukin -1-receptors; C – C - Coiled coil; TrD - Transmembrane domain; PEST - Protein degradation domain (proline-glycine-serine-threonine); ECS - Endocytosis cell signalling domain; NLS - Nuclear localization signal; WRKY - Amino acid domain; HM1 - *Helminthosporium carbonum* toxin reductase enzyme (Gugurani et al. 2012).

1.4.1 Plant resistance gene classes

Plant resistance genes can be broadly divided into eight main groups based on their amino acid motif organisation and their membrane spanning domains (Gururani et al. 2012). LRRs (Leucine rich repeats) are present in the majority of R proteins, which play an important role in recognition. The first class of resistance genes consists of cytoplasmic proteins, which have LRR and NBS motifs and an N-terminal domain with homology to the mammalian toll-interleukin-1-receptor (TIR) domain. The tobacco N gene, Flax L6 gene (Lawrence et al.1995) and the gene responsible for *Vr2 (Rvi15)* apple scab resistance also belong to the TIR-NBS-LRR class (Schouten et al. 2014). The second main class of R genes includes genes encoding for the cytoplasm protein with a nucleotide binding site (NBS), a C terminal leucine rich repeat (LRR) and a putative coiled coil (CC) domain at the N terminus. Examples of this type of resistance gene include the *RPS2* gene of *Arabidopsis,* conferring resistnce to *P.syringae* and the resistance gene 12 of tomato,

conferring the resistance to *Fusarium oxysporium*. The candidate gene for fire blight resistance *(Erwinia amylovora*) in *Malus* × *robusta* 5 also codes for a CC-NBS-LRR gene (Fahrentrapp et al. 2013). The third main class of resistance genes devoid of the NBS motif consists of extra cytoplasmic leucine rich repeats (eLRR), attached to a transmembrane domain (TrD) (Gururani et al. 2012). eLRRs domains are known to play an important role in certain defence proteins, such as poly galacturonase inhibiting proteins (PGIPs) (Jones and Jones 1997) even though they are not directly involved in pathogen recognition and activation of defence genes (Jones 2001 and Stotz et al. 2000). The *HcrVf2* gene conferring scab resistance in the apple belongs to the eLRR class (Belfanti et al. 2004). The fourth class of resistance genes consists of an extracellular LRR domain, a transmembrane domain (TrD) and an intracellular serine-threonine kinase (KIN) domain (Song et al. 1995). The rice Xa21 resistance gene for *Xanthomonas* is an example of this class of resistance gene. The fifth class of resistance genes contains a membrane protein domain (TrD), fused to a putative coiled coil domain (CC) (Wang et al. 2009). The *Arabidopsis* RPW8 protein is an example of the fifth main class of resistance genes. The sixth main class of resistance genes contains putative extracellular LRRs, along with a PEST (Pro-Glu-Ser-Thr) domain for protein degradation, and short protein motifs (ECS) that might target the protein for receptor mediated endocytosis (Thomma et al. 2011). The seventh main class of resistance genes instead includes the *Arabidopsis RRS1*-R gene, conferring resistance to the bacterial phyto-pathogen *Ralstonia solanacearum*, and this is a new member of the TIR-NBS-LRR R protein class. *RRS1*-R has a C-terminal extension with a putative nuclear localization signal (NLS) and a WRKY domain (Deslandes et al. 2002 and 2003). The WRKY domain is a 60 amino acid region that is defined by the conserved amino acid sequence WRKYGQK at its N terminal end, together with a novel zinc-finger-like motif. The last main class of resistance genes includes enzymatic R-genes that contain neither LRR nor NBS groups, for example the maize *Hm1*

gene, which provides protection against southern corn leaf blight caused by the fungal pathogen *Cochliobolus carbonum* (Johal and Briggs. 1999). Unlike other resistance genes, *Hm1* encodes the enzyme HC toxin reductase, which detoxifies a specific cyclic tetra-peptide toxin produced by the fungus (HC toxin) that is essential for pathogenicity. Therefore, cereal resistance genes like *Hm1* can be seen to encode a range of different proteins that in some cases have obviously very different functions.

Table 3: Main classes of plant resistance genes
LRR- Leucine rich repeats; NBS -Nucleotide-binding site; TIR -Toll/Interleukin-1-receptors; CC- Coiled coil; Trd- Trans membrane domain; PEST- Amino acid domain; ECS- Endocytosis cell signalling domain; NLS -Nuclear localization signal; WRKY- Amino acid domain; HC- toxin reductase *Helminthosporium carbonum* toxin reductase enzyme.

S. no	Major R-gene classes	Domains										Example
		LRR	NBS	TIR	Kinase	CC	TrD	PEST	ECS	NLS	WRKY	
I	NBS–LRR–TIR	✓	✓	✓	X	X	X	X	X	X	X	*N, L6, RPP5*
II	NBS–LRR–CC	✓	✓	X	X	✓	X	X	X	X	X	*I2, RPS2, RPM1*
III	LRR–TrD	✓	X	X	X	X	✓	X	X	X	X	*Cf-9, Cf-4, Cf-2*
IV	LRR–TrD–Kinase	✓	X	X	✓	X	✓	X	X	X	X	*Xa21*
V	TrD–CC	X	X	X	X	✓	✓	X	X	X	X	*RPW8*
VI	TIR–NBS–LRR–NLS- WRKY	✓	✓	✓	X	X	X	X	X	✓	✓	*RRS1R*
VII	LRR–TrD–PEST–ECS	✓	X	X	X	X	✓	✓	✓	X	X	*Ve1, Ve2*
VIII	Enzymatic R-genes	X	X	X	✓	X	X	X	X	X	X	*Pto, Rpg1*
		X	X	X	X	X	X	X	X	X	X	*Hm1*

1.5 Apple scab resistance breeding

The first report on the origin of human crossed scab resistance apples dates back to the early nineteenth century (Brown 1975), when Charles S Crandall (1926) started an apple breeding program at the University of Illinois. He explored the potential of various species of *Malus* as breeding material. Crandall produced crosses of crab apple–like species with commercial apple cultivars, but he never released any cultivars. In 1943, a severe epidemic of apple scab which defoliated all susceptible, unsprayed apple trees occurred due to unusually cool, wet weather. Fred Hough (1944), who was evaluating Crandall's work, found one progeny deriving from the cross (*Malus floribunda* 821 × 'Rome Beauty') × (*M. floribunda* 821 × 'Rome Beauty') had an approximate segregation ratio of 1 scab resistant: 1 scab susceptible. Further crosses with two resistant selections from this cross indicated that resistance was conferred by a single qualitative dominant gene, or by a block of closely linked quantitative genes, that was subsequently named *Vf* (*Venturia* resistance from *floribunda).* Since then breeders have repeatedly crossed scab resistant apples with commercial cultivars to produce high quality, resistant apples (Gessler et al. 2006).

In traditional apple breeding, selection of the best phenotypes from seedlings grown from open pollinated seeds is based on phenotypic evaluation. However, initially little progress was made in improving apple cultivars through controlled crossing, which was attributed to poor selection of parents (Janick et al. 1996). The apple is self-incompatible and highly heterozygous, which results in very diverse progeny with only a few individuals being a major improvement on the parents. As most characteristics have a polygenic control, low efficiency in terms of the genetic improvement of breeding lines, together with a long juvenile period, make breeding this crop a slow and expensive process.

Due to the development of genetics and genomic studies it has been possible to improve the efficiency of traditional breeding programs with the help of molecular markers.

Molecular markers allow genetic variability at molecular level to be identified, based on changes in the DNA. These variations may have direct effects on the phenotype, or more often they are simply genetically linked to a trait. Molecular markers are biological features that are determined by allelic forms and can be used as tags to keep track of a chromosome region or a gene. In modern genetics, polymorphism is the relative difference at any genetic locus across a genome. Desirable genetic markers should meet the following criteria: (i) high level of genetic polymorphism; ideally markers should be highly polymorphic in terms of breeding material, especially core breeding material (ii) reliability; markers should be closely linked to the target loci, preferably at a genetic distance of less than 5 cM. (iii) Clear distinct allele features: required to recognise different alleles easily, (iv) even distribution over the entire genome; (v) neutral selection (without pleiotropic effects); (vi) technical procedure; the level of simplicity and the time required for the technique are critical considerations. High-throughput simple and quick methods are highly desirable (vii) cost; marker assay must be cost-effective in order to use MAS (viii) high reproducibility; the reproducibility of markers is essential for data accumulation and sharing between laboratories (Xu 2010).

1.5.1 MAS (Marker Assisted Selection) for scab resistance

The term MAS (Marker Assisted Selection) describes an indirect selection process for the trait concerned with the help of a molecular marker linked to the trait. For example, if MAS is being used to select individuals with scab resistance, the presence or absence of the allele coupled with the considered trait is assessed. The theory behind this is that the marker used for selection is associated with the gene or quantitative trait locus (QTL) concerned with a high frequency, due to genetic linkage (close proximity of the marker locus and the disease resistance determining locus on the chromosome,). MAS can be very useful for efficiently selecting traits that are difficult or expensive to measure, show low

heritability and are expressed late in development. Although genetics and genomic studies have made tremendous progress in many crops, the application of MAS is still limited to a very few traits (Collard and Mackill 2008). In apple breeding, MAS is used in most breeding programs aiming at introducing resistance against important orchard diseases. In MAS for the apple, molecular markers have evolved from Restriction Fragment Length Polymorphism (RFLP) (Roche et al. 1997a, b) to a variety of Polymerase Chain Reaction (PCR) based markers (Koller et al. 1994; Gianfranceschi et al. 1996; Tartarini et al. 1999; Gardiner et al. 2007). The most widely used molecular markers in apple scab resistance are simple sequence repeats (SSR); they are highly reliable, co-dominant, relatively simple and cheap to use and generally highly polymorphic. Today a large number of SSR based genetic framework maps are available for the apple (Guilford et al. 1997; Liebhart et al. 2002; Vinatzer et al. 2004; Silfverberg-Dilworth et al. 2006), most of which include SSRs that are linked to apple scab resistance.

The reasons that may affect the limited uptake of MAS in breeding programs were described by Collard and Mackill (2008), the following applying to the apple: 1) the uncertainty of map positions for some resistance loci; 2) lack of markers that are closely linked to resistance; the availability of closely linked markers is a prerequisite for successful MAS. The ideal distance when using a single marker is about 1cM, while having two markers flanking either side of the trait concerned will make MAS more precise and efficient. Despite this, the availability of markers co-segregating with the trait concerned also increases the efficiency of MAS; 3) the lack of marker polymorphism exhibited in some breeding populations; 4) most of the traits controlled by QTLs have not been validated and many QTLs have too wide an interval of confidence. 5) the lack of markers suitable for high-throughput analysis.

However, Single Nucleotide Polymorphism (SNP) markers are also becoming popular in apple scab resistance breeding (thanks to the availability of the ‘Golden Delicious’

genome sequence) due to their high frequency (between 4.5 to 20 markers /kbp) (Velasco et al. 2010; Micheletti et al. 2011) and the possibility of simultaneous genotyping from a few to thousands of markers per DNA sample. The development of SNP markers for five major apple scab resistance genes (*Rvi2, Rvi4, Rvi6, Rvi11, Rvi15*) commonly used in apple scab resistance breeding programmes has been described by Jänsch et al. 2015.

Table 4: Molecular markers for the main scab resistance genes suitable for MAS (Gessler et al.2006)

Gene (synonyms)	Linkage group	Marker (s)	Distance from the gene (cM)	Reference
Va	1	B398-480$_{(RAPD)}$	16.0	Hemmat *et al.*, 2003
Vb	12	Hi02d05$_{(SSR)}$	7.8	Erdin *et al.*, 2006
		Hi07f01$_{(SSR)}$	13.7	
Vbj	2	CH05e03$_{(SSR)}$	0.6	Gygax *et al.*, 2004
		Z13$_{(SCAR)}$	3.3	
Vd	10	OPAF07-880$_{(RAPD)}$	2.0	Tartarini *et al.*, 2004
		CH2b07$_{(SSR)}$	9.0	
Vf	1	M18$_{(CAPS)}$	0.2	Gianfranceschi *et al.*, 1996
		CH-Vf1$_{(SSR)}$	0.0	Vinatzer *et al.*, 2004
		AL07$_{(SCAR)}$	0.9	Tartarini *et al.*, 1999
				Patocchi *et al.*, 1999b
Vg	12	MC105$_{(RFLP)}$	3.0	Durel *et al.*, 2000;
		CH01D03$_{(SSR)}$	0.5	Calenge *et al.*, 2004
Vh2	2	OPL19$_{(SCAR)}$	1.0	Bus *et al.*, 2002
Vh4 (Vr_1, V_x)	2	CH02c02$_{(SSR)}$	5.0	Bus *et al.*, 2005a
		S22$_{(SCAR)}$	4.0	Hemmat *et al.*, 2002
Vh8	2	OPL19$_{(SCAR)}$	1.3	Bus *et al.*, 2005b
Vm	17	Hi07h02$_{(SSR)}$	0.0	Patocchi *et al.*, 2005
		OPB12$_{(SCAR)}$	5.4	Cheng *et al.*, 1998
Vr_2	2	CH02c02a$_{(SSR)}$	0.0	Patocchi *et al.*, 2004

1.5.2 Gene pyramiding for durable resistance

Due to the varying reported evidence of different resistance genes having been overcome by the pathogen, no one can say whether any form of monogenic resistance is durable, even if still unbreached. Pyramiding is the process of combining several genes together in a single genotype (Lespinasse et al. 1999). This process is also possible with conventional breeding, but it is usually not easy to detect plants carrying more than one gene of interest.

DNA markers can greatly facilitate the detection of pyramided genes using a single DNA sample without phenotyping. The most widespread application of gene pyramiding is combining multiple resistance genes to acquire durable resistance. The reason for this is the development of 'durable' or stable disease resistance, since pathogens frequently overcome single major genes due to the emergence of new pathogen races, but the pathogen's ability to overcome two or more effective genes by mutation is considered to be much lower as compared to the breakdown of resistance controlled by a single gene. However, in conventional breeding programmes obtaining cultivars with pyramided R genes with a good impact on the market requires many years and more complex crosses. Recently, researchers have developed in vitro strategies, such as 'cisgenesis', to insert R genes, avoiding the difficulties of classic breeding (Jacobsen and Schouten 2007). In this approach, R genes found in crossable species are inserted into the cultivars with their native promoters and terminators. Foreign genes are used during the transformation process, but they are removed in the final product, so it has been argued that the plants obtained using this method are as safe as plants obtained with traditional breeding techniques (Jacobsen and Schouten 2007). However, to proceed with 'cisgenesis' the availability of physically isolated, functionally characterised R genes is a prerequisite.

A successful strategy for the isolation of R genes is the map based cloning procedure, which relies on the availability of markers closely linked to the R gene. With the help of closely linked markers and chromosome walking, Bacterial Artificial Chromosome (BAC) or Yeast Artificial Chromosome libraries created from the cultivar concerned are screened to discover clones covering the region of interest. Sequencing of clones and bioinformatic analysis of the sequences will then provide the sequences of candidate R genes. Many R genes in different species have been isolated by using this strategy: *Pro* in the tomato (Martin et al. 1993), *RPS2* in *Arabidopsis thaliana* (Bent et al. 1994) *Xa21* in rice (Song et al. 1995) and specifically the apple scab resistance genes of *Rvi6* from *M.floribunda*

(Patocchi et al. 1999; Vinatzer et al. 2001; Belfanti et al. 2004) and *Rvi15* of GMAIL 2473 (Galli et al. 2010a).

1.6 The *Rvi5* (Vm) apple scab resistance gene

The *Rvi5* (Vm) apple scab resistance gene derives from the *Malus atrosanguinea 804* accession (Dayton and Williams 1970), however this accession is reported to have two dominant scab resistance genes (Shay et al. 1953). The major gene from the accession, which produces a pit type (Type 1) resistance reaction, is called as *Vm* (Dayton and Williams 1970), while the other gene, generating a few restricted sporulating lesions (Type 3), is referred to as a 'masked' gene, because its phenotype is not expressed in plants containing the pit type gene (William and Brown 1968). A second source of *Vm* has been reported in *Malus micromalus 245-38;* in this accession the presence of a second 'masked 'gene is also reported (Dayton and Williams 1970). The *Vm* gene is not allelic to the *Vf* gene ((Dayton and Williams 1970) or the "pit-type" gene *Va* (Dayton and Williams 1968). However, no information is available about the allelic relationship between *Vm* and other major scab resistance genes.

The *Vm* gene confers resistance to *V.inaequlis* races 1-4, but is vulnerable to race 5. The resistance breakdown of *Vm* was first reported in England (William and Brown 1968). However, recent surveys conducted in different European regions by the VINQUEST initiative (www.vinquest.ch/monitoring/publications.htm) have reported that this virulence is not very widespread. According to the survey results, *V.inaequalis* race 5 was only reported in two sites in Germany and Belgium (Patocchi et al. 2009, www.vinquest.ch/monitoring/publications.htm). There is however evidence of a possible breakdown of *Rvi5* resistance reported in North America (Beckerman 2009).

The *Vm* gene has already been introduced into the two apple cultivars; 'Murray' and 'Rouville', by removing the majority of the undesired crab apple traits. The limited diffusion of *V.inaequalis* race 5 and the different reaction type (hypersensitivity) of plants carrying the *Vm* gene as compared to *Vf*, make this gene interesting for pyramiding with other apple scab resistance genes.

The *Vm* gene induces a hypersensitive response (Type 1) for *V.inaequalis* races 1-4 3 days after inoculation.

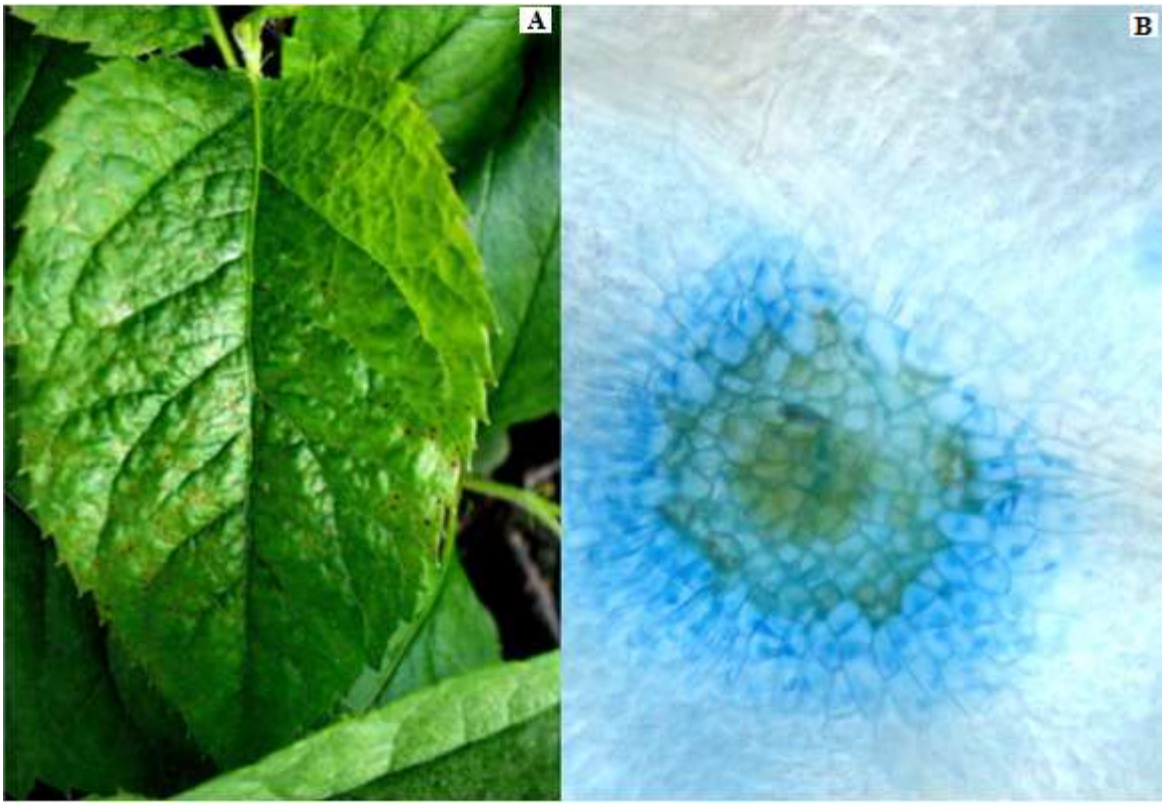

Figure 6: *Rvi5 (Vm)* resistance response on 'Murray' leaf for *V.inaequalis*.
A) 'Murray' leaf with typical hypersensitive response for *V.inaequalis*, B) Light microscopic observation of hypersensitive response of 'Murray' leaf with magnification of X40.

Four major genes, including *Rvi5 (Vm),* induce a hypersensitive response (HR) characterised by a rapid response on the upper surface of young infected leaves, leading to circular to oval-shaped necrotic zones of dead epidermal and mesophyll cells under and

around the pathogen penetration site (Galli et al. 2010). According to studies conducted by Chevalier et al. (1991), the hypersensitive response is a localized depression of 100-500 μm in diameter, where the epidermal cells have collapsed. In the centre of the depression a conidium remains, with a very restricted subcuticular stroma. Numerous germinated conidia forming one or few appressoria without formation of subcuticular stroma can be observed in areas remaining healthy. Histological studies of the hypersensitive response have revealed that the hypersensitive response is a localised depression with 4-20 modified epidermal cells.

1.7 Scope of the thesis

The key objective of this study was to isolate and characterise the *Rvi5 (Vm)* apple scab resistance gene from the *Malus* × *domestica* 'Murray' genotype. All the experiments were conducted to achieve this goal. The research described in this dissertation was guided by the following hypotheses and objectives.

1) Fine mapping of the *Rvi5 (Vm)* apple scab resistance locus in the 'Murray' apple genotype.

The *Rvi5* locus of the 'Murray' was fine mapped using two F1 populations created from cross between 'Golden Delicious'× 'Murray' and 'Galaxy'× 'Murray', consisting of a total of 1243 progeny plants. Fine mapping of the *Rvi5* locus is an essential step for recognising the precise map position of the R gene and the development of flanking markers linked to resistance.

2) Isolation of the *Rvi5 (Vm)* locus from 'Murray' *Malus* × *domestica*.

Once determination of the precise map position of the R locus with two flanking markers delimiting the margins of the R locus was completed, the putative *Rvi5* locus was isolated

by screening of a bacterial artificial chromosome library (BAC) of the resistant parent‘Murray’ using chromosome walking.

3) Identification of a TIR-NBS-LRR gene in the *Rvi5 (Vm)* apple scab resistance locus of *Malus* × *domestica* ‘Murray’ genotype.

Isolated BAC clones lying between the flanking molecular markers were fully sequenced and assembled to obtain the sequence of the *Rvi5* locus. The sequence obtained was subjected to Open Reading Frame (ORF) prediction to recognise possible candidate genes for *Rvi5* apple scab resistance. Only one gene was identified as a possible candidate gene for *Rvi5* resistance, the gene belongs to the TIR-NBS-LRR gene resistance family.

4) Cloning and transformation of the identified candidate gene

The possible candidate gene identified was cloned and transformed in the ‘Gala’ scab susceptible cultivar using *Agrobacterium* mediated transformation technology, with the help of binary gateway vectors. The first five transgenic plants with the inserted candidate gene concerned have been obtained and the plants are currently in in-vitro conditions. Transgenic plants are being multiplied using a regeneration process to obtain sufficient plants for future inoculation experiments with *Venturia inaequalis*.

CHAPTER 1

Fine mapping of the *Rvi5 (Vm)* apple scab resistance locus in the 'Murray' apple genotype.

Fine mapping of the *Rvi5* (*Vm*) apple scab resistance locus in the 'Murray' apple genotype

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Abstract

Apple scab, caused by the fungal pathogen *Venturia inaequalis*, is the most devastating pathogen in the apple growing industry. In the last two decades, many studies have been initiated to identify new resistances to apple scab and to introgress them into new cultivars through breeding. The *Rvi6* gene from *Malus floribunda 821* has been the one most intensively used in breeding programs worldwide, but the identification of new pathogen strains that are virulent to *Rvi6* has increased the need for pyramiding of more than one resistance gene to obtain cultivars with durable resistance. Here we report on the fine mapping of the *Rvi5* apple scab resistance locus using two large segregating populations. A region of about 1 cM at the distal end of LG17 carrying the *Rvi5* resistance gene was detailed by developing and mapping ten molecular markers, including SCAR, SSR and SNP markers. The *Rvi5* locus was restricted to a region of 228kb on the 'Golden Delicious' reference genome between the two flanking SSR markers FMACH_Vm4 and FMACH_Vm2. Three co-segregating molecular markers were also developed (SSR FMACH_Vm3, Vm–SCAR1 and Vm-SNP5). All these markers will facilitate the development of Marker Assisted Selection (MAS) protocols for this gene using both low-cost methods and high-throughput systems. The findings of this study will thus be useful for further investigation of the *Rvi5* resistance locus of 'Murray', aimed at candidate gene identification and the physical isolation of the resistance gene.

Keywords: *Venturia inaequalis*; apple scab; *Rvi5*; fine mapping; *Malus* x *domestica*; Murray.

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Introduction

The cultivated apple (*Malus* × *domestica)* is widely grown worldwide and is susceptible to several fungal diseases. Of these, *Venturia inaequalis* (Cooke) Winter, the causal agent of apple scab, is highly damaging, especially in temperate areas with high spring and summer rainfall (MacHardy 1996). The pathogen damages both the fruit and leaves, and causes extensive yield losses as a result of fruit infection. Characteristic black scabby blotches are the main symptoms observable on affected fruits and leaves, but severe pathogen infections result in deformed fruits and these make the fruit unmarketable (Gessler et al. 2006). The two main disease control methods currently used involve either intensive use of fungicides (up to 15-25 treatments per season) or the planting of scab-resistant cultivars. Growing resistant cultivars can also help to reduce production costs and contributes to increasing the environmental sustainability of apple production by reducing the chemical footprint. To date, more than eighteen race-specific apple scab resistance genes (Bus et al. 2011; Jha et al. 2009; Soriano et al. 2009) have been identified in old cultivars and wild *Malus* species, most of which have been mapped on different chromosome regions of the apple genome. Specifically, the *Rvi6, Rvi10* and *Rvi17* genes were mapped on three different regions of linkage group (LG) 1, and *Rvi2, Rvi4, Rvi8, Rvi9, Rvi11* and *Rvi15* genes on different positions of LG2, *Rvi1* and *Rvi12* on different regions of LG12, and *Rvi16, Rvi3, Rvi1, Rvi7, Rvi13* and *Rvi5* genes on LGs 3, 4, 6, 8, 13 and 17, respectively (Bus et al. 2011). In addition to these genes, the apple scab resistance gene *Rvi18* has recently been mapped on LG11 (Soriano et al. 2014).

To date, only three R genes have been cloned in apple, *Rvi6* derived from *Malus floribunda 821* (Vinatzer et al. 2001, Belfanti et al. 2004, Joshi et al. 2011), *Rvi15* from

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accession GMAL 2473 (Galli et al. 2010a; Schouten et al. 2014) and *Rvi1* discovered in 'Golden Delicious' (Bénaouf and Parisi 2000; Cova et al. 2015).The *Rvi6* gene was isolated by means of map based cloning (Vinatzer et al. 2001), and named *HcrVf2*, referring to the homology to *Cladosporium fulvum* (*Cf*) resistance genes in tomato, exist as the second gene in a cluster of resistance gene analogs at the *Rvi6* locus. Later, it was functionally analysed and demonstrated, that the *HcrVf2* confers the *Rvi6* scab resistance (Belfanti et al. 2004). Other two apple scab resistance locus, *Rvi15* and *Rvi1*, have been recently investigated (Galli et al. 2010; Cova et al. 2015) and contain cluster of TIR-NBS-LRR (TNL) genes. Schouten et al. (2014) demonstrated, one of the three TNL genes carrying the *Rvi15* locus is confers the full resistance for *Rvi15*, by giving a clear hypersensitive response.

Scab resistance differs not only according to chromosomal location, but also in terms of the different type of resistance symptoms after pathogen infection. At least 5 different reaction classes have been described and named as class 0, 1, 2, 3a, 3b and stellate chlorosis or necrosis (Shay and Hough 1952; Chevalier et al. 1991; Bus et al 2011). Some resistance genes, such as *Rvi6*, elicit different responses ranging from no symptoms to chlorotic (class 2) and necrotic/chlorotic lesions with different degrees of sporulation (class 3a and 3b); other genes, such as *Rvi2*, *Rvi8* and *Rvi13*, show a more specific response of stellate necrosis. Furthermore, scab resistance genes also differ according to their spectrum of action against the various *V. inaequalis* races: when they are effective against only a few isolates of the pathogen population are defined as "narrow spectrum" resistance genes (Ex: *Rvi1*- shows resistance only against the race *6*), while the R genes that are effective against the most, like *Rvi18* (Soriano et al. 2009), are defined as "broad spectrum" resistance genes (Bus et al. 2011).

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The apple scab resistance gene *Rvi5* (*Vm*) is historically associated with the typical hypersensitive response and resistance symptoms are clearly visible on the leaf surface as small pinpoint pits that appear within three days of inoculation (Shay and Hough 1952; Williams and Kuc 1969). This kind of reaction was named as class 1 (Chevalier et al. 1991). This apple scab resistance gene derives from *Malus micromalus* 245-38 and *M. atrosanguinea* 804, and has been overcome by the *V. inaequalis* race (5), which was first reported in England (Williams and Brown 1968). However, recent surveys conducted in different European regions by the VINQUEST initiative (www.vinquest.ch/monitoring/publications.htm) show that this virulence is not very widespread. According to these survey results, *V. inaequalis* race (5) has only been reported at two sites in Germany and in Belgium (Patocchi et al. 2009, www.vinquest.ch/monitoring/publications.htm). Further evidence of a possible breakdown of *Rvi5* resistance has been reported in North America (Beckerman 2009).

The genetics of *Rvi5* resistance was investigated by different authors and two molecular markers closely linked to the R gene were described: the Sequence Characterized Amplified Region (SCAR) marker $OPB12_{687}$ (Cheng et al. 1998) and the SSR Hi07h02 (Patocchi et al. 2005). The $OPB12_{687}$ SCAR marker is located at approximately 5 cM distance from the *Rvi5* gene, while the SSR Hi07h02 has been reported as co-segregating with the resistance (R) gene.

The *Rvi5* gene remains especially interesting for investigation because of its counterpart, which is responsible for the specific gene-for-gene relationship. Indeed, the product of the *AvrRvi5* avirulence gene matching the *Rvi5* (*Vm*) protein was the first protein isolated from *V. inaequalis* in liquid culture and able to elicit HR-necrosis in the *Rvi5* host, but not

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in susceptible hosts (Win et al. 2003). The elicitor is likely to be a secreted protein and Win and collaborators (2003) narrowed down the necrosis-inducing proteins to three major candidates.

In this article, we report the fine mapping of the region around the *Rvi5* locus and development of new molecular markers closely linked to resistance on the distal end of LG17. The development of improved *Rvi5*-linked molecular markers will better facilitate marker assisted selection (MAS) in apple breeding programs. The 'Golden Delicious' genome sequence was used to search for possible candidate genes in the *Rvi5* region. The results of this study can be considered as an initial step to physically isolating the *Rvi5* candidate gene.

Materials and Methods

Plant material and DNA extraction

Two progenies segregating for the *Rvi5* gene with a total of 1242 plants (consisted 701 seedlings of 'Galaxy' × 'Murray' and 541 seedlings of 'Golden Delicious'× 'Murray') were created by manually pollinating flowers of 'Golden Delicious' and 'Galaxy' with pollen collected from the resistant cultivar 'Murray'. This apple cultivar is an advanced selection from the Canadian apple breeding program carrying the *Rvi5* resistance gene from *M. atrosanguinea* 804 (Warner and Potter 1988), while 'Golden Delicious' and 'Galaxy' are susceptible to scab, even if 'Golden Delicious' carries the *Rvi1* resistance ephemeral gene (Bénaouf and Parisi 2000) and a broad-spectrum QTL for scab resistance has been detected in 'Gala' (Soufflet-Freslon et al. 2008). A panel of twelve apple cultivars including parental cultivars of segregating populations were used to test the

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marker specificity of developed SNP markers, of which in addition to cultivar Durello di Forli and Fiesta other eight cultivars were according to Jänsch et al. 2015.

Newly emerged young leaves from progeny plants and parental cultivars were collected and freeze dried using a Scan-Vac freeze dryer (LaboGene, Denmark). Genomic DNA was extracted using a modified CTAB protocol according to Maguire et al. (1994). DNA concentrations were estimated using a Nanodrop 8000 spectrophotometer (Thermo Scientific, USA).

Resistance evaluation

Young leaves of actively growing shoots were inoculated twice in the greenhouse with a suspension of *V. inaequalis* conidia (at least $2x10^5$ conidia/ml). The inoculum was prepared from scabbed leaves of 'Fuji', 'Red Chief' and 'Golden Delicious' varieties grown in an untreated orchard. The temperature was set at 19 ± 5 °C and relative humidity was kept at 100% for at least 2 days.

Symptoms were evaluated 3, 6 and 10 days after each inoculation as described by Chevalier et al. (1991) using a two-class system: class 1 for hypersensitive response and class 4 for extensive sporulation. Scab resistance evaluation was continued for other two years on the whole progeny and on parental cultivars in the field under natural infection conditions. In the fourth year, three sprouts from recombinant individuals and parentals, were grafted onto three different M.9 rootstocks and artificially inoculated in glasshouse using an inoculum prepared as described above. Scab scores were repeatable over the four years of evaluation.

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Genotyping and preliminary mapping of the R gene

A sub-set of 96 plants from the 'Golden Delicious' x 'Murray' population was used for preliminary mapping of the *Rvi5* gene. The available SSR markers located at the distal end of LG17 (CH04f08 and CH05d08 from Liebhard et al. (2002); Hi02f12 and Hi07h02 from Silfverberg-Dilworth et al. (2006)) and the $OPB12_{687}$ SCAR marker (Cheng et al. 1998) were used to genotype the mapping population. Forward primer of all SSR markers were labelled with either 6-FAM or NED fluorescent dyes in PCR reaction mixtures. The cycling profiles were according to Patocchi et al. (2005).

The multi-locus SSR marker CH05d08 (Silfverberg-Dilworth et al. 2006) was converted into a single locus marker by optimizing PCR conditions as follows: a final volume of 25µl containing 67 mM Tris-HCl (pH 8.8), 16 mM of $(NH_4)_2SO_4$; 1.5 mM of $MgCl_2$; 0.24 mM of each dNTP; 0.8 µM of each forward and reverse primer; 5 ng of DNA and 1U of BIOTAQ DNA polymerase. The temperature profile used was: 94 $^{\circ}$C for 3 minutes, then 25 cycles at 94 $^{\circ}$C for 1 minute, 55 $^{\circ}$C for 1 minute and 72 $^{\circ}$C for 2 minutes, with a final extension cycle of 72 $^{\circ}$C for 10 minutes. Amplified PCR products were fractioned and sized by capillary electrophoresis using a 3730xl Genetic Analyser (Applied Bio-systems, Foster City, CA) and generated data were analysed using GeneMapper (Applied Bio-systems, Foster City, CA) software.

For R gene mapping, progeny plants showing only hypersensitive reaction were considered to be resistant, while all the other plants with sporulating scab lesions were considered susceptible. Resistant plants were denominated as heterozygous for the *Rvi5* gene and susceptible plants were homozygous for the recessive allele. Phenotypic and genotypic data of the screened progenies were used for linkage analysis using Joinmap 4.0

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software (Van Ooijen 2006), employing the Kosambi mapping function with the following parameters: a minimum LOD score threshold of 2.0, a recombination fraction threshold of 0.35, a ripple value of 1.0 and a jump threshold value of 3.0. Constructed maps were visualised using MapChart 2.0 (Voorrips 2002).

Development of new molecular markers in the *Rvi5* region and fine mapping

The sequences of SSR Hi07h02 and CH05d08 were BLASTed (*Altschul* et al. 1990) against the 'Golden Delicious' genome sequence v1.0 contig (Velasco et al. 2010; http://www.rosaceae.org/gb/gbrowse/malus_x_domestica) to identify the physical position of the markers on the reference genome. The sequences of contigs between the two markers were then used to develop new SSR, SCAR and SNP markers. All primers were designed using Primer 3 (Rozen and Skaletsky 2000). SSR sequences were identified using SSRIT - Simple Sequence Repeat Identification Tool (Temnykh et al. 2001; http://archive.gramene.org/db/markers/ssrtool). SSR amplification and capillary electrophoresis were set up as described before for the other SSR markers.

To design SCAR markers, locus-specific primer pairs were designed using Primer 3 (Rozen and Skaletsky 2000) and the sequences of the contigs between the two flanking markers. Primers were first used to amplify parental genomic DNA ('Golden Delicious', 'Galaxy' and 'Murray') in a 20 µl volume containing 50 ng of genomic DNA, 0.1 µM of primers, 1.5 mM $MgCl_2$, 200 µM dNTPs, 1U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA) and 1x reaction buffer. The cycling profile was as follows: 10 min denaturation at 95 ℃, followed by 30 cycles of 45 s at 60 °C, 2 min at 72 ℃, and 30 s at 95 ℃, with a final extension of 7 min at 72 ℃. Amplicons were electrophoresed on 2% TAE agarose gel to check amplification and the presence of length

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polymorphisms among parental cultivars. Polymorphic fragments were retained and used for mapping while those not showing length polymorphism were searched for SNP markers. These latter amplicons obtained for parental genomic DNA were purified using the Wizard® SV Gel and PCR Clean-Up System Kit (Promega, Madison, USA) following the manufacturer's instructions and sequenced at Bio-Fab Research srl (Pomezia, Italy) with both forward and reverse specific primers. SNP detection was performed by visualizing obtained sequences on Chromas version 2.22 (Technelysium Ltd, Brisbane, Australia) as double peaks. The final sequence assembly was manually performed with the SeqMan software (Lasergene v8.0). For SNP detection, the TSP approach described by Hayden et al. (2009) was chosen. The locus- and allele-specific TSP primers of each SNP are reported in Table 4. TSP assays were performed using 1.5 U AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA), 100 ng/µl bovine serum albumin Fraction V (Thermo Fisher Scientific, Waltham,USA), 1 x reaction buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.1 µM each locus-specific primer, 0.5µM allele-specific primer and 50 ng genomic DNA in a total reaction volume of 10 µl. Thermal cycling was optimized for each TSP on an Applied Biosystems 2720 thermal cycler (Supplementary Table 2). PCR products were analysed on 2% agarose gel and stained with ethidium bromide.

To verify the location of the *Rvi5* locus we arbitrarily chose to work on the region between the SSR CH05d08 and Hi07h02 first by genotyping the two segregating populations with these two SSR markers. Subsequently, phenotypically confirmed recombinant individuals were genotyped using the newly designed polymorphic SSR, SCAR and SNP markers. The number of recombination events occurring in the interval between two flanking

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markers was assessed and the location of the *Rvi5* locus was inferred using a graphical mapping approach.

Gene prediction on the 'Golden Delicious' genome

The contig sequences between the two SSR markers flanking the *Rvi5* locus were downloaded from the reference genome sequence of 'Golden Delicious' (http://www.rosaceae.org/gb/gbrowse/malus_x_domestica) (Velasco et al. 2010) and used for open reading frame (ORF) prediction by using FGENESIS 2.6 (Salamov and Solovyev 2000). Deduced protein sequences resulting from ORF prediction were compared using BLASTX against all deposited protein sequences in the NCBI database.

Results and Discussion

Phenotypic evaluation of plants and segregation of *Rvi5* resistance

Progenies and parental cultivars showed consistent and precise phenotypic reactions in all the resistance evaluations performed under different conditions (glasshouse and field). Despite the use of mixed inocula, the parental 'Murray' plants only showed a resistant reaction to scab with the typical hypersensitive response/pinpoint pits (class 1), while 'Golden Delicious' and 'Galaxy' showed strong sporulation (class 4), as expected. These observations confirmed the absence of a *V. inaequalis* strain(s) able to overcome the *Rvi5* resistance gene in the mixed inoculum used for the experiment.

Following the resistance evaluation carried out in the greenhouse for a total of 1242 plants of two segregating populations, 369 out of 701 plants (52.6%) of the 'Galaxy' × 'Murray' progeny showed class 1 symptoms, while 332 plants showed extensive sporulation. In the 'Golden Delicious' × 'Murray' progeny, 315 out of 541 plants (58.1%) showed the class 1

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resistance reaction, while 226 plants had extensive sporulation. In this case, the results of the greenhouse infection were again confirmed in the following two years under natural infection conditions.

The segregation data observed in the 'Galaxy' × 'Murray' progeny strongly support the inheritance of a single dominant gene (X^2 = 1.957). A different situation was observed in the 'Golden Delicious' × 'Murray' progeny, for which the resistant/susceptible ratio was significantly skewed (X^2 = 13.831) towards the resistant genotypes. A similar trend was observed by Patocchi et al. (2005), who found 78 resistant (54.9%) and 64 susceptible plants in a 'Golden Delicious' × 'Murray' progeny, which however was not significantly different (X^2 = 1.38) from the expected 1:1 ratio. The observed skewed segregation in the 'Golden Delicious' × 'Murray' progeny may be the result of the effects of the self-incompatibility locus located on the same chromosome in a region quite close (~160kbp) to the *Rvi5* gene (Antanaviciute et al. 2012).

Genotyping and preliminary mapping

A subset of 48 resistant and 48 susceptible 'Golden Delicious' x 'Murray' progeny and the parental cultivars were genotyped using five molecular markers available at the distal end of LG17 (CH04f08, Hi02f12, OPB12 SCAR, CH05d08 and Hi07h02).

The conversion of the multi-allelic SSR marker CH05d08, which was not polymorphic in 'Murray' (Patocchi et al. 2005), into an informative single-locus marker segregating in 'Murray' resulted in the preferential amplification of the SSR allele of 'Murray' from the LG17 locus with a size of 127bp associated with the resistance. 'Murray' was heterozygous for SSR Hi07h02, with the 224bp allele linked to resistance and the 271bp allele linked to susceptibility. A three base pair shift of allele size compared with the allele

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sizes reported by Patocchi et al. (2005) was observed. This may have been the consequence of the use of a different technique for fragment size separation. For all the markers used to create the partial linkage map of LG17 of 'Murray', allele sizes linked with the *Rvi5* resistance are reported in Supplementary Table 1. Genotypic and phenotypic data comparison showed one plant with genotype-phenotype incongruence (GPI; Gygax et al. 2004). The presence of a few GPI plants in large progenies was expected, as already reported in various mapping experiments, but these plants are usually removed with the aim of improving fine mapping efficiency (Patocchi et al. 1999; Erdin et al. 2006; Soriano et al. 2009). A partial genetic linkage map of LG17 of 'Murray' covering a genetic distance of 35.3cM was built. *Rvi5* was mapped 1cM from SSR marker CH05d08 and co-segregating with SSR Hi07h02, with the genetic distance between the two SSR markers being 1cM (Supplementary Figure 1).

Development of new molecular markers and fine mapping of the *Rvi5* region

In order to obtain fine mapping of the region surrounding the distal part of LG17, all the available segregating progenies were genotyped by the two closely linked SSRs (CH05g08 and Hi07h02). Nine recombination events between the R gene and the two SSR markers were observed, of which seven recombination events were reported between the R gene and SSR Hi07h02, while two recombination events were found towards the SSR CH05d08 from the R gene. Hence, these results indicated that the *Rvi5* locus is probably more closely linked to SSR CH05d08 than SSR Hi07h02.

BLAST analysis of the sequences of SSRs CH05d08 and Hi07h02 on the 'Golden Delicious' genome sequence identified a region of 540Kbp. The sequences of contigs between the two markers were used to design new SSR markers having at least ten

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dinucleotide repeats or more than six tri-nucleotide repeats. Nine new SSRs were designed, of which only four were polymorphic among the parental cultivars (Table 2).

Table 2: Newly developed SSR markers used for the fine mapping of *Rvi5* resistance. Alleles in coupling with the *Rvi5* resistance are shown in bold.

Marker	**Sequences**	**Annealing temperature**	**'Murray' alleles**
FMACH_Vm4	For-GAAATTGATTGGGGGTTTGA	60°C	208, **212**
	Rev-GAAAGCCCAACGACGTAAAG		
FMACH_Vm3	For-GTTCCCTGCAGTTTCATGGT	60°C	**355,** $
	Rev-CTAGCATTGGCCTCAGATCC		
FMACH_Vm2	For-TGGTGAAAGAAAATATGCCAAG	60°C	144, **158**
	Rev-TCCATTTCTCCATTTGGTGTT		
FMACH_Vm1	For-GGTTTGAGCTGGGAGACAAA	60°C	**416**, 426
	Rev-ATTGGTGCAAAGGTCAAAGG		

$ null allele

Six locus-specific primer pairs were also designed using the same region used to develop SSR markers, of which only one was polymorphic between the parental cultivars (Table 3).

The amplicons of non-polymorphic locus-specific primer pairs were sequenced for SNP mining. Five polymorphic SNP markers were identified in 5 different amplicons (Table 4). All polymorphic markers were used to screen the nine recombinant plants and the genotyping results were used to genetically fine map both the markers and *Rvi5* (Table 1). The *Rvi5* gene was mapped between the FMACH_Vm2 and FMACH_Vm4 markers, while the SSR marker FMACH_Vm3, SCAR marker Vm-SCAR 1 and the SNP marker Vm-SNP5 co-segregated with the resistance locus. The map order of the markers was then

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compared with their physical order based on the ‘Golden Delicious’ genomic sequence (Velasco et al. 2010). The genetic and physical orders were coherent.

The identified markers will ensure a more efficient application of MAS for the *Rvi5* gene than those used to date both in laboratories with few resources as well as in those with high-throughput systems. In addition, the SNP markers developed in this study can be used to screen progenies segregating for *Rvi5*, because they were tested on a panel of 12 apple genotypes and confirmed the allele in coupling with the resistance as highly specific for the wild donor species of the resistance gene (Supplementary Table 3).

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Table 3: Details of SCAR marker used in this study detailing primer sequence, position on Golden Delicious genome and PCR product sizes on parental cultivars.

SCAR name	Position in the GD genome	Locus specific primer	Allele size (bp)		
			'Murray'	'Gala'	'Golden D.'
Vm-SCAR1	MDC018385.173 chr17:23960508..23978623	For-GTTTGCGAGACAAACACCTG	1037	~ 700	~ 700
		Rev-CTGCAGTGGATGAGATCGAG			

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Table 4: SNP characteristics details of locus- and allele-specific primers and allele sizes.

Marker name	SNP alleles [a]		Locus specific primer		Size (bp)	TSP primer [c]
	Murray	Galaxy/Golden D.				
Vm-SNP1	**T**/A=W	A/A	For	CACATCAAAAGCCGGAAAAA	740 and 900 [b]	GGCTTCTCAGCTCAATAAAT
			Rev	GCGCGATAGAGGATTAGACG		
Vm-SNP2	**C**/G=S	G/G	For	GCCAAAAATGTTCACGAGGT	777	CCGTAGTCAATTTCGAGTC
			Rev	ATGCCAAGATTTTGGACTGC		
Vm-SNP3	C/**T**=Y	G/G	For	AAAGGTGTGGCATCGGTAAG	843	CCGCATACTCTTAATTTGTT
			Rev	CTGAAAACCGCCATTAGAGC		
Vm-SNP4	A/**G**=R	T/T	For	AATCTCCTGGTTTTGATACATTCA	850	CGAAGATCAGTTATCTACTGG
			Rev	CAATATGAATGGGGGAGGAG		
Vm-SNP5	**A**/G=R	G/G	For	TTGTAAGTGATCAGTTGTGGCAGT	1150	GGAATCTCTCAAGGTTAGGA
			Rev	TCCTTCATATAGCCAGTTTCTTCA		

[a] The allele specific base identified by the TSP primer is in bold

[b] Two fragments of about 740 and 900 bp were obtained with locus specific primers. The 740 bp fragment was then gel purified and sequenced for SNP detection

[c] the additional 2-bp non-complementary tails, added to the 5' end of each TSP the primer, are underlined

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Table 1: Schematic diagram showing the recombination events identified in 'Golden Delicious' × 'Murray' and 'Galaxy' × 'Murray' populations in the *Rvi5* mapping interval of 1 cM. Recombinant plant names are mentioned in the column named 'Recombinants', plants named as Q22, O32, O30, O13, R20 are belong to the 'Golden delicious' × 'Murray' progeny, while recombinant plants N47,B39,M42,I05 are belong to 'Galaxy'× 'Murray' progeny. 'S' in the figure represents plants being homozygous for the allele in coupling with susceptibility of apple scab and 'R' represents heterozygous plants carrying the allele in coupling with the resistance. The column named '*Rvi5'* contains the phenotypic data correspondent to each plant. Recombinant phenotypes observed for each molecular marker are showed in Red color. The raw named 'number of recombinations' represents the number of recombination events observed for each molecular marker.

Recombinants	CH05D08 SSR	FMACH _Vm4 SSR	Vm-SCAR 1	Vm-SNP5	FMACH _Vm3 SSR	*Rvi5*	FMACH _Vm2 SSR	Vm-SNP4	Vm-SNP3	FMACH_ Vm1 SSR	Vm-SNP2	Vm-SNP1	Hi07h02 SSR
Q22	S	S	S	S	S	S	S	S	R	R	R	R	R
N47	S	S	S	S	S	S	S	S	R	R	R	R	R
O32	S	S	S	S	S	S	R	R	R	R	R	R	R
B39	R	R	R	R	R	R	R	R	S	S	S	S	S
O30	R	R	R	R	R	R	R	R	S	S	S	S	S
O13	R	R	R	R	R	R	S	S	S	S	S	S	S
R20	R	R	R	R	R	R	S	S	S	S	S	S	S
M42	R	R	S	S	S	S	S	S	S	S	S	S	S
I05	R	S	S	S	S	S	S	S	S	S	S	S	S
Number of recombinations	2	1	0	0	0		3	3	7	7	7	7	7

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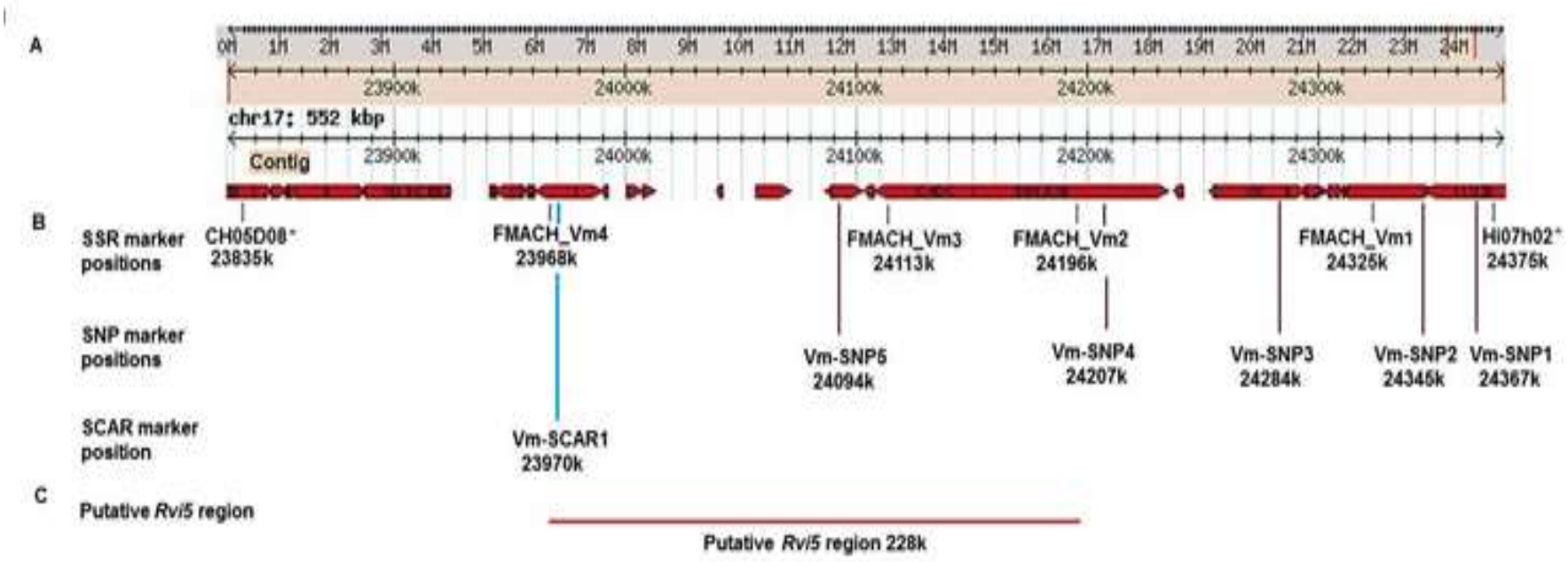

Figure 1: Schematic representation of the region surrounding the *Rvi5* resistance gene. **A**) Representation of genomic region spanning the *Rvi5* locus in 'Golden Delicious' as available from the apple genomic sequencing project (http://www.rosaceae.org/gb/gbrowse/malus_x_domestica);**B**) Positions of SSR, SNP and SCAR markers on the corresponding region of 'Golden Delicious' genome. The lines show the position of the SSR, SNP and SCAR marker positions on the corresponding region of the 'Golden Delicious' genome. **C)** The putative *Rvi5* region spanning a 228 kb area in between two flanking SSR markers FMACH_Vm4 and FMACH_Vm2 on the 'Golden Delicious' genome sequence.

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‘Golden Delicious’ ORF prediction

In order to estimate the physical size of the *Rvi5* region delimited after fine mapping, the genetic map was projected on the genomic sequence of ‘Golden Delicious’ (http://www.rosaceae.org/gb/gbrowse/malus_x_domestica) and the positions of all markers were checked and localised (Figure 1). The *Rvi5* region between the SSR markers FMACH_Vm2 and FMACH_Vm4 flanking the *Rvi5* region is about 228 kbp. The actual size determination of the *Rvi5* gene should be treated with caution because the distal end of LG17, where *Rvi5* is located, is highly homologous to the distal part of LG9 (Velasco et al. 2010). There are also uncovered areas (gaps) due to the highly repetitive nature of the region and some possible local miss-assemblies may be present, as already reported by Michael and Jackson (2013) and Baldi et al. (2013). The sequence of ‘Golden Delicious’ homologous to the *Rvi5* locus was searched for open reading frames (ORFs). A total of 33 putative ORFs were identified in the Consensus Gene Set, of which one TIR-NBS-LRR (TNL) and three leucine rich receptor-like proteins were similar to CLAVATA in *Pyrus*, and to *HcrVf* homologues in *Malus* (Vinatzer et al. 2004). Furthermore, four more putative ORFs associated with TNL were identified in the ‘Golden Delicious’ ‘‘alternative haplotype” (See NCBI database; Supplementary Table 4).

TNL proteins have been found to elicit resistance to many pathogens in different species, for example, against fungal pathogens, such as RPP5 and RPP1 for downy mildew in *Arabidopsis* (Parker et al. 1997; Botella et al. 1998), and M and L6 for rust in flax (Anderson et al. 1997; Lawrence et al. 1995). Three transcribed TNL genes have been identified at the *Rvi15* locus (*Vr2*) in apple (Galli et al. 2010b), which also induces a hypersensitive response, and a single ORF has been identified as the functional *Rvi15*

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resistance gene (Schouten et al. 2014). Four TNLs have also been recently identified at the *Rvi1* (*Vg*) locus (Cova et al. 2015) and Bastiaanse et al. (2015) have been identified the genes encoding for TIR-NBS-LRR proteins in the sequence of 'Golden Delicious' homologous to the complex resistance locus of 'Geneva' for the *Rvi3* resistance. A NBS-LRR gene may be involved in *Erwinia amylovora* resistance in the apple accession 'Evereste' (Parravicini et al. 2011), while a CC-NBS-LRR gene in *Malus* 'Robusta 5' (Fahrentrapp et al. 2013) was found to induce resistance against the same pathogen (Broggini et al. 2014). The *HcrVf2* gene, identified at the *Rvi6* locus, is coding for a protein with a putative extracellular LRR that could act as the receptor for the *Avr* gene product (Belfanti et al. 2004; Joshi et al. 2011). This gene showed a high level of similarity with members of the *Cladosporium fulvum (Cf)* resistance gene tomato family. A number of homologs to *HcrVf*-like genes have been isolated interspersed in the apple genome (Broggini et al. 2009; Velasco et al. 2010).

Thus, either a single gene or a few genes from the predicted putative resistance genes on the syntenic region of 'Golden Delicious' could be candidate gene(s) for *Rvi5*. However, it is important to consider that 'Golden Delicious' does not carry *Rvi5* resistance or lacks a functional *Rvi5* protein.

The reported fine mapping of the *Rvi5* region is a preliminary but necessary step towards future isolation and functional characterization of *Rvi5* candidate genes. These aims may be achieved by screening already constructed Bacterial artificial chromosome (BAC) library of 'Murray, which permits to identify candidate genes of *Rvi5* as already demonstrated for *Rvi6* and *Rvi15* (Vinatzer et al. 2004; Belfanti et al. 2004; Galli et al. 2010b; Schouten et al. 2014).

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Supplementary materials

Supplementary table 1: The SSR and SCAR markers used in the study for the linkage mapping of *Rvi5* resistance. Allele sizes coupling with *Rvi5* resistance is in bold letters.

Marker	**Sequences 5'-3'**	**Annealing temperature**	**'Murray' allele sizes in bp**
CH04f08 [a]	For-ATTTGAGATTGGGGGTGGAC	55°C	185**,189**
	Rev-ATTTCCCCGATTTAACCGTC		
Hi02f12 [b]	For-ACATGGCCGAAGACAATGAC	55°C	130, **$**
	Rev-GTTTCAACCTTTATCCCTCCATCTTTC		
OPB12 $_{687}$ [d]	For-CCTTGACGCAGCTT	58°C	**700**
	Rev-CCTTGACGCATCTACG		
CH05D08[a]	For-TCATGGATGGGAAAAAGAGG	55°C	120, **127**
	Rev-TGATTGCCACATGTCAGTGTT		
Hi07h02[c]	For-CAAATTGGCAACTGGGTCTG	60°C	**224**, 271
	Rev-GTTTAGGTGGAGGTGAAGGGATG		

$ null allele

[a] Liebhard et al. 2002

[b] Silfverberg-Dilworth et al. 2006

[c] Patocchi et al. 2005

[d] Cheng et al. 1998

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Supplementary table 2 - Optimal conditions used for SNP detection by the TSP method. Main differences are highlighted in bold

TSP program name	TSP	Parameter	TSP conditions								
			Denaturation	Locus-specific PCR			First step TSP PCR		Second step TSP PCR		
TSP 1	Rvi5-SNP1	Temperature	95 ℃	94 ℃	**62 ℃**	72 ℃	94 ℃	**45 ℃**	94 ℃	**54 ℃**	72 ℃
		Time	10 m	30 s	30 s	1 m 30 s	10 s	30 s	30 s	30 s	5 s
		Cycles	1	20			5		10		
TSP 2	Rvi5-SNP2 Rvi5-SNP3 Rvi5-SNP4	Temperature	95 ℃	94 ℃	60 ℃	72 ℃	94 ℃	**42 ℃**	94℃	53 ℃	72 ℃
		Time	10 m	30 s	30 s	1 m 30 s	10 s	30 s	30 s	30 s	5 s
		Cycles	1	20			5		10		
TSP 3	Rvi5-SNP5	Temperature	95 ℃	94 ℃	60 ℃	72 ℃	94 ℃	**48 ℃**	94 ℃	53 ℃	72 ℃
		Time	10 m	30 s	30 s	1 m 30 s	10 s	30 s	30 s	30 s	10 s
		Cycles	1	20			5		10		

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Supplementary table 3. Specificity of the SNP alleles in coupling with *Rvi5* resistance in a set of apple cultivars widely used as parents in many apple breeding programs.

Cultivar name	*Rvi5* gene presence	*Vm*-SNP1 165 bp	*Vm*-SNP2 244 bp	*Vm*-SNP3 243 bp	*Vm*-SNP4 635 bp	*Vm*-SNP5 951 bp
Murray	+	+	+	+	+	+
Braeburn	-	-	-	-	-	-
Cox's orange pippin	-	-	-	-	-	-
Delicious	-	-	-	-	-	-
F2-26829-2-2	-	-	-	-	-	-
Golden Delicious	-	-	-	-	-	-
Granny Smith	-	-	-	-	-	-
Jonathan	-	-	-	-	-	-
McIntosh	-	-	-	-	-	-
Durello di Forlì	-	-	-	-	-	-
Fiesta	-	-	-	-	-	-
Gala	-	-	-	-	-	-

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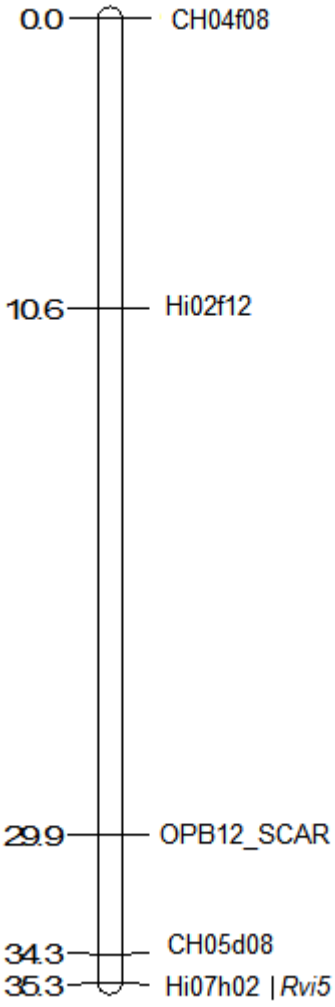
Supplementary table 4: Open reading frame predictions for the *Rvi5* homologous region in 'Golden Delicious'.

Protein coding gene prediction in Consensus Gene Set	Genome location-strand-Contig	Description	E value	Accession
MDP0000303653	chr17:23971804-23977288 (+ strand), Contig MDC010385.173:11296-16780	proline-rich receptor-like protein kinase PERK3 [Malus domestica]	0.00	XP_008343159.1
MDP0000283442	chr17:23982635-23986515 (- strand), Contig MDC005796.307:4011-10891	uncharacterized protein LOC103959942 isoform X2 [Pyrus x bretschneideri]	1.00E-19	XP_009370602.1
MDP0000285349	chr17:24001227-24002086 (+ strand), Contig MDC011148.172:535-1394	transcription initiation factor TFIID subunit 10-like [Malus domestica]	1.00E-51	XP_008343164.1
MDP0000269033	chr17:24010535-24011563 (+ strand), Contig MDC010976.137:2863-3891	DNA-directed RNA polymerase I subunit RPA2-like [Malus domestica]	0.00	XP_008365333.1
MDP0000269034	chr17:24012106-24012996 (- strand), Contig MDC010976.137:4434-5324	hypothetical protein ETSY2_09225 [Candidatus Entotheonella sp. TSY2]	0.11	ETX07780.1
MDP0000187085	chr17:24091603-24096103 (- strand), Contig MDC010450.947:5251-9751	leucine-rich repeat-containing protein 40-like [Malus domestica]	0.00	XP_008362557.1
MDP0000285146	chr17:24097161-24097748 (- strand), Contig MDC010450.933:8513-8900	uncharacterized protein LOC103941455 [Pyrus x bretschneideri]	2.00E-95	XP_009349923.1
MDP0000368275	chr17:24104099-24104234 (+ strand), Contig MDC015283.1:599-734	NO MATCHES		
MDP0000288644	chr17:24107988-24108974 (- strand), Contig MDC010450.929:806-2792	pentatricopeptide repeat-containing protein At1g02370, mitochondrial-like, partial [Malus domestica]	5.00E-141	XP_008343277.1
MDP0000288646	chr17:24116863-24125172 (+ strand), Contig MDC010450.929:9681-17990	heterogeneous nuclear ribonucleoprotein 1-like [Malus domestica]	0.00	XP_008342253.1
MDP0000757592	chr17:24125203-24127911 (- strand), Contig MDC008508.2191:540-4248	uncharacterized protein C05D11.9-like isoform X2 [Pyrus x bretschneideri]	0.00	XP_009367210.1
MDP0000288647	chr17:24125526-24126688 (+ strand), Contig MDC010450.929:10344-19506	acyl-coenzyme A thioesterase 9, mitochondrial-like [Malus domestica]	7.00E-24	XP_008372667.1
MDP0000190217	chr17:24131760-24136009 (- strand), Contig MDC012758.433:677-4826	hypothetical protein MIMGU_mgv1a019408mg [Erythranthe guttata]	5.00E-09	EYU23677.1
MDP0000319434	chr17:24131760-24136009 (- strand), Contig MDC012758.433:677-4826	hypothetical protein MIMGU_mgv1a019408mg [Erythranthe guttata]	5.00E-09	EYU23677.1
MDP0000302445	chr17:24136453-24137644 (+ strand), Contig MDC017873.436:3698-4889	3-hydroxy-3-methylglutaryl-coenzyme A reductase 1-like [Malus domestica]	1.00E-60	XP_008357826.1
MDP0000417061	chr17:24143748-24144357 (- strand), Contig MDC012758.433:12665-13274	dnaJ homolog subfamily B member 13-like [Malus domestica]	8.00E-105	XP_008368747.1
MDP0000638339	chr17:24144512-24145633 (- strand), Contig MDC010450.808:2403-3524	heterogeneous nuclear ribonucleoprotein 1-like [Malus domestica]	1.00E-142	XP_008362570.1
MDP0000281627	chr17:24145262-24148393 (+ strand), Contig MDC007283.1051:7238-10369	leucine-rich repeat receptor-like protein CLAVATA2 [Pyrus x bretschneideri]	0.00	XP_009347195.1
MDP0000281628	chr17:24150502-24151727 (+ strand), Contig MDC014045.406:223-1448	uncharacterized protein LOC103958869 isoform X2 [Pyrus x bretschneideri]	5.00E-80	XP_009347188.1
MDP0000296128	chr17:24150502-24151727 (+ strand), Contig MDC014045.406:223-1448	pentatricopeptide repeat-containing protein At1g02370, mitochondrial-like [Pyrus x bretschneideri]	0.00	XP_009367213.1
MDP0000199779	chr17:24152133-24156446 (- strand), Contig MDC014045.406:1854-6167	ribonucleases P/MRP protein subunit POP1 [Malus domestica]	0.00	XP_008343170.1
MDP0000279764	chr17:24155114-24157353 (- strand), Contig MDC007283.1045:8644-10883	protoheme IX farnesyltransferase, mitochondrial-like isoform X1 [Malus domestica]	1.00E-51	XP_008339489.1
MDP0000180236	chr17:24157005-24170037 (+ strand), Contig MDC014045.406:6726-19758	nodal modulator 1 [Malus domestica]	0.00	XP_008343168.1
MDP0000199780	chr17:24157005-24170037 (+ strand), Contig MDC014045.406:6726-19758	nodal modulator 1 [Malus domestica]	0.00	XP_008343169.1
MDP0000348307	chr17:24170879-24171005 (+ strand), Contig MDC019732.276:827-953	NO MATCHES		
MDP0000223990	chr17:24174886-24179030 (- strand), Contig MDC019732.276:4834-8978	receptor-like protein 12 [Malus domestica]	0.00	XP_008362544.1
MDP0000424798	chr17:24176336-24177934 (+ strand), Contig MDC015654.424:45-1643	LRR receptor-like serine/threonine-protein kinase FLS2 [Malus domestica]	0.00	XP_008359807.1
MDP0000252512	chr17:24177055-24184669 (+ strand), Contig MDC006656.912:001-9615	TMV resistance protein N-like [Malus domestica]	0.00	XP_008343279.1
MDP0000122961	chr17:24177055-24184669 (+ strand), Contig MDC006656.912:001-9615	TMV resistance protein N-like [Malus domestica]	0.00	XP_008343279.1
MDP0000185764	chr17:24181034-24183521 (- strand), Contig MDC010909.299:9246-11733	probable glycerol-3-phosphate acyltransferase 3 [Pyrus x bretschneideri]	0.00	XP_009367200.1
MDP0000173198	chr17:24185203-24187266 (+ strand), Contig MDC005749.250:2544-4329	protoheme IX farnesyltransferase, mitochondrial-like [Malus domestica]	0.00	XP_008362578.1
MDP0000257975	chr17:24188058-24192085 (- strand), Contig MDC008138.290:2095-6130	ABC transporter F family member 4-like isoform X2 [Pyrus x bretschneideri]	0.00	XP_009366245.1
MDP0000368178	chr17:24189578-24188812 (+ strand), Contig MDC008138.290:3623-3857	hypothetical protein [Asticcacaulis sp. AC466]	5.06	WP_023457946.1
Protein coding gene prediction in Alternative Gene Set	**Genome location-strand-Contig**	**Description**	**E value**	**Accession**
MDP0000283441	chr17:23978895-23982460 (+ strand), Contig MDC005796.307:271-3836	TMV resistance protein N-like isoform X1 [Malus domestica]	0.00	XP_008345162.1
MDP0000184980	chr17:24092323-24096394 (+ strand), Contig MDC010450.933:3475-7546	TMV resistance protein N-like [Malus domestica]	0.00	XP_008345165.1
MDP0000288649	chr17:24139561-24144096 (- strand), Contig MDC010450.929:32579-36914	TMV resistance protein N-like [Malus domestica]	0.00	XP_008345276.1
MDP0000190216	chr17:24125636-24128839 (- strand), Contig MDC010450.898:56-3259	TMV resistance protein N-like [Malus domestica]	0.00	XP_008362577.1

Supplementary figure 1: Partial genetic linkage map of the region surrounding *Rvi5* in the 'Golden Delicious' x 'Murray' progeny. Map distances are expressed in centimorgans (cM).



CHAPTER 2

Isolation of *Rvi5* (Vm) locus from *Malus* x *domestica* 'Murray'.

Isolation of *Rvi5* (Vm) locus from *Malus* x *domestica* 'Murray'.

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Keywords: Apple scab resistance, *Venturia inaequalis,* Bacterial artificial chromosomes, Candidate genes, Cisgenesis

Abstract

Scab resistance gene *Rvi5* (*Vm*) is derived from *Malus micromalus* or *Malus atrosanguinea* 804 was introgressed into the apple variety 'Murray'. This gene has the ability to induce a clear hypersensitive response after three days from *Venturia inaequalis* infection. Being 'Murray', a cultivar with an acceptable fruit quality compared to the old ancestors, this genotype could be used in breeding to pursue a durable resistance against *Venturia inaequalis*. This goal could be accomplished by two different approaches: resistance gene pyramiding by classical breeding or by a 'cisgenic' approach, once the resistance gene will be identified. The isolation of the locus that harbors the resistance is an essential step toward the identification of the candidate genes. A Bacterial Artificial Chromosome (BAC) pooling strategy enabled the identification of three overlapping BAC clones from a 'Murray' library that are covering the *Rvi5* region. Then, thanks to the

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availability of the *Malus* × *domestica* 'Golden Delicious' genome sequence, we were able to position the *Rvi5* locus on the 'Golden Delicious' genome sequence in a region estimated of about 228 kbps in size. These three clones will be sequenced and assembled to obtain the complete sequence of *Rvi5* locus. The assembled sequences will then be used for candidate gene identification.

Introduction

Apple scab disease cause by *Venturia inaequalis* is the most devastating pathogen in apple orchards, which leads to massive yield loss by fruit infections and growth retardation due to the foliar infection (Gesseler et al., 2006). Use of fungicides is the common control method that use against the pathogen, but it is not economically or environmentally sustainable. There are apple cultivars that naturally inherit resistance against the pathogen, but most of them carry dominant monogenic genes. New pathotypes or races have the ability to overcome the resistance of dominant single genes; it is the major problem of single dominant scab resistance genes (Galli et al., 2010a). To overcome this issue pyramiding two or more resistance genes into same cultivar has been suggested to delay or prevent the resistance breakdown (MacHardy et al., 2001). If breeding is conducted in classical way to obtain a plant combining two or more genes for a durable resistance will require at least couple of decades, but 'Cisgenesis' approach of adding natural genes from crosable donor plants without adding foreign genes, is a good alternative way that require less time and also it prevents the introgression of undesired alleles (Schouten et al., 2006a). More importantly Cisgenesis also allows to preserve the proven fruit quality and other desired characters of high quality cultivars (Schouten et al., 2013). The avilability of isolated functional scab resistance genes is a preliminary requirement of 'cisgenesis'.

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The *Rvi5* (*Vm*) apple scab resistance gene has been initially mapped on the distal end of linkage group 17 by analysing 95 plants of a cross population between 'Golden Delicious' and 'Murray' (Patocchi et al., 2005). Fine mapping of the *Rvi5* locus allowed to identify two flanking markers (SSR FMACH_Vm2 and FMACH_Vm4) and a co-segregating marker for the resistance (SSR FMACH_Vm3) (Cova et al.- pers. Commun). Isolation of the region that controls the resistance is a key step of identification of candidate genes. Here we describe the procedure that we followed to isolate BAC clones spanning the *Rvi5* region from 'Murray' BAC library. Results of this this study will undergo sequencing and assembling for the candidate gene identification procedure.

Materials and Methods

To identify the BAC clones containing theresistant allele of SSR markers FMACH_Vm2, FMACH_Vm3 and FMACH_Vm4, a BAC library of 'Murray' was screened by using colony PCR. Identified positive clones were cultured in LB media overnight and plasmid DNA was extracted according to Sambrook protocol (2001). By using PCR amplification and gel electrophoresis accuracy of identified clones were confirmed. PCR amplification and capillary electrophoresis were set up according to Cova et al., 2010.

BAC ends were sequenced using M13 forward and reverse primers and BAC end sequences were blasted against the apple genome sequence of Golden Delicious at (https://genomics.research.iasma.it/gb2/gbrowse/apple) to position the BAC contigs on 'Golden Delicious' genome sequence. Orientation and relative overlaps of BAC contigs were determined by using the position of forward and reverse BAC end sequences.

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Results and Discussion

RThree BAC clones spanning the *Rvi5* locus have been identified by screening 'Murray' BAC library. The screening procedure has been completed by using the resistance alleles of SSR FMACH_Vm2, SSR FMACH_Vm3 and SSR FMACH_4.

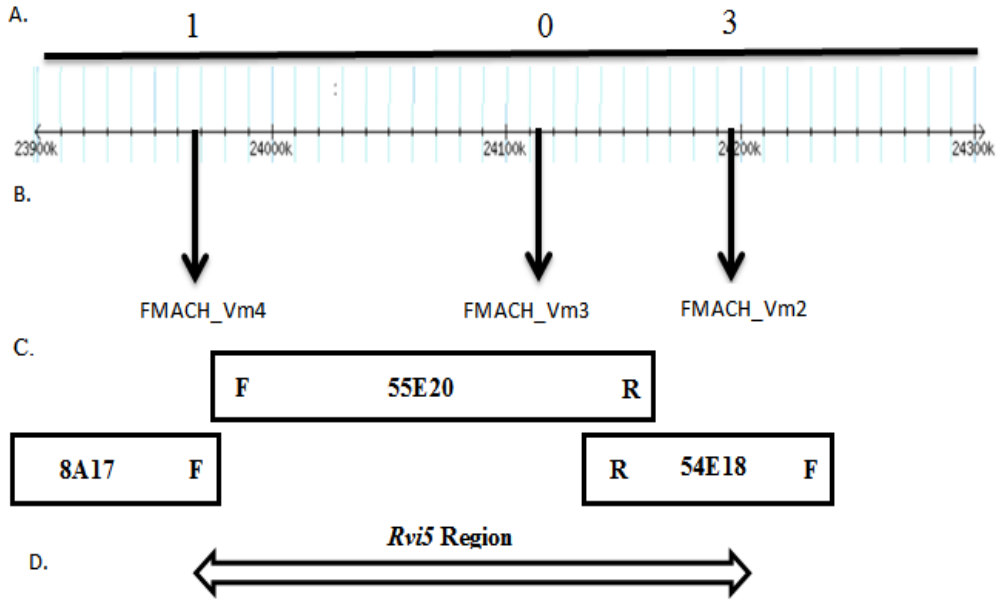

Figure 1: Schematic representation of *Rvi5* Region
A) Numbers indicate the number of recombinant plants found in the segregating population. B). Position of the SSR markers on the corresponding region of the 'Golden Delicious' genome genomic region spanning the *Rvi5* locus in 'Golden Delicious'. C) 'Murray' bacterial artificial chromosome (BAC) contigs cover the *Rvi5* region. F and R: are the M13 forward and M13 reverse BAC end sequences. D). Putative *Rvi5* region spanning from FMACH_Vm4 to FMACH_Vm2

BAC clone 8A17

BAC clone 8A17 was positive for the resistant allele of SSR FMACH_Vm4. Forward and reverse BAC end sequences were found in the 'Golden Delicious' reference genome sequence in between 23978.624 kb to 23977.714 kb and 23713.548 to 23714.058 kb respectively. According to the above values estimated size of BAC 8A17 was 265kb and orientation was reverse to forward, according to the technical data provided by the

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manufacturer of ‘Murray’ BAC library BAC contigs are within the range of 70kb to 260kb. Estimated size of BAC 8A17 is bit higher than the expected range, but influence of available unsequenced areas on ‘Golden Delicious’ sequence may be the reason for this unexpected size. There are several unsequenced areas in the region covered by BAC 8A17 in ‘Golden Delicious’ sequence.

BAC clone 55E20

Clone 55E20 was positive for the resistant allele of SSR FMACH_Vm3 and the forward BAC end sequence was found in 23978.304kb to 23978.844kb on ‘Golden Delicious’ sequence. The reverse sequence was found in 24151.917 to 24152.836kb. Estimated size of BAC contig was 174kb and orientation was forward to reverse. However, the size of this BAC clone is in acceptable range, but there are a lot of unsequenced areas in reference sequence in the area covered by BAC 55E20 also.

BAC clone 54E18

BAC clone 54E18 was positive to resistant allele of SSR FMACH_Vm2, the forward and reverse BAC end sequences were found in 24237.134 to 24238.000 kb and 24125.849 to 24126.431kb respectively. The orientation of the BAC 54E18 is reverse to forward and size should be 112 kb.

The forward BAC end sequences of BAC 8A17 and 55E20 are positioned in the same contig of ‘Golden Delicious’ sequence having 450bp region in common. That indicates the BAC 8A17 and BAC 55E20 overlapping each other. Like that, reverse BAC end sequence of BAC 55E20 and reverse BAC end sequence of BAC 54E18 also overlaps each other by

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sharing a region of 26.987kb in size. These results indicate the complete coverage of *Rvi5* region by identified BAC contigs.

According to the 'Golden Delicious' genome sequence, size of the identified *Rvi5* region is about 228kb. To find out the actual size of 'Murray' *Rvi5* region, it is essential to recognize the sizes of identified BAC contigs by Pulsed field Gel Elecrophoresis (PFGE). Results of this study will be used in future work of identification of *Rvi5* candidate genes. Complete sequence of *Rvi5* region will be obtained by constructing paired end libraries of identified BACs. Once the complete sequence is available gene prediction will be done to identify the candidate genes.

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CHAPTER 3

Identification of a TIR-NBS-LRR gene in the *Rvi5* (Vm) apple scab resistance locus of *Malus* × *domestica* genotype 'Murray'.

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Identification of a TIR-NBS-LRR gene in the *Rvi5* (Vm) apple scab resistance locus of *Malus* × *domestica* genotype 'Murray'

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Abstract

Apple scab, caused by fungal pathogen *Venturia inaequalis*, is a severe disease of cultivated apple (*Malus* × *domestica* Borkh.). It is the most studied plant–pathogen interaction of woody plant species using genetic, genomic, proteomic and bioinformatics approaches in both species. Until now 17 monogenic resistances against the disease have been identified in different *Malus* species and some of them are currently used in scab resistance breeding programs. However, the evolution of virulent pathogen strain/s has the ability to overcome the monogenic resistance, thus raising the need to define new strategies to obtain a durable resistance in apple breeding. Gene pyramiding becomes a successful method to obtain plants with durable resistance. The isolation of functionally different resistance genes is an essential prerequisite for gene pyramiding. Recently, the *Rvi5* (Vm) apple scab resistance from the cultivar 'Murray' was fine mapped and the region carrying the resistance was restricted into a region of 1 cM, flanked by two SSR markers (FMACH_Vm4 and FMACH_Vm2). Three bacterial artificial chromosome (BAC) clones spanning the resistance locus were identified, completely sequenced and assembled, which allowed identifying the putative *Rvi5* locus in a region of 154kb in size. The open reading frame prediction and functional annotation of the identified region revealed the presence of one putative gene homologous to TMV resistance protein of *Malus* × *domestica*, characterized by a Toll and mammalian interleukin-1 receptor protein nucleotide-binding site leucine-rich repeat structure.

Keywords Apple scab, *Venturia inaequalis*, Candidate gene, *Rvi5*, Vm, TNL genes

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Introduction

Apple (*Malus* × *domestica* Borkh.) is one of the most important fruit crop in the world with an annual worldwide production of over 80 million tons in 2013 (FAO;http://faostat.fao.org), which is second to the world banana production. Apple is host to a wide range of pest and diseases, most of which are present in all apple producing regions in the world (Way et al. 1990). Apple scab disease, caused by an ascomycete fungus *Venturia inaequalis* Cooke Wint., is a devastating pathogen present in apple cultivating countries in temperate zone with cool humid growing seasons (MacHardy et al. 1996). Almost all commercial apple varieties are susceptible for the disease. Maintaining good hygienic conditions in orchards, especially the removal of leaf litter, helps to control the disease (Gomez et al. 2007), but for the prevention, it is necessary either to spray fungicides 20-30 times per season or to cultivate scab resistance cultivars. The most common control method currently used is the extensive fungicide treatments, which eventually leads to increase the cost of production and generates long-term residues in food and in the environment. Breeding for resistance and the use of resistant varieties for the apple scab could help to avoid the problems associated with the use of fungicides.

Up to now, 17 major scab resistance genes and several QTLs have been discovered from wild *Malus* species and old apple varieties (Calenge et al. 2004; Gardiner et al. 2006; Gessler et al. 2006; Bus et al. 2011). The apple scab resistance *Rvi6* gene has been extensively used in apple breeding programs (Laurens 1999) but almost about a century of breeding efforts, breeders were unable to produce scab resistance apple variety with a considerable fruit quality (Gessler and Pertot 2011). The appearance of *V. inaequalis* races 6 and 7, which are able to overcome the *Rvi6* resistance (Parisi et al. 1993; Trapman

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2006), pointed out the need of new breeding strategies to obtain durable resistance. Gene pyramiding, integration of two or more resistance genes (R) in the same cultivar is the best strategy to obtain a durable resistance (MacHardy et al. 2001). This approach either delays the time required to overcome the resistance or prevents the resistance breakage. Due to the self-incompatibility and the long generation time of *Malus* spps plus high fruit quality demanded by the fresh market, breeding for high quality resistant cultivars may be a long and expensive task. Using traditional breeding techniques, the development of varieties with pyramided R genes and quality fruits may require many years and complex crosses. Researches recently highlighted that transferring genes between species of the same genera,the so called 'cisgenesis' (Schouten et al. 2006a; Schouten et al. 2006b; Schouten et al. 2009), permits to introduce genes from one crossable donor plant to other without adding foreign genes (from sexually incompatible species) and allows to incorporate only the favorable alleles to receiver plant. By using this 'clean vector technology', many apple resistance genes controlled by their own promoters could be combined to generate genetically modified-plants without containing foreign genes in the final products (Krens et al. 2004). This is opening the door to the creation of resistant cisgenic apples (Vanblaere et al. 2011) that are considered more acceptable than those obtained by the traditional transgenic approach. However, the isolation of functionally different R genes is a prerequisite to develop cisgenic apple cultivars with pyramided R genes.

Until now, three apple scab resistance genes have been cloned, of which two were functionally isolated, the *Rvi6* gene located on the linkage group (LG) 1 (Gianfrancheschi et al.1996; Tartarini et al.1999) and the *Rvi15* apple scab resistance gene located on LG 2 (Patocchi et al. 2003). The *Rvi6* encodes for leucine- rich repeat receptor like family

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protein (LRR-RLP) similar to the tomato *Cf* like resistance gene(Vinatzer et al. 2001; Belfanti et al.2004), while the *Rvi15* encodes for a nucleotide-binding site leucine –rich repeats (TIR-NBS-LRR) family proteins (Galli et al. 2010b). More recently, a candidate genes for the apple scab resistance *Rvi1* on LG 2 were reported by Cova et al. (2015) while nine NBS-LRR resistance genes on LG 4 were reported by Bastiaanse et al. (2015) for the scab resistance cultivar 'Geneva'.

The apple scab resistance gene *Rvi5*, mapped on the distal end of LG 17 (Patocchi et al. 2005) believed to be inherited from *Malus micromalus 245-38* and *Malus atrosanguinea 804* (Shay and Hough 1952). Dayton and Williams (1970) described it as a pit type gene induces hypersensitive response for the pathogen of apple scab after 3-5 days of inoculation. *V.inaequalis* race 5 has overcome the gene, for the first time discovered in England (William and Brown 1968). However, recent surveys conducted under the frame of VINQUEST initiative (www.vinquest.ch, Patocchi et al. 2009a), suggested that this virulence is not widely widespread. The well-studied apple scab resistance gene *Rvi15* also induces the hypersensitive response for the pathogen, but *Rvi15* elicits slow response compared to the *Rvi5*, which requires 15 days to elicit the resistance symptoms after the inoculation. The time differences required to elicit the symptoms in between *Rvi15* and *Rvi5* may be due to the different genes responsible for the resistance reaction. The limited spread of *V.inaequalis* race 5 and the differences of time required to elicit the resistance reaction with respect to *Rvi15*, makes this gene interesting to study aiming resistance gene pyramiding. The *Rvi5* resistance was found to segregate for a single dominant locus (Cheng et al. 1998; Patocchi et al. 2005). Further, fine mapping of the locus using 1243 progeny plants of 'Golden Delicious' x 'Murray' and 'Galaxy' x 'Murray' has delimited

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the locus controlling the resistance to a 1 cM in 'Murray' genome and developed three co-segregating markers for the resistance gene (Cova et al. 2015b).

In this article, we report the isolation of *Rvi5* locus from 'Murray' bacterial artificial chromosome (BAC) library using molecular markers tightly linked to the *Rvi5* gene. The *Rvi5* resistance locus was covered by three overlapping BAC clones. The identified BAC clones were sequenced and Open Reading Frames (ORF) found on the *Rvi5* region was characterized. The candidate gene responsible for the *Rvi5* resistance has been identified as a TIR-NBS-LRR gene. The finding of this study will lead to the cloning and functional characterization of *Rvi5* apple scab resistance in near future.

Materials and Methods

BAC library construction, pooling, and screening

A BAC library of 'Murray' was constructed at the Amplicon Express (Pulmann, WA) starting from frozen 'Murray' leaves according to Baldi et al. (2013). A BAC 'pooling' strategy was applied in order to reduce the number of steps needed to screen the whole library for single BAC identification. The original BAC library was stored in 96 plates of 384 well plates which were replicated twice to form two pools denominated 'plate pool' and 'well pool'. The "plate pool" was simply obtained by mixing all the 384 clones of each plate into one well of a new 96 well plate, while the 'well pool' was obtained by virtually stacking the 96 plates and mixing all the 96 clones located in the same "vertical position" of the plate. This step was accomplished by using the 96-pipetting system of a *Freedom EVO® robotic* workstation (Tecan Group, Männedorf, CH). Pooled BAC library was screened using two set of colony PCRs to identify the BAC clones carrying the sequences of interest: i) on "plate pool" to identify the BAC library plate that contains the

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positive clone, and ii) on the ‘‘well pool” to identify the well coordinates within each plate.

BAC screening was carried out using the direct fluorescent primer method (Schuelke 2000). A forward primer with a fluorescent dye at the 5’ end was synthesized (Sigma-Aldrich, St. Louis, MO) for each set of SSR primers reported in Cova et al. 2015b. Colony PCR amplification was carried out in a final volume of 50µl with 5 µl of 10X PCR buffer,3 µl of 25mM $MgCl_2$, 1 µl of 10mM dNTPs, 1 µl of each primer diluted to 20mM and 0.2 U of Taq polymerase (Hot start Taq; Applied Biosystems, Foster City, CA). The cycling regime consisted of a denaturing step at 95^0C for 15 min, followed by 35 cycles at 95^0C, 60^0C for 1 min and 72^0C for 2 min, with a final extension of 7 min at 72^0C. The PCR products were separated and detected using a 3730xl DNA analyser (Applied Biosystems). The SSR FMACH_Vm 2 marker (Cova et al. 2015b) was used as the entry point of BAC screening; BAC pools were screened by using resistance allele of SSR FMACH_Vm 2 (158bp). Once positive clones have been identified for that specific SSR marker, BAC DNA was extracted using the plasmid DNA extraction kit of Sigma Aldrich (Sigma Aldrich, St. Louis, MO) according to the manufacture instructions. BAC ends were sequenced using the M13 forward (GTAAAACGACGGCCAGT) and reverse primers (AACAGCTATGACCATG), on the 3730xl Genetic analyzer (Applied Biosystems, Foster City, CA.) according to Baldi et al. (2013). When several positive clones have been identified for a single molecular marker, BAC end sequences were visualized used Chromas v 2.2 (Technelysium Ltd Brisbane, Australia) to check their quality and BLAST (*Altschul* et al. 1990) against the ‘Golden Delicious’ genome sequence v1.0 contig (Velasco et al. 2010; (http://www.rosaceae.org/gb/gbrowse/malus_x_domestica) to recognize the largest BAC

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clone. The largest BAC clone covering maximum area of the reference genome has been selected for the chromosome walking. New markers were designed on the sequenced BAC ends of the selected clone to continue the chromosome walking. Newly developed molecular markers on BAC end sequences of identified BAC clone were used to screen the BAC pools as second chromosome walking step. Once positive BAC clones have been identified, identified BAC clones were screened for the resistant allele of SSR FMACH_Vm3 (355bp). BAC clones that were positive for the both molecular markers used for the BAC screening were used for the BAC DNA extraction and end sequencing. As described in previous chromosome walking step, the largest BAC clone was selected to continue the chromosome walking. The BAC pool screening and chromosome walking continued a BAC clone carrying resistant allele of SSR FMACH_Vm4 was found

BAC clone sequencing and assembly

All BAC clones covering the resistance locus were sequenced using the 454 shotgun sequencing protocol (GS FLX (Titanium), Roche applied science, Mannheim, Germany) by preparing 3kb paired end libraries to reach 30X coverage. Vector trimmed BAC sequences were assembled using Mira-4.0.2 Software (Chevreux et al. 1999) with high stringency parameters (99% match, 20bp minimum overlap). After assessing the sequence quality and aligning the markers linked to the *Rvi5* resistance locus, specific primers were designed to close the gaps. To find the orientation of BAC clones, BAC ends were sequenced following the same conditions reported above. To assess the relative overlaps of each BAC, specific probes were designed on the BAC ends sequences using Primer3 software (Rozen and Skaletsky 2000) and probe hybridization experiments were done according to the Cova et al. 2015a. Once the assembly of each BAC has been completed,

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BAC end sequences were blasted against the assembled BAC sequences at a high stringency (99% similarity) to confirm the complete coverage of *Rvi5* resistance locus.

ORF prediction and Identification of *Rvi5* candidate genes

Candidate genes were predicted using a mixed ab-initio/knowledge based approach. All scab resistance genes available at the NCBI database were collected and aligned against the sequence identified between the two SSR markers (FMACH_Vm 2- FMACH_Vm 4). All matched sequences together with the BAC sequence were submitted to the web service of FGENESH software (http://www.softberry.com/ Salamov and Solovyev 2000); three plant gene models were used to tuning the search parameters: Arabidopsis, grapevine and tomato. All predictions were then further analyzed with Blastx (Altschul et al. 1990) similarity search against UniProt database and InterProScan search to determine protein domains.

Transcription analysis of *Rvi5* candidate genes

Total RNA was extracted from un-inoculated 'Murray' plants using iRIPE_RNA isolation protocol (Sigma Aldrich, St. Louis.) according to the manufacture instructions. First strand cDNA was synthesized by SuperScript^{TM} III Reverse Transcriptase and Oligo(dt)20 primer (Invitrogen, CA) following manufacture instructions. Specific primer pairs were designed by using Primer3 (Rozen and Skaletsky 2000) for the identified candidate genes. Candidate gene amplification was done using a long PCR amplification protocol as follows: 2µl of undiluted cDNA solution was mixed with 5ul of 10x long PCR buffer with MgCl2, 2.5Mm of each dNTP, 5Um of each primer and 1Uof long PCR enzyme mix (Thermo scientific, MA.) in a total volume of 50µl. The thermal cycling conditions were as follows: Initial denaturation at 94^0C 30 seconds; followed by 35 cycles of 10 seconds at

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94^0C, 30 seconds at 60^0C and 3 minutes at 72^0C and 10 minutes of final extension step at 72^0C. The amplicons obtained were sub cloned using TOPO-TA cloning kit with one shot TOP10 chemically competent cells (Invitrogen, CA). Plasmid DNA was extracted using plasmid DNA extraction kit of Sigma Aldrich and the candidate gene sequence was obtained by primer walking.

Results

BAC construction, pooling, and screening

The BAC library of 'Murray' consisted of 36,864 BAC clones with an average insert size of 130Kb, representing approximately 3.5 times the diploid apple genome (Velasco et al. 2010). The BAC pooling strategy used in this study helped to reduce the number of samples to screen from 38640 to 480. Three chromosome walking steps were used to recognize the BAC clones spanning the *Rvi5* resistance locus starting from SSR FMACH_Vm2. At the end of chromosome walking three overlapping BAC clones (54E18, 55E20, and 8A17) were identified (Figure 1).

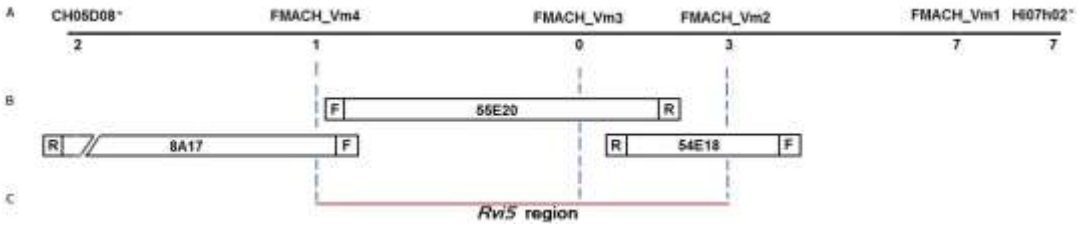

Figure 1: Schematic representation of the region surrounding *Rvi5* resistance gene. **A)** SSR markers flanking the putative *Rvi5* locus and the number recombinant plants found for each SSR marker in the segregating populations are indicated by numbers. **B)** 'Murray' bacterial artificial chromosome (BAC) contigs spanning the *Rvi5* region. Lines show the position of the SSR markers on the (BAC) contigs. **F** and **R**: are the M13 forward and M13 reverse BAC insert-end; **C**) The putative *Rvi5* region spanning in between two flanking SSR markers FMACH_Vm4 and FMACH_Vm2.

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The BAC clone 54E18 was the largest BAC clone in coupling with the resistance allele of SSR marker SSR FMACH _Vm2 marker; hence the clone 54E18 was selected as the first clone of chromosome walking. The primers designed at the reverse BAC end sequences of clone 54E18 (Table 1) permitted to identify the BAC clone 55E20. The BAC clone 55E20 was positive for the primer 54E18 Rev and also in coupling with the resistance allele of primer FMACH_Vm3, hence it has been selected as next BAC clone to proceed with chromosome walking. By following the same procedure, the BAC clone 8A17 was identified, which was positive for the primers designed at the forward end sequence of BAC 55E20 and in coupling with the resistant allele of SSR FMACH-Vm4.

Table 1: Molecular markers developed for the end sequences of BAC contigs for the chromosome walking. The markers used to identify the BAC clones are highlighted in bold.

Primer name	Forward sequence	Reverse sequence	Annealing Temperature	Product size
54E18 For	**5'-AACGGTGTAGATCGGTGAGG**	**5'-AAGGGGATGTCGTTGTTTGC**	**55 ^{0}C**	**210bp**
54E18 Rev	**5'-TATTCAATCTGGTCGCGGGT**	**5'-ATGCTCTCTGGTAGGCTTGT**	**55 ^{0}C**	**200bp**
55E20 For	**5'-TGCAAGCTTTTCTCCGTTTCA**	**5'-CCAACCGAGAAGTTTTCCTCA**	**55 ^{0}C**	**175bp**
55E20 Rev	**5'-GGACATCTCACTGTGGACCA**	**5'-AAGCCTTCATCCTGGTCCAG**	**55 ^{0}C**	**158bp**

The complete sequence of the three BACs was obtained by creating 3kb paired end libraries and sequences were assembled using Mira 4.0.2 software. The mean average

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coverage obtained after the assembly was 66% and the largest contig obtained was 18913 bp in size. The BAC assembly ended with BAC clone 8A17 assembled in 9 contigs, BAC 55E20 in 7 contigs and 54E18 in 6 contigs. Gap filling of the contigs within the same BAC clone was done by designing new molecular markers at the end of the each contig sequence and by sequencing the PCR product. Hence 15 primers for the gap fill of BAC 8A17, 12 primers for the BAC 55E20 and 10 primers for the BAC 54E18 were designed respectively (Supplementary table 1). Single amplicons obtained by PCR amplification were extracted from gel after electrophoresis and sequenced. The sequence information was used to close the gap. BAC end sequences were BLAST against the complete BAC sequence of adjacent BACs to find the orientation of each BAC. Sequences of the two SSR markers flanking the *Rvi5* resistance locus (FMACH_Vm2 and FMACH_Vm4) were BLAST on the sequences of the BAC clones 55E20 and 8A17 respectively to define the margins of *Rvi5* region. The putative *Rvi5* region of 'Murray' was isolated by removing overlapping regions and resulted in 154kb in size.

Gene prediction and identification of candidate gene

FGENESH results obtained with the three gene models were quite different by whole number of predictions obtained and their features. 41 ORFs were obtained with Arabidopsis parameters, 33 for the grapevine parameters and 38 with tomato. Predictions with a CDS were 22, 23 and 16 for the Arabidopsis, grapevine and tomato optimization parameters respectively.

Functional annotation; especially the conserved domains identification helped to select the most probable prediction. Since some CDS did not had any conserved domains (A.36, A.38, V.23, V28) or not all the mandatory domains were present (A.2, A.9 and A.26, V.2,

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V.7, V.25); (details are reported in supplementary table 2), there were just one complete prediction common for the three different parameters and it was designed as A.27 in Arabidopsis predictions V.25 for Grapevine and T.25 for tomato. The genomic sequence of the identified R gene consisted with TIR domain, NB-ARC domain and LRR domains, in between NB-ARC and LRR domains there was a intron about 5kb in size. It is the only R gene found in the putative *Rvi5* region having full length of coding DNA sequence and that can be identified by its functionality, hence that gene was considered as the putative resistance gene (*Rvi5*_Murray) of *Rvi5* scab resistance.

Transcription analysis of *Rvi5* candidate genes

The transcript of the putative resistance gene was amplified with the primers designed on the start (5'-ATGGATGCATTCCGAGATTC) and stop codons (5'-GATTTGTGCATCACCAACATT). The amplicon obtained was about 3.5kb in size and it has been cloned to TOPO-TA vector for sequencing. The full ORF of the transcript was sequenced and the sequence deposited at NCBI.

Discussion

BAC sequencing

The BAC library of 'Murray' consisted of 38640 clones arranged in 96 plates of 384 wells per plate. The BAC pooling strategy adopted in this study allowed us to increase the efficiency of BAC screening by reducing the time, and the number of PCRs required to identify the positive clones. After making two separate pools called 'plate pool' and 'well pool', the 'plate pool' was screened first using the marker of interest. The position/s of the positive clone/s in 96 well 'plate pool' indicates the plate number carrying the positive clone. The screening results of 'well pool' indicate the position of positive clone on the

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identified plate. The position of positive clone was determined by combing 'plate pool' and 'well pool' results. Different pooling techniques have been reported in different studies to simplify the complex BAC screening procedure (Xia et al. 2009; You et al. 2010). Xia et al. 2009 have been used a similar BAC pooling procedure similar to ours which allows to pool the complete BAC library manually, But You et al. (2010) described a more complex high-throughput five dimensional pooling strategy supported by the Illumina GoldenGate assay for the screening of the pooled BAC library. BAC sequencing was performed using the 454 paired-end sequencing technique, which sequences both ends of the fragments and generates high quality alignable sequence data, in particular for highly repetitive sequence regions (Fullwood et al. 2009). Paired-end sequencing reads produce *both* ends of a DNA fragment, and is capable of pairing ends together, hence it helps to recognize the ends of the fragments, even if each individual read doesn't overlap with its mate. This allows getting sequences of the ends of larger pieces, which means that any piece that contains an entire repetitive element may give a pair of reads that identifies both flanking sequences. According to Velasco et al. (2010) apple genome has a relatively high number of repeated sequences, which are difficult to assemble or anchor. The distal end of LG 17, where the *Rvi5* is mapped, in 'Golden Delicious' is also highly repetitive and the current apple genome assembly showed numerous sequence gaps. To help assembling this complex region carrying the *Rvi5* resistance gene we used the 454 paired-end sequencing method.

Gene prediction and candidate gene identification

Genes are not equally spread throughout the genomes (Barakat et al. 1999; Bernardi 2004); hence it is difficult to compare the gene densities between different genomes and their genomic regions. In the apple genotype GMAL 2473, Galli et al. 2010b found 17

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ORFs in 48.6kb region, three of which were predicted to be related with the plant resistance genes. Fahrentrapp et al. (2013) and Parravicini et al. (2011) have predicted the genes responsible for the fire blight resistance in *Malus* x *robusta* and ornamental apple cultivar 'Everest' respectively. The number of genes predicted in *Malus* x *robusta* was 47 in 162 kb region and observed only one candidate gene. In a 78kb region of the cultivar 'Everest' they have found 23 ORFs, of which eight genes were associated with the resistance. In our study we observed 33 to 41 ORFs in a region of 154kb (number of ORFs observed were varies according to the plant model used), of which only one candidate gene was found (*Rvi5*_Murray). Although the number of resistance genes we have identified in 154kb region is low, gene density is more than three times compared to the gene density observed in 'Golden Delicious' (0.78 genes per 10kb) by Velasco et al (2010). Usually, plant resistance genes are found in clusters of R genes and R gene analogs (RGA) (Meyers et al. 2003), which is also true for apple (Baldi et al. 2004; Broggini et al. 2009; Calenge et al. 2005). However, in our study we identified one candidate gene not embedded in a cluster of paralogs.

The candidate gene we found belongs to the TNL proteins, which consist of N-terminus with homology to the intracellular domain of *Drosophila* Toll and interleukin-1 receptor proteins (TIR) (Whitham et al.1994), a central nucleotide-binding site (NBS), and a C-terminal leucine –rich repeat (LRR) domain (Figure 2)

The ORFs predicted on the sequence of 'Golden Delicious' homologous to the *Rvi5* identified one TNL gene (MDP0000252512) and three leucine rich receptor-like proteins. However, we haven't found receptor-like protein class of plant resistance genes (called *HcrVf* genes in *Malus*), that were found in *Rvi6* apple scab resistance. The ClustalW

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pairwise alignment of the candidate gene of *Rvi5* (*Rvi5*_Murray) with the scab resistance gene of *Rvi15* (Vr2-C) and the TNL gene found in the *Rvi5* homologous region of 'Golden Delicious' (MDP0000252512) was resulted 22% of identity in between amino acid sequences of TNL gene MDP0000252512 and *Rvi5*_Murray, while Vr2-C and *Rvi5*-Murray has only 37% of identity, hence can't made any conclusion about the mode of action of *Rvi5*-Murray based on ClustalW pairwise alignment results.

> *Rvi5*_Murray

a)

MDAFRDSSSSSYRCSYHAFLSFRGQDTRKGFTDHLYRALELAGIHTFRDDDEIKRGENIESELDKAIQESQVSIIV
FSKDYASSRWCLNELLKIVERRNTDHRHVVLPVFYDVDPSDVRKQSGPFAEAFARHEERFSTEMDKVEQWRR
ALGDVASLGGMVLGDRYEGQFIQEIVEEIRNKVGHAALDVAPFSVGMD

b)

NRVQDLNMWLQDGSNDVGVAVICGMGGIGKSTIAKAAYNRNFDRFQGSSFLADIRESSEQPNGFACLQRKLLS
DIQKGKAKRVYNMDEGTIRIKQAVGYKKVLIVLDDVSNQDQLNAILGMQEWLHRGSKIIITTRHEHLLNAHEV
FEKFMVPELNEYESLELLSWHAFGQSHPVEGYMQLSKHVVQHCGGLPLALQVLGSSLSGKSVEVWQSALKKL
DVIPNDKIQKVLRISFDSLQDDHDKNLFLHIASFFTGNTMD

c)

YTITILDSLDFCTRIGIENLVDICLVEIQGSKLVMHQLVRNMAMAIIRKESPHDPGKRSRVMQKDASNILRKLSGT
ENIKGLMLNLASKRTFVGSDKKRCHVEDYDGNCSRRRRLGFFSWKPVSFSSTNSASASNEVDFKSEAFRRMHN

d)

LEILMLNNVNVRGNYEEFSKN

LVCLSWRGFHLKSIPENLYLGN

LVALNMRNSSLQHVWNGIRFLPR

LKILNLSHSHGLRTTPDFSGLPN

LERLILKDCINLVEVDESIGYLEK

LVVLNLEGCKNLMKLPKLRS

IQDLILSGCSKLVLGANTTATTGHLQSTACEMKKLNLLSAKSWYSIWSRVSPRKNIEPSSFSLASLPHS

LTSLRLDSCNLSEIPSALTMLSS

LEYLDLNNNPITSLPESMNNLVK

LRTLEIYCCRNLTMLPELPHSLKRLFAHSCRSLKRITNL

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e)

LNADISLDSDHHIALESNLWYCGKLVDVESLFNTLPLSSFDIKLLKDNDLFDLKPNGGVE
VQNCASRREFKGLYGCGIFSIFVHESKIPDRFNYRSMGNTVFSIIVPSQPNIKIVGLNACILYARQSDRSPEIARGY
RRHHSLVLRNETKGLKWNLSKLRGVEWKVMNEDMLWLSRWIMGNNELECGEKVCFEIIGEQDGFLTKEIGVQ
FVYEQKNKDEEDVLSSSEDTMIQCHRSPAKFSWGFDRLGQCIPKKKVMSTPVCAADYFLCNPFLCNPMQCIRK
TQFNSN

Figure 2: Amino acid sequence of the predicted full length transcript of *Rvi5*_Murray candidate gene. The amino acid sequence has been divided into five domains **a**) N_ terminus *Drosophila* Toll and mammalian interleukin-1 receptor (TIR); **b**) Nucleotide binding site **c**) connecting region **d**) Leucine-rich repeats **e**) The C terminal region. Alignment of the LRR domain has been performed following cytoplasmic LRR consensus 'LxxLx[IL]xxCxxLxxL'(Jones and jones 1997) ,amino acids of the LRR like motif are highlighted :L-blue, C-yellow, I-green, residues doesn't follow the consensus - gray .

In total, 10 hydrophobic LRR like motifs were found to fit the consensus of LxxLx[IL]xxCxxLxxL (Jones and jones 1997). The LRR like motifs is also putatively involved in protein-protein interaction, like classic LRRs. Furthermore, in the consensus LRR like motif, aliphatic I and L could be substituted by hydrophobic as well as non-polar I, C, L (Kyte and Doolittle 1982; Taylor 1986) due to their shared characteristics. Different numbers of LRR motifs have been found in different R genes. Galli et al. (2010b) found 15, 29 and 15 imperfect LRRs in three *Rvi15* (Vr2-A,Vr2-B,Vr2-C) scab resistance protein candidates. The fire blight resistance CNL genes, the MdE-EaN gene identified in *Malus* cultivar 'Evereste' possess 11-12 imperfect LRRs (Parravicini et al. 2011 and FB_MR5 gene found in *Malus* × *robusta* had 23 LRR motifs (Fahrentrapp et al. 2013). However, apple scab resistance gene Vr2-C was the first functionally identified TNL resistance gene for the apple scab (Schouten et al. 2014).

Assumed mode of function of *Rvi5*-Murray

Different TNL proteins have been found to elicit resistance against many pathogens in various species. The *RPP5* (Parker et al. 1997) and *RPP1* (Botella et al.1998) genes that act against the fungal pathogen of downy mildew and rust resistance *L6* (Lawrence et al.

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1995) gene in *Arabidopsis* also belongs to the TNL proteins. The TNL gene family contains an LRR domain that has the ability to recognize the effector proteins of the pathogen directly or indirectly. We haven't found transmembrane domains or signal peptide in the *Rvi5* region, which suggests this protein reside inside the host cell. This implies the spatial separation of the pathogen and the recognition protein of the host. This is typical for TNL proteins (McHale et al. 2006). To facilitate the host pathogen recognition, an effector protein from *V.inaequalis* should enter to the host cell, which might allow binding to the LRR domain of *Rvi5*, or binding to a symplastic host protein that is guarded by *Rvi5*. Houterman et al. (2009) have studied the interaction in between *I-2* resistance protein in tomato, which is also a NBS-LRR protein and the effector protein Avr2 from its pathogen *Fusarium oxysporum.* They hypothesized that the Avr2 effector protein might suppress or counteract on host defenses, which makes Avr2 a virulent gene and it induces the sending of effector in to the host cell. The same hypothesis was used to describe the resistance mechanism behind the *Rvi15* (Shouten et al. 2014). Both *Rvi5* and *Rvi15* share the same class of proteins (TNL) and induce hypersensitive response after the infection of *V.inaequalis.* We can therefore hypothesize that the pathogen sends an effector protein into the plant cell for suppression or counteracting host defense. The resistance protein *Rvi5*, which resides inside the host cell, recognizes the effector protein or it recognizes the effector protein which is interacted with a symplastic host protein guarded by the *Rvi5*, would results local hypersensitive response.

Conclusion

The *Rvi5*_Murray is the second TNL candidate resistance gene of *Malus* x *domestica* to be cloned. However, its functionality can be proven only after a complementation or

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silencing experiments, which is pre-requisite for the gene pyramiding of apple scab resistance in a single background by genetic modification.

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Supplementary materials

Supplementary table 1: Molecular markers used for the gap filling of BAC contigs

Primer name	Sequence	Annealing temperature
For the BAC 55E20		
55E20_c9.1	5'-tcagcctcggaagaaactg	60 ^{0}C
55E20_c1.1	5'-ctccgctacacccattttgt	60 ^{0}C
55E20_c1.2	5'-tcttcagccctcttgcatct	60 ^{0}C
55E20_c2.1	5'-aagttagcggcttacgacga	60 ^{0}C
55E20_c2.2	5'-gcctgagagggttgtcagag	60 ^{0}C
55E20_c6.1	5'-caacatgccaaacacacaca	60 ^{0}C
55E20_c6.2	5'-gagattgggaacgaggttca	60 ^{0}C
55E20_c13.1	5'-tgggtctttgggaaattgag	60 ^{0}C
55E20_c13.2	5'-ggcaatgcctacatccagtt	60 ^{0}C
55E20_c16.1	5'-acgcttgaccaagcctaaaa	60 ^{0}C
55E20_c16.2	5'-ccaacaccgtttctcgttct	60 ^{0}C
55E20_c8.1	5'-tttgttgaatttcccaatttcc	60 ^{0}C
For the BAC 54E18		
54E18_c758.1	5'-tgatgaggggactggtgatt	60 ^{0}C
54E18_c758.2	5-ctgataaatcggctgcacaa	60 ^{0}C
54E18_c759.	5'-cttcgggatcttctttgtgc	60 ^{0}C
54E18_c759.2	5'-tccagtgtcatggcaaaatg	60 ^{0}C
54E18_c760.1	5'-tgcaagcctcttccgttact	60 ^{0}C
54E18_c760.2	5'-acagttgaggaaccattgcac	60 ^{0}C
54E18_c766.1	5'-actggaaggggataacgatg	60 ^{0}C
54E18_c766.2	5'-gtggaattcagaggggaaca	60 ^{0}C
54E18_c771.1	5'-gaacgaaacagtcgggaaaa	60 ^{0}C
54E18_c771.2	5'-cgcggctgattactttctct	60 ^{0}C
54E18_c772.1	5'-ttgcagtgtaccaaggcaga	60 ^{0}C
54E18_c772.2	5'-cagcccctgaagacgttaaa	60 ^{0}C
54E18_c776.1	5'-ttatttcttgtgggcgaagg	60 ^{0}C
54E18_c776.2	5'-gctttttgcatccgttttgt	60 ^{0}C
54E18_c856.1	5'-ggggtgaaaataccccttcgt	60 ^{0}C

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For the BAC 8A17		
8A17_c9.1	5’-gtctctcaaaggggcacac	60 ^{0}C
8A17_c9.2	5’-gtctctcaagggcacacagg	60 ^{0}C
8A17_c28.1	5’-aggtaattgcatttgggtaca	60 ^{0}C
8A17_c28.2	5’-tcaaaacctgatgcgctaaa	60 ^{0}C
8A17_c24.1	5’-ttagcgcatcaggtttttga	60 ^{0}C
8A17_c24.2	5’-ggtcgctttttgtggtcttc	60 ^{0}C
8A17_c21.1	5’-ccaaacatttataccacctttcc	60 ^{0}C
8A17_c21.2	5’-gaagcgaaaattgcacatca	60 ^{0}C
8A17_c27.1	5’-tgaagccttccaaaacaagg	60 ^{0}C
8A17_c27.2	5’-gcagccatgagttgtgaaga	60 ^{0}C

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Supplementary table 2: Predicted ORFs of the putative *Rvi5* region for the algorithms of Dicot plants, *Vitis vinifera* and Tomato. The ORF gave homology for the known resistance genes are highlighted in yellow.

Predicted genes for Algorithm of Dicot plants

Predicted ORF name	*Homologous gene*	e.Value	Query coverage	Available Domains
A.1	DEAD-box ATP-dependent RNA helicase 24-like [Pyrus x bretschneideri]	0.0		
A.2	TMV resistance protein N-like isoform X2 [Malus domestica]	4,00E-52	44%	NBARC
A.3	uncharacterized protein LOC103432406 [Malus domestica]	0.0		
A.4	putative COBL7 (COBRA-LIKE 7) [Malus domestica]	5,00E-22		
A.5	hypothetical protein [Malus domestica]	5,00E-102		
A.6	putative RNA-directed DNA polymerase (Reverse transcriptase) [Malus domestica]	2,00E-140		
A.7	hypothetical protein [Malus domestica]	4,00E-06		
A.8	DEAD-box ATP-dependent RNA helicase 24-like [Malus domestica]	1,00E-29		
A.9	PREDICTED: TMV resistance protein N-like isoform X1 [Pyrus x bretschneideri]	1,00E-95		TIR ONLY
A.10	uncharacterized protein LOC103433180 [Malus domestica]	2,00E-71		
A.11	pentatricopeptide repeat-containing protein At1g02370, mitochondrial-like [Pyrus x bretschneideri]	0.0		
A.12	ribonucleases P/MRP protein subunit POP1 [Malus domestica]	3,00E-29		
A.13	predicted protein [Malus domestica]	1,00E-52		
A.14	uncharacterized protein LOC103934706 [Pyrus x bretschneideri]	3,00E-67		
A.15	putative RNA-directed DNA polymerase (Reverse transcriptase) [Malus domestica]	0.0		
A.16	T4.14 [Malus x robusta]	2,00E-152		
A.17	predicted protein [Malus domestica]	4,00E-34		
A.18	putative RNA-directed DNA polymerase (Reverse transcriptase) [Malus domestica]	0.0		
A.19	T4.14 [Malus x robusta]	1,00E-109		
A.20	predicted protein [Malus domestica]	2,00E-55		
A.21	ribonucleases P/MRP protein subunit POP1 [Malus domestica]	7,00E-143		

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A.22	pentatricopeptide repeat-containing protein At4g01990, mitochondrial-like [Malus domestica]	4,00E-84		
A.23	copia LTR rider [Solanum lycopersicum]	2,00E-120		
A.24	LOW QUALITY PROTEIN: uncharacterized protein LOC103444267 [Malus domestica]	5,00E-64		
A.25	hypothetical protein VITISV_003191 [Vitis vinifera]	0.0		
A.26	TMV resistance protein N-like [Malus domestica]	1,00E-76	15%	TIR-NBARC-LRR
A.27	no match			
A.28	no matcyh			
A.29	hypothetical protein VITISV_004062 [Vitis vinifera]	1,00E-130		
A.30	uncharacterized protein LOC103452283, partial [Malus domestica]	8,00E-144		
A.31	uncharacterized protein LOC103434364 [Malus domestica]	2,00E-47		
A.32	lysosomal alpha-mannosidase-like [Malus domestica]	1,00E-46		
A.33	hypothetical protein VITISV_000226 [Vitis vinifera]	1,00E-08		
A.34	Reverse transcriptase (RNA-dependent DNA pol [Malus domestica]	2,00E-64		
A.35	uncharacterized protein LOC103958468 [Pyrus x bretschneideri]	3,00E-167		
A.36	TMV resistance protein N-like [Malus domestica]	7,00E-53	12%	No protein coding domains
A.37	copia LTR rider [Solanum lycopersicum]	0.0		
A.38	TMV resistance protein N-like [Malus domestica]	3,00E-87	22%	No protein coding domains
A.39	pentatricopeptide repeat-containing protein At4g01990, mitochondrial-like [Malus domestica]	3,00E-74		
A.40	ribonucleases P/MRP protein subunit POP1 [Malus domestica]	9,00E-68		
A.41	transcription factor UPBEAT1 [Malus domestica]	2,00E-70		

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Predicted genes of Algorithm of Vitis

Predicted ORF name	Homologous gene	e.Value	Query coverage	Available Domains
V.1	DEAD-box ATP-dependent RNA helicase 24-like [Pyrus x bretschneideri]	0.0		
V.2	TMV resistance protein N-like isoform X2 [Malus domestica]	0.0	74%	nbarc-lrr8
V.3	uncharacterized protein LOC103432406 [Malus domestica]	0.0		
V.4	putative COBL7 (COBRA-LIKE 7) [Malus domestica]	0.0		
V.5	uncharacterized protein LOC103417687 [Malus domestica]	0.0		
V.6	DEAD-box ATP-dependent RNA helicase 24-like [Malus domestica]	4,00E-35		
V.7	TMV resistance protein N-like [Malus domestica]	1,00E-95	20%	tir only
V.8	uncharacterized protein LOC103425749 [Malus domestica]	1,00E-124		
V.9	beta-carotene isomerase D27, chloroplastic [Malus domestica]	9,00E-35		
V.10	pentatricopeptide repeat-containing protein At1g02370, mitochondrial-like [Malus domestica]	0.0		
V.11	ribonucleases P/MRP protein subunit POP1 [Malus domestica]	3,00E-29		
V.12	predicted protein [Malus domestica]	6,00E-37		
V.13	ribonucleases P/MRP protein subunit POP1 [Malus domestica]	5,00E-110		
V.14	putative COBL7 (COBRA-LIKE 7) [Malus domestica]	4,00E-143		
V.15	uncharacterized protein LOC103417687 [Malus domestica]	3,00E-65		
V.16	putative RNA-directed DNA polymerase (Reverse transcriptase) [Malus domestica]	0.0		
V.17	putative COBL7 (COBRA-LIKE 7) [Malus domestica]	0.0		
V.18	uncharacterized protein LOC103417687 [Malus domestica]	0.0		
V.19	putative COBL7 (COBRA-LIKE 7) [Malus domestica]	1,00E-144		
V.20	uncharacterized protein LOC103956881 isoform X3 [Pyrus x bretschneideri]	4,00E-145		
V.21	pentatricopeptide repeat-containing protein At1g02370, mitochondrial-like [Pyrus x bretschneideri]	5,00E-159		
V.22	putative gag-pol polyprotein [Fragaria x ananassa]	0.0		
V.23	TMV resistance protein N-like [Malus domestica]	4,00E-76	17%	No protein coding domains
V.24	copia LTR rider [Solanum lycopersicum]	0.0		
V.25	TMV resistance protein N-like [Malus domestica]	9,00E-140	55%	TIR-NBARC-LRR

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V.26	hypothetical protein VITISV_035070 [Vitis vinifera]	0.0		
V.27	uncharacterized protein LOC103435525 [Malus domestica]	0.0		
V.28	TMV resistance protein N-like [Malus domestica]	8,00E-99	21%	No protein coding domains
V.29	copia LTR rider [Solanum lycopersicum]	0.0		
V.30	uncharacterized protein LOC103941455 [Pyrus x bretschneideri]	7,00E-42		
V.31	pentatricopeptide repeat-containing protein At4g01990, mitochondrial-like [Malus domestica]	3,00E-74		
V.32	ribonucleases P/MRP protein subunit POP1 [Malus domestica]	8,00E-96		
V.33	transcription factor UPBEAT1-like [Pyrus x bretschneideri]	3,00E-72		

Predicted genes of Algorithm of Tomato

Predicted ORF name	Homologous gene	e.Value	Query coverage	Available Domains
T.1	DEAD-box ATP-dependent RNA helicase 24-like [Pyrus x bretschneideri]	0.0		
T.2	TMV resistance protein N-like isoform X2 [Malus domestica]	0.0	51%	ONLY NBARC
T.3	uncharacterized protein LOC103432406 [Malus domestica]	0.0		
T.4	hypothetical protein [Cecembia lonarensis]	3,00E-77		
T.5	uncharacterized protein LOC103417687 [Malus domestica]	5,00E-80		
T.6	PREDICTED: TMV resistance protein N-like [Malus domestica]	1,00E-95	20%	TIR ONLY
T.7	uncharacterized protein LOC103425749 [Malus domestica]	2,00E-110		
T.8	pentatricopeptide repeat-containing protein At1g02370, mitochondrial-like [Pyrus x bretschneideri	1,00E-121		
T.9	uncharacterized protein LOC103956881 isoform X3 [Pyrus x bretschneideri]	5,00E-30		
T.10	putative COBL7 (COBRA-LIKE 7) [Malus domestica]	1,00E-17		
T.11	hypothetical protein [Cecembia lonarensis]	2,00E-80		
T.12	PREDICTED: uncharacterized protein LOC103417687 [Malus domestica]	4,00E-65		
T.13	uncharacterized protein LOC103417687 [Malus domestica]	0.0		
T.14	putative RNA-directed DNA polymerase (Reverse transcriptase) [Malus domestica]	0.0		

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T.15	predicted protein [Malus domestica]	2,00E-136		
T.16	predicted protein [Malus domestica]	3,00E-32		
T.17	uncharacterized protein LOC103417687 [Malus domestica]	0.0		
T.18	predicted protein [Malus domestica]	1,00E-97		
T.19	predicted protein [Malus domestica]	1,00E-52		
T.20	uncharacterized protein LOC103956881 isoform X3 [Pyrus x bretschneideri]	5,00E-145		
T.21	PREDICTED: pentatricopeptide repeat-containing protein At1g02370, mitochondrial-like [Pyrus x bretschneideri	6,00E-92		
T.22	uncharacterized protein LOC103444267 [Malus domestica]	4,00E-33		
T.23	putative gag-pol polyprotein [Fragaria x ananassa]	0.0		
T.24	uncharacterized protein LOC103941455 [Pyrus x bretschneideri]	1,00E-67		
T.25	hypothetical protein VITISV_003191 [Vitis vinifera]	0.0		
T.26	TMV resistance protein N-like [Malus domestica]	0.0	28%	TIR-NBARC-LRR
T.27	putative polyprotein (retrotransposon protein) [Malus domestica]	1,00E-155		
T.28	no match			
T.29	unnamed protein product [Coffea canephora]	8,00E-77		
T.30	putative polyprotein [Oryza sativa Japonica Group]	2,00E-36		
T.31	lysosomal alpha-mannosidase-like [Malus domestica]	8,00E-52		
T.32	uncharacterized protein LOC103958468 [Pyrus x bretschneideri]	7,00E-135		
T.33	TMV resistance protein N-like [Malus domestica]	8,00E-99	21%	No protein coding domains
T.34	putative RNA-directed DNA polymerase (Reverse transcriptase) [Malus domestica]	0.0		
T.35	copia LTR rider [Solanum lycopersicum]	0.0		
T.36	uncharacterized protein LOC103941455 [Pyrus x bretschneideri]	2,00E-33		
T.37	pentatricopeptide repeat-containing protein At4g01990, mitochondrial-like [Malus domestica]	3,00E-74		
T.38	ribonucleases P/MRP protein subunit POP1 [Malus domestica]	9,00E-68		
T.39	transcription factor UPBEAT1-like [Pyrus x bretschneideri]	3,00E-72		

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CHAPTER 4

Cloning and transformation of the identified candidate gene.

Introduction

The introgression of scab resistance genes in apple cultivars has a history dating back about a hundred years; it was initiated by Crandall in 1914. Crandall first made a cross between the scab susceptible cultivar 'Rome beauty' and the resistant crab apple cultivar *Malus floribunda 821* (Crandall 1926; Hough et al. 1953; Gessler and Pertot 2011). Then putative resistant and susceptible siblings of the progeny were crossed and 1:1 segregation for resistance observed, indicating the presence of monogenic resistance (Hough 1944). The gene responsible for resistance was named *Vf* (Williams et al. 1966). Due to the very poor fruit quality of *Malus floribunda 821,* it took approximately 80 years to release an apple cultivar with *Vf* resistance and reasonable fruit quality on the market, although these cultivars still occupy small market niches, because the fruit quality is not yet at the highest level (Gessler and Pertot 2011). However, to date the *Vf* apple scab resistance gene has been the most widely used apple scab resistance gene in breeding programmes (Janick et al. 1996) and it has been introduced into many commercial apple cultivars.

Identification of *Venturia inaequalis* strains that have ability to overcome *Vf* resistance (Parisi et al. 1993; Trapman 2006) in north western Europe (Parisi et al. 2006), where the weather is optimal for apple scab, has highlighted the need for new strategies to obtain durable resistance. To date, 17 major apple scab resistance genes and several QTLs have been discovered (Calenge et al. 2004; Gessler et al. 2006; Bus et al. 2011). Normally, the major resistance genes have a considerable effect on the pathogen, which lacks matching virulence. However, this effect also applies high selection pressure to the pathogen population. According to MacHardy et al. (1996), most of major resistance genes remain resistant until a pathogen strain appears with matching virulence and overcomes the resistance. A promising way to reduce the risk of resistance breakdown is to combine several functionally different resistance genes in a single cultivar, this procedure being

called gene pyramiding (MacHardy et al. 2001). This strategy should delay or even prevent resistance breakdown and create cultivars with durable resistance to apple scab. If the breeding is performed using traditional methods, development of a cultivar carrying pyramided R genes with high quality fruits will require several decades and more complex crosses.

Recently, researchers have invented an alternative way of introducing resistance genes into susceptible cultivars by means of genetic modification called 'cisgenesis', without simultaneous introgression of unwanted alleles. This also avoids the difficulties of classic breeding methods (Jacobsen and Schouten 2007). With this genetic modification approach, R genes found in crossable species are inserted into cultivars with their native promoters and terminators and preserve the proven fruit quality and other desired traits of high quality cultivars. During the transformation procedure foreign genes are utilized, but they are removed from the final product. It has therefore been argued that plants produced using this technology are as safe as plants obtained using traditional breeding techniques.

A prerequisite for cisgenesis is the availability of different isolated resistance genes. To date only two R genes have been cloned in apple. *Rvi6 (Vf*), the apple scab resistance gene located on linkage group (LG) 1, was the first functionally isolated R gene in apple. Two independent series of studies provided similar results using different bacterial artificial chromosome (BAC) libraries for chromosome walking. The first series of studies started with identification of the *Rvi6* resistance locus in 550kb (Patocchi et al. 1999) from the 'Florina' BAC library created using the genomic DNA of 'Florina' (Vinatzer et al. 1998). By analysing the cDNA library of the identified region, three putative genes (*HcrVf1*, *HcrVf2* and *HcrVf3)* homologous to the cloned *Cladosporium fulvum* (*Cf*) R gene in the tomato were identified by Vinatzer et al. (2001). Subsequently, expression of one of the

candidate resistance genes (*HcrVf2)* in the cauliflower mosaic virus 35S promoter demonstrated that *HcrVf2* alone is sufficient to confer *Rvi6* scab resistance (Belfanti et al. 2004). Xu and Korbon (2002) started with construction of a smaller BAC contig of 290kb, spanning the resistance locus of *Rvi6,* as the second series of studies. Sequencing of positive BAC clones allowed the isolation of three genes expressed in the region (*Vfa1*, *Vfa2, Vfa4),* of which *Vfa1* and *Vfa2* are identical to the *HcrVf1* and *HcrVf2* previously identified by Vinatzer et al. (2001).

The second functionally isolated apple scab R gene was *Rvi15 (Vr2*), which provides full resistance to apple scab, and no breakdown of resistance has yet been reported. The *Rvi15 (Vr2)* apple scab resistance gene, which elicits a slow hypersensitive response (HR) (Galli et al. 2010a), was mapped at the top of LG 2 in the GMAL 2473 accession (Patocchi et al. 2004). Furthermore, Galli et al (2010b) isolated the BAC spanning the *Rvi15* region and sequenced it. Analysis of the 48.5kb sequence between two flanking markers of *Rvi15* revealed three transcribed putative resistance gene analogs (*Vr2-A*, *Vr2-B and Vr2-C*) with a Toll and mammalian interleukin-1 receptor protein nucleotide – binding site and leucine rich repeat structure (TIR-NBS-LRR) (Galli et al. 2010b). The studies carried out by Schouten et al. (2014) functionally isolated the gene responsible for *Rvi15* as *Vr2-C,* using gene transformation studies.

Furthermore, candidate genes for *Rvi5 (Vm*), *Rvi1 (Vg)* apple scab resistance are currently available (Lewke Bandara et al. (unpublished data); Cova et al. 2015).

The apple scab resistance gene *Rvi5* is believed to be inherited from *Malus atrosanguinea* 804 and *Malus micromalus*. Dayton and Williams (1970) described the *Rvi5* gene as a gene conditioning the pit-type hypersensitive response to *V. inaequalis*. The allelelism studies conducted on the resistance loci of *Rvi5* demonstrated that the pit-type genes

carried by these two apple species are allelic and independent from the *Vf* locus. However, *M. micromalus* carries a second, masked resistance gene (Shay and Hough 1952) inducing a type 3 resistance reaction (restricted necrotic lesions with sporulation). Molecular marker analysis indicated that the masked gene is mostly likely *Vf* (Vinatzer et al. 2004). The *Rvi5* gene induces the hypersensitive reaction following inoculation with *V. inaequalis* races 1 to 4. Race 5 of *V. inaequalis* overcame the *Rvi5* gene, which was first reported in England (William and Brown 1968). However, the studies carried out to 'monitor *V. inaequalis* virulence' with the VINQUEST initiative demonstrated that resistance breakdown of *Rvi5* is not widely spread (www.vinquest.ch/monitoring/publications.htm). *V. inaequalis* race 5 has been reported at a few sites in Germany and Belgium (Patocchi et al. 2009, www.vinquest.ch/monitoring/publications.htm), while further evidence of the possible breakdown of resistance has been reported in North America (Beckerman 2009). The *Rvi5* gene has been introduced into two apple cultivars *(*'Murray' and 'Rouville'), by removing the majority of the undesired crab apple traits, but these two cultivars have not been used in commercial apple orchards. A sequence–characterised amplified marker (SCAR) $OPB12_{687}$, was the first molecular marker developed for *Rvi5* resistance using bulked segregant analysis (Cheng et al. 1998).The marker was mapped approximately 5 cM from the *Rvi5* gene. Later, Patocchi et al. (2005) were able to map *Rvi5* on the distal end of LG 17 with the help of co-segregating simple sequence repeat (SSR) marker Hi07h02. Fine mapping and development of additional molecular markers around the *Rvi5* region was done by Cova et al. (2015) and limited the genetic region to 1cM. Furthermore, Lewke Bandara et al. (2015) isolated three BACs spanning the *Rvi5* locus, using the BAC library created for the *Rvi5* resistant 'Murray' apple genotype. The tree BAC clones spanning the *Rvi5* region were sequenced, and the genetic region carrying putative *Rvi5* resistance was

isolated using flanking markers. Analysis of the 154kb sequence between the flanking markers revealed the presence of one putative resistance gene carrying a Toll and mammalian interleukin-1 receptor protein nucleotide binding site leucine-rich repeat structure (TIR-NBS-LRR). Amplification of identified candidate gene in first strand cDNA with gene specific primers confirmed the identified gene has been transcribed.

Here we describe the cloning and transformation of the identified candidate gene; by inserting it into the scab susceptible cultivar 'Gala' using *Agrobacterium tumefaciens* mediated transformation.

Materials and Methods

Sequencing of the candidate gene from BAC DNA

Molecular markers were designed for the genomic DNA sequence of the identified candidate gene using Primer3 software (Rozen and Skaletsky 2000). Eight primer pairs (Table 1) were designed to cover the complete sequence of the candidate gene to sequence the candidate gene using primer walking. The genomic sequence of the identified candidate gene was 10288bp in size (Figure 1); amplification of the complete gene as a single amplicon was difficult and thus the gene was amplified as five overlapping fragments with seven primer pairs (1F with 1R, 2F with 2R, 2F with 3R, 3F with 2R, 4F with 4R, 4F with 5R, and 5F with 4R). PCR mixture and amplification conditions were set according to Cova et al. 2015a. Amplified PCR fragments were sequenced using the Applied Biosystems 3730xl sequencer using a big dye terminator kit.

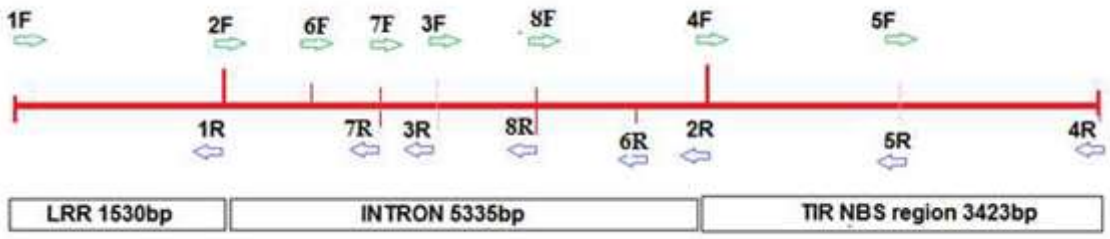


Figure 1: Molecular markers designed for the identified candidate gene for the purpose of sequencing. F indicates the forward primer and R the reverse primers.

Table 1: Newly designed molecular markers for the identified candidate gene sequence

Primer name	DNA sequence	Annealing temperature
TMV_1F	5'-cacctgatttatccagtgcaca	60^0C
TMV_1R	5'-gcaaaatttgagctgctgtga	60^0C
TMV_2F	5'-agagagagaacctggcatcg	60^0C
TMV_2R	5'-atatcaccggccccagattc	60^0C
TMV_3F	5'-ggcacttcacggttaggga	60^0C
TMV_3R	5'-gcccaaaatgccttgcctaa	60^0C
TMV_4F	5'-actccgtcgtctccaaacta	60^0C
TMV_4R	5'-caattgtcctaggccttgca	60^0C
TMV_5F	5'-gcatgaccaacctgttccat	60^0C
TMV_5R	5'-ttcacaggaaagacgatggat	60^0C
TMV-6F	5'-gaacagtgcgagtagatggc	60^0C
TMV-6R	5'-agaagaacatctccctcgtca	60^0C
TMV-7F	5'-cactgggtgcattggaatcc	60^0C
TMV-7R	5'-tgacagctgctccaccaata	60^0C
TMV-8R	5'-gcttctttgtaggcggatgg	60^0C
TMV-8F	5'-gatgaggcacctacccaaga	60^0C

First strand cDNA synthesis and candidate gene amplification on cDNA

First strand cDNA was synthesized using Superscript™ III Reverse transcriptase (Invitrogen, CA), oligo $(dT)_{20}$ primer and extracted total RNA. The reaction mixture was as follows; 1µl of oligo(dT)20 primer, 3µg of total RNA, 1µl of 10mM dNTP mix and up

to 13µl of sterile, distilled water were added to a nuclease free micro-centrifuge tube. The mixture was heated to 65^0C for 5 minutes and incubated on ice until use. Then the content of the tube was briefly centrifuged and 4µl of 5X First-Strand buffer, 1µl of 0.1M DTT, 1µl of RnaseOUT TM Recombinant RNase inhibitor and 2µl of Superscript™ III Reverse transcriptase were added. The mixture was gently mixed by pipetting up and down and incubated using the following conditions. 25^0C for 5 minutes, 50^0C for 30 minutes, 55^0C for 30 minutes and 70^0C for 15 minutes. Synthesized first strand cDNA was stored at -20^0C until use.

A new set of primers were designed on the start (5'-ATGGATGCATTCCGAGATTC-3') and end codon (5'-GATTTGTGCATCACCAACATT-3') of the candidate gene using Primer 3 software. The candidate gene was amplified using synthesized first strand cDNA using the following PCR mixture and conditions. 5µl of 5X Phusion high fidelity buffer, 0.5µl of 10mM dNTP mix, 1.25µl of 10µM forward primer, 1.25µl of 10µM reverse primer, 0.25µl of Phusion DNA polymerase, 2µl of Synthesized first strand cDNA, Nuclease free water up to 25µl were mixed together and amplified using the following thermo cycling conditions; initial denaturation at 98^0C for 30 seconds, then 35 cycles at 98^0C for 10 seconds, 60^0C for 30 seconds and 72^0C for 2.5 minutes, and final extension at 72^0C for 10 minutes. Before performing the sub cloning procedure, 3'- ends of the amplicons were modified by adding poly Adenine (Poly A) overhangs. 1 unit of *Taq* polymerase was added to the tube containing the amplicon and incubated at 72^0C for 10 minutes. Poly A-overhangs added amplicon was immediately used for sub cloning. The amplified gene was sub-cloned to the TOPO-TA sequencing vector according to the manufacturer's instructions (Invitrogen, CA) and sequenced using additional sequencing primers designed with Primer 3 software. The fully sequenced candidate gene was

converted to its amino acid sequence using the ExPASy translate tool (http://web.expasy.org/translate/).

Table 2: Primers used to sequence the cDNA sequence of the candidate gene

Primer Name	Sequence	Annealing Temperature
cDNA-1F	5'atggatgcattccgagattc	60^0C
cDNA-1R	5'-cattcctcccaaagatgcaa	60^0C
cDNA-2F	5'atgttgcatctttgggagga	60^0C
cDNA-2R	5'-caaacattgtacttgtataatccatcg	60^0C
cDNA-3F	5'-agccagtttcttcacaggaaag	60^0C
cDNA-3R	5'-tagtttcccaaaataccataaatctg	60^0C
cDNA-4F	5'-aacaaatttaccgaacttggaca	60^0C
cDNA-4R	5'-ccaacctcctttgtgaggaa	60^0C
cDNA-S5R	5'-gatgaactgcccctcatacc	60^0C
cDNA-S6F	5'-tctgggatcttctctatctggaa	60^0C
cDNA-S6R	5'-ccgacgtcgtcttgaacaat	60^0C
cDNA-S7F	5'-cgcatggccttaggacaac	60^0C
cDNA-S7R	5'-cgaatagatcgatatctttgagga	60^0C
cDNA-S8F	5'-ggtattgtgggaaactagttgatg	60^0C
cDNA-1.8	5'- gatttgtgcatcaccaacatt	60^0C

Preparation of *Rvi5*_Murray constructs: insertion of the gene of interest into the entry vector

The amplicon obtained from cDNA amplification with gene specific primers of *Rvi5*_Murray was analysed with gel electrophoresis and purified using a gel elution kit. The eluted product was quantified with Nanodrop 8000 (Thermoscientific) and the concentration of eluted DNA was increased by using the following procedure. $1/10^{th}$ volume of 3M NaOH at pH 5.2 and 3 volume of 96% Ethanol was added to eluted DNA and incubated at -20^0C for 20 minutes then centrifuged at 13000rpm for 20 minutes. The pellet was washed with 70% ethanol and suspended in 10µl of distilled water. Concentrated DNA was used as a template for the entry vector.

The selected entry clone was pENTER^{TM}/ D-TOPO (Invitrogen,CA). To facilitate directional cloning with the entry clone, the 5'-end of the gene specific forward primer was modified by adding a four base pair sequence (CACC). For amplification of the candidate gene with the added CACC sequence, phusion high fidelity DNA polymerase (New England Bio Labs, MA) was used, producing blunt ended PCR products. The TOPO cloning reaction was set as follows for the chemical transformation protocol: the reaction mixture of 6 µl consisted of 3 µl of gel eluted concentrated PCR product, 1 µl of salt solution, 1 µl of water and 1 µl of TOPO vector. All the components were inserted into a 0.5 ml PCR tube and incubated at room temperature for 5 minutes. The incubated mixture was placed on ice until required for further reactions. 2 µl of the cloning reaction was added to a vial of one shot chemically competent TOP10 cells and incubated on ice for 30 minutes. The incubated mixture was heat shocked at 42^0C for 30 seconds and immediately incubated on ice for 2 minutes. 250 µl of room temperature S.O.C medium was added to the mixture and incubated at 37^0C for one hour with shacking at 225 rpm. The incubated culture was spread on pre-warmed selective plates with 50ng/ml Kanamycin as a selective antibiotic and incubated overnight at 37^0C.

The colonies were checked using gene specific primers of the candidate gene with colony PCR to check the precense of the insert using the following conditions; 38 µl of sterile distilled water, 5 µl of 10x PCR buffer, 3 µl of 25mM $MgCl_2$, 1 µl of 10mM dNTPs , 1 µl of 20 µM forward primer (cDNA-1F), 1 µl of 20 µM reverse primer (cDNA-1.8) and 0.5 µl of Hot Start Taq polymerase were mixed together for a 50 µl PCR. A small amount from each colony grown on the selective plates was added to each PCR tube using a 10µL pipette tip and mixed well by pipetting up and down, and the colony PCR conditions were as follows; one cycle at 95^0C for 15 minutes as initial denaturation and 35 cycles at 95^0C for 1 minute, 66^0C for 1 minute, 72^0C for 2 minutes and 72^0C for 7 minutes. Positive

colonies identified using PCR were used to extract plasmid DNA using a plasmid extraction kit (Sigma Aldrich, Missouri). The positive colonies with insert were cultured overnight in 200 ml of LB media with 50ng/ml Kanamycin and plasmid DNA was extracted using a plasmid DNA extraction kit from Sigma Aldrich.

Insertion of the gene of interest into the destination vector

The extracted plasmid DNA from the entry vector was diluted to obtain a dilution of 150ng/µl to perform the LR recombination reaction. The following mixture was prepared in a 1.5mL Eppendorf tube at room temperature: 1µl of 150ng/µl concentration entry clone, 1µl of 150ng/µl destination vector (pK7WG2) and 6µl of TE buffer at PH 8.0. Then 2µL of LR Clonase II was added to the above mixture and briefly vortexed for 2 seconds, after which the mixture was incubated at 25^0C for two hours. 1µl of Proteinase K solution was added and incubated at 37^0C for 10 minutes. Transformation was carried out using One Shot TOP10 chemically competent *E. coli* cells, using the procedure described in the previous paragraph. Two culture plates containing 50ng/ml Streptomycin as a selective antibiotic were cultured using 50 µl and 100 µl of transformed chemically competent cells and the selective plates were incubated at 37^0C overnight.

Positive colonies were checked with colony PCR and plasmid DNA was extracted from positive colonies. Additional PCR amplification was performed to confirm the incorporation of the gene of interest into the destination vector backbone and its orientation.

Plant transformation and regeneration

Destination vector pK7WG2 with the inserted *Rvi5*_Murray gene was transformed into the *Agrobacterium tumefaciens* strain EHA105 (Chetty et al. 2013). Apple transformation was basically performed as described by Joshi et al. (2011). The four youngest 3-4 week old

leaves from in vitro propagated ‘Gala’ shoots were used as explants. The leaves were cut into 2-3 mm strips and incubated on a sterile Petri dish filled with *A. tumefaciens* suspension for 30 minutes. Then the explants were transferred to co-cultivation media consisting of MS salts and vitamins for three days (Murashige and Skoog 1962). After co-cultivation the explants were transferred to selective regeneration media with kanamycine to select transformants and cefotaxime to eliminate *A. tumefaciens.* The explants were cultured in the dark for 3 weeks and sub-cultured on fresh selective regeneration medium at 2 week intervals. When the callus emerged from the explants and shoot-like structures started to emerge from the calli, clusters were transferred on elongation medium for several weeks.

Molecular analysis of transformants

Genomic DNA from putative transformants was extracted using a Qiagen plant DNA extraction kit and analysed for the presence of the inserted gene of interest using a pair of gene specific primers (cDNA-1F and cDNA 5R) of *Rvi5*_Murray. The PCR reaction mixture and PCR conditions were similar to those for candidate gene amplification.

Results

Amplification of the gene of interest on ‘Murray’ BAC DNA

The identified candidate gene was 10288bp in size in the genomic ‘Murray’ sequence. Due to its size, it was difficult to amplify as a single amplicon, so the complete gene was amplified using five overlapping fragments. The LRR part of the gene was amplified by using primer combinations 1F and 1R, primer combination 2F and 2R doesn’t amplify, but splitting the region in to two with a middle primer pair (3F and 3R) allowed to amplify the region by producing two overlapping fragments. The last part of the gene

having NBS and TIR domains was amplified using the primer combination of 4F with 4R, but amplicon was a faint band on agarose gel. PCR amplification carried out by splitting the region into two fragments (4F with 5R and 5F with 4R) resulted in two clear bands on the gel (Figure 2).

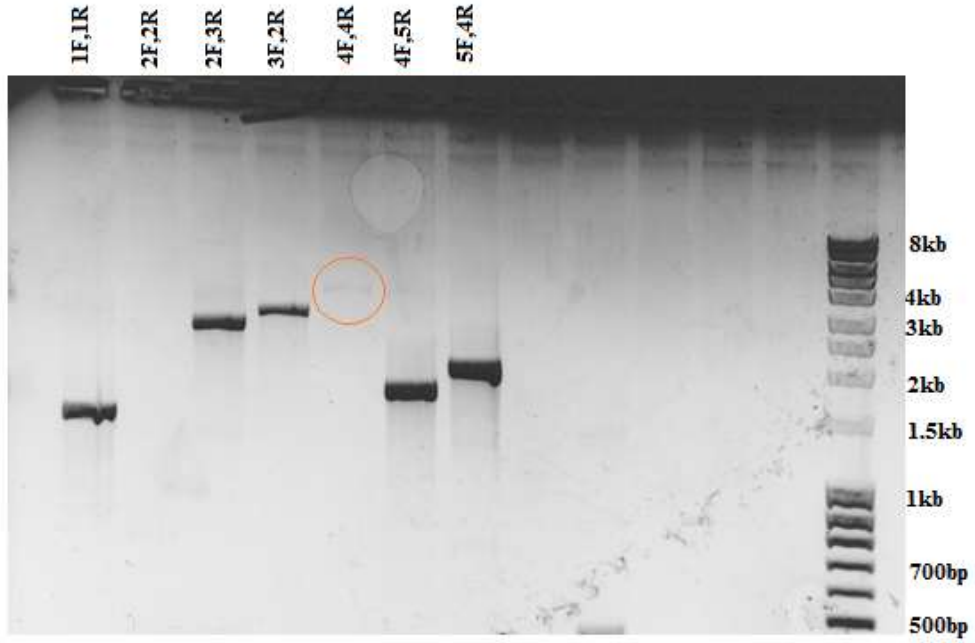
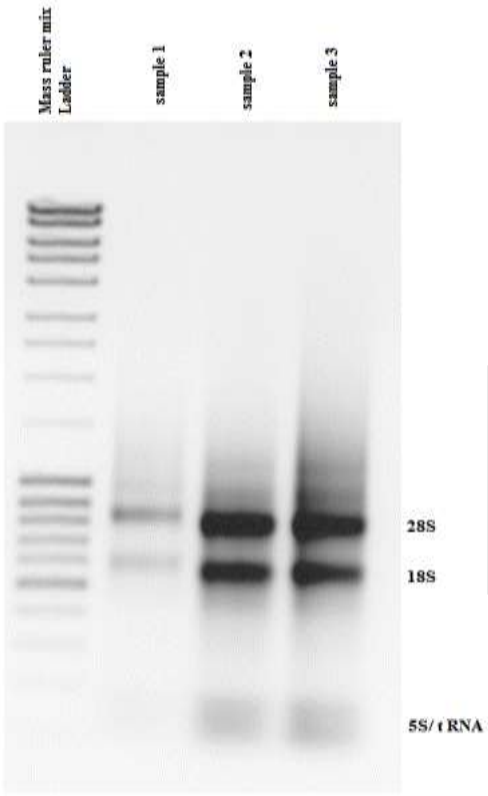


Figure 2: Amplicons of the identified candidate gene. Due to the size of the candidate gene the gene was amplified as five overlapping amplicons

Sequencing of amplicons with primer walking confirmed the complete sequence of the candidate gene obtained with BAC assembly.

First strand cDNA synthesis and candidate gene amplification on cDNA

Total RNA was extracted from young leaves of un-inoculated, healthy 'Murray' plants grown in greenhouse conditions. The RNA concentration and quality of the extracted RNA were measured using a Nanodrop 8000 spectrophotometer and 1.5% agarose gel (Figure 3).



Total RNA concentration of sample 1 – 348ng/µl

Total RNA concentration of sample 2 -830ng/µl

Total RNA concentration of sample 3 -906ng/µl

Figure 3: Quality checking of extracted RNA on 1.5% agarose gel

The concentration of synthesized cDNA mainly depends on the quality and concentration of extracted total RNA. The concentration of synthesized first strand cDNA can be determined by using the amount of total RNA used to synthesize cDNA.

Example: if the amount of total RNA used to synthesize cDNA is 3.5µg in 20µl of first strand cDNA synthesis reaction mixture, the synthesized cDNA concentration should be 3500ng/20 µl = 175ng/µl.

The candidate gene was amplified using forward primer 5'-ATGGATGCATTCCGAGATTC-3' and reverse primer 5'-GATTTGTGCATCACCAACATT-3', using first strand cDNA and Phusion high fidelity Taq polymerase. The amplicon was 3500bp in size (Figure 4).

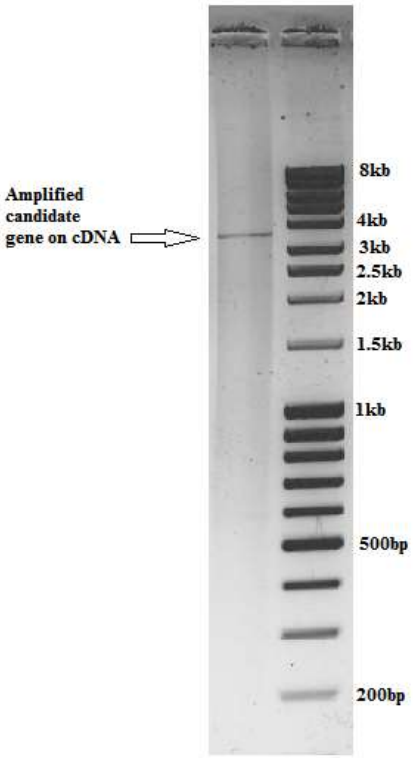


Figure 4: Amplified candidate gene of *Rvi5*_Murray on first strand cDNA.

The adenine tail added amplicon was sub-cloned into the TOPO vector using a TOPO-TA sequencing kit and the complete sequence of the candidate gene was obtained by sequencing. The candidate gene sequence was converted to its amino acid sequence using the ExPASy translate tool.

>Candidate gene sequence (*Rvi5*_Murray)

ATGGATGCTTTCCGAGATTCTTCTTCCTCCTCTTACCGCTGTTCCTATCATGCCTTCTTGAGTTTC
AGAGGCCAGGACACGCGCAAGGGCTTTACTGATCACCTCTATAGAGCCTTGGAGCTAGCAGGA
ATCCACACGTTTAGAGACGATGATGAAATCAAGAGAGGGGAGAATATTGAGTCGGAGTTAGAC
AAGGCGATACAGGAGTCACAAGTATCGATAATTGTCTTCTCCAAGGACTACGCATCCTCAAGG
TGGTGTTTGAACGAACTTCTGAAGATCGTGGAACGTAGAAATACTGATCATAGACATGTGGTTC
TGCCCGTTTTCTATGATGTGGATCCATCCGATGTCAGGAAGCAGAGTGGTCCTTTTGCTGAAGC
ATTTGCCAGACATGAGGAACGATTCAGTACGGAGATGGACAAGGTGGAGCAGTGGAGAAGAG
CTCTAGGAGATGTTGCATCTTTGGGAGGCATGGTTTTAGGAGATCGGTATGAGGGGCAGTTCAT
CCAAGAAATTGTTGAAGAGATTAGAAATAAAGTGGGTCACGCAGCTTTAGACGTCGCTCCCTT

TTCAGTTGGAATGGATAATCGTGTGCAAGACCTAAACATGTGGCTACAAGATGGATCCAATGA
TGTTGGTGTAGCTGTCATCTGTGGGATGGGTGGAATTGGCAAGAGCACCATCGCGAAAGCCGC
GTATAACCGCAACTTTGATAGATTTCAAGGTAGCAGCTTTCTTGCAGATATACGAGAATCTTCA
GAACAACCCAATGGTTTTGCTTGCTTGCAAAGAAAACTTCTTTCAGATATCCAAAAGGGGAAG
GCAAAGAGAGTATACAATATGGATGAAGGAACAATCAGGATCAAACAAGCCGTAGGCTATAA
AAAAGTTCTTATTGTTCTTGATGATGTGAGCAACCAGGATCAACTCAATGCAATTCTTGGAATG
CAAGAGTGGCTTCATCGAGGAAGTAAAATTATCATAACAACTAGACATGAGCATTTGTTAAAT
GCTCATGAAGTTTTTGAAAAGTTTATGGTTCCAGAACTGAATGAGTATGAATCACTTGAGCTTT
TAAGTTGGCATGCCTTCGGACAATCCCATCCAGTAGAAGGTTACATGCAGCTTTCAAAACATGT
GGTGCAGCACTGTGGAGGGCTTCCATTAGCTCTTCAAGTTCTGGGATCTTCTCTATCTGGAAAA
AGTGTAGAAGTATGGCAAAGTGCATTGAAGAAGTTAGACGTGATCCCTAATGACAAAATTCAA
AAAGTTCTGAGGATAAGTTTTGATTCTTTACAAGATGATCATGACAAAAATTTATTCCTTCATA
TAGCCAGTTTCTTCACAGGAAACACAATGGATTATACAATTACAATCTTGGACAGCTTAGACTT
TTGCACAAGGATTGGGATTGAAAATCTAGTTGACATATGCCTAGTGGAAATTCAAGGGTCGAA
GTTGGTCATGCATCAATTGGTGAGAAACATGGCAATGGCAATTATCCGCAAAGAATCACCTCA
TGACCCTGGAAAACGTAGTAGAGTGATGCAGAAAGATGCCTCTAATATTTTGAGAAAATTAAG
TGGCACGGAAAATATTAAGGGCCTCATGCTCAACCTTGCTAGCAAAAGAACGTTTGTTGGAAG
TGATAAAAAACGATGCCATGTTGAAGATTATGATGGAAATTGTTCAAGACGACGTCGGCTTGG
TTTCTTCTCTTGGAAGCCAGTTAGCTTTTCCTCCACAAACTCAGCTTCTGCATCAAATGAGGTAG
ACTTCAAATCTGAGGCATTTAGAAGGATGCACAATCTTGAAATTCTCATGCTCAACAACGTCAA
CGTCAGGGGAAACTACGAAGAATTTTCAAAAAATTTAGTATGTTTATCTTGGCGAGGATTCCAC
TTAAAATCAATACCAGAAAATTTATATTTGGGAAACCTGGTTGCTCTTAACATGCGAAATAGTA
GCCTACAACATGTTTGGAATGGGATCAGGTTTCTTCCGAGGCTGAAAATTCTTAATCTCAGTCA
TTCGCATGGCCTTAGGACAACCCCGGACTTCTCAGGACTCCCTAACCTTGAGAGATTGATTCTT
AAAGATTGCATAAATTTGGTTGAGGTTGATGAGTCCATCGGATACCTAGAGAAACTTGTTGTTT
TGAATCTAGAAGGCTGCAAAAATCTCATGAAGCTTCCAAAATTGAGATCTATTCAGGATCTTAT
TCTTTCTGGTTGCTCAAAGCTTGTGCTTGGTGCCAATACGACTGCCACAACTGGTCACTTACAA
TCTACAGCTTGTGAAATGAAGAAACTGAATCTGCTATCAGCTAAATCATGGTATTCAATCTGGT
CGCGGGTGTCGCCAAGAAAAAATATTGAACCAAGCAGTTTCTCATTGGCAAGTTTACCACATTC
CTTGACAAGTTTACGTCTGGATAGCTGCAATCTATCAGAGATTCCGAGTGCCCTTACTATGCTG
TCTTCGTTAGAGTACTTGGATCTGAATAACAATCCAATTACAAGCCTACCAGAGAGCATGAAC
AATCTTGTTAAGCTCCGTACTCTTGAAATATATTGTTGCAGAAACCTCACAATGCTTCCAGAGC
TCCCACATAGTTTGAAGAGATTGTTTGCTCACTCTTGCAGATCATTGAAAAGAATAACAAATTT
ACTGAACGCGGACATATCACTTGACTCAGATCACCACATAGCATTGGAGTCAAATCTTTGGTAT
TGTGGGAAACTAGTTGATGTTGAAAGCTTGTTCAATACACTACCATTGAGCAGCTTTGACATAA
AACTCCTCAAAGATAACGATCTATTTGATTTGAAACCCAACGGTGGCGTTGAGGTCCAAAATTG
TGCAAGTAGAAGGGAATTCAAGGGATTATATGGATGCGGCATATTTAGCATTTTTGTTCATGAG
AGCAAGATTCCAGATCGGTTCAATTACAGGAGCATGGGTAACACAGTGTTCTCTATTATTGTTC
CTTCACAGCCTAATATCAAGATCGTAGGTTTAAATGCATGTATATTGTATGCCCGGCAATCGGA
TCGAAGCCCTGAGATTGCACGTGGTTATCGTCGTCACCATTCGCTTGTACTTAGGAATGAGACA
AAGGGCCTTAAGTGGAACTTAAGCAAGTTGAGAGGGGTTGAATGGAAAGTGATGAACGAAGA
TATGTTATGGCTAAGCCGTTGGATCATGGGGAACAATGAATTGGAGTGCGGGGAAAAAGTGTG
TTTTGAAATAATCGGAGAGCAAGACGGTTTCCTCACAAAGGAGATAGGAGTCCAGTTTGTGTA
CGAGCAGAAAAATAAGGATGAGGAGGATGTCCTATCAAGTAGTGAAGATACAATGATCCAAT
GTCACAGATCGCCTGCCAAATTTAGCTGGGGTTTCGATAGACTTGGGCAATGCATCCCTAAGAA
GAAAGTTATGTCAACTCCCGTGTGCGCGGCTGATTACTTTCTCTGTAACCCGTTTCTCTGTAACC
CGATGCAATGCATCCGTAAGACCCAGTTTAATTCAAATTGATGTTGGTGATGCACAAATCA

>Amino acid sequence of candidate gene (*Rvi5*_Murray)

MDAFRDSSSSSYRCSYHAFLSFRGQDTRKGFTDHLYRALELAGIHTFRDDDEIKRGENIESELDKAQ
ESQVSIIVFSKDYASSRWCLNELLKIVERRNTDHRHVVLPVFYDVDPSDVRKQSGPFAEAFARHEER
FSTEMDKVEQWRRALGDVASLGGMVLGDRYEGQFIQEIVEEIRNKVGHAALDVAPFSVGMDNRV
QDLNMWLQDGSNDVGVAVICGMGGIGKSTIAKAAYNRNFDRFQGSSFLADIRESSEQPNGFACLQ
RKLLSDIQKGKAKRVYNMDEGTIRIKQAVGYKKVLIVLDDVSNQDQLNAILGMQEWLHRGSKIIIT
TRHEHLLNAHEVFEKFMVPELNEYESLELLSWHAFGQSHPVEGYMQLSKHVVQHCGGLPLALQVL
GSSLSGKSVEVWQSALKKLDVIPNDKIQKVLRISFDSLQDDHDKNLFLHIASFFTGNTMDYTITILDS
LDFCTRIGIENLVDICLVEIQGSKLVMHQLVRNMAMAIIRKESPHDPGKRSRVMQKDASNILRKLSG
TENIKGLMLNLASKRTFVGSDKKRCHVEDYDGNCSRRRRLGFFSWKPVSFSSTNSASASNEVDFKS
EAFRRMHNLEILMLNNVNVRGNYEEFSKNLVCLSWRGFHLKSIPENLYLGNLVALNMRNSSLQHV
WNGIRFLPRLKILNLSHSHGLRTTPDFSGLPNLERLILKDCINLVEVDESIGYLEKLVVLNLEGCKNL
MKLPKLRSIQDLILSGCSKLVLGANTTATTGHLQSTACEMKKLNLLSAKSWYSIWSRVSPRKNIEPS
SFSLASLPHSLTSLRLDSCNLSEIPSALTMLSSLEYLDLNNNPITSLPESMNNLVKLRTLEIYCCRNLT
MLPELPHSLKRLFAHSCRSLKRITNLLNADISLDSDHHIALESNLWYCGKLVDVESLFNTLPLSSFDI
KLLKDNDLFDLKPNGGVEVQNCASRREFKGLYGCGIFSIFVHESKIPDRFNYRSMGNTVFSIIVPSQP
NIKIVGLNACILYARQSDRSPEIARGYRRHHSLVLRNETKGLKWNLSKLRGVEWKVMNEDMLWLS
RWIMGNNELECGEKVCFEIIGEQDGFLTKEIGVQFVYEQKNKDEEDVLSSSEDTMIQCHRSPAKFSW
GFDRLGQCIPKKKVMSTPVCAADYFLCNPFLCNPMQCIRKTQFNSN

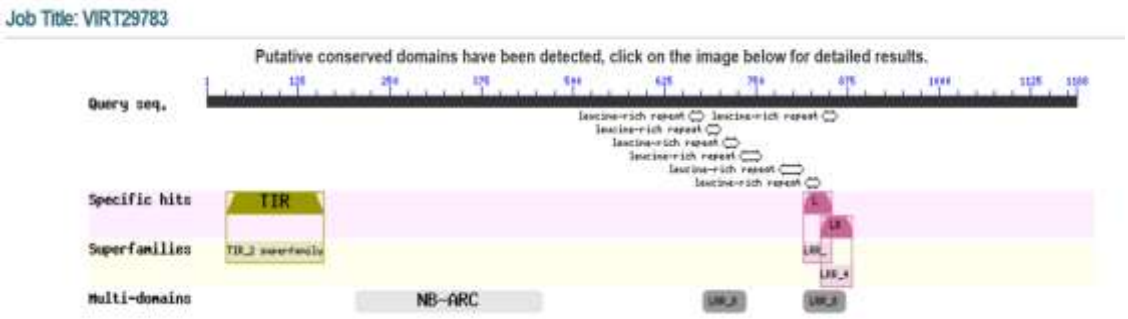


Figure 5: Structure and protein domains of the *Rvi5*_Murray candidate gene

Preparation of vector constructs carrying the *Rvi5*_Murray gene

Insertion of the gene of interest into the entry vector

The four base pair sequence (CACC), which is necessary for directional cloning, was added to the 5' end of the forward gene specific primer. The amplicon obtained using PCR amplification with first strand cDNA and gene specific primers was purified using gel elution and the concentration of eluted amplicon was increased. The purified, concentrated

amplicon was used as a template for the next PCR amplification step, which makes it possible to add a CACC four base pair sequence to the 5' end. After adding the CACC sequence to the 5' end of the gene of interest the complete sequence was cloned into the pENTR™/D-TOPO entry vector and incubated overnight at 37^0C on plates containing kanamycin as a selective antibiotic. Six colonies were grown on selective plates. Colony PCR was performed for the colonies grown on selective plates using gene-specific primers. Only four colonies were positive for the gene of interest (Figure 6).

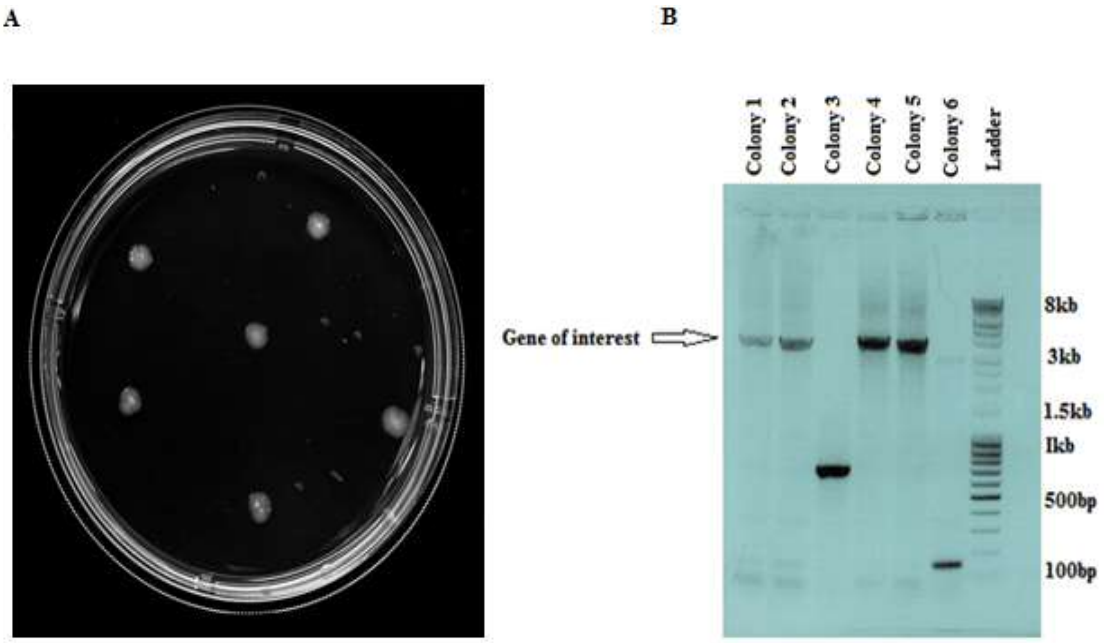


Figure 6: A) pENTER/D-TOPO colonies grown on selective media with Kanamycin B). Results of colony PCR used to recognise the entry vector colonies with the gene of interest.

Plasmid DNA was extracted from the positive colonies and the gene of interest was sequenced. Sequences were used to check the mutations and the orientation of the gene of interest on the pENTR/D-TOPO vector. All four colonies were positive for insertion of the gene of interest and correctly oriented on the pENTR/D-TOPO vector. Plasmid DNA extracted from the pENTR/D-TOPO vector was used to incorporate the gene of interest in the destination vector.

Insertion of the gene of interest into the destination vector

Plasmid DNA of the entry vector diluted to 150ng/µl was mixed with the same concentration of destination vector with TE buffer and LR clonase II enzyme. The prepared destination vector was inserted into one shot TOPO chemically competent cells and incubated at 37^0C overnight on a media containing streptomycin as selective antibiotic. Only three colonies grew on the selective media plate; colony PCR using gene specific primers confirmed integration of the gene of interest (Figure 7). In addition to colony PCR, further PCR amplification was performed to confirm the vector backbone of pK7WG2 using primers from the 35S promoter and 35S terminator regions. PCR amplification carried out using the primer combination of T35s and P35s was negative, but PCR performed using the T35s primer with cDNA 5R and the P35S primer with cDNA 4F produced their amplicons, which confirmed the gene of interest was incorporated into the pK7WG2 vector backbone in the correct orientation.

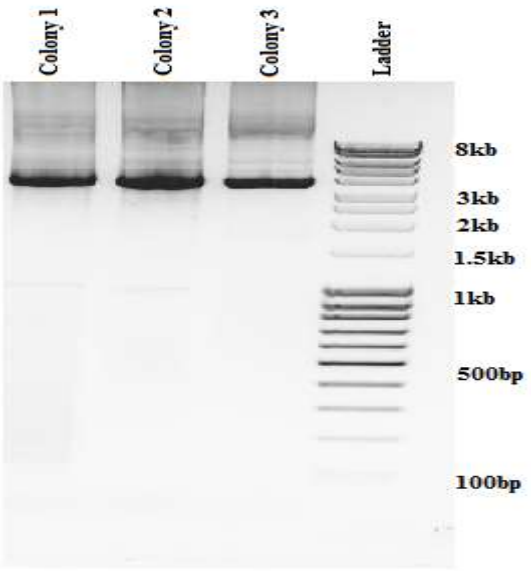


Figure 7: Results of colony PCR used to recognise destination vector colonies with the gene of interest.

Plant transformation and regeneration

The confirmed destination vector for the introgression of the gene of interest was used to create transgenic plants using *Agrobacterium tumefaciens*. The transgenic plants obtained (Figure 9) after the transformation and regeneration procedure were tested for the inserted gene of interest. DNA was extracted from transgenic plants using a Qiagen plant mini DNA extraction kit (Qiagen Ltd, Manchester, UK) according to the manufacturer's instructions and gene insertion was confirmed using PCR amplification with a 35S forward primer (5'-GCTATCGTTCAAGATGCCTCT-3') and cDNA 5R primer (5'-GATGAACTGCCCTCATACC-3'; one of the reverse primers designed 1000bp away from the start codon of the gene of interest (Figure 8).

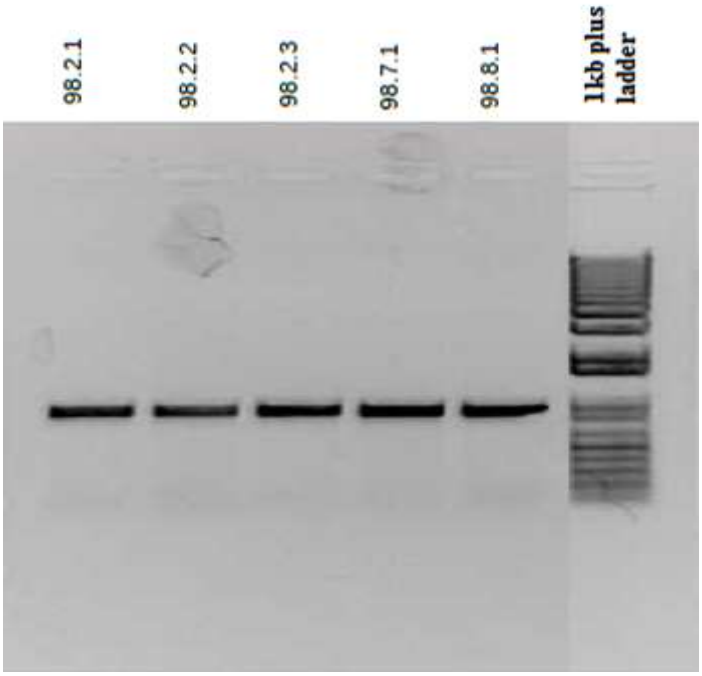


Figure 8: PCR confirmation of transgenic plants for the presence of the gene of interest

Figure 9: Transgenic plants with the inserted *Rvi5*_Murray gene

Discussion

Candidate gene sequencing and analysis

Amplification of the identified candidate gene in BAC DNA was performed to confirm the candidate gene sequence obtained from BAC assembly, Thus the candidate gene was amplified as five overlapping fragments in BAC DNA and the sequences obtained from PCR amplification of the candidate gene were used to correct the mismatches present in the gene sequence obtained by BAC assembly. Then the candidate gene was amplified on cDNA using the new set of primers designed for the start and end codons of the gene. The amplified gene was 3500kb in size and the gene was sequenced using the primer walking technique. The cDNA sequence of the gene confirmed the presence of 5kb intron in between the LRR and NBS domains and the cDNA sequence was used to predict the amino acid sequence of the gene using the ExPASy translate tool.

For the cloning and transformation procedure we used directional gateway cloning technology.

Directional gateway cloning technology

Gateway technology is a universal cloning method that takes advantage of the site-specific recombination system for bacteriophage lambda, which facilitates the integration of lambda into the *E. coli* chromosome. Gateway technology was developed by improving the specificity and efficiency of components in the lambda recombination system (Bushman et al. 1985). Lambda recombination occurs between site-specific attachments (*att*) sites. The *att* sites serve as the binding site for recombination proteins and were well characterised by Weisberg and Landy in 1983. Gateway technology provides several advantages in cloning: 1) enable rapid and highly efficient DNA sequence transfer into multiple vector systems for protein expression and functional analysis, while maintaining orientation and reading frame; 2) permit use and expression from multiple types of DNA sequences (e.g. PCR products, restriction fragments and cDNA clones); 3) easily accommodates and transfers a large number of DNA sequences to multiple destination vectors; 4) can be adapted to high–throughput formats; 5) allow easy conservation of the favourite vector in a gateway destination vector.

Lambda recombination is catalysed by a mixture of enzymes that bind to specific sequences (*att* sites).

The entry vector

We used pENTR /D-TOPO vector as the entry vector (Figure 10). It was 2580bp in size, with *attL1* and *attL2* sites, which allow re-combinational cloning of the gene of interest in the entry construct with a gateway destination vector. In addition to the *att* sites, several important sites and sequences are embedded in the pENTER/D-TOPO vector: rrnB T1 and T2 transcription sequences which reduce potential toxicity in *E.coli* by preventing basal

expression of the PCR product; M13 forward and reverse sites to facilitate sequencing of the insert; TOPO directional cloning site to allow rapid, directional cloning of the inserted PCR product; T7 promoter site for in vitro transcription and sequencing of the insert; Kanamycin resistance gene, which allows selection of the plasmid in *E.coli*; pUC origin of replication (ori), which allows high copy replication and maintenance in *E.coli* .

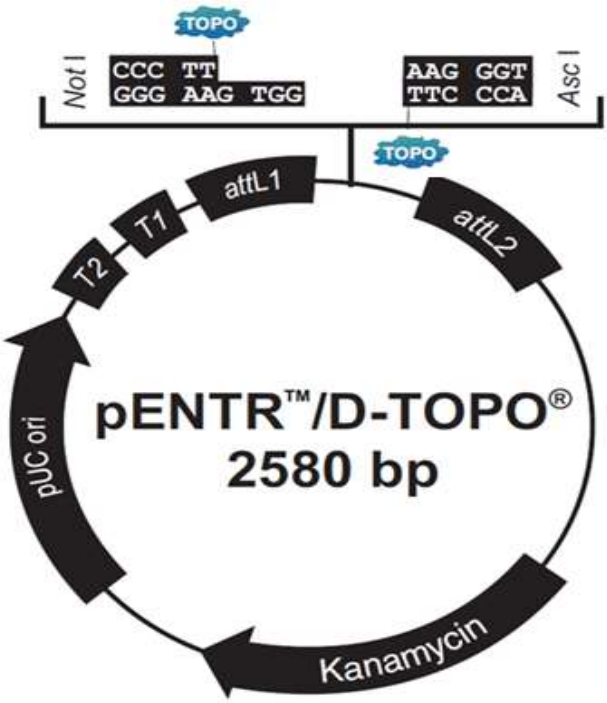


Figure 10: Map and features of pENTR/ D-TOPO vector

The directional cloning procedure is facilitated by the Topoisomerase I enzyme, which binds to duplex DNA at specific sites (CCCTT) and cleaves the phosphodiester backbone of one strand (Shuman 1991). Directional joining of double strand DNA using TOPO – charged oligonucleotides occurs by adding a 3'single–stranded end to the incoming DNA. This single stranded overhang is identical to the 5'end of the TOPO charged DNA

fragment. In TOPO cloning kits, a 4 nucleotide (GTGG) overhang sequence was added to the TOPO – charged DNA.

PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang of the cloning vector (GTGG) invades the 5' end of the PCR product, anneals to the added bases and stabilises the PCR product in the correct orientation.

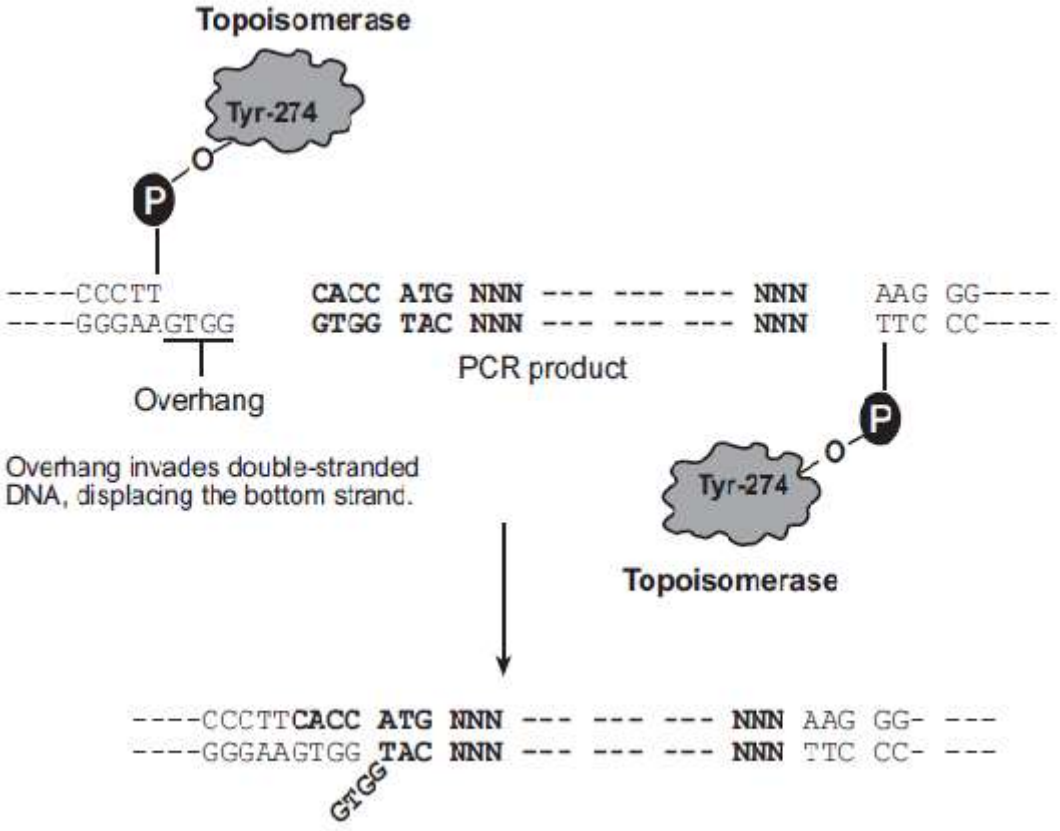


Figure 11: Schematic representation of how directional TOPO cloning works

The destination vector

The destination vector we used was pK7WG2 and was 11159bp in size (Figure 12). The vector contains compatible recombination sites (*attR*) for the att sites' presence in the

entry vector (*attL*). The recombination reaction is mediated by the GATEWAY LR clonase enzyme. In addition to the *attR* sites, there are also other important sites embedded in the destination vector. The *ccdB* gene is located between the *attR1* and *attR2* sites, which allows negative selection of the donor and destination vectors in *E.coli* following recombination and transformation. The *ccdB* protein interferes with *E.coli* DNA gyrase and inhibits the growth of most *E.coli* stains. When recombination occurs between the entry vector and destination vector, the *ccdB* gene is replaced by the gene of interest. Cells that carry the vector with no insertionnot grow. This procedure allows highly efficient recovery of the desired clones. The GATEWAY site is located between the promoter and the terminator of the cauliflower mosaic virus (CaMV) 35S transcript, because this promoter is highly active in most plant cells of transgenic plants. The upper part between the LB and RB sites is T-DNA and the lower part is the vector backbone.

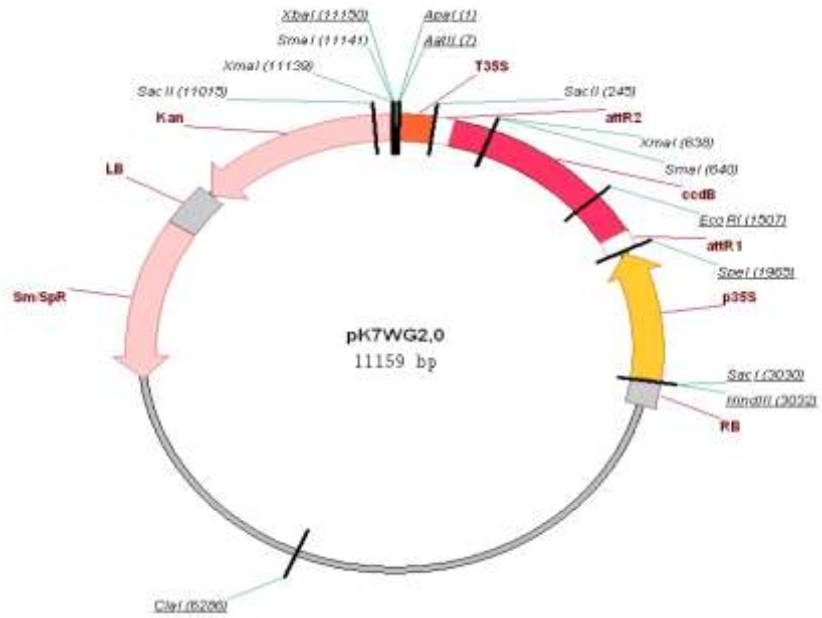


Figure 12: Map and features of the pK7WG2.0 destination vector

Regeneration and transformation of the apple

Transformation and regeneration of the apple are considered to be difficult tasks, due to low transformation efficiency and the long regeneration period compared to *Arabidopsis*. In *Arabidopsis* within a period of one year it is possible to obtain at least 3-4 regeneration cycles, but for the apple it takes a year to complete a single regeneration cycle. The gene transformation procedure of the apple mainly depends on the availability of the tissue culture technique, the regeneration of shoots, selection of transformants and the propagation of transgenic plants. Increasing leaf regeneration efficiency is critical for the development of a Rosaceae family transformation system using an *Agrobacterium tumefaciens* vector or biolistic process (Aldwinkle and Malnoy 2009). In many instances, the lack of an efficient regeneration system is the main limiting factor preventing the development of gene transfer technologies for perennial crops (Dandekar 1992).

The first apple cultivar to be transformed was 'Greensleeves' (James et al. 1989); the transformation efficiency was very low, 0.1-0.5%. However, the efficiency of apple transformation and regeneration capacity is highly dependent on the genetic background of the apple cultivar (De Bondt et al. 1994). In early stages of apple transformation, the efficiency of transformation reported is between 0.2 and 15%. After the modifications and improvements made by Aldwinckle and Malnoy (2009) it was possible to increase the rate of transformation to arrive at 80%. To increase the transformation efficiency of *Malus*, several modifications have been made, including use of: 1) plant phenolic compounds to increase the expression of several virulence genes in *A. tumefaciens;* 2) gelling agent; 3) nitrogen source; 4) concentration of $AgNO_{3;}$ 5) binary vectors. The first report of transformed apple plants in 1989 showed promise for a new apple cultivar that would be superior in taste, healthier and easier to grow. Although many traits have been introduced

successfully to the apple, transformed apple cultivars are not yet available for commercial production. Most of the time, apple transformation has been used in 'proof of concept' experiments. In gene activation experiments, researchers have relied on a well-characterised constitutively expressing CaMV 35S promoter.

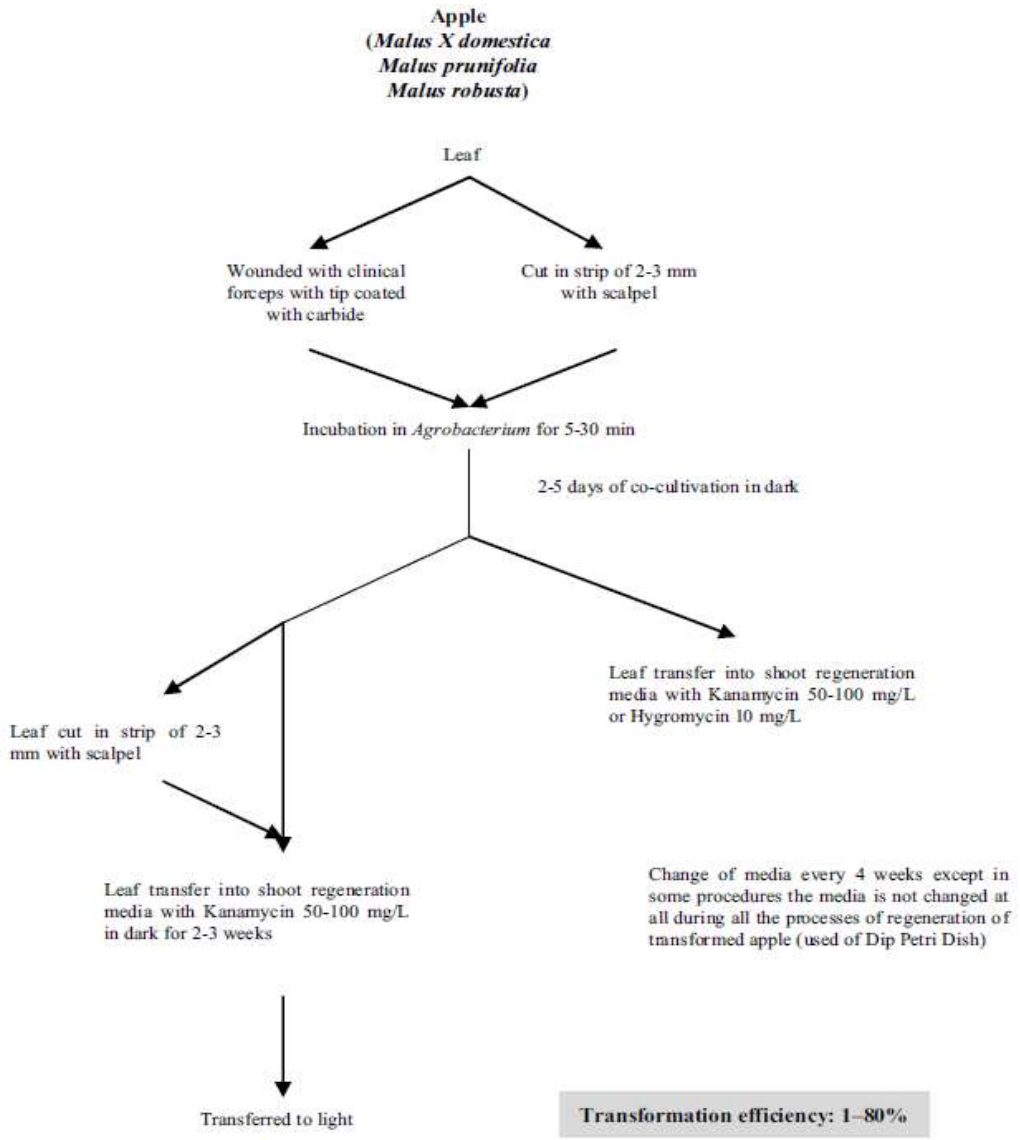


Figure 13: Representation of the apple (*Malus ×domestica, M.prunifolia, M.robusta*) transformation procedure (Aldwinckle and Malnoy 2009)

In the last decade apple transformation has been used for functional testing of traits of potential commercial interest, such as production and consumer traits. Production traits such as bacterial, fungal and pest resistance dwarfing, propagation, stress resistance, precocity, storage life and self-fertility (Malnoy et al. 2007; Bolar et al. 2000; Maheswaran et al. 2007; Zhu et al. 2008; Artlip et al. 2007) and consumer traits such as improved taste, reduced browning after slicing and reduced allergenicity traits (Schaffer et al. 2007; Murata et al. 2000; Gilissen et al. 2004) have been checked using gene transformation.

The development of fire blight (*Erwinia amylovora*) resistant apples using gene transformation technology was the first reported effort of the use of gene transformation in disease resistance experiments (Aldwinckle and Malnoy 2009). Different genes originating from different sources including bacteriophage, *A. tumefaciens* and apple (Aldwinckle et al. 2003; Viss et al. 2003; Malnoy et al. 2007) were used for the development of fire blight resistant transgenic apples. In addition to fire blight resistance, several groups of researchers are working on apple scab resistance using highly susceptible apple cultivars of 'Gala' (Belfanti et al. 2004), 'Galaxy' (Malnoy et al. 2008), and McIntosh (Bolar et al. 1999). The first apple scab resistance gene *(HcrVf2*) was characterised using overexpression of the gene in the apple scab susceptible cultivar 'Gala' with the constitutive promoter CaMV35S (Belfanti et al. 2004). Very recently, Schouten et al. 2014 characterised the second apple scab resistance gene of *Rvi15* (Vr2-C) from GMAL 2473 using gene transformation technology.

For characterisation of the *Rvi5 (Vm)* apple scab resistance gene, we also used gene transformation technology. We transformed the identified candidate gene of *Rvi5 (Vm)* from its resistant parent 'Murray' in the apple scab susceptible cultivar 'Gala' using

Agrobacterium mediated transformation with the CaMV35S promoter. The transgenic plants obtained were checked for insertion of the gene of interest using PCR amplification on DNA extracted from transgenic plants with specific primers designed on the gene of interest. All the plants obtained were positive for insertion of the gene of interest, and transgenic plants are undergoing multiplication and the regeneration procedure necessary to obtain sufficient plants for future inoculation experiments with *Venturia inaequalis*.

General discussion and conclusions

Positional cloning or map-based cloning has been successfully used for the isolation of resistance (R) genes in different crop species. The *Pto* and *Cf-2* genes of the tomato (Martin et al. 1993; Dixon et al. 1996), the *Mlo* gene of barley (Büschges et al. 1997), the *Xa26* gene of rice (Sun et al. 2004), RAC1 from Arabidopsis (Borhan et al. 2004) and the apple scab resistance genes *HcrVf,* and *Rvi15* from *Malus floribunda 821* and apple accession GMAL 2473 (Vinatzer et al. 2011 and Schouten et al. 2014) are a few examples of the isolation of R genes using positional cloning from different crop species. This method consists of three essential steps (Gessler et al. 2006): 1) identifying the clones of a large-insert library spanning the region between two molecular markers flanking the gene of interest; 2) recognising candidate genes among all the expressed sequences on these clones; 3) demonstrating that one of the candidates is the gene of interest by transformation in the susceptible cultivar.

However, this methodology relies on the availability of markers closely linked to the R gene, from which chromosome walking is undertaken to isolate the genomic clones from a library with a large insert to span the region of the gene of interest.

Rvi5 apple scab resistance was studied for the development of molecular markers linked to the resistance (R) gene to recognise the position of the R gene in the apple genome by Cheng et al. (1998) and Patocchi et al. (2005) respectively. Two molecular markers were available for *Rvi5* resistance: the $OPB12_{687}$ SCAR (Cheng et al. 1998) marker was found to be linked at about 5 cM from the R gene and the SSR marker Hi07h02 was reported to be co-segregating (Patocchi et al. 2005). For the isolation and characterisation of the *Rvi5* gene from the 'Murray' apple genotype, available information about the map position of the gene and the availability of molecular markers linked to resistance was useful, but not

sufficient to start screening of large insert libraries. Hence, we decided to start with preliminary mapping of *Rvi5* for the purpose of determining the precise map position and developing more molecular markers linked to the R gene.

Preliminary mapping of the *Rvi5* locus using a subset of the F1 population with 96 progeny plants

Initial efforts to map *Rvi5* resistance were carried out using 95 progeny plants of a cross between 'Golden Delicious' and 'Murray', consisting of 52 susceptible and 43 resistant plants, which made it possible to map *Rvi5* on the distal end of LG 17 (Patocchi et al. 2005) with SSR Hi07h02 as a co-segregating marker. The molecular marker $OPB12_{687}$ SCAR was linked to resistance, with a distance of 5 cM. Hence, we decided to use a new mapping population with 96 progeny plants of 'Golden Delicious' × 'Murray' for the purpose of preliminary mapping of *Rvi5*, consisting of 48 resistant and 48 susceptible plants with already available molecular markers on the distal end of LG 17. The SSR marker CH05D08 was reported to be non-polymorphic for the *Rvi5* gene, but after manipulating the PCR conditions of the marker, it was converted to make it polymorphic. Genotyping and phenotyping of the selected mapping population made it possible to construct a preliminary genetic map for *Rvi5*. The *Rvi5* gene was mapped 1 cM from the SSR marker CH05d08 and co-segregating with SSR Hi07h02 (Chapter 1 -Supplementary Figure 1). The genetic distance between the two markers was 1cM. The results of preliminary mapping indicated the location of the *Rvi5* locus; it should be in the region below SSR CH05d08. The idea behind the use of preliminary mapping was to identify the position of *Rvi5* on the distal end of LG 17 by delimiting two flanking markers. Compared to use of a large population, the use of 96 plants is less time-consuming and does not require much effort. However, the available molecular markers were not sufficient to

delimit the R locus. Hence, we decided to screen the complete F1 population with newly developed molecular markers.

Use of a large mapping population for fine mapping of the locus

Two F1 progenies, with a total of 1243 plants, were used to specifically recognise the *Rvi5* locus. 701 F1 plants of 'Galaxy' × 'Murray' and 542 plants of 'Golden Delicious' × 'Murray' were inoculated with *Venturia inaequalis* to evaluate scab resistance symptoms. The scab evaluation procedure was repeated for two years in greenhouse conditions and third year evaluation was carried out only for identified recombinant plants. According to the scab evaluation results, inheritance of a single dominant gene ($X^2 = 1.957$) in 'Galaxy' × 'Murray' progeny is strongly supported. However, in 'Golden Delicious' × 'Murray' progeny the availability of single dominant gene ($X^2 = 13.831$) was not supported, which is significantly different from the expected 1:1 ratio. The observed skewness of the segregation may be due to the effect of the self- incompatibility locus located on the same chromosome, quite close to the *Rvi5* locus (Antanaviciute et al. 2012). In order to explain this distorted segregation we investigated the self-incompatibility (S-) locus, searching for similarities of the S alleles in 'Golden Delicious' and 'Murray'. It is known that fertilisation is blocked when the S- allele carried by the pollen matches with the one of the two alleles expressed in the pistil, therefore cultivars bearing both S-alleles in common are incompatible, while those bearing one S-allele in common are partially fertile. 'Gala' contains S2S5 alleles, while 'Golden Delicious' contains S2S3 alleles (Broothaertz et al. 1995). The common S2 allele from both 'Gala' and 'Golden Delicious' could not make any difference to these two cultivars at pistil level. The S3 allele borne by 'Golden Delicious' is almost identical to the S10 allele and 'Murray' is probably inherited with the S10 allele from McIntosh. The experiment conducted to prove this hypothesis with the

specific primers for the S2 and S10 alleles (Broothaertz et al. 1995) and three parental cultivars showed that ‘Gala’ and ‘Golden Delicious ’ had the expected amplicon for the S2 allele, but ‘Murray’ showed no specific amplification, while the S10 allele was amplified only in ‘Murray’ and ‘Golden Delicious ’. Therefore, the ‘Golden Delicious ’ pistil (S2S3) receiving the ‘Murray’ pollen (SxS10) probably blocks all the S10 allele bearing gametes and this may be the reason for the observed distorted segregation in the ‘Golden Delicious’ × ‘Murray’ population.

The large population size of the F1 progeny made it possible to recognise recombinant plants, which was essential for precise determination of the *Rvi5* locus. We identified seven recombination events for the SSR marker Hi07h02, which showed that the *Rvi5* locus does not co-segregate with SSR Hi07h02, and two recombination events for SSR CH05d08, which showed that the *Rvi5* locus is closer to SSR CH05d08 (Chapter 1 - Table 1). As a first step, we randomly selected the region between SSR Hi07h02 and CH05d08 to develop new molecular markers.

Development of new molecular markers and fine mapping of the *Rvi5* region

The availability of the *Malus* × *domestica* reference genome (Velasco et al. 2010) was an advantage for the development of new primers. The sequences between SSR Hi07h02 and CH05d08 were downloaded from the apple GDR (https://www.rosaceae.org/gb/gbrowse/malus_x_domestica) and used for marker development using Primer3 software. Three different types of molecular markers were designed for the region of interest using sequences of the reference genome (Chapter 1- Tables 2, 3 and 4), of which only polymorphic markers were used for genotyping. Even in the reference genome of *Malus* × *domestica* the region of interest was not fully sequenced, as there were gaps and un-sequenced regions. Due to the presence of gaps in the reference

genome region, the number of markers developed was limited. In any case, we developed 10 informative molecular markers, including four SSR markers, five SNP markers and one SCAR marker. These new SSR markers helped to narrow down the *Rvi5* region further. Phenotypic and genotypic evaluation of F1 population segregating for the *Rvi5* resistance, allowed to identify the resistant locus in between two flanking markers (SSR FMACH_Vm4 and SSR FMACH_Vm2). One recombination event was identified for SSR FMACH_Vm4 and three recombination events were identified for SSR FMACH_Vm2 (Chapter 1 - Table 1). In addition, three new co-segregating molecular markers (Vm-SCAR 1, Vm-SNP 5 and SSR FMACH-Vm3) were identified (Chapter 1 - Table 1).

Screening of a large insert library of 'Murray' to recognise BAC clones between two flanking molecular markers

After identifying two flanking molecular markers (SSR FMACH_Vm4 and SSR FMACH_Vm2) delimiting the *Rvi5* locus, the next step was screening of a large insert library to recognise the clones spanning the resistance locus. The BAC library of 'Murray' consisted of 36864 contigs in 96 plates of 384 wells each. If we had screened the BAC library plate by plate it would have been a time-consuming and difficult task. For this reason we used a BAC pooling strategy, which reduces the number of PCRs required to screen the whole BAC library. With BAC pooling we prepared two BAC pools called the 'plate pool' and 'well pool'; in total the number of samples to be checked was reduced to 484. The 'plate pool' was created by adding all the BAC clones to one plate in a single well of 96 well plates. In the end, the 'plate pool consisted of one plate with 96 samples. Likewise, the 'well pool' was created by bringing together all the BAC clones located in the same well position of each plate, hence the 'well pool' consisted of 384 samples in one 384 well plate. After completing BAC pooling, chromosome walking was started from SSR FMACH_Vm2 and development of new molecular markers for the BAC ends helped

to continue chromosome walking until a BAC clone carrying the SSR CH05d08 was recognised. During screening, the BAC pool first screens the 'plate pool' to recognise the number of plates carrying positive clones and then screens the 'well pool' to recognise the well number carrying positive clones for the molecular marker used. Three overlapping BAC clones were identified spanning the *Rvi5* locus (Chapter 2-Figure 1). The size of the *Rvi5* resistance locus was approximately 228kb in the 'Golden Delicious' reference sequence. However, this estimate must be considered with caution, because there are un-sequenced areas in the reference genome, although the actual size of the putative *Rvi5* locus could be obtained by sequencing the three BAC clones identified spanning the *Rvi5* locus of 'Murray'.

BAC sequencing and assembly

BAC DNA was extracted from the three identified BAC clones spanning the putative *Rvi5* locus of 'Murray' with an increased BAC DNA concentration. At least 3mg of BAC DNA needs to be used for the construction of 3kb paired end libraries. Instead of using the normal BAC DNA extraction protocol, a few modifications were applied to obtain increased BAC DNA concentration, including doubling of the volume of the bacterial culture used for DNA extraction and extending the incubation time at 37 ^{0}C from 24 hrs to 48 hrs. The putative *Rvi5* region of 'Murray' located on the distal end of LG 17 is known to be a highly repetitive region. Hence, sequencing of this region needs to be carried out with caution. We decided to use the paired end sequencing technique to sequence the three identified BAC clones. Paired-end DNA sequencing reads provide superior alignment across DNA regions containing repetitive sequences and produce longer contigs for *de novo* sequencing, by filling gaps in the consensus sequence. Paired-end DNA sequencing also detects rearrangements such as insertions, deletions and inversions. However, there

are also some disadvantages associated with paired end sequencing; unlike the PACBIO smart sequencing technique, the obtainable average read size is about 500 bp and after assembly the possibility of obtaining a complete BAC sequence as a single contig is low. Normally with the paired end sequencing technique several contigs could be obtained for a single BAC clone and the gaps between the contigs need to be filled with additional marker development and Sanger sequencing. In our study, we obtained nine contigs for BAC clone 8a17, and seven and six contigs for the BAC clones of 55e20 and 54e18 respectively. To fill in the gaps between the contigs, molecular markers were designed (Chapter 3 - Supplementary Table 1) and gap sequences were obtained using PCR amplification and Sanger sequencing. After obtaining complete sequences of BAC clones, the putative *Rvi5* locus was extracted by removing overlapping sequences between the BAC contigs. The size of the putative *Rvi5* resistance locus of 'Murray' was 154 kb.

ORF prediction and identification of candidate genes

Once the sequence of putative *Rvi5* locus had been identified, the sequence of the *Rvi5* putative resistance locus was subjected to ORF prediction using FGENESH software. ORF prediction was carried out using the algorithms of dicot plants, *Vitis vinifera* and the tomato. In a region of 154 kb we found 41 ORFs, of which only one ORF was associated with plant resistance. However, the identified candidate gene belonged to the TIR-NBS-LRR family of resistance genes, which is a common group of resistance genes found only in dicot plants (Meyers et al. 1999). Usually, plant resistance genes are found as resistance gene clusters or resistance gene analogs (RGA), which is also true for the apple (Baldi et al. 2004). However, in our study, the observed resistance gene was not embedded in a cluster of paralogs. A similar situation was also reported for fire blight resistance in *Malus*

× *robusta*. Fahrentrapp et al. (2013) observed a single candidate gene for fire blight resistance in a *Malus* × *robusta* resistance locus of 162 kb in size.

The candidate gene we found consisted of a N-terminus *Drosophila* Toll and interleukin - 1 receptor protein domain (TIR), a central nucleotide–binding site (NBS), and a C terminal leucine-rich repeat domain. The LRR domain consisted of ten hydrophobic LRR motifs.

Cloning and transformation of the '*Rvi5*_Murray' candidate gene

The identified candidate gene was amplified on first strand cDNA and the amplified product was sub cloned to the TOPO-TA vector for sequencing. The completely sequenced amplicon was used to correct mismatches of the candidate gene sequence obtained by BAC assembly. The corrected candidate gene sequence was translated to its amino acid sequence and primers were designed using the amino acid sequence for amplification of first strand cDNA.

The amplified candidate gene on first strand cDNA was used to construct the vectors for gene transformation through *Agrobacterium tumefaciens. Venturia inaequalis* infects only plants of the *Malus* species; hence for the transformation experiments we could not use other model plants such as *Arabidopsis* or *Nicotiana.* Apple transformation studies are associated with some disadvantages, especially due to the long transformation and regeneration time, as it takes a year to obtain transgenic plants, and plants at least seven months old are required in order to confirm whether the plants are transgenic or not, while other model plants used for transformation studies require only two months to complete a transformation cycle.

In our study we used binary vectors with gateway cloning technology to ensure the integration of the candidate gene in a predefined order with correct orientation. The entry vector we used was pENTR/D-TOPO with *attL1* and *attL2* sites, which allow directional cloning of the gene of interest into the entry vector. The destination vector we used consisted of compatible sites for *attL1* and *attL2* (*attR1* and *attR2*), the recombination reaction between those sites being mediated with the GATEWAY LR clonase enzyme. When the recombination procedure is completed, the destination vector is ready for the transformation procedure with *Agrobacterium tumefaciens*. Three to four week old scab susceptible 'Gala' apple leaves were used as starting material for transformation.

Conclusions and future works

The *Rvi5* apple scab resistance locus was fine mapped and restricted to a region of 1cM, flanked by two molecular markers on the distal end of LG 17 of the 'Murray' *Malus* × *domestica* genotype. The *Rvi5* resistance locus of 'Murray' was isolated from the 'Murray' BAC library using three overlapping BAC clones. The identified BAC clones spanning the *Rvi5* resistance locus were completely sequenced and assembled to obtain the sequence of the *Rvi5* resistance locus. The sequence of the *Rvi5* resistance locus was subjected to Open Reading Frame (ORF) prediction and candidate genes were identified using functional analysis of predicted open reading frames. One ORF belonging to the plant resistance genes was found among the predicted ORFs and was considered to be a *Rvi5*-Murray candidate gene. The *Rvi5*-Murray candidate gene was transformed into scab susceptible 'Gala' leaves using *Agrobacterium* mediated transformation (Figure 9). Five transgenic apple plants with the inserted gene of interest were identified and they are still growing in tissue culture containers in sterile conditions.

Before conducting the scab inoculation experiments, transgenic plants are needed in order to undertake multiplication and regeneration to obtain sufficient plant materials for inoculation experiments. Once a sufficient amount of plant material has been obtained from transgenic plants, the shoots will be micro-grafted onto 'Golden Delicious' rootstocks. When the micro-grafted shoots reach the ten leaf stage, scab inoculation experiments will be continued with monoconidial *V. inaequalis* isolates, with differential interaction with *Rvi5.*

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