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**GENETIC CHARACTERIZATION OF *Rvi12* BASED SCAB RESISTANCE
FROM *Malus baccata* 'HANSEN'S BACCATA # 2'**

presentata da

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List of Abbreviations

AFLP	Amplified Fragment Length Polymorphism
Avr	Avirulence
BAC	Bacterial Artificial Chromosome
bp	Base pair
BSA	Bulked Segregant Analysis
CC	Coiled coil
cDNA	Complementary DNA
Chl	Chlorosis
cM	centi Morgan
DNA	Deoxyribonucleic acid
EST	Expressed Sequence Tags
FAO	Food and Agriculture Organization
GfG	Gene for gene
GPI	Genotype to Phenotype Incongruence
GSA	Genome Scanning Approach
HB2	Hansen's baccata #2
HGAP	Hierarchical Genome Assembly Process
HR	Hypersensitive response
kb	kilobase
LG	Linkage group
LRR	Leucine-rich repeat
MAB	Marker-assisted breeding
MAS	Marker-assisted selection

N	Necrosis
NBS	Nucleotide-binding site
NLS	Nuclear Localization Signal
PacBio	Pacific Biosciences
qRT-PCR	Quantitative real time polymerase chain reaction
QTL	Quantitative trait loci
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RGA	Resistance gene analogue
R-gene	Resistance gene
RLKs	Receptor like kinases
RLP	Receptor-like protein
RNA	Ribonucleic acid
Rvi	Resistance to <i>Venturia inaequalis</i>
SC	Stellate chlorosis
SCAR	Sequence Characterized Amplified Regions
SMRT	Single Molecule Real Time
SN	Stellate necrosis
SNP	Single nucleotide polymorphism
SSR	Single sequence repeat
TIR	Toll interleukin1 receptor

Abstract

Apple scab caused by *Venturia inaequalis* is one of the most prevalent and devastating diseases of apple (*Malus × domestica*) worldwide. Breeding programs are attempting to introgress scab resistance genes from wild apples into commercial cultivars for sustainable disease control. Most of the commercially available scab-resistant varieties rely on the *Rvi6* resistance gene from *Malus floribunda* 821. The evolution of new pathotypes of *V. inaequalis*, which have caused the breakdown of *Rvi6*-based resistance, highlights the need for the characterization and pyramiding of different scab resistance genes for durable scab resistance. This study was performed to characterize another scab resistance locus *Rvi12* from *Malus baccata* ‘Hansen’s baccata #2’ (HB2). The *Rvi12* locus was fine-mapped to a physical interval of approximately 882 kb by the use of ‘Golden Delicious’ reference genome guided marker development and a large mapping population. Through a BAC-library-based chromosome walking, additional markers were developed and a single BAC clone spanning the *Rvi12* interval was identified. Following PacBio RS II sequencing and the use of the hierarchical genome assembly process, the BAC clone sequence was assembled. The *Rvi12* resistance locus was further narrowed to a 62.3 kb genomic region of the HB2 genome. Gene prediction and *in-silico* characterization identified five putative candidate genes showing homology to known disease resistance genes or genes involved in disease resistance signaling. Protein domain analysis and quantitative real-time PCR (qRT-PCR) of the five putative candidate genes indicated a LRR receptor-like serine/threonine kinase as the most likely candidate gene for *Rvi12* resistance. Overall, the novel markers developed from this study will be useful for the marker-assisted selection of apple genotypes carrying the *Rvi12* resistance. Further the identification of candidate gene for *Rvi12* based scab resistance will provide the initial resources required for developing scab resistant transgenic or cisgenic apple lines with the *Rvi12* resistance.

Chapter 1

General introduction

1.1. Apple production, nutritional facts and health benefits

Domesticated apple (*Malus × domestica* Borkh) is one of the world's most economically important fruit crops. It belongs to the Rosaceae family, which includes other important fruit crops such as pear, peach and soft fruit species such as strawberry and raspberry (Way et al, 1991; Janick 2005). Apple is the second largest produced fruit crop after banana with over 80 million metric tons of apples harvested in 2013. It is cultivated mainly in the temperate and subtropical climatic regions and in some higher altitude areas of tropical regions. In the year 2013, the People's Republic of China was the world's major apple producer, with approximately 39 million metric tons of fruits harvested, and followed by the United States of America which produced approximately four million metric tons. Apple production in Europe totals approximately 16 million metric tons, and Italy is the second largest producer in Europe, and the fifth largest in the world (FAO statistics, 2013).

Table 1.1 Top five apple producers in the world

Country	Production (In million metric tons)
China	39.68
United States of America	4.08
Turkey	3.13
Poland	3.09
Italy	2.22

Data was obtained from the FAO statistics (2013)

Apart from the economic importance to the producer, apple fruits are rich in nutrients and their consumption has been shown to provide various health benefits to consumers and fresh apples are also low in calories, containing just 50 calories per 100g of fresh fruit (National nutrient database

for standard reference, USDA, 2015). Nutritionally, they contain large quantities of dietary fibers, antioxidants, flavonoids, polyphenols and vitamin C and do not contain cholesterol (National nutrient database for standard reference, USDA, 2015). Additionally, the phytochemicals present in apple and apple products have been shown to help reduce colon cancer cell proliferation *in vitro* (Gosse et al, 2005). A hospital-based case control study conducted in Italy with 6,000 participants found a clear association between the quantity of apples consumed and a reduction in risk of contracting various types of cancer (Gallus et al, 2005). There was also a clear inverse correlation between apple flavonoid consumption and coronary mortality in Finnish women (Knekt et al, 1996). The presence of high dietary fibers was shown to improve the cardio vascular health of patients in clinical studies by reducing the LDL cholesterol levels in their bloodstream (Bazzano et al, 2003). The vitamin C and other flavonoids present in apple act as strong antioxidants and help neutralize free radicals and boost immunity against diseases (Avci et al, 2007). Thus apple is a high value superfood providing numerous health benefits to the consumer. However, due in part to its widespread cultivation, apple production is severely affected by many pests and diseases.

1.2. Pests and diseases of apple

Apple trees are affected by numerous pests and diseases leading to reductions in yields of varying levels. The most important insect pests of the apple are codling moth (*Cydia pomonella*), rosy apple aphid (*Dysaphis plantaginea*) and wooly apple aphid (*Eriosoma lanigerum*) (McVay et al, 1993). Codling moth and Rosy apple aphid are the main causal insects for fruit damage. The damages caused by these insect pests lead to unmarketable fruits and a drastic reduction in yield of infested trees (McVay et al, 1993).

Apple trees are also affected by a number of bacterial, fungal and viral diseases. The major diseases of apple include apple scab, fire blight, powdery mildew and European canker (Jones and Aldwinkle, 1990; Jones, 1996). Fire blight is caused by the bacteria *Erwinia amylovora* and is

responsible for major yield losses in apple production (Jones and Aldwinkle, 1990). *Podosphaera leucotricha* is the causative fungal agent of powdery mildew in apple trees. Powdery mildew infection on young fruit leads to net-like russeting phenotype on mature fruits. Thus, powdery mildew disease reduces both the marketable quality of fruits and the yield of the trees (Jones, 1996). Apple canker or European canker is caused by the fungus *Neonectria ditissima*. Apple canker is a serious problem causing a drastic reduction in yield of apple fruits and death of the apple trees (Jones, 1996).

1.3. Apple scab disease

Among all the diseases, apple scab is the main threat to the apple production in all growing areas where humid and wet conditions prevail. Apple scab is caused by *Venturia inaequalis*, a fungus from the ascomycetes family (MacHardy, 1996; Bowen et al, 2011). *Venturia inaequalis* infects all parts of the tree including leaves, petioles, flowers, fruits, pedicels and young shoots (MacHardy, 1996; Villaincourt and Hartman, 2000 and 2005).

1.3.1. Susceptibility symptoms

The symptoms of apple scab are clearly visible on the leaves and fruits of scab-susceptible trees. The symptoms initially appear on the abaxial side of the leaf as small circular lesions that are light green in color. Upon opening of the leaves, the infection and symptoms start to appear also on the adaxial side. The circular lesion increases in size and becomes olive brown in color because of asexual spore (conidia) formation. Once conditions are favorable, the pathogen colonizes more young tissues, leading to secondary infections. Old tissues are always resistant to apple scab and this is known as ontogenic resistance (MacHardy, 1996). In the final stages of infection of the leaves and fruits, symptoms appear as olive-brown colored velvety, scabby blisters (**Figure 1.1A, B**) (Villaincourt and Hartman, 2000; 2005). The affected tissues usually become distorted and puckered. In severely infected trees, leaves and fruits drop off, which leads to increased

susceptibility of the apple trees to freezing damage, insect injury and other diseases such as fire blight or canker (Jones, 1996). The infected leaves can also act as inoculum for subsequent infections in the following growing seasons if not managed properly (MacHardy, 1996).

1.3.2. Lifecycle of *Venturia inaequalis*

Venturia inaequalis is a successful pathogen because of its sophisticated life cycle reproducing both sexually and asexually (**Figure 1.1C**) (Villaincourt and Hartman, 2000 and 2005). Towards the end of the apple season, the apple scab fungus overwinters in fallen leaf tissue (Holb et al, 2004). Before leaf abscission, mating of two different mating types takes place and sexual spore (ascospore) formation occurs in specialized fruiting bodies called pseudothecia. Thus, the pathogen is able to survive the winter in fallen leaves and form sexual spores with different combinations of (a)virulence genes in a timely manner. Ascospore maturation and release coincides with the bud break stage of the apple trees, which gives an adaptive advantage for the pathogen to be able to land on the young leaves and cause a successful primary infection (Stensvand et al, 1997; MacHardy, 2001). Once the ascospores land on the leaves, they germinate, penetrate the cuticle and form specialized subcuticular structures called stroma or haustoria (Ortega et al, 1998). These subcuticular structures are thought to play a role in nutrient acquisition from the host plant. Then conidiospores (asexual spores) are formed on specialized short hyphae named conidiophores. Conidiophore and conidiospore formation is the cause of the velvety, scabby appearance of infected plant tissue (MacHardy, 2001; Bowen et al, 2011). The release and germination of both ascospores and conidiospores requires moist conditions, with a humidity of at least 60 percent (Villaincourt and Hartman, 2000). Thus, if the conditions are favorable, the pathogen can complete one cycle of ascospore formation and multiple cycles of conidiospore formation in a single growing season, which can lead to substantial reduction in yields, sometimes with losses in production of up to 70 percent (MacHardy, 2001).

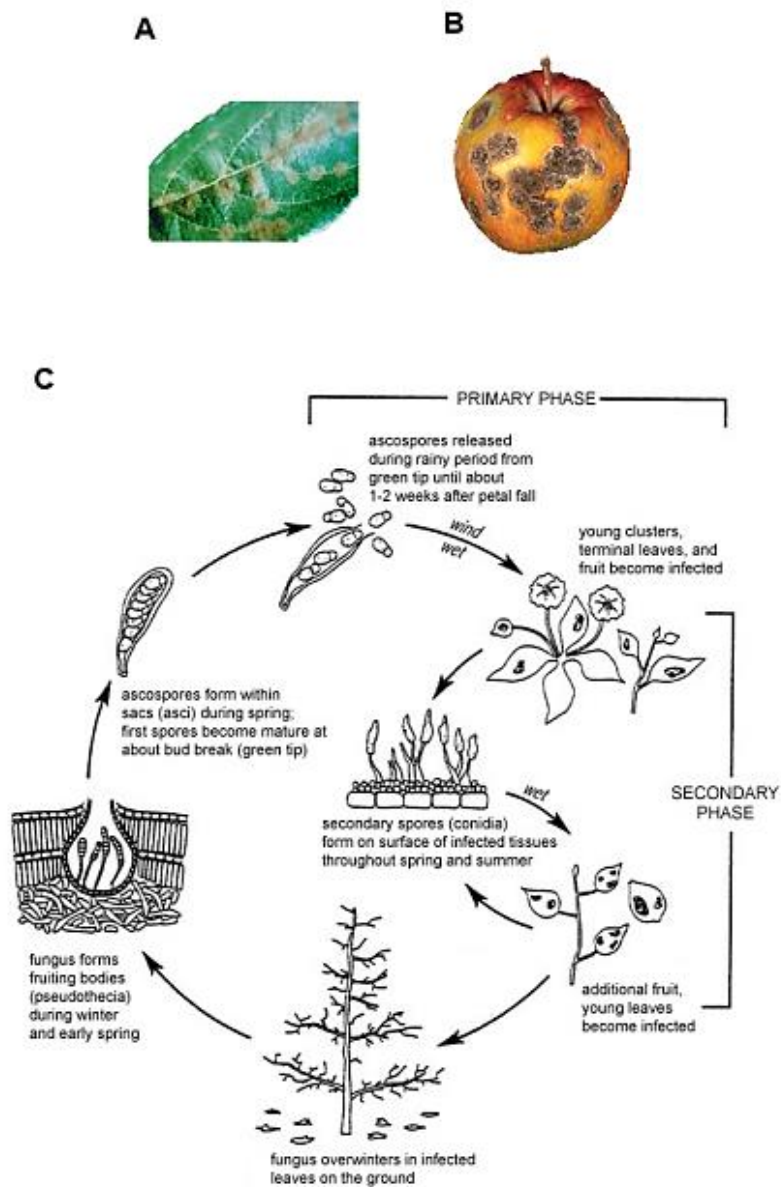


Figure 1.1 Apple scab symptoms on the susceptible leaves and fruits and the lifecycle of the causal agent *Venturia inaequalis*. (A) Apple scab symptoms on susceptible leaves, olive brown colored scabby lesions on the leaf; (B) Apple scab symptoms on susceptible fruit, brown colored scabby blisters on the affected fruit; (C) Sophisticated lifecycle of the apple scab pathogen *Venturia inaequalis* with its sexual and asexual reproduction. Images for this figure were adapted and modified from Villaincourt and Hartman (2000).

1.4. Control of apple scab

1.4.1. Cultural practices

The first strategy to prevent apple scab damages was based on cultural practices targeting the reduction of the primary infection from ascospores (Villaincourt and Hartman, 2000). Removal and disposal of fallen leaves was used to prevent the buildup of ascospores, but it was not practical to perform in large commercial-scale orchards (Holb, 2006). Thus, other cultural practices such as the application of urea to trees before leaf fall, tilling of the fallen leaves into the soil, or chopping of leaves into small pieces were recommended to improve the leaf decomposition and to reduce the ascospore population for infection in the subsequent season (Gupta, 1992; Sutton et al, 2000; Carisse and Dewdney, 2002). Regular pruning of the trees to increase air circulation and to allow penetration of sunlight was also recommended to help with the drying of the leaves and to reduce the primary inoculum load (Holb, 2008; Hetherington, 2010). The difficulty with the application of these cultural practices to control apple scab is that they are time consuming and labor intensive.

1.4.2. Chemical control

Chemical fungicide application is currently the main strategy for control of apple scab, targeting the prevention of germination of spores and growth of fungal hyphae. Several fungicides including fixed copper, Bordeaux mixtures, copper soaps, sulfur, mineral or neem oils, and mycobutanol have been used for the prevention and control of apple scab (Jamar et al, 2008). In the early days, the application of chemical fungicides was performed once every 5-7 days at the beginning of the season and once every 7-10 days in late season to control the disease. Thus up to 30 treatments were required for a single growing season (Holb, 2008; Villaincourt and Hartman, 2000). In recent years, the application of chemical fungicides has been reduced to 15-20 rounds per growing season and these are applied on a timely basis, based on the knowledge gained about the pathogen's requirements for successful infection and prevailing environmental conditions (Villaincourt and

Hartman, 2000). Fungicides are applied during the bud break stage to prevent the ascospore infection and later during the periods when the environmental conditions are favorable for fungal infection. Even though chemical fungicides are effective in the control of the disease, the pathogen could evolve and confer resistance against multiple fungicides because of the generation of different novel genotypes through sexual reproduction. *Venturia inaequalis* strains showing resistance to dodine, methyl benzimidazole carbamate (MBC), demethylation inhibitors (DMIs) and Quinoline outside inhibitor (QoI) fungicides have been identified from different apple growing regions (Koller, 1994; Koller and Wilcox, 2001; Koller et al, 2004). The substantial cost of chemical fungicides, and the ability of *Venturia* to evolve resistance to the fungicides, along with the non-sustainable nature of chemical application has led to a search for other alternative strategies to control the disease.

1.5. Breeding for scab resistance - Resistant sources and historic scab resistance genes

Breeding for scab resistance started with the observation that some of the small fruited wild accessions of crab apples were resistant to apple scab (Hough, 1944). Thus a breeding program aimed at identifying scab resistance sources and producing scab resistant cultivars was initiated in 1945 by J.R. Shay at Purdue University and L.F. Hough at University of Illinois and later at Rutgers University. This collaboration, named the PRI (Purdue University, Rutgers University and Illinois University) breeding program, was the first official attempt aimed at developing apple scab resistant cultivars.

Further studies from Shay and Hough (1952), Shay et al, (1966), Dayton and Williams (1968) and Williams and Kuc (1969), identified 25 different *Malus* species and selections showing high levels of genetic resistance to apple scab. Intercrosses were performed between these lines followed by test crosses with susceptible genotypes to test for allelism between the resistant loci. The results obtained from the study of Williams and Kuc (1969), indicated that 11 of the 25 tested lines

contained a single scab resistance locus *Vf*. Following allelism tests, six different gene pools of scab resistance were identified. The historic apple scab resistance sources identified were *Malus floribunda* 821 carrying the *Vf* locus, *Malus micromalus* containing the *Vm* resistance locus, *M. pumila* R12740-7A containing the *Vr* locus, *M.baccata jackii* containing the *Vbj* locus, *M.baccata* ‘Hansen’s baccata #2’ containing the *Vb* locus and ‘Antonovka PI 172623’ containing the *Va* locus (Dayton and Williams, 1968, 1970). In the following years, with the help of apple breeding programs throughout the world, and through the study of *Malus-Venturia* interactions, at least 18 scab resistance genes were identified from different *Malus* germplasm collections (Bus et al, 2011; Soriano et al, 2014) and these sources of resistance are used in developing scab resistant apple cultivars.

1.6. *Venturia inaequalis* inoculation methods

Venturia inaequalis inoculation and phenotyping of *Malus* germplasm collections is the first step in identifying novel resistance sources. Apple scab resistance screening by inoculation of seedlings with a *Venturia inaequalis* conidial suspension was mainly done at two stages, as seedlings (2-6 leaves stage) and as grafted plants in greenhouse conditions or field conditions. Seedling screening performed at the two leaf stage (Lamb and Hamilton, 1969) may lead to death of the susceptible seedlings and plants often show weak resistance responses in genetically resistant seedlings because of juvenility effects. Additionally, in the case of death of seedlings following inoculation, it would not be possible to get plant material for molecular analyses. Thus seedlings at the six leaf stage are preferred for scab inoculation. Inoculation in a greenhouse provides a conducive environment for scab infection and for uniform scoring of resistant responses (Gardiner et al, 1996).

Spray inoculation

Conidial suspension from individual isolates or a mixture of isolates is usually prepared in double distilled water at a concentration of 1×10^5 (Gardiner et al, 1996) or 2.5×10^5 conidia ml⁻¹ (Patocchi et

al, 2005) and used for spray inoculation on the young leaves of seedlings. Following inoculation, the seedlings are kept at 100% humidity for the first 48 hours, followed by 80% humidity for 14-21 days post inoculation. After 14 and 21 days post inoculation, the resistance reactions are scored on the seedlings. The leaves from the susceptible seedlings showing abundant sporulation are collected, dried and stored at -20°C and used as inoculum for the next season. The downside of the spray inoculation method is that it can lead to the death of susceptible seedlings after just a single inoculation (Bus et al, 2005). Thus using this method, it is not possible to inoculate the same seedlings with multiple isolates of *Venturia inaequalis* to study differential interactions.

Droplet inoculation

To circumvent the above mentioned problem, Bus and colleagues (2005), developed a novel inoculation method named droplet inoculation. A small inoculation chamber made up of 8mm diameter microcentrifuge tube caps with 2mm holes was constructed. The inoculation chambers were attached to the adaxial side of the leaves with the help of hairclips. One hundred microliters of conidial suspension at a concentration of 4×10^4 conidia ml⁻¹ was pipetted into the inoculation chamber. The inoculated seedlings were kept at 100% humidity for 48 hours followed by 80% humidity for 14 to 21 days before scoring the resistance reactions. By using this method more than one isolate of *Venturia* can be inoculated on same leaf of the seedling or on different newly emerging young leaves.

In the earlier studies, detached leaf inoculation methods were developed and used in analyzing resistance reactions of apple leaves from tissue culture (Yepes and Aldwinckle, 1993; Barbieri et al 2003) or leaves from whole plants (Nicholson et al, 1973) or by the use of leaf discs (Martinez-Bilbao and Murillo, 2005).

1.7. Phenotyping – Classes of resistance and susceptibility reactions

Scoring of resistance reactions is usually performed 14 to 21 days after inoculation. Different classification systems have been developed to group the resistance response of the seedlings at the greenhouse conditions (Hough 1944, Chevalier et al, 1991). Based on the microscopic and macroscopic observation of scab infection and the resistance responses of the apple leaves, Chevalier et al (1991) grouped the reactions into five classes starting with complete resistance through to complete susceptibility. Class 1 was described as a hypersensitive response or pin point pits due to the programmed cell death of the cells surrounding the conidia penetration site. Class 2 was described as the symptoms of chlorosis and/or very small necrosis on the leaves without sporulation. Class 3A was described as resistance reactions such as chlorosis or necrosis that could also display a weak, sparse sporulation. Class 3B was described as abundant sporulation in specific areas of the leaf, but with a leaf surface that had become strongly curled and crinkled after pathogen development. Class 4 was described as complete susceptibility with abundant sporulation on the entire leaf surface and without any sign of leaf reaction. When studying the segregation of resistance genes based on the resistance reactions observed, plants carrying the Class 1 – 3A reactions are considered as fully resistant to scab. Plants classified as 3B are also showing weak resistance reaction but with a high likelihood of mis-classification (Tartarini et al, 1999). Class 4 is considered as susceptible. While studying the differential interactions of the TSR35T15 accession containing the *Vh2* (*Rvi2*) resistance gene, Bus and colleagues (2005) observed star shaped necrosis and chlorosis reactions, which were subsequently named as stellate necrosis (SN) and stellate chlorosis (SC) and were sub-grouped under class 2 of Chevalier resistance. Patocchi et al (2005) included class 0 to the phenotypic classes which include seedlings without any macroscopic reactions (**Figure 1.2**). The resistance reactions exhibited by each of the resistance varieties and the duration at which the resistance is exhibited vary based on the resistance gene(s) the variety possesses. It can vary also between greenhouse and field conditions.

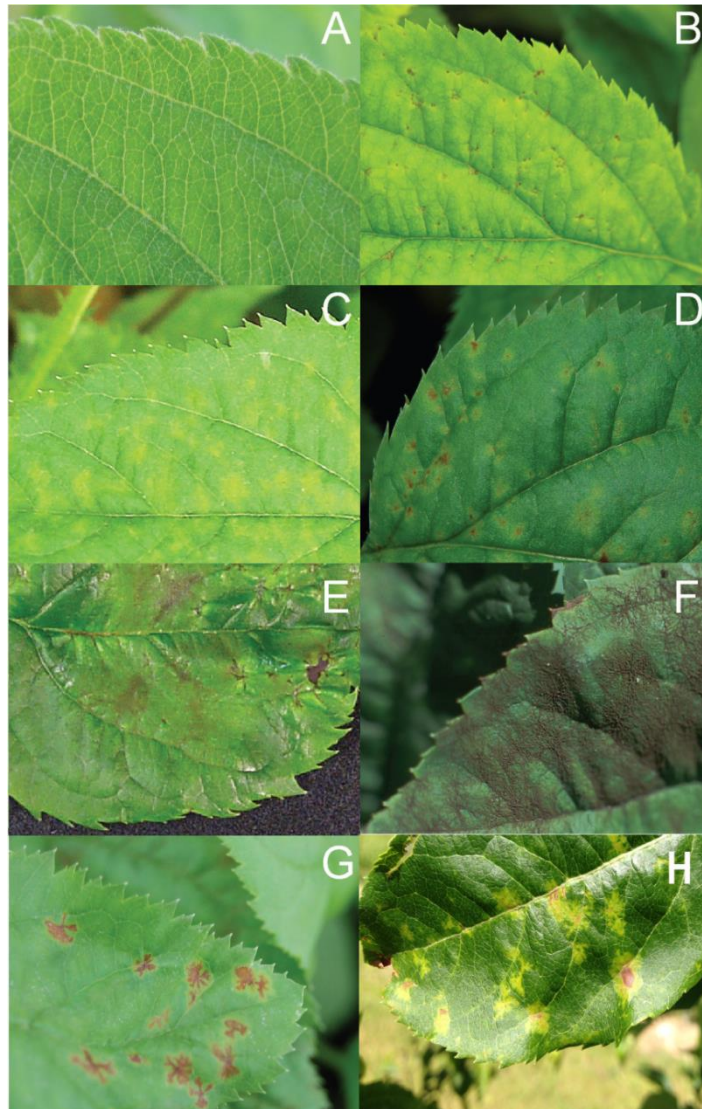


Figure 1.2 Resistance and susceptibility reactions of apple leaves to *Venturia inaequalis* inoculation. (A) Class 0 or no symptoms; (B) Class 1 - ‘pin point pits’; (C) Class 2 – chlorosis and very small necrosis; (D). Class 3A – chlorosis and necrosis with sparse sporulation; (E) Class 3B – chlorosis and necrosis with restricted sporulation; (F) Class 4 – complete susceptibility; (G) SN – stellate necrosis; (H) SC – stellate chlorosis. Images from A-G were adapted from Gessler et al, (2006) and the image of stellate chlorosis reaction (H) was obtained from Patocchi A (ACW, Wädenswil, Switzerland).

Field screening of resistance reactions can be performed using natural infections during favorable environmental conditions or with artificial inoculation of conidial suspension (Liebhard et al, 2003).

Field scoring of amount of infection is calculated by the quantitative methods developed from Croxall et al (1952) and Lateur and Populer (1994). But field screening methods do not allow for the scoring of specific resistance reactions because of the presence of multiple isolates of *Venturia inaequalis*, leading to varying resistant responses against each of the isolates.

Phenotyping methods developed for scab resistance screening in germplasm collections and biparental mapping populations have allowed researchers to study the genetic basis of apple scab resistance.

1.8. Genetics of scab resistance and test of allelism

Following identification of resistant sources, the next step is to study the genetics of resistance and to map the resistance genes. Studying the genetics and inheritance of qualitative characters (Mendelian characters) which are controlled by major genes is rather a straightforward procedure. The inheritance of major resistance gene(s) in a particular variety can be studied by generating a F_1 population from a cross between a resistant variety heterozygous at the resistance locus, and a susceptible variety. Inoculation of the resultant segregating F_1 progenies and scoring the resistance responses can help with inferring the mode of inheritance and number of genes involved in scab resistance. In the case of resistance based on a single dominant gene, ($Rr \times rr$), 1:1 (resistant to susceptible) segregation would be observed in F_1 population, whereas if the resistance is controlled by two unlinked dominant resistance genes ($R_1r_1/r_2r_2 \times r_1r_1/R_2r_2$; or $R_1r_1/R_2r_2 \times r_1r_1/r_2r_2$), a 3:1 segregation is observed. So by analyzing the segregation of the scab resistance data in F_1 populations, the inheritance of resistance and number of genes responsible for scab resistance can be inferred (Williams et al, 1966; Dayton and Williams, 1968; Williams and Kuc, 1969). In some varieties, resistance is controlled by multiple genes with smaller additive effects derived from the individual resistance genes, and this is termed polygenic resistance (Bastiaanse et al, 2016). The resistance conferred by multiple genes does not follow the simple Mendelian segregation ratios.

The easiest way to differentiate if the resistance from two different sources is controlled by the same gene (or two different alleles at the same locus) or by two independent genes is by simply observing the type of resistance reactions provided by the plant material with each particular resistance gene following pathogen inoculation (Shay and Hough, 1952). The resistance exhibited by different resistance genes may vary in the type of resistance reaction and the time at which the reactions appear. In some cases the type of resistance reaction observed is a range of resistance reactions (for example from class 0 to class 3A) instead of a single clear class of phenotype (Class 2). So it is complicated to check for allelism with just the resistance phenotype data. An alternate and powerful strategy to check for allelism in scab resistance sources is to perform pseudo-test crosses (Williams et al, 1966; Dayton and Williams, 1968; Williams and Kuc, 1969). A more detailed procedure for allelism tests is described in Gessler et al, (2006). In short, pseudo-test crosses between a F_1 heterozygous resistant genotype (generated by crossing two resistant sources) (Rr or R_1R_2/r_1r_2) and a susceptible parent (rr) are needed to check if the two resistance sources carry the same resistance gene (or two alleles at the same locus) or by two unlinked resistance genes. If two putative resistance genes are allelic ($R_1=R_2=R$), 1:1 segregation ($Rr:rr$) is observed whilst if the resistance is controlled by two different alleles (not allelic) ($R_1 \neq R_2$), a 3:1 segregation (R_1R_2/r_1r_2 , R_1r_2/r_1r_2 , r_1R_2/r_1r_2 and r_1r_2/r_1r_2) would be observed. Even though pseudo-testcrossing can help with understanding allelism between scab resistance genes, they are time consuming and require a minimum of two generations of crosses to obtain results.

Based on the type of resistance reactions observed, allelism tests and from the studies of Gene for Gene interactions (GfG) in the *Malus – Venturia* pathosystem, at least 18 major scab resistance genes were identified (Bus et al, 2011; Soriano et al, 2014).

1.9. Gene for Gene interactions and revised nomenclature for major scab resistance genes

Flor (1956) conceptualized the gene for gene hypothesis (GfG) when studying flax – flax rust interactions. Based on the GfG hypothesis, for each gene that condition resistance (Resistance gene) in the host, there is a corresponding gene that conditions pathogenicity (avirulence gene) in the pathogen. Mutation in either the resistance gene in the host or the avirulence gene in the pathogen can stimulate an incompatible interaction which leads to the disease symptom development. Based on recent molecular studies, the GfG hypothesis is defined as the specific recognition event of Avr protein (or effectors) from the pathogen or the host protein modified by the pathogen effectors, by the corresponding resistance protein of the host plant (Jones and Dangl, 2006). This specific recognition leads to downstream signaling and activation of defense genes that brings about the resistance reactions.

Gene for Gene interactions between the scab resistance genes in *Malus* and avirulence genes in *V. inaequalis* have been studied using a set of differential hosts and differential races of *V. inaequalis*. Based on the results obtained from studies of differential interactions, a new nomenclature system was proposed by Bus et al (2011). The new nomenclature system comprised 17 different GfG interactions described with 17 differential hosts and 17 races of *V. inaequalis* (**Table 1.2**). The resistance genes identified in differential hosts were named as *Rvi1* to *Rvi17* (*Rvi* stands for Resistance to V. *inaequalis*) with the possibility of including novel resistance genes when they will be identified. The *Rvi18* scab resistance gene was added later to this list as a new source of resistance to apple scab (Soriano et al, 2014). Seven out of the 18 differential hosts were placed as temporary differential hosts because of the uncertainty of monogenic scab resistance (Bus et al, 2011). The loss of the corresponding avirulence genes in *V. inaequalis* can lead to incompatible interactions and susceptibility.

Table 1.2 Nomenclature of the Gene-for-Gene interactions between *Malus* – *Venturia inaequalis*.

The races are defined by the avirulence genes they are lacking, hence resulting in susceptibility on complementary hosts. This table was adapted from Bus et al, (2011) with the addition of *Rvi18* which was described recently in Soriano et al, (2014)

<i>Malus</i>						<i>Venturia inaequalis</i>			
Differential host		Phenotype ^a	Resistance locus			Avirulence locus			
No.	Accession		Histori--cal	LG	New	New	Old	LG ^e	Race
h(0)	Royal Gala	Susceptibility							(0)
h(1)	Golden Delicious	N	<i>Vg</i>	12	<i>Rvi1</i>	<i>AvrRvi1</i>			(1)
h(2)	TSR34T15	SN	<i>Vh2</i>	02	<i>Rvi2</i>	<i>AvrRvi2</i>	p-9	A6	(2)
h(3)	Geneva ^b	SN	<i>Vh3</i>	04	<i>Rvi3</i>	<i>AvrRvi3^d</i>	p-10		(3)
h(4)	TSR33T239	HR	<i>Vh4=</i> <i>Vx=Vr1</i>	02	<i>Rvi4</i>	<i>AvrRvi4^d</i>		4	(4)
h(5)	9-AR2T196	HR	<i>Vm</i>	17	<i>Rvi5</i>	<i>AvrRvi5</i>			(5)
h(6)	Priscilla	Chl	<i>Vf</i>	12	<i>Rvi6</i>	<i>AvrRvi6</i>		2	(6)
h(7)	<i>Malus</i> × <i>floribunda</i> 821	HR	<i>Vfh</i>	08	<i>Rvi7</i>	<i>AvrRvi7</i>			(7)
h(8)	B45	SN	<i>Vh8</i>	02	<i>Rvi8</i>	<i>AvrRvi8</i>		A4	(8)
h(9)	K2	SN	<i>Vdg</i>	02	<i>Rvi9</i>	<i>AvrRvi9</i>	p-8	A6	(9)
h(10)	A723-6 ^b	HR	<i>Va</i>	01 ^c	<i>Rvi10</i>	<i>AvrRvi10^d</i>			(10)
h(11)	A722-7	SN/Chl	<i>Vbj</i>	02	<i>Rvi11</i>	<i>AvrRvi11^d</i>			(11)
h(12)	Hansen's baccata #2 ^b	Chl	<i>Vb</i>	12	<i>Rvi12</i>	<i>AvrRvi12^d</i>			(12)
h(13)	Durello di Forli	SN	<i>Vd</i>	10	<i>Rvi13</i>	<i>AvrRvi13^d</i>			(13)
h(14)	Dulmener Rosenapfel	Chl	<i>Vdr1</i>	06	<i>Rvi14</i>	<i>AvrRvi14^d</i>			(14)
h(15)	GMAL2473	HR	<i>Vr2</i>	02	<i>Rvi15</i>	<i>AvrRvi15^d</i>			(15)
h(16)	MIS op 93.051 G07-098 ^b	HR	<i>Vmis</i>	03	<i>Rvi16</i>	<i>AvrRvi16^d</i>			(16)
h(17)	Antonovka APF22 ^b	Chl	<i>Val</i>	01	<i>Rvi17</i>	<i>AvrRvi17^d</i>			(17)
h(18)	1980-015-025 ^b	HR	<i>V25</i>	11	<i>Rvi18</i>	<i>AvrRvi18</i>			(18)

^a Phenotype of resistance reactions. HR-Hypersensitive response; N-necrosis; Chl-Chlorosis; SN-Stellate necrosis.

^b Temporary differential host, until the host has been confirmed as monogenic, or a monogenic progeny from the polygenic host is selected

^c Provisional placement based on the assumption that the resistance in sources PI172623 and PI172633 are identical

^d Gene for Gene relationship not confirmed to date

^e Position of Avr genes from Broggin et al, (2011). A4 and A6 refer to minor LG in *Venturia inaequalis*.

Studies on pathogenicity genes from *V. inaequalis* identified 19 Avr genes that were corresponded to both broad spectrum and narrow spectrum resistance genes, which were named as p-1 to p-19 (Boone et al, 1971). In a study conducted by Win et al, (2003), three proteins with low isoelectric point ($pI < 4.5$) were identified as the elicitors of *Rvi5* based necrosis reaction however the avirulence gene encoding for the protein elicitor was not identified. Studies of Brogini et al, (2011) showed segregation and mapping of 15 avirulence genes from *V. inaequalis*. The avirulence genes from the pathogen were also renamed based on gene-for-gene interaction using the new nomenclature system accordingly as *AvrRvi1* to *AvrRvi18*. The new nomenclature system allows researchers around the world to use the same designation for same resistance genes, which helps with the preservation of known genes and the identification of novel resistance genes (**Table 1.2**).

1.10. Durable scab resistance

1.10.1. Breakdown of *Rvi6* resistance

Even though many major scab resistance genes have been identified, only the *Rvi6* resistance gene was widely used in the apple scab resistance breeding programs for the whole of 20th century. The use of *Rvi6* for scab resistance breeding started with the cross ‘*Malus floribunda* 821’ × ‘Rome Beauty’ performed by Prof. Crandall (1926). The progenies from that cross acted as the starting material for the PRI breeding program in 1944. From that time, selections containing *Rvi6* resistance were solely used for scab resistance throughout the world. Gessler and Pertot (2012) reported that more than 80 scab resistant varieties developed to date carry just the *Rvi6* gene as the sole source of resistance. So the scab resistant apple cultivation in the twentieth century relied mainly on monoculture orchards with varieties carrying *Rvi6* resistance.

The first observation of scab infection on the cultivar ‘Prima’ carrying *Rvi6* resistance was noticed in Ahrensburg, Germany in the year 1984. Thus to check for breakdown of *Rvi6* resistance and the possible development of a new race of *V. inaequalis* that can overcome *Rvi6* resistance, Parisi et al

(1993) inoculated a collection of *Malus* selections carrying *Rvi6* with the Ahrensburg inoculum and a local inoculum from INRA. The selections carrying *Rvi6* resistance locus were susceptible to the Ahrensburg inoculum and resistant to the INRA scab inoculum. Parisi et al (2000) and Durel et al (2003) inoculated a mapping progeny of ‘Prima’ × ‘Fiesta’ with the same two scab inocula. The progeny was completely susceptible to the inoculum from Ahrensburg but segregated as 1:1 (resistance:susceptible) to the INRA inoculum. These results clearly indicated the presence of a pathotype of *V. inaequalis* from Ahrensburg named later ‘race 6’ that can overcome *Rvi6* based resistance. In the same way, race 7 of *V. inaequalis* that can overcome *Vfh* (*Rvi7*) and *Rvi1* based resistance from ‘Golden Delicious’ was also identified (Benaouf and Parisi, 1997). The EU-DARE project conducted by Parisi et al (2004) found the presence of race 6 and race 7 of *V. inaequalis* in the whole of Northern Europe and the breakdown of *Rvi6* and *Rvi7* based resistance.

In plant-pathogen systems, the plants and their corresponding pathogens evolve together in a form of co-evolution (McDonald and Lindt, 2002). The co-evolutionary arms-race between the plant resistance genes and the pathogen avirulence genes leads to boom and bust cycles of disease resistance and their breakdown by new races of pathogen over time (Hulbert et al, 1997; Ellis et al, 1999; Bus et al, 2011)). In the *Malus-Venturia inaequalis* pathosystem, because of the co-evolution and high selection pressure on the *Venturia* populations from the single *Rvi6* resistance source, the pathogen has evolved in some environments and the breakdown of *Rvi6* resistance has occurred (Durel et al, 2000, 2003).

1.10.2. Pyramiding of resistance genes for durable resistance

Plant pathologists and breeders have long suggested pyramiding of major resistance genes and the use of polygenic resistance in breeding programs as an alternative to prevent or delay the breakdown of disease resistance and bring about durable resistance (Pedersen and Leath, 1988; Poland et al, 2008). Thus to bring about durable scab resistance, pyramiding of multiple scab

resistance genes into a single cultivar is needed. Pyramiding of scab resistance through traditional pseudo-back cross breeding combined with phenotypic selection is complex and time consuming (Ibanez and Dandekar, 2007; Joshi et al, 2009). Phenotypic selection of seedlings carrying multiple scab resistance genes is often impossible because of the similar resistance reactions exerted by different scab resistance genes. Thus development of molecular markers closely linked to each of the scab resistance genes is required for marker assisted selection of the seedlings carrying multiple scab resistance genes (Patocchi et al, 2009). An alternative strategy would be to identify the candidate gene responsible for scab resistance from different sources and generate scab resistance transgenic or cisgenic plants carrying multiple scab resistance genes (Schouten et al, 2006, 2014; Vanblaere et al, 2011).

1.11. Mapping of major scab resistance genes

Bi-parental populations are mainly used for the mapping of the scab resistance loci because of the simple Mendelian segregation pattern of major-gene based scab resistance. In the initial years of scab resistance breeding, bulked segregant analysis (BSA) along with the available molecular markers at that time such as RFLPs, AFLPs and RAPDs were used to map resistance loci (Michelmore, 1991). Bulked segregant analysis involves screening for differences between two pooled DNA samples derived from a segregating population that originated from a single cross. Each pool, or bulk, contains individuals selected to have identical genotypes for a particular genomic region ("target locus or region") but random genotypes at loci unlinked to the selected region. Therefore, the two resultant bulked DNA samples differ genetically only in the selected region and are seemingly homozygous and thus monomorphic for all other regions. Markers that show polymorphism between the resistance and susceptible bulked DNA are thus likely to be the closely linked to the scab resistance locus (Gianfranceschi et al., 1994; Tartarini, 1996).

The second and most widely used approach for mapping scab resistance loci is the genome scanning approach (GSA) which uses approximately equally spaced SSR markers covering the entire apple genome (Patocchi et al, 2005). Using this approach, a subset of resistant and susceptible seedlings along with parental lines are genotyped using the genome spanning marker panel. The map position of the R-gene is determined based on the segregation distortion between the genotypic data and phenotypic data. In the first round of GSA, the linkage group or chromosome in which the scab resistance locus is located can be identified. Subsequently, by using more seedlings of the segregating progeny and saturating the resistance LG with an abundance of novel markers, scab resistance genes can be mapped to a narrow genetic interval. This approach has been favored in the past as it reduces the cost of SSR marker screening.

Molecular markers have revolutionized the field of conventional plant breeding. With the availability of molecular markers, studies of genetic diversity in germplasm collections, selection of parental lines for crossing, selection of seedlings with desirable characters in breeding programs, and the mapping and map based cloning of genes responsible for desirable characters has become possible. At the end of 20th century, apple breeders did not have sufficient molecular markers for saturated genetic linkage mapping of traits. RAPD, RFLP and isozyme marker systems were initially used in the mapping studies of *Rvi6*, *Rvi10* and *Rvi12* scab resistance gene mapping (King et al, 1998; Hemmat et al, 2003). RAPD markers were not codominant in nature and not highly reproducible between the laboratories. RFLP markers are hybridization based markers and their implementation is laborious and time consuming and requires handling of radioactive material. Microsatellites or simple sequence repeat (SSR) markers were adopted as a marker of choice because of their ease of use and highly efficient co-dominant nature. Because of the lack of sequence information, the studies of Hemmat et al, (1997), Guilford et al, (1997) and Liebhard et al, (2002) used sequence capture of apple DNA fragments with oligonucleotide probes containing repetitive sequence to enrich for the sequences of microsatellite repeats and from these libraries,

primer pairs were designed flanking SSR markers. Patocchi et al (2005) and Silfverberg-Dilworth et al (2006) used apple EST sequences to design SSR markers for linkage mapping. The markers developed were a valuable genetic resource for mapping studies in apple but they were not sufficient for developing high-density, saturated linkage maps, and for fine mapping of resistance loci. The time and effort taken to develop the markers was very high because of the unavailability of whole genome sequence information for apple.

The advent of next generation short-read sequencing technologies such as Illumina and 454 sequencing and with them the reduced cost of sequencing compared to Sanger sequencing, allowed the whole genome sequencing of plant genomes. In an international effort, Velasco et al, (2010) sequenced the 'Golden Delicious' genome as the reference genome for apple by using the combination of Sanger and 454 sequencing. Following the iterative assembly of the sequences, 81.3% (603 Mb out of 743.2 Mb) of the sequence of the apple genome could be assembled into meta-contigs (sequence scaffolds). Over 71.2% of the metacontigs were anchored to a genetic map of 'Golden Delicious' using 1643 genetic markers. This provided the much required sequence information for the rapid and robust development of markers specific to particular regions of the genome for gene mapping, fine-mapping and map based cloning studies. It also helped with functional characterization of genes and gene families in apple through the use of reverse genetic approaches.

Scab resistance genes identified by Dayton and Williams (1968) were the first scab resistance genes to be mapped. *Rvi6* (*Vf*) was identified from a small fruited wild accession of '*M. floribunda* 821'. Plants carrying the *Rvi6* gene show resistance symptoms varying from class 2 to class 3B after artificial inoculation under greenhouse conditions. Researchers from different groups mapped the *Rvi6* locus to narrower genetic intervals using different marker systems such as isozymes, RAPDs, RFLPs (Gianfranceschi et al., 1994; Tartarini, 1996, King et al, 1998), SCARs (Tartarini et al, 1999) and AFLPs (Xu and Korban, 2000). The first linkage map of the apple genome was

developed from the cross ‘Prima’ × ‘Fiesta’ by Maliepaard et al (1998) using 155 seedlings and 208 markers including isozyme markers, SSRs, RAPDs and RFLPs. Linkage maps of each parental genome containing 17 linkage groups were obtained and the *Rvi6* locus was mapped to Linkage Group 1 (LG1) of ‘Prima’.

The *Rvi5* (*Vm*) locus from ‘*M. micromalus*’ confers a strong hypersensitive response following *V. inaequalis* artificial inoculation. Plants carrying the *Rvi5* resistance gene develop a very small necrosis reaction, 2-3 days post inoculation and these symptoms are known as pin point pits (Shay and Hough, 1952; Willams and Kuc, 1969; Patocchi et al, 2005). The *Rvi5* locus was mapped to LG17 of the resistant variety ‘Murray’ using SCAR markers (Cheng et al, 1998), and subsequently using SSRs and GSA approach (Patocchi et al, 2005). The *Rvi5* locus co-segregated with the SSR marker Hi07h02 and mapped closely to the SCAR marker OPB12_SCAR.

The *Rvi10* (*Va*) locus from ‘Antonovka’ was mapped using RAPD markers and BSA (Hemmat et al, 2003) to LG1 of the apple genome. *Rvi10* conferred hypersensitive response phenotype when inoculated with mixed races of *V. inaequalis* (Hemmat et al, 2003; Dunemann and Egerer, 2010).

The *Rvi11* (*Vbj*) gene from ‘*Malus baccata jackii*’ was mapped to LG2 using SSR and SCAR markers (Gygax et al, 2004). *Rvi11* was flanked by the markers T6-SCAR and CH05e03 in a short genetic distance.

The *Rvi1* (*Vg*) gene, a narrow spectrum resistance gene from ‘Golden Delicious’ was mapped to LG12 using the RFLP and SSR markers (Durel et al, 2000). More isolate specific resistance genes such as *Rvi2* (*Vh2*) and *Rvi4* (*Vh4*) were identified from the progenies of ‘Russian apple R12740-7A’ from the studies of differential interactions. *Rvi2* and *Rvi4* were mapped to LG2 using SSR markers and GSA (Bus et al, 2005). *Rvi2* exerted a typical Stellate Necrosis (SN) resistance reaction. *Rvi4* conferred hypersensitive pinpoint pit symptoms. *Rvi8* (*Vh8*) which confers hypersensitive response was mapped to LG2 from ‘*M. sieversii* W193B’ (Bus et al, 2005b).

The *Rvi13* (*Vd*) gene from ‘Durello di Forlì’ was mapped to LG10 by using the SSR and AFLP markers by using the race 6 isolate of *V. inaequalis* for inoculation and phenotyping (Tartarini et al 2004).

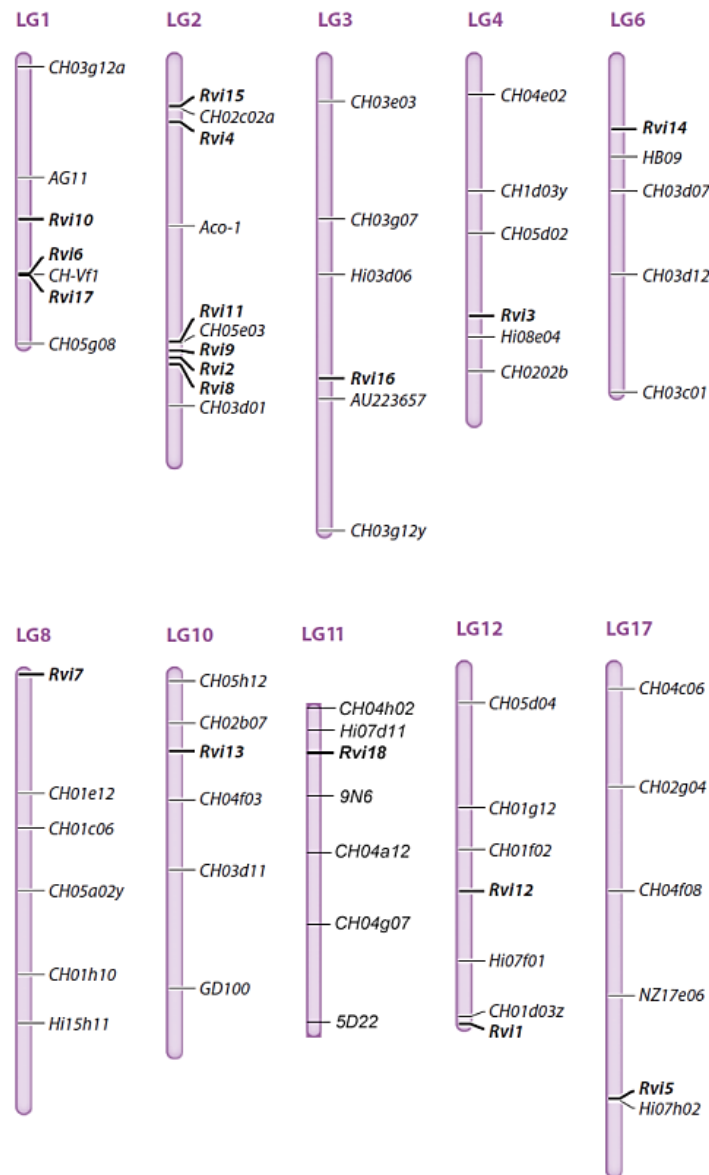


Figure 1.3 Genetic map positions of the eighteen apple scab resistance genes identified on the apple linkage map. The image was adapted and modified from Bus et al, (2011). The LG11 was included to the image from Bus et al, (2011) to show the genetic map position of the recently identified scab resistance gene *Rvi18* (Soriano et al, 2014).

By using different marker systems and differential interaction studies, 18 major scab resistance genes have been mapped onto 10 linkage groups of the apple genome (**Figure 1.3**) and the closely linked markers have been developed for many of them (Bus et al, 2011; Soriano et al, 2014). The closely linked markers to scab resistance loci have been successfully used for marker assisted selection of resistant seedlings in apple breeding programs (Patocchi et al, 2009).

1.12. Plant disease resistance genes

Map based cloning is the method used for identification of genes responsible for disease resistance. Based on the studies from Arabidopsis, tobacco, tomato, flax and rice, many different disease resistance genes have been identified against bacterial, fungal and viral diseases. These disease resistance genes were grouped into five classes based on the proteins encoded by the candidate genes and the domains present in them (Hammond-Kosack and Jones DJ, 1997). Later with the advancement in the knowledge, disease resistance genes were grouped into eight classes (Gururani et al, (2012). Class 1 resistance genes encode cytoplasmic proteins containing Toll1-interleukin like domain, nucleotide binding site and leucine rich repeats (TIR-NBS-LRR or TNL). Class 2 resistance genes encode cytoplasmic proteins with coiled coil domain containing nucleotide binding site and leucine rich repeats (CC-NBS-LRR or CNL). Class 3 resistance genes code for receptor like proteins (RLPs) with extracellular leucine rich repeats, a single transmembrane domain and a short cytoplasmic carboxyl terminal domain. Receptor like kinases (RLKs) makes up the class 4 resistance genes. RLKs contain an extracellular LRR domain, a single transmembrane domain and a cytoplasmic protein kinase domain. Fifth class of resistance genes contain genes encoding for proteins with extracellular LRR, PEST domain (Pro-Glu-Ser-Thr) responsible for protein degradation and short protein motif (ECS – Endocytosis Cell Signaling domain) for receptor mediated endocytosis. Protein with transmembrane domain and a coiled coil domain comprise the sixth class of resistance genes. Seventh class of resistance proteins are basically TIR-NBS-LRR proteins with a C-terminal extension containing nuclear localization signal (NLS) and WRKY

domain. Enzymatic R-genes like *Hm1* from Maize which lack both the NBS and LRR domains were grouped into class 8 of resistance genes. **Figure 1.4** describes the cloned R-genes from different plant species and their specific protein domains. LRR domains, and to an extent NBS and protein kinase domains have been shown to be responsible for specific pathogen recognition. The LRR domains have been found to be hypervariable compared to the other domains of R-proteins and it was hypothesized that this domain is responsible for pathogen recognition specificity (Michelmore, 1998). The intracellular domains such as protein kinase domains have been shown to be involved in the phosphorylation of downstream proteins such as mitogen activated protein kinases (MAP Kinase) and lead to signal transduction and activation of plant defense related genes. So identification of resistance genes and studying the molecular basis of their resistance expression can help breeders select and combine specific R-genes in bringing about resistance against multiple races of pathogens and multiple pathogens.

Classical genetics and molecular data suggest that most of the R-genes identified in plants to date are contained in gene clusters (Michelmore, 1998). For example, the resistance genes *Cf4*, *Cf9* were clustered in the same region of tomato genome with at least five copies of LRR-TM domain coding genes (Parniske et al, 1997; Thomas et al, 1997). Similarly the *Dm* resistance locus from lettuce, *M* from flax, *N* from tobacco, *Pto* from tomato and *Xa21* from rice contain four to 24 copies of genes of respective family members. Synonymous and non-synonymous substitutions of amino acids in each of the domains of R-proteins along with presence of multiple copies of R-genes in particular genomic regions makes it difficult to sequence the region and study the function of specific genes. Divergent selection and the 'birth' and 'death' of R-genes was suggested as the model for clustering of plant R-genes in the genome (Michelmore, 1998).

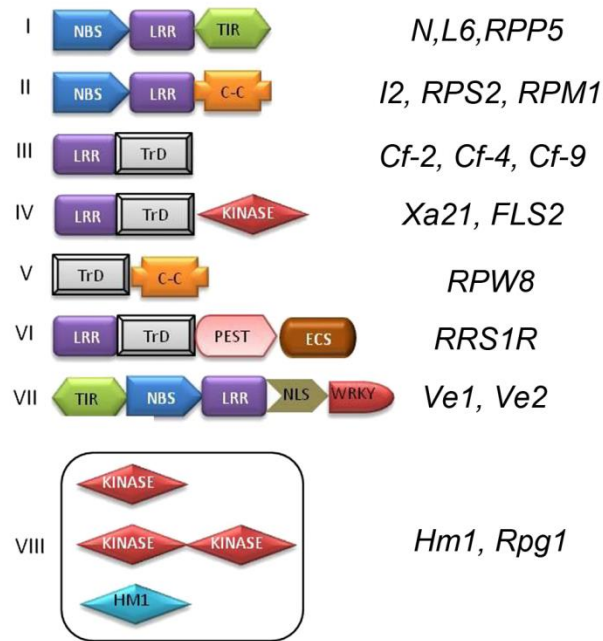


Figure 1.4. Major classes of cloned R-genes based on their protein domains. The names of the cloned R-genes for each class of the resistance genes are given next to them. This figure was adapted and modified from Gururani et al, (2012).

1.13. Fine mapping and candidate resistance genes for apple scab resistance

Despite 18 scab resistance genes have been mapped and closely linked markers having been identified for marker assisted selection, there is little information on the genes responsible for scab resistance. Usually, the steps involved in the identification of candidate resistance genes are,

1. Fine-mapping of the resistance locus using large mapping populations and development of novel, tightly linked molecular markers;
2. Construction and screening of large insert BAC library for identification of overlapping BAC clones covering the resistance gene mapping interval;
3. Sequencing, assembly and analysis of BAC clone sequences for the identification of candidate genes;

4. Validation of the candidate gene functions and its involvement in the resistance response by genetic transformation

Following this approach, five scab resistance genes, *Rvi6*, *Rvi15*, *Rvi18*, *Rvi1* and *Rvi5* were fine-mapped (Patocchi et al, 1999; Xu and Korban, 2000; Galli et al, 2010; Soriano et al, 2014; Cova et al, 2015a, 2015b). Candidate R genes were identified for four scab resistance genes *Rvi6*, *Rvi15*, *Rvi1* and *Rvi5*.

In the study of *Rvi6*, fine-mapping was performed by using over 2000 seedlings from different mapping populations. Following BAC library based chromosome walking, a 550kb contig covering the entire *Rvi6* interval was constructed. Then, by designing novel markers from BAC-end sequences, the *Rvi6* interval was further narrowed down to a 350 kb region covered by five contiguous BAC clones. For the identification of candidate R genes for *Rvi6* resistance, a different approach involving a cDNA library was used because of the high sequencing costs during that time. A cDNA library from resistance parent was constructed and it was hybridized to the BAC clones covering the *Rvi6* mapping interval. The hybridized cDNA clones were sequenced using Sanger sequencing and the candidate genes were identified. A cluster of four genes coding for *Cladosporium fulvum* like resistance genes named *HcrVf1*, *HcrVf2*, *HcrVf3* and *HcrVf4* were identified (Patocchi et al, 1999; Vinatzer et al, 1999, 2001). Reverse transcription PCR confirmed the expression of three full length candidate genes, *HcrVf1*, *HcrVf2* and *HcrVf4* and one truncated mRNA for *HcrVf3* (Xu and Korban, 2002). Cloning of *HcrVf2* under the CaMV35S promoter via *Agrobacterium*-mediated transformation of ‘Gala’ plants was performed. The transgenic ‘Gala’ plants carrying *HcrVf2* showed resistance against *V. inaequalis* inoculation, which confirmed *HcrVf2* as the gene responsible for *Rvi6* based resistance (Belfanti et al, 2004). Further studies from Malnoy and colleagues (2008) confirmed the role of *Vfa1* (*HcrVf1*) and *Vfa2* (*HcrVf2*) in *Rvi6* based resistance and showed that *Vfa4* (*HcrVf4*) was not responsible for the scab resistance. The native promoter of each scab resistance gene was characterized (Silfverberg et al, 2005). Further

studies from Vanblaere et al, (2011) used the native promoter and native terminator to develop the cisgenic ‘Gala’ lines with *Rvi6* scab resistance. Both the transgenic and cisgenic lines developed using the *Rvi6* gene was characterized by expression profiling, phenotypic, molecular and biochemical characterization (Paris et al, 2009; Jansch et al, 2014).

Rvi15 was originally thought to be inherited from the Russian apple variety ‘R12-740-7A’ but it was later identified as a new resistance gene from the *Malus* accession GMAL2473. *Rvi15* conferred a strong hypersensitive response upon *V. inaequalis* inoculation. Initial mapping of *Rvi15* was performed by BSA using the genotypic data obtained from 168 AFLP and RAPD markers. Three AFLP markers showed an association with the scab resistance. A further 33 SSR markers were designed in the mapping interval and tested for association with *Rvi15* scab resistance. The SSR marker CH02c02a was found to co-segregate with the *Rvi15* locus in a segregating progeny of 89 seedlings (Patochi et al, 2004). *Rvi15* fine mapping was performed using 989 progeny plants from the cross between ‘Golden Delicious’ and ‘GMAL2743’. By genotyping the population with CH02c02a and novel markers designed from NBS sequences surrounding *Rvi15*, the *Rvi15* interval was narrowed down to 1.5 cM. Subsequent BAC library based chromosome walking of a ‘Florina’ BAC library identified a single clone that covered the entire *Rvi15* interval. By sequencing the BAC clone, ten additional markers were developed and the *Rvi15* interval was further narrowed down to 0.5cM (Galli et al, 2010a). Screening of two BAC libraries from the resistance accession GMAL2743 with flanking markers identified a single BAC clone covering the entire *Rvi15* interval. Shotgun Sanger sequencing of the BAC clone to a 10x depth of coverage was performed and the sequences were assembled into seven contigs. Following PCR based gap filling, the BAC clone sequences were assembled into four contigs. The flanking markers were present in a single contig of 48.6 kb in length. Analysis of this sequences found a cluster of three TIR-NBS-LRR coding genes as candidates for *Rvi15* (Galli et al, 2010b). Cloning of these candidates *Vr2-A*, *Vr2-B* and *Vr2-C* with its own promoter and terminator was performed. *Agrobacterium*-mediated transformation of

'Gala' plants was performed. The transgenic 'Gala' plants were inoculated with *V. inaequalis* and the lines containing the *Vr2-C* candidate gene were resistance to the infection, confirming that *Vr2-C* is the gene responsible for *Rvi15* scab resistance (Schouten et al, 2014).

Rvi18 scab resistance was identified from the selection '1980-015-025' of Wageningen UR. This resistance selection was found to contain three scab resistance genes, *Rvi6*, *Vd3* and *Rvi18* (*V25*) (Soriano et al, 2014). An F₁ mapping progeny of 261 seedlings was used for initial mapping of *Rvi18*. A monoconidial isolate of EU-NL05 (10⁵ conidia ml⁻¹) which was virulent against *Rvi6* and *Vd3* was used for phenotyping. Following initial mapping with SSR markers and markers from a DArT array, the *Rvi18* was mapped to LG11 in an interval of 15.6 cM flanked by Hi07d11 and DArT-9N6 (Soriano et al, 2014). Fine-mapping was performed with a population of 894 seedlings. Ten AFLP markers were found to map in the *Rvi18* interval by testing of 254 AFLP primer combinations. By using these closely linked AFLP markers, *Rvi18* was mapped to an interval of 0.9 cM flanked by AFLP-362 and AFLP-314. These two flanking markers covered a physical interval of 434 kb based on the 'Golden Delicious' reference genome. Novel SSR markers were designed in the *Rvi18* mapping interval using the 'Golden Delicious' reference genome sequence. *Rvi18* was localized in a physical interval of 34 kb flanked by SNP marker Kn28621 and *Rvi18*-SSR-478. *In silico* analysis of transcriptional units in the 34 kb sequence of the 'Golden Delicious' genome sequence revealed a Lectin like receptor kinase (LRK) as the unique candidate for *Rvi18* (Soriano et al, 2014). It may be possible that the candidate gene predicted is the susceptible allele from the 'Golden Delicious' background which lacks the functional *Rvi18* allele. Thus, to get a functional resistance allele of *Rvi18*, it would be necessary to perform BAC library based chromosome walking of BAC library from resistant selection '1980-015-025' and identify the resistant alleles of the candidate gene.

The *Rvi1* scab resistance gene from 'Golden Delicious' was initially mapped to the distal end of LG12 (Durel, 1999, 2003; Calenge et al, 2004). *Rvi1* exhibits a necrotic reaction with crinkling of

leaf after ten days of inoculation with isolates carrying *AvrRvi1*. For the fine mapping of *Rvi1*, a population of 1983 progeny plants from the cross ‘Golden Delicious’ × ‘Granny Smith’ and novel SSR markers designed from the ‘Golden Delicious’ genome sequence were used. Following fine mapping, *Rvi1* was mapped between Vg12-SSR and Vg15-SSR in a physical interval of 110 kb. Screening of a ‘Golden Delicious’ BAC library with the flanking markers identified one resistant BAC clone and one susceptible BAC clone covering the entire *Rvi1* interval. 454-sequencing technology was used for sequencing and assembly of the BAC sequences. Gene prediction using GeneScanW and FGENESH predicted 13 to 20 genes in the *Rvi1* interval. From the *in silico* analysis of predicted genes from resistant BAC clone, five candidate genes for *Rvi1* were identified. The candidates identified encoded for a serine threonine phosphatase 2A protein, three TIR-NBS-LRR protein and another candidate pseudogene coding for prematurely truncated TIR-NBS-LRR protein (Cova et al, 2015a). Further complementation studies are needed to validate the function of candidates and identify the resistance gene responsible for *Rvi1* based scab resistance.

The *Rvi5* locus from the cultivar ‘Murray’ was mapped to the distal end of LG17 between the co-segregating SSR marker Hi07h02 and CH05d08 which flanked the locus at a genetic distance of 1 cM (Patocchi et al, 2005). *Rvi5* elicited a hypersensitive response 2-3 days post inoculation with *V. inaequalis*. Fine mapping of the *Rvi5* gene was performed using two mapping population consisting of 701 and 541 seedlings and using novel SSR, SCAR and SNP markers. Following fine mapping, *Rvi5* was mapped to an interval of 228 kb based on the ‘Golden Delicious’ reference genome and it was flanked by the markers Fmach_Vm4 and Fmach_Vm2. ORF prediction analysis of ‘Golden Delicious’ sequences covering the entire *Rvi5* interval revealed the presence of 33 putative ORFs. *In silico* analysis revealed one TNL coding gene and three genes coding for LRR receptor like proteins as the putative candidates for *Rvi5* scab resistance (Cova et al, 2015b). However the candidates identified were from the ‘Golden Delicious’ genome sequence which lacks the *Rvi5*

resistance allele. So further studies using a 'Murray' BAC library and physical mapping is required for identification of resistance genes of the *Rvi5* locus.

To date, five major scab resistance genes have been fine mapped and the putative candidate genes were identified using each BAC library based physical mapping and sequencing and analysis of BAC sequences using various sequencing technologies. Such an approach has therefore been shown to be a suitable and efficient method for identification of scab resistant candidate genes.

The identified scab resistance candidate genes and putative candidates for *Rvi6*, *Rvi15* and *Rvi1* were present as clusters which is common for disease resistance genes. Perazzoli and colleagues (2014) characterized resistance gene analogues (RGA) from the whole genome sequence of apple to study the evolutionary history in the Rosaceae family. They identified 868 RGAs from the cultivar 'Golden Delicious' which accounted for 1.51% of the total genes. More than 56% of RGAs were anchored to six chromosomes: Chromosomes 2, 7, 8, 10, 11 and 15. Chromosome 2 alone was found to contain 25% of all RGAs. More than 80% of RGAs (622 out of 868) were found to be clustered together in the apple genome with an average of four RGAs per cluster. Chromosome 2 was found to contain a RGA cluster composed of 21 RGAs. The RGAs identified were found to co-localize with major resistance genes and QTLs responsible for disease resistance (Perazzoli et al, 2014). The large number of resistance gene analogues and resistance genes in the apple genome and their clustered complex genome organization makes it difficult to sequence and identify all the causal alleles responsible for disease resistance using short read shotgun sequencing and assembly methodologies.

Chapter 2

Aim of the thesis

One of the long term objectives of the apple breeding community is the development of durable apple scab resistance varieties. This can be achieved by pyramiding of major scab resistance genes through marker assisted breeding or by developing transgenic or cisgenic apple lines with multiple scab resistance genes. The prerequisite for this is to develop molecular markers closely linked to each of the scab resistance genes for MAB and identification of candidate scab resistance genes for cisgenesis or transgenesis approaches. This thesis project focused on the molecular genetic characterization of *Rvi12* scab resistance locus.

2.1. Background research on *Rvi12* scab resistance

The *Rvi12* scab resistance locus which was previously referred to as *Vb* was identified from Siberian crab apple *M. baccata* ‘Hansen’s baccata#2’ (Dayton and Williams, 1968). From the testcross studies of Dayton and Williams (1969), *Rvi12* was found to be a novel resistance source which is not allelic to *Rvi6* from *M. floribunda* ‘821’.

The first mapping study of *Rvi12* was conducted by Hemmat and colleagues (2003). A population of 120 seedlings from the cross ‘Empire’ × ‘Hansen’s baccata #2’ was used for mapping. Inoculation of mapping population was performed with mixed culture of *V. inaequalis* containing races 1 to 5. The scab resistance gene that segregated in the progeny exhibited chlorotic wrinkled reactions with or without sporulation. By using RAPD markers and BSA, the *Rvi12* locus was mapped to LG1 of ‘Hansen’s baccata#2’. However, the mapping results obtained from Hemmat et al (2003), contradicted with the allelism test results of Dayton and Williams (1968).

Subsequent mapping of *Rvi12* was conducted by Erdin et al, (2006) using a population of 96 seedlings from the cross ‘Golden Delicious’ × ‘Hansen’s baccata #2’. A local inoculum with mixed

isolates of *Venturia inaequalis* was used for inoculation. The seedlings conferred the resistance reactions of chlorosis (class 2), chlorosis with sparse sporulation (class 3A) and abundant sporulation (class 3B). By using SSR markers and employing GSA, *Rvi12* was mapped to LG12 of the genome. The *Rvi12* locus was flanked by Hi02d05 and Hi07f01 markers at a genetic distance of 7.8 cM and 13.7 cM respectively.

The results obtained from the two mapping studies pointing to two different map positions for the *Rvi12* locus. The seemingly contradictory map positions from these studies raised the possibility that more than one scab resistance gene was present in ‘Hansen’s baccata #2’. Thus further studies were needed to analyze the segregation of scab resistance genes in a large mapping population.

The closest marker developed from the mapping studies of Hemmat et al (2003), B220₇₀₀ mapped 12 cM from the *Rvi12* resistance locus. From the other mapping study, the *Rvi12* locus was mapped in a genetic interval of 21.5 cM (Erdin et al, 2006). Because of the large genetic distance between the flanking markers and the *Rvi12* locus, there is more chance of recombination events happening between the marker and the resistance locus. Thus these markers could not be efficiently used in selection of seedlings carrying *Rvi12* resistance in apple breeding programs. So more closely linked markers (<5cM from resistance locus) were needed for efficient marker assisted selection. Following marker development, the next step would be to fine-map the *Rvi12* scab resistance locus and identify the candidate genes.

2.2. Final objectives

The final objectives of this thesis work were thus to,

- analyze the genetic segregation of scab resistance genes in *M. baccata* ‘Hansen’s baccata #2’
- develop markers closely linked to the *Rvi12* scab resistance locus

- fine map the *Rvi12* locus using a large mapping population and develop closely linked markers
- identify & perform a preliminary characterization of candidate genes for *Rvi12* based resistance by using BAC library based chromosome walking

Chapter 3

Fine mapping of the apple scab resistance locus *Rvi12* (*Vb*) derived from

‘Hansen’s baccata #2’

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3.1. Abstract

Apple scab is a disease caused by the fungus *Venturia inaequalis*, which leads to significant economic losses in apple production especially in temperate regions. Breeding programs are attempting to introgress scab resistance genes from wild apple into commercial cultivars to control the disease. Most of the commercially available scab-resistant varieties to date rely on the *Rvi6* (*Vf*) resistance gene from *Malus floribunda* 821. The evolution of new pathotypes of *V. inaequalis*, which have caused the breakdown of *Rvi6* based resistance, at least in northern Europe, highlights the need for the characterization and pyramiding of scab resistance genes from different sources for durable disease resistance. In this study, the scab resistance gene *Rvi12* from *Malus baccata* ‘Hansen’s baccata #2’ was confirmed as mapping to apple linkage group 12 in the cross ‘Gala’ × ‘Hansen’s baccata #2’ in an interval between SSR markers Hi02d05 and CH02h11b. Using the ‘Golden Delicious’ genome sequence, novel SSR markers and SNPs were identified in the *Rvi12* mapping interval, and mapped in an extended mapping population of 1,285 plants. *Rvi12* was fine-mapped to an interval spanning 958 kb of the ‘Golden Delicious’ genome sequence. The 18 SNPs fine-mapped to the *Rvi12* interval were screened in eight apple breeding founders and for 16 of the 18 SNPs, the alleles linked in coupling with the *Rvi12* resistance locus were found only in ‘Hansen’s baccata #2’. The SNPs identified will thus be useful for the efficient identification of apple genotypes carrying the *Rvi12* resistance locus.

Key words: SNP, SSR, marker assisted breeding, Rosaceae, pome fruits, genomics

3.2. Introduction

The cultivated apple (*Malus pumila* Mill.) is one of the most economically-important fruit crops with an annual worldwide production of over 76 million tonnes (<http://faostat.fao.org>). Apple production is significantly affected by a number of pests and pathogens, which include the fungal disease apple scab (*Venturia inaequalis*) amongst many others. *Scab is a devastating disease in almost all apple producing areas of the world, and crop losses of up to 70% mainly due to fruit spoilage have been reported (MacHardy 1996).* The disease affects both the leaves and fruits, and causes characteristic black spots, scabby blotches and in severe cases deformation of the fruits, all of which make the harvest unmarketable. *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 dodine, benzimidazole (Koller and Wilcox 2001), kresoxim-methyl and trifloxystrobin (quinoline outside inhibitor) fungicides (Koller et al. 2004).* 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 (Bus et al. 2011; Crandall 1926).

Breeding for apple scab resistance primarily involves the introgression of resistance genes from varieties of cultivated and wild apple species, and to date, a total of 17 scab resistance loci have been characterised (Bus et al. 2011). The most extensive studies on these resistance genes have focused on *Rvi6* (*Vf*) derived from the Asiatic wild apple accession *M. floribunda* 821 (Crandall 1926; Dayton et al. 1970; Gessler et al. 2006; Hough et al. 1953; Kellerhals and Furrer 1994). At the same time, most of the commercial scab-resistant cultivars currently available carry *Rvi6* resistance (Gessler and Pertot 2012). However, it has been reported that isolates of *V. inaequalis* can overcome natural *Rvi6* based resistance (Parisi et al. 2004; Parisi and Lespinasse 1996; Parisi et

al. 1993) as well as in apple varieties overexpressing *HcrVf2* (Joshi et al. 2011; Silfverberg-Dilworth 2004). Thus, the breakdown of *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.

Apple seedlings carrying multiple resistance genes can be identified in a breeding population through the screening of molecular markers closely linked to each resistance gene and selection of genotypes carrying only alleles linked in coupling to the resistance genes, a process known as marker-assisted selection (MAS). Molecular markers have been developed that are linked, to a greater or lesser degree, to many of the identified scab resistance genes (Patocchi et al. 2009; Bus et al. 2010; Dunemann and Egerer 2010).

The Siberian crab apple *M. baccata* ‘Hansen’s baccata #2’ (HB2) is resistant in all, or nearly all, locations monitored by the VINQUEST initiative (Patocchi et al. 2009; the verification of the possible breakdown of resistance at two sites is ongoing; <http://www.vinquest.ch/monitoring/publication.htm>), making this source of resistance attractive for use in durable resistance breeding. The scab resistance conferred by HB2 was attributed to a single major resistance gene by Dayton and Williams (1968), who denoted the locus *Vb. Rvi12*, the revised name for *Vb* (Bus et al. 2011), has been the study of two independent mapping studies. Hemmat et al. (2003) reported that the *Rvi12* locus was located on LG1 of the *Malus* genome, loosely associated with *Rvi6*. In a more recent study, *Rvi12* was mapped to linkage group 12 (LG12) of the *Malus* genome between two SSR markers Hi02d05 and Hi07f01 (Erdin et al. 2006), which were located 7.8 cM and 13.7 cM from the *Rvi12* locus respectively. Whilst the study of Erdin et al. (2006) provided an approximate map position for *Rvi12*, the associations with the two closest-flanking SSRs were not strong enough for the efficient selection of resistant seedlings using MAS. The seemingly contradictory map positions of the gene in the two published studies raises the

possibility that more than one scab resistance gene is present in the HB2 genotype, although neither study reported the possibility of such an eventuality.

In this investigation, a progeny from the cross ‘Gala’ × HB2 was raised and used to confirm the previously published map position of the *Rvi12* locus (Erdin et al. 2006). The genetic interval containing *Rvi12* was fine-mapped using an extended mapping progeny, employing a set of newly-developed, tightly linked SSR and SNP markers. The SSR and SNP markers developed will be a useful and effective tool for the efficient selection of resistant seedlings, and the fine-mapping and map-based cloning of the *Rvi12* resistance gene.

3.3. Materials and methods

3.3.1. Plant material and phenotypic evaluation

A controlled cross between the apple varieties ‘Gala’ (susceptible to scab) and HB2 (resistant to scab) was performed at the Agroscope research station, Wädenswil, Switzerland in 2010. A total of 305 seedlings from the cross (denoted G×HB2) were used for initial mapping and development of closely linked markers. For fine-mapping of the *Rvi12* gene, an additional population of 980 plants from the same cross (denoted G×HB2-980) was used. In the spring of 2011, 146 seedlings of the G×HB2 progeny were spray-inoculated with a conidial suspension (2×10^5 conidia/ml) containing mixed strains of *V. inaequalis* originating from orchards of the region of Zürich, Switzerland. In 2012, an additional 159 seedlings of the same progeny were also inoculated as described above. After inoculation, the plants were kept for 48 hours at ca. 100% relative humidity and a temperature of around 20°C. After one week of incubation at ambient humidity, the humidity was again increased to ca. 100% for a further 48 h. Phenotypic evaluation was performed 14 days after inoculation. Resistance reactions were grouped into the classes described by (Chevalier et al. 1991): class 0 - no visible symptoms; class 2 - chlorosis; class 3a - small necrotic spots with sparse sporulation; class 3b - chlorosis with abundant sporulation and class 4 – abundant sporulation with

no resistance reaction, with the addition of a further class, containing plants showing stellate chlorosis (SC) symptoms. In 2012, the 119 surviving seedlings raised in 2011, and in 2013 the 100 seedlings raised in 2012 that did not display stellate chlorosis symptoms as seedlings, were grafted onto ‘M.9’ rootstocks and screened again for apple scab resistance to confirm their phenotypes. Phenotyping of the G×HB2-980 population was performed in the spring of 2013 following the procedure described above. Plants of the G×HB2-980 showing recombination between the SSR markers flanking the *Rvi12* locus (see methods below) were grafted onto ‘M.9’ rootstocks and phenotyping was repeated as indicated above in the Spring of 2014.

3.3.2. Microsatellite marker development

The SSR markers spanning LG12 described by Erdin et al. (2006) with the addition of a further SSR marker (CH02h11b; Liebhard et al. 2002) identified from the linkage map of Fernandez-Fernandez et al. (2008) were used for initial genotyping of the G×HB2 family. The sequences of markers Hi02d05 and CH02h11b flanking *Rvi12* were used as queries for BLAST against the ‘Golden Delicious’ genome sequence v1.0 contigs (Velasco et al. 2010) to identify the physical position of the markers on the reference genome. Next, the sequences of the contigs located between the two markers were mined for SSR sequences using the SSR server on the Genome Database for Rosaceae (GDR; <http://www.rosaceae.org/node/55>; Jung et al. 2013). Microsatellite motifs with a minimum of ten dinucleotide repeats or six trinucleotide repeats were identified. Primer pairs were designed for 48 of the SSRs identified that were distributed evenly throughout this region using Primer3 (Untergasser et al. 2012) with the following design criteria: a PCR product between 100 and 350 bp in length, a T_m of 55–65°C (optimum 60°C), a primer length of 20–24 bp (optimum 22 bp) and a GC-clamp of 2 bp.

3.3.3. DNA extraction, marker amplification and screening

Young, newly emerging leaves from plants of the parents and progeny were collected, frozen in liquid nitrogen, and subsequently freeze-dried using a ScanVac freeze drier (LaboGene, Denmark). DNA extraction for G×HB2 was performed using the DNeasy Plant mini kit (Qiagen GmbH, Germany) following the manufacturer's recommendations. DNA extraction for G×HB2-980 was performed using the CTAB method described in Chen and Ronald (1999) after the leaves were ground to a fine-powder using a TissueLyser (Qiagen GmbH, Germany).

All PCRs were performed in a final volume of 12.5 µl using 1× PCR buffer (Qiagen GmbH, Germany), 1.5 mM Mg²⁺, 200 µM dNTPs, 0.5 µM of primers and 0.5 U Hotstart *Taq* polymerase (Qiagen GmbH, Germany). The cycling profile described by Patocchi et al. (2005) was used for the amplification of SSRs. All products were initially assessed for amplification and heterozygosity in the parental genotypes of the progeny after electrophoresis through a 2% TAE agarose gel containing GelRed (Biotium, Hayward, CA, USA) for 1.5 h at 110 V and they were visualised over UV light. Microsatellite markers putatively heterozygous in the HB2 parental genotype were labelled on the forward primer with either 6-FAM or HEX fluorescent dyes and the products were fractionated and sized by capillary electrophoresis using a 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Data generated were analysed using the GeneMapper (Applied Biosystems, Foster City, CA, USA) software application.

3.3.4. Data analysis and map construction

Segregation data for the SSRs and the *Rvi12* gene were used for linkage analysis with JOINMAP 4.0 (Kyzma, NL) employing the Kosambi mapping function and using 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. The maps presented were visualised using MapChart 2.0 (Voorrips 2002).

3.3.5. SNP development

Gene sequences identified in the ‘Golden Delicious’ genome sequence interval, between the SSR markers most closely flanking the *Rvi12* locus was obtained from the GDR. A total of 96 exon-primed-intron-crossing (EPIC) primer pairs (Palumbi and Baker 1994) were designed using Primer3 with the following parameters: a PCR product of between 400 and 850 bp in length spanning at least one intron, a T_m of 57–63°C (optimum 60°C), a primer length of 18–27 bp (optimum 20 bp) and a GC-clamp of 2 bp. The PCRs were performed with the parental lines with the following cycling profile: an initial denaturation at 94°C for 15 minutes was followed by 30 cycles of 94°C for 45 seconds, 62°C for 30 seconds and 72°C for 45 seconds, and a final extension at 72°C for 7 minutes. Amplification of specific PCR products were checked by electrophoresis through a 2% agarose gel containing GelRed (Biotium, Hayward, CA, USA) for 1 h at 110 V following which products were visualised over UV light. Post-PCR product purification was performed using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) following the manufacturer’s directions. Then the PCR products were sequenced using the BigDye Direct Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s directions.

The resultant sequence data were visualized and SNPs were identified using the SeqMan software package (DNASTAR, Madison, WI, USA). The segregation of SNPs was checked by amplification and sequencing of eight progeny plants (four resistant and four susceptible) and the two parents of the G×HB2 population. Those SNPs that were heterozygous only in the HB2 genotype and which putatively fell within the *Rvi12* mapping interval based on the eight genotypes scored, were used for the genotyping of 115 individuals of the G×HB2 progeny that did not show a SC phenotype and unambiguously displayed either resistance class 0 or susceptible class 4 phenotypes in the inoculations as seedling and grafted plants.

3.3.6. Fine-mapping of the *Rvi12* locus

Genotyping of the G×HB2-980 population was performed initially with the two SSR markers (Ch02h11b and SSR-23.03) flanking the *Rvi12* locus according to the map generated using the data of the 115 individuals of the G×HB2 progeny. Recombinant individuals were identified, which were then phenotyped in the same way as the G×HB2 progeny. Subsequently, the recombinant individuals from the G×HB2-980 population for which a reliable phenotype was scored were genotyped with the closely flanking SNP markers, and the location of the *Rvi12* locus in relation to these markers was inferred.

3.3.7. Screening of founder lines with the closely linked SNPs

Six apple varieties and selections reported to be founder lines of the majority of modern apple cultivars, namely ‘Golden Delicious’, ‘Red Delicious’, ‘Cox’s Orange Pippin’, F2-26829-2-2, ‘Jonathan’ and ‘McIntosh’, and two of significant importance for breeding, ‘Braeburn’ and ‘Granny Smith’ (van de Weg, personal communication), were genotyped with the closely linked SNPs by direct sequencing of PCR products as described above in order to assess the degree of specificity of the SNP alleles associated to the *Rvi12*.

3.4. Results

3.4.1. Mapping populations and evaluation of the resistance reaction

Plants of the G×HB2 progeny were first phenotyped as seedlings. Of these, 90 were scored as resistant and showed symptoms either 0, 2, 3a or 3b (0 to 3b), 108 were considered resistant, too, but showing SC, in some cases associated with limited sporulation, and the remaining 107 were scored as class 4 and considered as susceptible. The number of seedlings from the G×HB2-980 displaying resistant (0 to 3b), SC and susceptible phenotypes after one year’s phenotyping were 493, 260 and 227, respectively. Taken together, the total number of seedlings displaying resistant (0

to 3b or SC) and susceptible phenotypes were 959 and 316 respectively, indicating the independent segregation of two unlinked resistance genes ($3:1 \chi^2=0.859$). Thus, from the phenotypic segregation, it was hypothesized that the resistance displayed in the seedlings analysed was controlled by two unlinked resistance genes, *Rvi12*, and a second, yet uncharacterized scab resistance gene conferring the SC phenotype. The second putative resistance gene was shown to be unlinked to the *Rvi12* locus (see “*Mapping of Rvi12*” section below) but was not characterized further in this investigation.

To enable fine mapping and molecular characterization of the *Rvi12* resistance locus, part of the G×HB2 progeny was phenotyped at least a second time as grafted plants. To ensure precise mapping, only plants not displaying SC at any developmental stage (seedling or grafted plant) and in general being unambiguously class 0 when inoculated as grafted plants, or class 4 (susceptible, not carrying *Rvi12*) plants were used. In total, 119 seedlings of the G×HB2 progeny were selected for mapping of *Rvi12*, 53 were susceptible (class 4), and 66 were resistant (class 0). These seedlings were used to identify markers flanking the *Rvi12* locus and to determine an accurate map position for the gene.

3.4.2. Mapping of *Rvi12* in the G×HB2 progeny

The seven SSR markers (**Table 3.1**) that had previously been shown to map to LG12 (Erđin et al. 2006; Fernandez-Fernandez et al. 2008) were scored in the selected 119 seedlings of the G×HB2 population. Comparison of genotypic and phenotypic data revealed that four out of the 119 progeny plants displayed Genotype-Phenotype Incongruence (GPI) (Erđin et al. 2006; Gyğax et al. 2004). All four GPI plants carried the marker alleles linked to susceptibility for all seven SSRs but were scored as resistant based on phenotypic characterization. Thus, these four plants were removed from further analysis. The seven SSR markers and the *Rvi12* resistance gene coalesced into a single linkage group, covering a genetic distance of 58.4 cM and the *Rvi12* locus mapped between SSR

markers Hi02d05 and CH02h11b (**Figure 3.1**). To confirm that the genetic locus conferring the SC phenotype was not linked to the *Rvi12* resistance locus, 248 plants displaying an SC phenotype were genotyped with SSR markers CH02h11b and SSR-23.03 (see “*Novel SSR marker development*” section below for details of this marker). Mendelian segregation ratios for each marker in the SC plants were 156:92 and 138:110 for CH02h11b and SSR-23.03 respectively and their associated chi-squared values were 3.16 and 16.52, demonstrating distorted, but independent segregation of the two linked SSR loci from the second resistance locus.

3.4.3. Novel SSR marker development around the *Rvi12* locus

BLAST analysis of the ‘Golden Delicious’ genome sequence revealed 582 contigs between Hi02d05 and CH02h11b. These contigs comprised a total of 2.57 Mb of ‘Golden Delicious’ genome sequence and contained 238 SSRs with a dinucleotide repeat $\geq (XX)_{10}$, or a trinucleotide repeat $\geq (XXX)_6$. Primer pairs were designed from 48 SSRs that were distributed on average approximately every 50 kb between Hi02d05 and CH02h11b, of which 15 were putatively heterozygous following agarose gel electrophoresis, and five were mapped to the *Rvi12* region in the G×HB2 progeny (**Table 3.1, Figure 3.1**). Of the remaining ten, four were monomorphic, and six were heterozygous and segregating, but did not map to LG12. The SSR that mapped closest to *Rvi12* in the G×HB2 progeny was SSR-24.91, which co-segregated with the resistance gene in the 115 individuals tested, whilst SSR-23.03 and CH02h11b flanked either side of the gene at 0.9 cM and 3.5 cM, respectively. A further two markers, SSR-23.17 and SSR-23.29 co-segregated with SSR-23.03. A total of five recombinant individuals were identified between CH02h11b and SSR-23.03.

3.4.4. Identification and fine-mapping of SNPs closely linked to *Rvi12*

Analysis of sequences obtained from the screening of the ‘Gala’ and HB2 parental lines with the 96 EPIC primer pairs revealed 23 amplicons containing at least one SNP that was heterozygous only in

HB2. By comparing the sequences obtained from eight progeny plants, eight sequences contained a total of 18 SNPs that putatively segregated in the G×HB2 progeny in the *Rvi12* mapping interval, and their positions were confirmed following scoring in the 115 seedlings of the G×HB2 progeny (**Table 3.2, Figure 3.1**).

Screening of the G×HB2-980 progeny with the SSR markers flanking *Rvi12* (SSR-23.03 and CH02h11b) identified 14 recombinant individuals. Following phenotypic evaluation, four plants were classified as susceptible and two as resistant (unambiguously class 0), whilst eight were classified as SC, or gave an ambiguous phenotype. Only the six genotypes clearly identified as susceptible or resistant but not showing SC were used to construct a fine map of the *Rvi12* interval. Genotyping of the 11 recombinant individuals from both the G×HB2 and G×HB2-980 progenies with the 18 SNPs enabled the construction of a fine map around the *Rvi12* locus (**Figure 3.2**). Genotyping of the recombinant individuals with the newly developed SNP markers placed the *Rvi12* resistance gene between markers SNP_23.523 and SNP_24.482. Based on the ‘Golden Delicious’ genome sequence (Velasco et al 2010), this corresponded to an interval of 958 kb.

3.4.5. Screening of founder lines of apple with closely linked SNPs

The 18 heterozygous SNPs fine-mapped to the *Rvi12* mapping interval were screened in eight apple breeding founder lines. These lines carried only the allele linked in repulsion to the *Rvi12* resistance locus for 16 of the 18 SNPs. The closely linked markers, SNP_23.523 and SNP_24.482, contained five segregating SNP alleles and only those alleles linked to *Rvi12* in repulsion were found in the eight founder lines. The segregating SNP alleles in each of the eight founder lines along with the alleles in coupling to scab resistance in HB2 are given in **Table 3.3**.

LG12

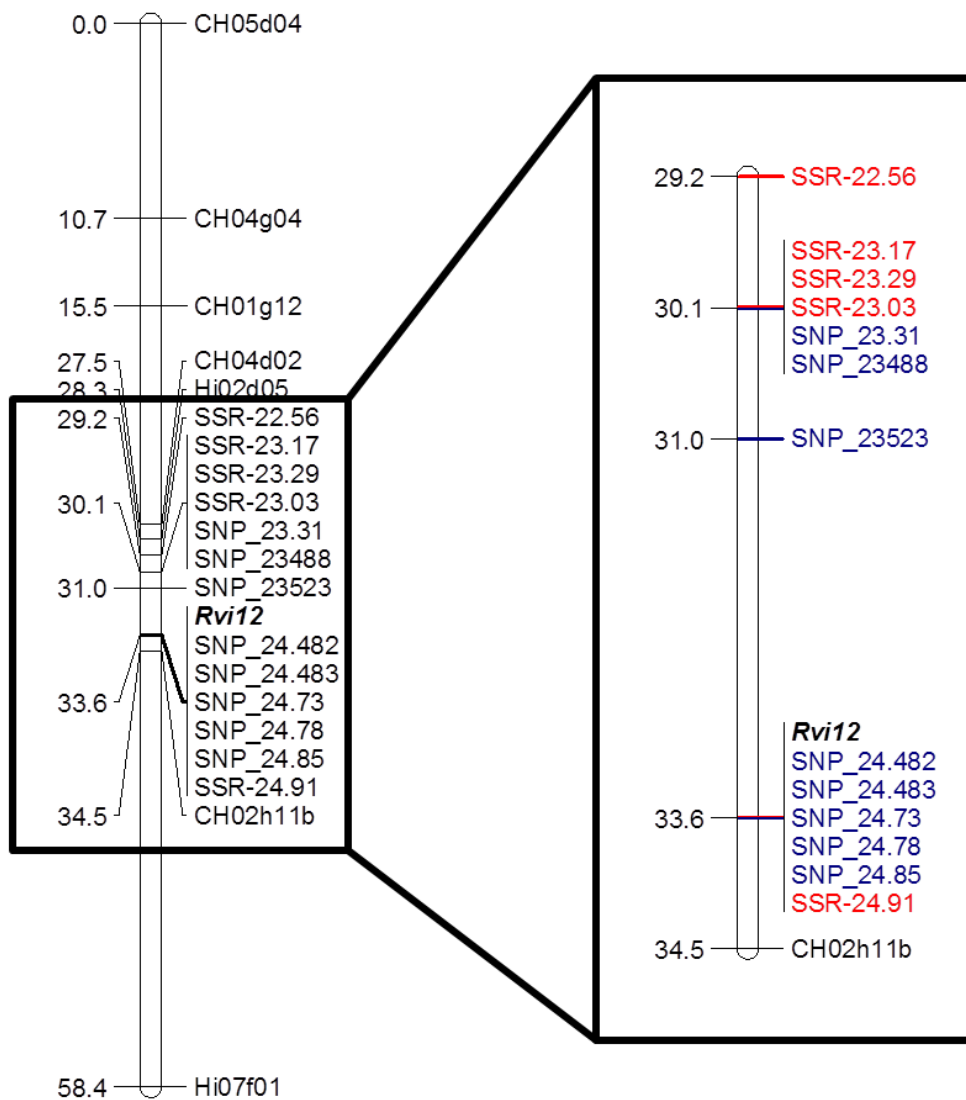


Figure 3.1. Genetic map of apple linkage group 12 of the G×HB2 mapping population. CH- and Hi-markers are those previously reported by Liebhard et al. (2002) and Silfverberg-Dilworth (2004), respectively; SSR- markers are the five novel SSR developed in this study and SNP_ are the eight amplicons containing the 18 novel SNP markers developed in this study. Each SNP locus name denotes the physical position of the amplicon sequenced on the ‘Golden Delicious’ genome.

Table 3.1. The microsatellite markers used in the study detailing the primer sequences, segregation ratios observed in the G×HB2 population, allele sizes obtained in the parental lines and the map positions of the markers, along with the *Rvi12* locus.

Marker name	Map position (cM)	Class ^a	Observed	X ^{2b}	Forward primer 5' to 3'	Reverse primer 5' to 3'	Allele sizes (nt)		EMBL accession number
							Gala	HB2 ^d	
CH05d04	0.0	aa:ab	61:54	0.43	ACT TGT GAG CCG TGA GAG GT	TCCGAAGGTATGCTTCGATT	180/187	154/178	
CH04g04	10.7	aa:ab	58:57	0.01	AGTGGCTGATGAGGATGAGG	GCTAGTTGCACCAAGTTCACA	173 /null	169/175	
CH01g12	15.5	aa:ab	57:58	0.01	CCCACCAATCAAAAATCACC	TGAAGTATGGTGGTGCGTTC	145/150	131/137	
CH04d02	27.5	aa:ab	54:61	0.43	CGTACGCTGCTTCTTTTGCT	CTATCCACCACCCGTCAACT	114/126	null/122	
Hi02d05	28.3	aa:ab	53:62	0.70	GAGGGAGAATCGGTGCATAG	CATCCCTCAGACCCTCATTG	151/191	null/165	
SSR22.56	29.2	aa:ab	54:61	0.43	AAGGGGAGCAATAACAGTGC	AGGCCGAGGAGAATATAGCC	235 /null	null/243	LM651405
SSR23.03	30.1	aa:ab	53:62	0.70	CAGTGCTGGCTTTAAGTTTGG	AATACAACGCCAGATGAGAGG	127 /null	106/115	LM651406
SSR23.17	30.1	aa:ab	53:62	0.70	GTTGCCCGTTAGAATTTTGC	CTAGTGTAGTGTGTGGGTGTGG	271/285	242/272	LM651407
SSR23.29	30.1	aa:ab	53:62	0.70	AGAGGGCGTTTGGATTAAGG	CTCGAAGCTTGTCCAGTTGC	245/246	232/249	LM651408
Rvi12	33.6	aa:ab	53:62	0.70	n/a	n/a	n/a	n/a	
SSR24.91	33.6	aa:ab	53:62	0.70	CTTGCTAGGGTTGTGCTTGG	CCACATAAAAGAAAGCCTTGG	217/235	209/201	LM651409
CH02h11b	34.5	aa:ab	54:61	0.43	GGGACGTAAACAGGTATTCTCTC	ATGGTTAGCCAAGCACATC	220/226	null/212	
Hi07f01	58.4	aa:ab	47:68	3.83 ^c	GGAGGGCTTTAGTTGGGAAC	GTTTGAGCTCCACTTCCAACTCC	200/205	199/195	

^a All markers were expected to segregate with a 1:1 ratio

^b All marker chi-square values were calculated with 1 df.

^c Segregation distortion significant at 0.05

^d First listed allele in coupling with resistance

	SSR-23.03	SSR-23.17	SNP_23.31	SNP_23488	SNP_23523	<i>Rvi12</i> (Vb)	SNP_24482	SNP_24483	SNP_24.73	SNP_24.78	SNP_24.85	SSR_24.91	CH02h11b
1	ab	ab	ab	ab	aa	aa	aa	aa	aa	aa	aa	aa	aa
2	ab	ab	ab	ab	ab	aa	aa	aa	aa	aa	aa	aa	aa
3	ab	ab	ab	ab	ab	aa	aa	aa	aa	aa	aa	aa	aa
4	ab	ab	ab	ab	ab	aa	aa	aa	aa	aa	aa	aa	aa
5	ab	ab	ab	ab	ab	ab	aa	aa	aa	aa	aa	aa	aa
6	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	aa
7	aa	aa	aa	aa	ab	ab	ab	ab	ab	ab	ab	ab	ab
8	aa	aa	aa	aa	aa	ab	ab	ab	ab	ab	ab	ab	ab
9	aa	aa	aa	aa	aa	ab	ab	ab	ab	ab	ab	ab	ab
10	aa	aa	aa	aa	aa	aa	ab	ab	ab	ab	ab	ab	ab
11	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	ab	ab

Figure 3.2. A schematic diagram showing the recombination events identified in the G×HB2 and G×HB2-980 populations in the *Rvi12* mapping interval. ‘ab’ in the figure represents the allele in coupling, and ‘aa’ represents the allele in repulsion to the resistance gene *Rvi12*.

Table 3.2. SNP markers used in this study detailing primer sequences, PCR product sizes and the SNP alleles observed in the amplicons from parental lines.

Marker name	Forward primer 5' to 3'	Reverse primer 5' to 3'	Product size (nt)	SNP alleles in amplicons		EMBL accession number
				Gala	HB2	
SNP_23.31	GACTTATCCATGTCCGAAAAGC	AGGTTGCTACCGACCCTACC	574	(C/C) ₁₀₂ , (T/T) ₁₉₉ , (T/T) ₂₂₇ , (C/C) ₂₉₇	(T*/C) ₁₀₂ , (T/C*) ₁₉₉ , (T/C*) ₂₂₇ , (T*/C) ₂₉₇	LM999991
SNP_23.488	ATGGCGTCACTGATACTCG	TTCAACCTTGACAATGGTATCG	526	(C/C) ₁₇₃ , (T/T) ₂₁₁	(T*/C) ₁₇₃ , (A*/T) ₂₁₁	LM999992
SNP_23.523	CTTCTGCTTACTCGGCATGG	ATTTCTTCGTTTTTCGGTTTCG	434	(A/A) ₁₀₉ , (C/C) ₁₇₀	(A/G*) ₁₀₉ , (A*/C) ₁₇₀ (A/G*) ₂₈₁ , (A*/G) ₃₁₈ ,	LM999993
SNP_24.482	TTTTGTTGGGGATTCTTTGC	GCTTCCAATTCTTCGTACCC	698	(A/A) ₂₈₁ , (G/G) ₃₁₈ , (C/C) ₄₆₅	(T*/C) ₄₆₅	LM999994
SNP_24.483	GCCCAGATGAGCTAGTAAAGC	TCCGAAAAATTTACAAATTC	670	(A/A) ₉₆	(A/T*) ₉₆	LM999995
SNP_24.73	GAGACCCATGGAATGCTACC	GGTGGTGGCTTCCTTTACC	586	(C/C) ₂₁₈ , (A/A) ₂₂₀	(T*/C) ₂₁₈ , (A/G*) ₂₂₀ (G/C*) ₁₈₀ , (A/G*) ₃₁₉ ,	LM999996
SNP_24.78	ATGCCTAGGCTCCGATGG	AAGATGGCCTTGACGATCC	591	(G/G) ₁₈₀ , (A/A) ₃₁₉ , (C/C) ₄₂₁	(C/A*) ₄₂₁	LM999997
SNP_24.85	AAGGGCAAGGAGATTGACG	TCATTCATAGCTCCCAAACC	599	(T/T) ₂₇₈	(A*/T) ₂₇₈	LM999998

*Allele in coupling with resistance

Table 3.3. The eight founder lines of modern apple cultivars genotyped in this study with SNP markers closely linked to the *Rvi12* locus and the SNP alleles detected.

Accession	SNP_23.31	SNP_23.488	SNP_23.523	SNP_24.482
Golden delicious	(T*/C) ₁₀₂ , (T/T) ₁₉₉ , (T/T) ₂₂₇ , (C/C) ₂₉₇	(C/C) ₁₇₃ , (T/T) ₂₁₁	(A/A) ₁₀₉ , (C/C) ₁₇₀	(A/A) ₂₈₁ , (G/G) ₃₁₈ , (C/C) ₄₆₅
Delicious	(T*/C) ₁₀₂ , (T/T) ₁₉₉ , (T/T) ₂₂₇ , (C/C) ₂₉₇	(C/C) ₁₇₃ , (T/T) ₂₁₁	(A/A) ₁₀₉ , (C/C) ₁₇₀	(A/A) ₂₈₁ , (G/G) ₃₁₈ , (C/C) ₄₆₅
Braeburn	(T*/C) ₁₀₂ , (T/T) ₁₉₉ , (T/T) ₂₂₇ , (C/C) ₂₉₇	(C/C) ₁₇₃ , (T/T) ₂₁₁	(A/A) ₁₀₉ , (C/C) ₁₇₀	(A/A) ₂₈₁ , (G/G) ₃₁₈ , (C/C) ₄₆₅
Cox	(C/C) ₁₀₂ , (T/T) ₁₉₉ , (T/T) ₂₂₇ , (C/C) ₂₉₇	(C/C) ₁₇₃ , (T/T) ₂₁₁	(A/A) ₁₀₉ , (C/C) ₁₇₀	(A/A) ₂₈₁ , (G/G) ₃₁₈ , (C/C) ₄₆₅
F2-26829-2-2	(T*/C) ₁₀₂ , (T/T) ₁₉₉ , (T/T) ₂₂₇ , (C/C) ₂₉₇	(C/C) ₁₇₃ , (T/T) ₂₁₁	(A/A) ₁₀₉ , (C/C) ₁₇₀	(A/A) ₂₈₁ , (G/G) ₃₁₈ , (C/C) ₄₆₅
GrannySmith	(C/C) ₁₀₂ , (T/T) ₁₉₉ , (T/T) ₂₂₇ , (C/C) ₂₉₇	(C/C) ₁₇₃ , (T/T) ₂₁₁	(A/A) ₁₀₉ , (C/C) ₁₇₀	(A/A) ₂₈₁ , (G/G) ₃₁₈ , (C/C) ₄₆₅
Jonathan	(C/C) ₁₀₂ , (T/T) ₁₉₉ , (T/T) ₂₂₇ , (C/C) ₂₉₇	(C/C) ₁₇₃ , (T/T) ₂₁₁	(A/A) ₁₀₉ , (C/C) ₁₇₀	(A/A) ₂₈₁ , (G/G) ₃₁₈ , (C/C) ₄₆₅
McIntosh	(C/C) ₁₀₂ , (T/T) ₁₉₉ , (T/T) ₂₂₇ , (C/C) ₂₉₇	(C/C) ₁₇₃ , (T/T) ₂₁₁	(A/A) ₁₀₉ , (C/C) ₁₇₀	(A/A) ₂₈₁ , (G/G) ₃₁₈ , (C/C) ₄₆₅
Gala	(C/C) ₁₀₂ , (T/T) ₁₉₉ , (T/T) ₂₂₇ , (C/C) ₂₉₇	(C/C) ₁₇₃ , (T/T) ₂₁₁	(A/A) ₁₀₉ , (C/C) ₁₇₀	(A/A) ₂₈₁ , (G/G) ₃₁₈ , (C/C) ₄₆₅
Hansen's baccata 2	(T*/C) ₁₀₂ , (T/C*) ₁₉₉ , (T/C*) ₂₂₇ , (T*/C) ₂₉₇	(T*/C) ₁₇₃ , (A*/T) ₂₁₁	(A/G*) ₁₀₉ , (A*/C) ₁₇₀	(A/G*) ₂₈₁ , (A*/G) ₃₁₈ , (T*/C) ₄₆₅
Accession	SNP_24.483	SNP_24.73	SNP_24.78	SNP_24.85
Golden delicious	(A/A) ₉₆	(C/C) ₂₁₈ , (A/A) ₂₂₀	(G/G) ₁₈₀ , (A/A) ₃₁₉ , (C/C) ₄₂₁	(T/T) ₂₇₈
Delicious	(A/A) ₉₆	(C/C) ₂₁₈ , (A/A) ₂₂₀	(G/G) ₁₈₀ , (A/A) ₃₁₉ , (C/C) ₄₂₁	(T/T) ₂₇₈
Braeburn	(A/A) ₉₆	(C/C) ₂₁₈ , (A/A) ₂₂₀	(G/G) ₁₈₀ , (A/A) ₃₁₉ , (A*/C) ₄₂₁	(T/T) ₂₇₈
Cox	(A/A) ₉₆	(C/C) ₂₁₈ , (A/A) ₂₂₀	(G/G) ₁₈₀ , (A/A) ₃₁₉ , (A*/C) ₄₂₁	(T/T) ₂₇₈
F2-26829-2-2	(A/A) ₉₆	(C/C) ₂₁₈ , (A/A) ₂₂₀	(G/G) ₁₈₀ , (A/A) ₃₁₉ , (A*/C) ₄₂₁	(T/T) ₂₇₈
GrannySmith	(A/A) ₉₆	(C/C) ₂₁₈ , (A/A) ₂₂₀	(G/G) ₁₈₀ , (A/A) ₃₁₉ , (C/C) ₄₂₁	(T/T) ₂₇₈
Jonathan	(A/A) ₉₆	(C/C) ₂₁₈ , (A/A) ₂₂₀	(G/G) ₁₈₀ , (A/A) ₃₁₉ , (A*/C) ₄₂₁	(T/T) ₂₇₈
McIntosh	(A/A) ₉₆	(C/C) ₂₁₈ , (A/A) ₂₂₀	(T/G) ₁₈₀ , (A/T) ₃₁₉ , (C/C) ₄₂₁	(T/T) ₂₇₈
Gala	(A/A) ₉₆	(C/C) ₂₁₈ , (A/A) ₂₂₀	(G/G) ₁₈₀ , (A/A) ₃₁₉ , (C/C) ₄₂₁	(T/T) ₂₇₈
Hansen's baccata 2	(A/T*) ₉₆	(T*/C) ₂₁₈ , (A/G*) ₂₂₀	(G/C*) ₁₈₀ , (A/G*) ₃₁₉ , (C/A*) ₄₂₁	(A*/T) ₂₇₈

*Allele in coupling with resistance

3.5. Discussion

Previously, a population derived from a cross between ‘Golden Delicious’ and HB2 (GD×HB2) and consisting of 96 progeny, was used to locate the *Rvi12* scab resistance locus to LG12 of the apple genome (Erdin et al. 2006). In that investigation, the authors scored disease symptoms based on the scale reported by Chevalier et al. (1991), but did not report additional unexpected phenotypic classes, and mapped *Rvi12* to an interval of 21.5 cM flanked by two SSR markers, Hi02d05 and Hi07f01. In the present investigation, through careful observation of plant reactions after *V. inaequalis* inoculation over a number of successive growing seasons, an additional unexpected SC phenotype was identified that was observed to be segregating in the G×HB2 progeny. Why the presence of this second R-gene was not identified in the work of Erdin et al. (2006) is unclear. One possible explanation could be the different inocula (Swiss vs Italian) used in the two studies. If this is the reason, the value for resistance breeding of the R-gene inducing SC symptoms is low as it is likely that this gene has already been overcome at least in the Bologna region of Italy. In another study, Hemmat et al. (2003) reported the map position of *Rvi12* to be on LG1, loosely linked to the *Rvi6* resistance locus, but these authors did not report evidence of the segregation of two resistance genes in their cross. The data from investigation support the findings of Erdin et al. (2006), who mapped *Rvi12* to LG12, however, our conclusion of the presence of a second resistance gene in HB2 raises the possibility that this additional resistance locus may be the one mapped by Hemmat et al. (2003) on LG1. Hemmat et al. (2003) described the phenotype of the R-gene they mapped as “*chlorotic wrinkled with or without some sporulation*”. This description is very similar to what we called SC (see in Results in section “*Mapping populations and evaluation of the resistance reaction*”). If many conidia germinate very close to each other on a genotype carrying a SC inducing resistance gene, a deformation (wrinkling) of the leaf can be observed. As Hemmat et al. (2003) reported a segregation of resistant vs susceptible seedlings close to 1:1, it could be hypothesized that the inoculum they used may contain conidia able to overcome the *Rvi12*

resistance gene we mapped on LG12, however, it is also possible that the HB2 accession used in the study of Hemmat et al. (2003) may not be the same as the one used in our study . Additional, precise phenotyping of plants conferring the SC, along with further mapping work will need to be undertaken to determine the exact location of the additional resistance locus segregating in the G×HB2 progeny. In addition, screening of apple scab isolates from US would allow us to determine if *Rvi12* on LG12 has already been overcome in North America, however such experiments are beyond the scope of the current investigation.

The use of the ‘Golden Delicious’ genome sequence (Velasco et al. 2010) in this investigation permitted a higher degree of precision in sequence-characterised marker development than was previously only possible employing bulked-segregant analysis (BSA) combined with RAPD or ALFP markers. Once a relatively narrow mapping interval had been established using previously published SSR markers, additional SSR markers and SNPs were identified from the genome sequence covering this mapping interval, and recombinants from an extended G×HB2 progeny were screened to reduce the mapping interval to a size that would allow for effective marker assisted selection (MAS). Thus, a set of five novel SSR and 18 novel SNP markers were closely mapped to the *Rvi12* locus, spanning a physical distance of 1.54 Mb, with the two closest markers defining a physical interval of 958 kb. Since 16 of the SNPs identified are found only in coupling with *Rvi12* and are not present in any of the eight apple founder breeding lines tested, they will permit the selection of genotypes containing *Rvi12* from breeding progenies with a much higher degree of precision than the previously reported linked SSR markers.

3.6. Concluding remarks

In this investigation, the *Rvi12* mapping interval has been reduced from the previous report of Erdin et al. (2006) by employing a large mapping population, precise phenotypic characterisation and mining of the ‘Golden Delicious’ genome sequence for the purposes of marker development. The two closest-flanking SNPs fine-mapped in this investigation define a physical interval of 958 kb of the ‘Golden Delicious’ genome sequence which is characterised by seven recombinants (5 and 2 on each side of the *Rvi12* locus) in the G×HB2 seedlings screened. The newly developed SNP markers presented here will allow *Rvi12* to be included in a program of MAS for durable scab resistance breeding.

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Chapter 4

Identification and characterization of a candidate gene at the apple scab resistance locus *Rvi12* using BAC-based physical mapping and single molecule real-time PacBio RS II sequencing

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4.1. Abstract

Apple scab caused by *Venturia inaequalis* is one of the most prevalent and devastating diseases of apple (*Malus × domestica*) worldwide. Consequently, for sustainable control of scab, resistant cultivars containing various major scab resistance genes introgressed from wild varieties of apple are being used. The breakdown of the *Rvi6* major gene based resistance led to the identification and characterization of new scab resistance genes. By using a large segregating population, the *Rvi12* scab resistance gene was previously mapped between markers SNP_23599 and SNP_24482. Starting from these markers, using Hansen's *baccata* #2 (HB2) BAC-library-based chromosome walking, a single BAC clone spanning the *Rvi12* interval was identified. Following PacBio RS II sequencing and the use of the hierarchical genome assembly process (HGAP), assembly of the BAC clone sequence, the *Rvi12* resistance locus was localized to a 62.3 kb genomic region of the 'HB2' genome. Gene prediction and *in-silico* characterization identified five putative candidate genes showing homology to known disease resistance genes or genes involved in disease resistance signaling. Protein domain analysis and quantitative real-time PCR (qRT-PCR) of the five putative candidate genes indicated the likely candidate gene conferring scab resistance at the *Rvi12* locus to be a probable LRR receptor like serine/threonine kinase.

4.2. Introduction

Plant diseases impact heavily on crop productivity, threatening food security, and are thus of significant economic importance. Plants defend themselves against a wide range of pathogens through various defense responses. Plant resistance genes and signaling mechanisms are the major defense steps employed by plants in preventing plant pathogen attack (Jones and Dangl, 2006). However, pathogens overcome resistance responses through mutation of avirulence genes. Because of this co-evolutionary arms-race between the host and the pathogen population (McDonald and Lindt, 2002), pathogens can overcome resistance conferred by single major resistance genes in a

relatively short time-frame. Pyramiding of major disease resistance genes and/or quantitative trait loci (QTLs) conferring broad spectrum disease resistance (QDR) was proposed as an efficient strategy to develop broad spectrum durable resistance to plant pathogens (Pederson and Leath, 1988; Poland et al, 2009).

The identification and characterization of genes conferring resistance to plant pathogens is the initial step in developing durable resistance to major diseases in crop plants. Many genes conferring resistance to bacterial, fungal and viral pathogens have been identified using a map-based gene cloning approach. Most of the plant disease resistance genes identified to date belong to the family of nucleotide-binding site leucine-rich repeat-containing proteins (NBS-LRR) which include both Toll/interleukin1 domain-containing TIR-NBS-LRR genes (*N*, *L6*, *RPS4*; Dinesh-kumar et al, 1995; Gassmann et al, 1999; Lawrence et al, 1995) and coiled-coil (CC) domain containing CC-NBS-LRR genes (*RPS2*, *I2*; Mayers et al, 1999; Mindrinos et al, 1994). Other classes of resistance genes include receptor-like kinases (*Xa21*, *FLS2*; Song et al, 1995, Gomez-Gomez and Boller, 2000) and receptor-like proteins (*Cf-2*, *Cf-4*, *Cf-9*; Parniske et al, 1997). Varieties conferring durable broad spectrum resistance through the pyramiding of multiple R-genes have been developed with resistance to rice blast disease (Hittalmani et al, 2000; Fukuoka et al, 2015), and bacterial blight disease of rice (Huang et al, 1997; Pradhan et al, 2015) amongst others.

Apple scab, caused by the fungal pathogen *Venturia inaequalis*, is one of the major diseases of apple. Many scab resistance genes have been identified, however, breeding for scab resistance in the 20th century relied mainly on resistance derived from the *Rvi6* gene of *Malus floribunda* 821 (Crandall, 1926; Dayton et al, 1970; Hough et al, 1953). Evolution of the scab pathogen and the resultant breakdown of *Rvi6*-based resistance have obligated breeders to look for alternate scab resistance genes for use in breeding (Parisi and Lespinnasse, 1996; Parisi et al, 1993). In recent years, at least 18 scab resistance genes have been identified and mapped (Bus et al, 2011; Soriano et al, 2014). In an attempt to develop durable resistance, apple genotypes carrying three homozygous

scab resistance genes *Rvi6*, *Rvi2* and *Rvi4* were developed through marker assisted breeding, and these selections were also resistant to powdery mildew (*Pl1* and *Pl2*) and fire blight (*FBF7*) (Baumgartner et al, 2015). Gene pyramiding through traditional breeding in crops with a long vegetative phase such as apple takes a significant length of time and may lead to the inheritance of unwanted traits from the wild varieties used as donor parents through linkage drag. The use of high-speed breeding technologies based on transgenic early flowering varieties of apple and marker assisted selection for the introgression of traits can help reduce the time required for breeding, but linkage drag can still be a problem requiring numerous rounds of back-crossing to remove undesirable traits (Flachowski et al, 2011; Le Roux et al, 2012; van Nocker and Gardiner, 2014). Thus, the identification of resistance genes is extremely important for introgression of traits in a short time-frame through genetic engineering or cisgenesis (Jacobsen and Schouten, 2007; Krens et al, 2015).

Six scab resistance genes *Rvi6* (Patocchi et al, 1999; Xu and Korban, 2000), *Rvi15* (Galli et al, 2010a), *Rvi18* (Soriano et al, 2014), *Rvi12* (Padmarasu et al, 2014), *Rvi1* (Cova et al, 2015) and *Rvi5* (Cova et al, 2015) were fine-mapped, and candidate resistance genes for *Rvi6* (Patocchi et al, 1999a; Vinatzer et al, 1998, 2001; Xu and Korban, 2002), *Rvi15* (Galli et al, 2010b) and *Rvi1* (Cova et al, 2015) have been identified. The *Rvi6* scab resistance locus contained a cluster of four receptor-like proteins similar to *Cladosporium fulvum* resistance genes of tomato that were named *HcrVf1* to *HcrVf4* (Vinatzer et al, 2001). Scab resistant transgenic apple plants were developed by *Agrobacterium*-mediated transformation of *HcrVf2/Vfa2* and *Vfa1* in two independent studies (Belfanti et al, 2004; Malnoy et al, 2008). Subsequently, cisgenic ‘Gala’ plants containing *HcrVf2* were recently developed and characterized (Jansch et al, 2014; Vanblare et al, 2011, 2014). The *Rvi15* locus contained three TIR-NBS-LRR proteins coding candidate genes named *Vr2-A*, *Vr2-B* and *Vr2-C* (Galli et al, 2010b). Based on gene cloning and characterization of these candidates, it was determined that *Vr2-C* was responsible for *Rvi15*-based scab resistance (Schouten et al, 2014).

The presence of highly repetitive sequences and the clustering of resistance genes make it difficult to sequence and assemble these regions reliably using short-read sequencing technologies such as the Illumina and 454-sequencing platforms. The advent of long read sequencing technology such as Pacific Biosciences single molecule real-time (SMRT) sequencing has provided a robust solution for the sequencing and reliable assembly of complex genomic regions (Huddleston et al, 2014) and small plastid genomes (Ferrarini et al, 2013).

The *Rvi12* scab resistance from *Malus baccata* Hansen's baccata #2 (HB2) was one of the first apple resistance genes to be described (Dayton and Williams, 1968). The response of 'HB2' to *Venturia inaequalis* inoculation was reported as chlorosis (Class 2) or chlorosis with slight sporulation (Class 3a/3b) and infection did not elicit a hypersensitive response (HR) like those of the *Rvi5* and *Rvi15* resistance genes (Chevalier et al, 1991; Erdin et al, 2006; Hemmat et al, 2003; Patocchi et al, 2005; Galli et al, 2010a). *Rvi12* can be used in apple scab resistance breeding programs because of the weak, but broad spectrum nature of the resistance. *Rvi12* was mapped previously to LG12 of the apple genome between microsatellite markers Hi02d05 and Hi07f01 (Erdin et al, 2006). Subsequently, using a large segregating population of 1,285 seedlings of the cross Gala × HB#2 and employing newly-characterized markers, the *Rvi12* locus was fine-mapped between the SNP markers SNP_23523 and SNP_24492 in a physical interval of 958 kb (Padmarasu et al, 2014). The objective therefore of this study was to further fine map the *Rvi12* locus, identify and characterize candidate resistance genes for *Rvi12* based apple scab resistance through chromosome walking of a 'HB2' BAC library, the construction of a minimum tiling path of BAC clones covering the *Rvi12* interval, and PacBio sequencing and assembly of a BAC clone sequence containing the *Rvi12* resistance locus.

4.3. Materials and Methods

4.3.1. Novel SNP marker development and fine-mapping of *Rvi12*

The population of 1,285 plants that made up the ‘G×HB’ and ‘G×HB-980’ mapping populations and that were previously phenotyped by Padmarasu *et al*, (2014) was used for fine mapping in this experiment. Ninety eight Exon Primed Intron Crossing (EPIC) primer pairs (Palumbi and Baker, 1994) were designed from the DNA sequence of the ‘Golden Delicious’ genome sequence (Velasco *et al*, 2010) between the *Rvi12* flanking markers SNP_23523 and SNP_24482 as reported by Padmarasu *et al*, (2014). Novel SNPs mapping to the *Rvi12* interval (**Table 4.1**) were identified by using the protocol described in Padmarasu *et al*, (2014). Genotyping of recombinants from the ‘G×HB2’ and ‘G×HB2-980’ mapping populations was performed with the novel SNPs identified and the *Rvi12* locus was fine-mapped as described in Padmarasu *et al*, (2014).

4.3.2. Construction, three-dimensional (3D) pooling of Hansen’s *baccata* #2 BAC library

A BAC library was constructed from frozen leaves of Hansen’s *baccata* #2 (HB2) by Amplicon Express Inc, WA, USA following the protocol of Tao *et al*, (2002). Briefly, the BAC library was constructed by partial *HindIII* digestion and cloning into the copy control plasmid pCC1BAC from Epicentre, WI, USA. The average insert size of the library was estimated by *NotI* digestion of 28 random clones. The positive clones following blue/white selection were picked into 384-well plates containing storage media (LB lennox, 36 mM K₂HPO₄, 13.2 mM KH₂PO₄, 1.7 mM Sodium citrate, 0.4 mM MgSO₄, 6.8 mM (NH₄)₂SO₄, 4.4% Glycerol, 12.5 µg/ml chloramphenicol).

To create the pools, the four adjacent clones on each 384-well plate were pooled into a single well of a 96-well plate containing 150 µl of LB broth with 12.5 µg/ml of chloramphenicol using a 96-well replicator. The inoculated plates were incubated at 37°C at 50 RPM for 16 hours. The 96-well plates were divided into four stacks of 24 plates each for three-dimensional pooling. Ten microlitres of bacterial culture from each well of each row were pooled together from the group of 24 plates to

make a row pool. In the same way, 10 µl of bacterial culture from each well of each column were pooled together from a group of 24 plates to make a column pool. Ten microlitres of bacterial culture from all the wells of a plate were pooled together to make a plate pool. The pooled cultures (row pool, column pool and plate pool) were mixed thoroughly and inoculated in 10 ml of LB broth containing 12.5 µg/ml of chloramphenicol and grown for overnight at 37°C at 225 RPM (Bonet et al, 2009). Glycerol stocks of the pooled BAC clones were made by mixing 850 µl of bacterial culture with 150 µl of Glycerol and the stocks were stored at -80°C. BAC DNA was extracted from the rest of the culture following the protocol of Russell and Sambrook (2001), quantified using a ND8000 nanodrop spectrophotometer (Thermo Scientific Inc, MA, USA) and used for PCR screening of the library.

4.3.3. PCR screening of the BAC library pools

The BAC DNA extracted from the 3D pools was diluted 1:100 (i.e., approximately 50 ng/µl concentration of DNA). PCR was performed with markers of interest (**Table 4.1**) using the following cycling conditions: an initial denaturation step of 94°C for 10 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at melting temperature (T_m) of primer pairs -2°C for 30 seconds, extension at 72°C for 45 seconds and a final extension step of 72°C for 7 minutes. The PCR products were electrophoresed on a 1.5% agarose gel for 2 hours at 120 V and bands were visualized over UV light following staining with GelRed (Biotium, CA, USA). Based on the presence of expected bands in row, column and plate pools, the well position of putative positive clones were identified in the 96-well pooled plates. To identify a single positive clone, the four putative positive clones from the original 384-well plates were inoculated individually in LB broth containing 12.5 µg/ml of chloramphenicol and grown overnight as described above. PCR was performed as described above with 2 µl of overnight grown bacterial culture as template to identify positive clones.

4.3.4. Chromosome walking and construction of contiguous BAC clones covering the *Rvi12* interval

The 'HB2' BAC library was screened for positive clones with SNP markers, SNP_23599 and SNP_24482 which flanked the *Rvi12* locus, based on the protocol described above. From the positive clones, high quality BAC DNA was extracted from 400 ml of overnight grown bacterial culture using the Qiagen midi extraction kit (Qiagen GmbH, Germany) following the manufacturer's recommendations for maxi preparation. Both forward and reverse ends of the BAC clones were sequenced using BigDye chemistry (Applied Biosystems, CA, USA) using 15 µM of pCC1BAC sequencing primers (pCC1-Forward Sequencing Primer (FSP) 5' GGATGTGCTGCAAGGCGATTAAGTTGG 3'; pCC1-Reverse Sequencing Primer (RSP) 5' CTCGTATGTTGTGTGGAATTGTGAGC 3') and 750 ng/µl BAC DNA. The SeqMan application of the Lasergene software suite (DNASTAR, WI, USA) was used to remove vector sequences and to visualize the BAC-end sequences. Primer pairs were designed from the BAC-end sequences using Primer3 (Untergasser et al, 2012) using the following parameters: a PCR product between 100-350 bp in length, a T_m of 55-65°C (optimum 60°C), a primer length of 20-27 bp (optimum 22 bp) and a GC-clamp of 2 bp. Positive BAC clones were screened with all the primer pairs designed from all the BAC-end sequences. The primer pair which amplified only the expected PCR product from the clone from which it was designed was used for further screening of the BAC library to identify contiguous BAC clones. Screening of the library was performed with subsequent markers until the entire *Rvi12* interval was covered with contiguous BAC clones.

4.3.5. Designing of BAC end markers, closely linked markers and fine-mapping

The BAC-end sequences were screened for presence of microsatellite repeats using SSR server tool on the Genome Database for Rosaceae (<http://www.rosaceae.org>; Jung *et al*, 2014). If microsatellite repeats were found, flanking primers were designed using Primer3 (Untergasser *et al*, 2012) as

described in Padmarasu et al, (2014). If no repeats were found, primer pairs were designed from BAC-end sequences as described in the previous section and checked for novel SNP markers as described in Padmarasu *et al*, (2014). If no marker was identified, the BAC-end sequences were used as a query to BLAST the ‘Golden Delicious’ v1.0 genome sequence. The sequences of the contigs to which the BAC-end sequences matched were mined. Novel SSR markers were designed from the contig sequences as described in Padmarasu *et al*, (2014). The novel SSR markers designed from the ‘Golden Delicious’ contig sequences, along with SSR and SNP markers developed from BAC-end sequences were used for genotyping recombinants from G × HB2 and G × HB2 – 980 populations (Padmarasu *et al*, 2014) to narrow down *Rvi12* mapping interval. PCR was performed with these markers as described above on positive BAC clones, to identify resistant and susceptible alleles in the positive BAC clones and to identify minimum number of contiguous clones (minimum tiling path) required to cover the *Rvi12* interval.

4.3.6. Sequencing and assembly of 6F11 BAC clone sequences

BAC clone 6F11 was extracted from 400 ml of over-night grown bacterial culture as already described in 4.3.4. Forty microgram of the extracted BAC DNA was sent to the core genomics facility at Cold Spring Harbor Laboratory (CSHL) for single molecule real-time (SMRT) sequencing using the PacBio RS II platform (Pacific Biosciences, CA, USA). A 20 kb insert SMRT-bell library was prepared from the BAC DNA, and sequenced using two SMRT cells and the P5-C3 chemistry of PacBio RS II system. Raw data obtained were assembled using the custom hierarchical genome assembly program (HGAP) of SMRT analysis pipeline version 2.3.0 with whitelisting functionality. Whitelisting was performed by following the instructions from <https://github.com/PacificBiosciences/Bioinformatics-Training/wiki/HGAP-Whitelisting-Tutorial> with minor modifications. The whitelist containing read names of each read coming from BAC DNA and the reads which have homologous sequences between pCC1BAC vector and *E.coli* genomic DNA was prepared and used for HGAP assembly with the SMRT pipeline. The assembled

BAC sequence contig was analyzed for presence of entire BAC clone sequences by Align (bl2seq) analysis against the BAC-end sequences obtained from Sanger sequencing. The presence of flanking marker sequences in the BAC contig was checked by blasting the BAC contig against the sequence of SNP_32N11-RSP and the BAC-end sequence of 6F11-FSP. The sequence in-between the flanking markers was used for gene prediction.

4.3.7. Gene prediction and identification of candidate genes

The sequence present between flanking markers SNP_32N11-RSP and SCAR-6F11-FSP in the assembled BAC contig was used for gene prediction. The gene prediction was performed by using FGENESH 2.6 online program (Solovyev et al, 2006) using the *Arabidopsis thaliana* gene prediction training set. Predicted CDS sequences obtained were blasted against the non-redundant protein sequences database (blastx) of NCBI to check for homology against known proteins in the database. The predicted genes which showed homology to known disease resistance proteins or proteins involved in disease resistance signaling were used for further analysis. A conserved domain search (Marchler-Bauer et al, 2015) was performed to identify the domains and motifs present in translated protein sequences of the candidate genes. The Phobius protein function analysis tool from EMBL database was used to check for the presence of signal peptides and transmembrane domains in candidate proteins.

4.3.8. Scab inoculations, RNA extraction and expression analysis of candidate genes

Susceptible ‘Gala’ and resistant ‘HB2’ scions were grafted onto M9 rootstocks. Grafted plants at the same developmental stage were inoculated with *Venturia inaequalis* following the protocol detailed in Padmarasu et al (2014). Mock treatment with water was performed on a replicated set of grafted plants. Three biological replicates each of the inoculated and the mock treated leaves were collected at 0, 4 and 12 days post inoculation (dpi), frozen in liquid nitrogen and stored at -80°C. RNA extraction from the frozen leaves was performed using the Spectrum plant total RNA kit

(Sigma-aldrich, MO, USA) following the manufacturer's instructions. Quantification of RNA was done using a nanodrop spectrophotometer (Thermo Scientific Inc, MA, USA). Two micrograms of total RNA from each sample was treated with ten units of DNase I (Sigma-Aldrich, MO, USA) following the manufacturer's instructions. cDNA synthesis from the DNase I treated RNA was performed using SuperScript III reverse transcriptase (Life Technologies, CA, USA) and random primers (Invitrogen) following manufacturer's recommendations. qRT-PCR primers specific for the genes of interest were designed (**Table 4.3**) using the online tool Primique (Fredslund and Lange, 2007) with the following parameters: An expected product size of 50-120 bp, primer length of 18-24 bp, melting temperature of 55-63°C. The specificity of the primers was checked by blasting against the 'Golden Delicious' v1.0 genome sequence (Velasco et al, 2010). qRT-PCR was carried out using Fast SYBR green master mix (Life Technologies, CA, USA) using a ViiA7™ instrument (Life Technologies, CA, USA). The following qRT-PCR thermal conditions were used: an initial incubation at 95°C for 20 sec, 40 cycles of 95°C 1 sec and 60°C 20 sec followed by a cycle at 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec to determine the melting curve. The Ct results were obtained by averaging three independent normalized expression values for each sample. Relative gene expression was plotted as the mean of the normalized expression values using the Delta-Delta CT method (Livak and Schmittgen, 2001) and Md8283 was employed as housekeeping gene (Botton et al, 2011; Longhi et al, 2012) for normalization of data.

4.4. Results

4.4.1. Identification of novel SNPs and fine-mapping of *Rvi12*

Amplicons produced from 'Gala' and 'HB2' using a set of 98 EPIC primer pairs were sequenced using Sanger sequencing. Of these, 23 amplicons contained at least one heterozygous SNP in 'HB2'. Screening of eight progeny plants (four resistant and four susceptible) from the G × HB2 mapping population revealed only two amplicons (containing seven heterozygous SNPs);

SNP_23597 (3) and SNP_23599 (4) that were located in the *Rvi12* mapping interval. Genotyping of seven recombinant plants from the G × HB2 and G × HB2-980 mapping progenies previously reported in Padmarasu et al (2014), with the novel SNP markers, placed *Rvi12* between two loci; SNP_23599 (containing three SNPs) and the previously mapped SNP_24482 (containing three SNPs) (Table 4.1; Figure 4.1) which corresponded to an estimated physical interval of 882 kb on ‘Golden Delicious’ genome.

Markers/ Recombinants	SNP_23523	SNP_23597	SNP_23599	SSR- MDC005 174.220	SSR- MDC011 711.431	SSR- MDC005 797.286	SNP_3 2N11- RSP	<i>Rvi12</i> (Vb)	SCAR- 6F11- FSP	SSR- MDC012 922.528	SSR- 56H15	SNP_24482
10A1	ab	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa
131	ab	ab	ab	ab	ab	aa	aa	aa	aa	aa	aa	aa
269	ab	ab	ab	ab	ab	ab	ab	aa	aa	aa	aa	aa
8F4	ab	ab	ab	ab	ab	ab	ab	ab	aa	aa	aa	aa
218	aa	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab
4H4	aa	aa	aa	aa	aa	aa	aa	ab	ab	ab	ab	ab
9B1	aa	aa	aa	aa	aa	aa	aa	aa	aa	ab	ab	ab
Number of Recombinants	5	3	3	3	3	2	2		1	2	2	2

Figure 4.1 Schematic diagram showing recombination events identified in the G × HB2 and G × HB2-980 population in the *Rvi12* gene mapping interval. *ab* in the figure represents the allele in coupling, *aa* represents allele in repulsion to the resistance gene *Rvi12*.

Table 1 Markers used in this study detailing primer sequences, product sizes of SSR and SCAR markers, SNP alleles observed in parental lines

Marker	Forward Primer	Reverse Primer	Alleles	
			Gala	HB2
SNP_23523	CTTCTGCTTACTCGGCATGG	ATTCTTCGTTTTTCGGTTCG	(A/A) ₁₀₉ , (C/C) ₁₇₀	(A/G*) ₁₀₉ , (A*/C) ₁₇₀
SNP_23597	CTTCCAACACTGTGGACACACC	ACAAGCCCCAAGAATCTGC	(A/A) ₈₅ , (G/G) ₁₁₇ , (A/A) ₂₃₇	(A/C*) ₈₅ , (A*/G) ₁₁₇ , (A/G) ₂₃₇
SNP_23599	CCTTGTTGTCTGAGTATGTTGTG G	TAATCCAAGCAACGATCACG	(T/T) ₃₉ , (G/G) ₈₅ , (A/A) ₂₂₃ , (A/A) ₂₇₀	(A*/T) ₃₉ , (C*/G) ₈₅ , (A/T*) ₂₂₃ , (A/C*) ₂₇₀
SSR-MDC005174.220	GTAGTAATCCACCCCCATGC	TGTATGACTCGTCGCTCACG	219, 223 bp	209, 216* bp
SSR-MDC011711.431	TCCAACGGTTTTTCATTGAGC	GAATGATCACATACCTCGTAG GC	174, 183 bp	177, 187* bp
SSR-MDC005797.286	CCTGTTCGCAAACAAGAAGG	GCGTAGTCAATCAAAACATTC G	232, 252 bp	248, 258* bp
SNP_32N11-RSP	GAAACCGAACGTAGAATGAGC	CCAATTTTGGATTTTGGGAAGC	(C/C) ₂₄₃	(T*/C) ₂₄₃
SCAR-6F11-FSP	TCGTCTGACTCTTCCGTTCC	TGGCCACAGAAATTAAGATGG	~200 bp, null	150*, 200 bp
SSR-MDC012922.528	GAGGGCTCCAAATTGTTTCC	CTGTTTCGCGTGTCATGG	221, 225 bp	213*, 218 bp
SSR-56H15-RSP	TACACACACACACACACACA	CGCATCTCTCTCAAATGGCAA	120 - 150 bp	100-120*, 150 bp
SNP_24482	TTTTGTTGGGGATTCTTTGC	GCTTCCAATTCTTCGTACCC	(A/A) ₂₈₁ , (G/G) ₃₁₈ , (C/C) ₄₆₅	(A/G*) ₂₈₁ , (A*/G) ₃₁₈ , (T*/C) ₄₆₅

4.4.2. BAC library construction and pooling

A BAC library was constructed from the 'HB2' parental line which contained 36,864 clones arrayed into 96 plates of 384-wells. The average insert size of the BAC clones in the library was estimated to be 135 kb, and thus the library represented an estimated 6.7× coverage of the apple genome based on the previously-reported genome size of 742.3 Mb (Velasco *et al*, 2010). Following 3D-pooling of the BAC library, the 36,864 clones were condensed into 32 row pools containing 1152 clones per row pool; 48 column pools containing 768 clones per column pool; and 96 plate pools containing 384 clones per plate pool.

4.4.3. Screening of BAC library and identification of contiguous clones covering the *Rvi12* interval

Screening of the BAC library with flanking markers SNP_23599 and SNP_24482 identified one (69B16) and five (3K20, 21N16, 27F22, 45E3 and 56H15) positive clones with each marker respectively (**Figure 4.2**). Sequencing of the ends of all the identified BAC clones provided sequences from both forward (FSP) and reverse (RSP) ends of each insert. Subsequent screening of the library with a primer pair designed from the 69B16-Forward primer BAC-end sequence identified two positive clones (32N11 and 41B20). Screening of positive clones 32N11 and 41B20 with primer pairs designed from all four of their BAC end sequences identified the reverse primer sequence of clone 32N11 as that closest to *Rvi12*. The 32N11-RSP primer pair was used for the next round of screening and five positive clones (6F11, 14O3, 64A18, 68L24 and 84B19) were identified. On the other side of the *Rvi12* gene, the screening of the positive clones 3K20, 21N16, 27F22, 45E3 and 56H15 with primers designed from all their BAC-end sequences identified the reverse primer sequence end of 45E3 clone as that closest to *Rvi12*. The screening of the BAC library with 45E3-RSP identified a further eight (6F11, 20H12, 28C4, 64A18, 68L24, 84B19 and 88B4) positive clones. Four positive clones 6F11, 64A18, 68L24 and 84B19 obtained were

common when the library was screened with 32N11-RSP and 45E3-RSP, suggesting that the entire mapping interval containing *Rvi12* had been covered by the overlapping BAC clones. **Figure 4.2** depicts the schematic of contiguous clones covering the *Rvi12* region. **Supplementary table S4.1** provides the sequences of BAC end primers used for screening of the BAC library.

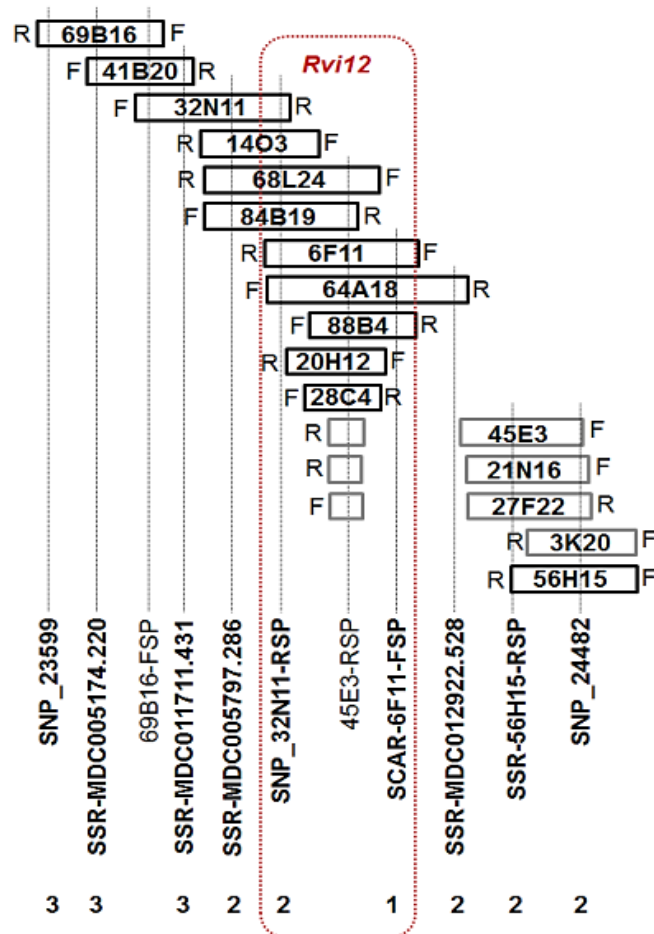


Figure 4.2 BAC tiling path spanning the *Rvi12* gene mapping interval. The BAC clones derived from the resistant chromosome are indicated by black boxes and the BAC clones from susceptible chromosome are indicated by grey boxes. It was not possible to group the BAC clones, 20H12 and 28C4 as ‘resistant’ or ‘susceptible’ clones. The names of each BAC clone are provided in their corresponding boxes. F, R on each side of the BAC clone rectangle represent the forward and reverse ends of the BAC clones. Dashed vertical lines represent the position of the novel markers developed. The numbers at the bottom indicate the number of recombinants present between the

corresponding marker and the *Rvi12* resistance locus. 69B16-FSP and 45E3-RSP were primer sequences for chromosome walking and not markers for fine mapping. The crimson rectangle contains the position of the narrowed down *Rvi12* interval.

4.4.4. Identification of BAC end markers and closely linked markers for fine-mapping

BAC-end sequences from 56H15-RSP were screened for the presence of SSR motifs and one SSR heterozygous in HB2 was identified and mapped to the *Rvi12* interval. A further SNP marker from 32N11-RSP and a SCAR marker from 6F11-FSP were identified and mapped to the *Rvi12* interval. Sequences from the BAC ends of 69B16-FSP and 21N16-FSP showed high similarity to contigs MDC005174.220 (chr13:16026888..16034647) and MDC012922.528 (chr13:15742986..15759642) of the ‘Golden Delicious’ reference genome sequence respectively. The sequences of the 17 contigs present between MDC005174.220 and MDC012922.528 on the ‘Golden Delicious’ genome sequence were mined, and four SSR markers (SSR-MDC005174.220, SSR-MDC011711.431, SSR-MDC005797.286 and SSR-MDC012922.528) which were heterozygous in ‘HB2’ were mapped in the *Rvi12* interval. **Table 4.1** lists the novel markers identified that were heterozygous in HB2 and the nature of the polymorphism identified.

4.4.5. Fine-mapping and identification of clones containing the *Rvi12* locus

The novel markers developed in this investigation were mapped in the following order within the *Rvi12* interval; SSR-MDC005174.220, SSR-MDC011711.431, SSR-MDC005797.286, SNP_32N11RSP, SCAR-6F11-FSP, SSR- MDC012922.528 and SSR-56H15-RSP. The *Rvi12* locus was fine-mapped between SNP_32N11-RSP (2 recombinants) and SCAR-6F11-FSP marker (1 recombinant) based on the comparison of genotypic and phenotypic data from the recombinants (**Figure 4.1**). Screening of positive BAC clones with the novel markers, revealed the identity of BAC clones as ‘resistant’ clones (69B16, 41B20, 32N11, 14O3, 68L24, 84B19, 6F11, 64A18, 88B4 and 56H15) and ‘susceptible’ clones (45E13, 21N16, 27F22 and 3K20). It was not possible to

group the BAC clones, 20H12 and 28C4 as resistance or susceptible clones because of the unavailability of molecular markers. Based on the data from the screening of the positive BAC clones, clones 6F11 and 64A18 both contained the resistant allele for flanking markers SNP_32N11-RSP and SCAR-6F11-FSP and covered the *Rvi12* locus (**Figure 4.2**). BAC clone 6F11 was used for sequencing and identification of candidate genes because of its smaller physical size, covering a shorter genetic distance around the *Rvi12* locus.

4.4.6. Sequencing and assembly of BAC clone sequence 6F11

BAC clone 6F11 was sequenced using the PacBio RSII sequencing platform. Sequence data derived from two SMRT cells generated 326,964 polymerase reads, 309,174 of which were retained after whitelisting. Following, subread filtering 32,295 subreads were recovered with a mean subread length of 10,214bp. This amounted to 329,891,600 bp of sequence. Following HGAP error correction and assembly of the sequence reads, the sequences were assembled into five contigs. Based on blast analysis of contigs against the *E.coli* K-12 strain MG1655 reference genome, the ‘Golden Delicious’ reference genome v1.0 and the pCC1BAC vector sequence, a single contig of 118.195 kb sequenced to a depth of 2795× with homology to sequences from 6F11 BAC clone was identified whilst four smaller contigs of sizes 26,346 bp, 34,113 bp, 29,844 bp and 20,235 bp contained sequences homologous to *E.coli* genomic DNA contamination. Because of the circular nature of the BAC clone, the assembled BAC contig contained an identical 17,414 bp sequence on both ends of linear contig. BAC-end sequences obtained from Sanger sequencing (6F11-FSP and 6F11-RSP) were identified in the BAC contig sequence immediately after the pCC1BAC vector *HindIII* cloning site flanking an ‘HB2’ sequence of 83,366 bp (**Figure 4.3**). This confirmed that the entire 6F11 BAC clone sequence was present in a single BAC contig. The 32N11-RSP marker sequence (412 bp) and 6F11-FSP end sequence (313 bp) from which SCAR-6F11-FSP was designed were found with 100% identity in the BAC contig. SNP_32N11-RSP and SCAR-6F11-FSP sequences flanked the *Rvi12* interval of 62,249 bp of sequence in the BAC contig (**Figure 4.3**).

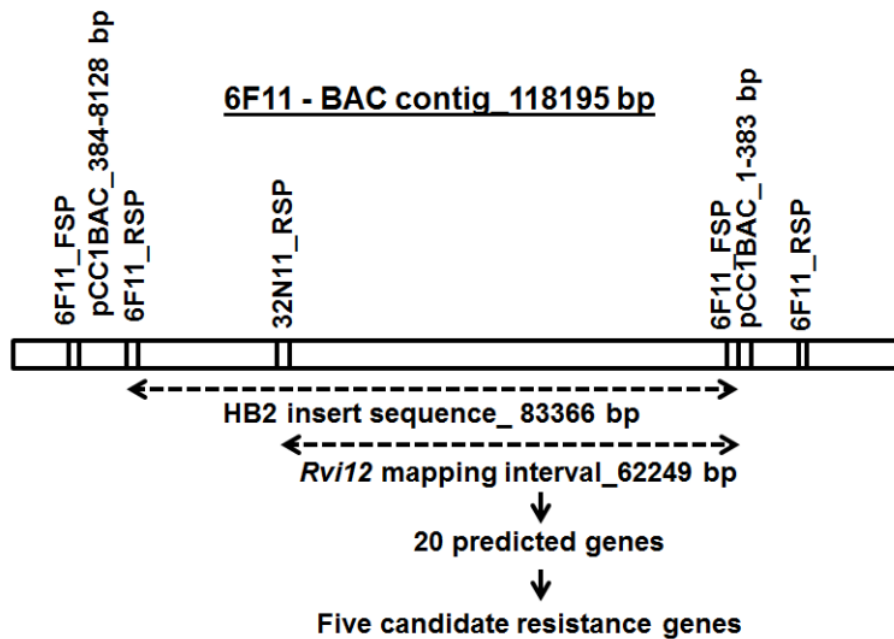


Figure 4.3 Schematic diagram showing the 6F11 BAC contig, ‘HB2’ insert sequence, the *Rvi12* gene mapping interval with the number of predicted genes and candidate genes it contains.

4.4.7. Gene prediction, identification and characterization of candidate genes

FGENESH 2.6 predicted 20 genes with 82 exons from the 62,249 kb sequence covering the entire *Rvi12* interval (**Supplementary table S4.2**). Five of the 20 predicted genes were homologous to proteins with structures known to be involved in disease resistance. Two candidates, *Rvi12_Cd1*, *Rvi12_Cd2* showed protein identity of 57% and 54% to a TIR-NBS-LRR protein of *Arachis hypogaea* (NCBI accession AEL30371.1). Furthermore, two candidates; *Rvi12_Cd3* and *Rvi12_Cd4* showed protein identity of 49-86% and 77-88% respectively to different members of the TGA-like transcription factor family from *Malus domestica* and *Pyrus × bretschneideri*. Another candidate gene, *Rvi12_Cd5* showed 92% protein identity to a probable LRR receptor like serine/threonine protein kinase from *Malus domestica* (NCBI accession XP_008360290). The conserved domain search analysis of translated protein sequences of candidates, *Rvi12_Cd1* (635 aa) and *Rvi12_Cd2* (357 aa) revealed the presence of a non-long terminal repeat (nLTR) retrovirus reverse transcriptase domain (RT_nLTR_like) and an endonuclease domain of the non-LTR retrotransposon LINE-1

(L1-EN) respectively (**Table 4.2**). Translated protein sequences of both *Rvi12_Cd3* (204 aa) and *Rvi12_Cd4* (221 bp) contained the delay of germination 1 (DOG1) domain (**Table 4.2**). A conserved domain search from NCBI and Phobius protein functional analysis tool from EMBL database, predicted *Rvi12_Cd5* (1026 aa) to contain an N-terminal signal peptide (amino acids 1-44), extracellular LRR repeats (aa 115-642), a transmembrane domain (aa 643-663), intracellular domain containing nucleotide binding sites (aa 705-728) and protein kinase domain with serine threonine specificity (aa 664-1024) (**Table 4.2 & Figure 4.4**).

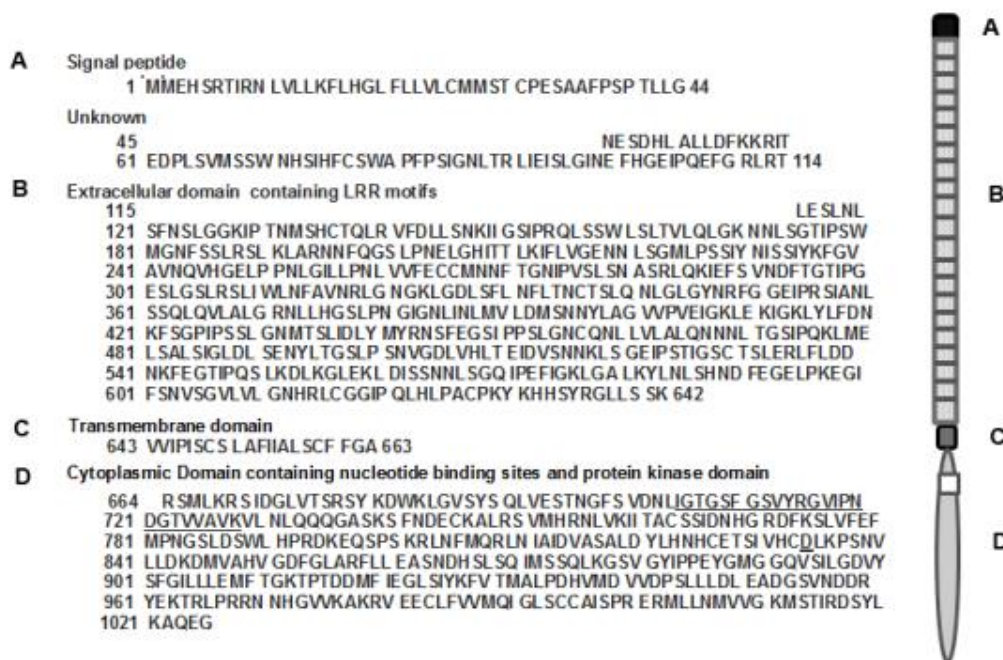


Figure 4.4 Predicted domains of *Rvi12_Cd5* protein and the corresponding amino acid sequences. (A) Signal peptide in the N-terminal region followed by amino acids with unknown function; (B) Extracellular LRR repeats; (C) Transmembrane domain; (D) Intracellular kinase domain with nucleotide binding sites. The numbers given correspond to the amino acid positions in the predicted protein. Underlined amino acids from positions 705-728 represent the nucleotide binding sites. Underlined amino acid Aspartate (D) at position 894 represents the proton acceptor in the active site of the kinase domain.

Table 4.2 Candidate resistance genes in *Rvi12* mapping interval, their homologous proteins and predicted protein domains

Candidate genes	Homologous proteins	Protein domains
<i>Rvi12_Cd1</i>	TIR-NBS-LRR protein from <i>Arachis hypogaea</i>	Non-long terminal repeat retrovirus reverse transcriptase domain (RT-nLTR like)
<i>Rvi12_Cd2</i>	TIR-NBS-LRR protein from <i>Arachis hypogaea</i>	Endonuclease domain of the non-long terminal repeat retrotransposon LINE-1 (L1-EN)
<i>Rvi12_Cd3</i>	Members of TGA-like transcription factors family	Delay Of Germination 1 (DOG1) domain
<i>Rvi12_Cd4</i>	Members of TGA-like transcription factors family	Delay of Germination 1 (DOG1) domain
<i>Rvi12_Cd5</i>	LRR receptor like serine/threonine protein kinase	18 imperfect LRR repeats, nucleotide binding sites, protein kinase-C domain

Table 4.3 Primer sequences of quantitative real-time PCR used for quantification of expression of *Rvi12* candidate resistance genes

Candidate genes	Forward primer	Reverse primer
<i>Rvi12_Cd1</i>	AACCAAGTGAGGTGCATCAAGG	CGCATTCTCTGTAGCAAGAACC
<i>Rvi12_Cd2</i>	ACTTGGTGCAAGGAATTGCTCAG	ACCTCCATAGTTGCCTGTCTCC
<i>Rvi12_Cd3</i>	TACAAGAAGAGGAAGACTGAGCAC	GATGAGGCAGGAAGAGAAGGC
<i>Rvi12_Cd4</i>	TCTAATGGCGTCTACCTGCACAC	CCAGAGGAGTTGAGGCTCAGAC
<i>Rvi12_Cd5</i>	CCAAGTACATGGAGAGCTACCAC	CGAGATTAGGAAGCAAGATGCC

4.4.8. Expression analysis of candidate genes

The expression of the five identified candidate genes was analyzed in RNA from leaves of ‘Gala’ (susceptible) and ‘HB2’ (resistant) at three time points (0, 4 and 12 dpi) that had been inoculated with either the mixed conidial inoculum of *Venturia inaequalis* or water as a mock treatment. Mean normalized expression of *Rvi12_Cd1* and *Rvi12_Cd2* in ‘Gala’ and ‘HB2’ were not significantly different either between any of the three time points (student t- test, $P > 0.05$) or between the mock and *Venturia inaequalis* treatments (Figure 4.5).

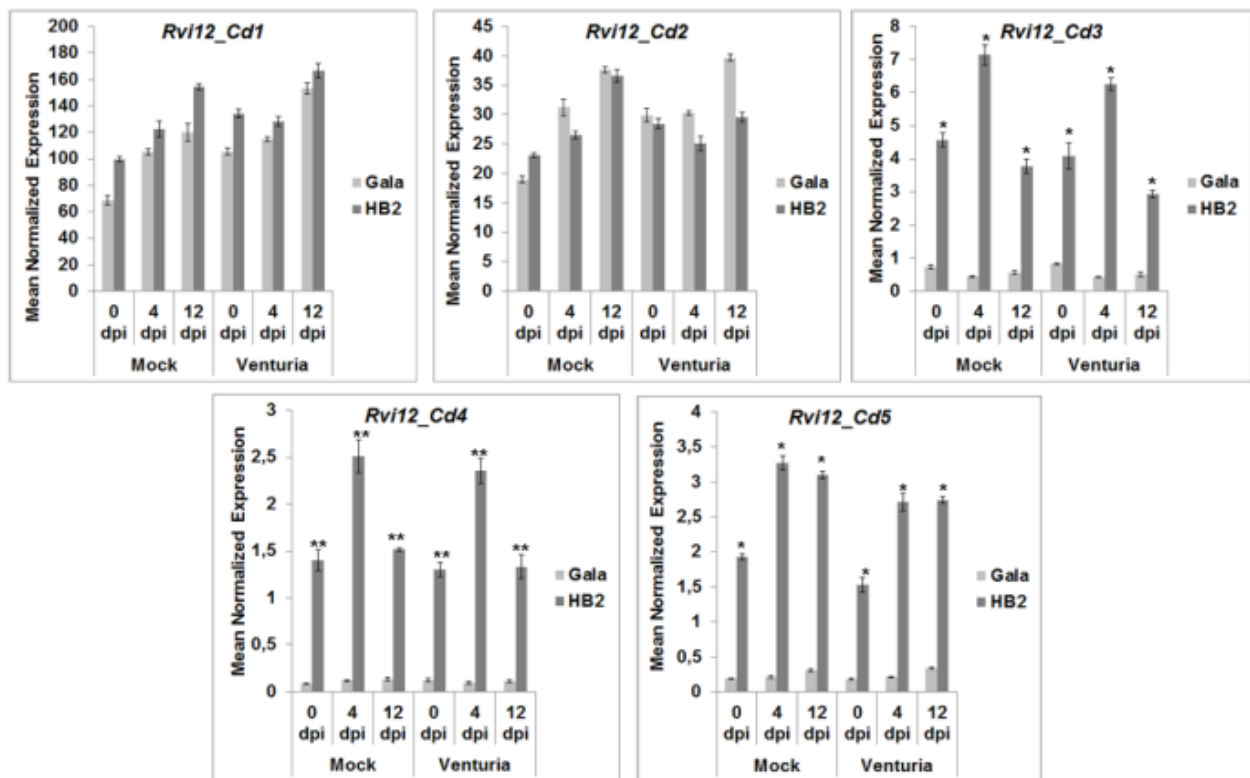


Figure 4.5 Quantitative real-time PCR-based expression of candidate *Rvi12* resistance genes. Dark grey bars and light grey bars represent mean normalized expression of candidates in ‘HB2’ and ‘Gala’ respectively. Mock treatment was performed with double distilled water. Dpi in the figure means days post inoculation; * represents statistical significant at 95% confidence; ** represents statistical significance at 99% confidence in student t-test.

Expression of *Rvi12_Cd3*, *Rvi12_Cd4* and *Rvi12_Cd5* were up to 16, 25 and 16 fold respectively higher in HB2 (Student t-test, $P < 0.005$) over the time course investigated but there was no significant difference in expression between the mock and *Venturia inaequalis* inoculation for any of the genes (student t- test, $P > 0.05$) (**Figure 4.5**). The results thus suggested that one or more of genes *Rvi12_Cd3*, *Rvi12_Cd4* or *Rvi12_Cd5* were the most likely candidates for the *Rvi12* resistance gene, and that resistance is constitutively expressed in ‘HB2’.

4.5. Discussion

A total of 23 amplicons containing at least one heterozygous SNP were identified that were putatively located in the *Rvi12* interval. However, only two of those amplicons, containing seven SNPs, mapped to the expected location, whilst the remaining 21 were located in other parts of the *Malus* genome. A subset of BAC clones were identified that spanned the *Rvi12* mapping interval during the chromosome walking steps of BAC library screening. The sequences of markers designed from the BAC ends of positive clones mapped to the *Rvi12* interval but they were physically located on the contigs of chromosome 13 of ‘Golden Delicious’ reference sequence, indicating a degree of mis-assembly in the ‘Golden Delicious’ genome sequence, as previously reported by Khan et al, (2012).

Using BAC-based chromosome walking, we located the *Rvi12* locus to a single BAC clone ‘6F11’. PacBio RS II long read sequencing technology, and HGAP assembly was used and the BAC clone sequence was assembled into a single contig. Long-read PacBio sequencing was chosen over short-read technologies for sequencing of the BAC clone, because of its superior performance in sequencing complex genomic regions (Huddleston et al, 2014) and small circular plastid genomes (Ferrarini et al, 2013). In the study of Galli et al (2010), identification of *Rvi15* candidates was done by 10× coverage shot-gun Sanger sequencing of BAC clones. Following sequence assembly, primer walking and gap filling of the sequences was then required. Even after gap filling, it was only

possible to assemble the sequence into four contigs (Galli et al, 2010). The problems faced in sequencing and assembly of resistance gene regions arise because of the highly repetitive nature of these regions and presence of clusters of resistance genes (Michelmore, 2000). Because of the cost-effectiveness of sequencing using PacBio technology versus Sanger sequencing, it was possible to sequence the BAC clone identified in this investigation to a very high coverage of over 2500×. The assembly of the sequences was straightforward and the entire BAC clone sequence was assembled into a single contig. This study demonstrates the power of the PacBio sequencing over other sequencing technologies for sequencing complex repetitive regions such as those containing resistance gene.

Five putative candidate resistance genes were identified in the *Rvi12* mapping interval. Despite blastx analysis of the *Rvi12_Cd1* and *Rvi12_Cd2* revealing homology of these genes to TIR-NBS-LRR proteins from *Arachis hypogaea*, the conserved domain search revealed the absence of TIR, NBS and LRR domains in these candidates which are essential for recognition of pathogen associated proteins or host proteins modified by pathogens and downstream signaling for disease resistance (DeYoung and Innes, 2006). Additionally, there was no significant difference in expression of *Rvi12_Cd1* and *Rvi12_Cd2* genes between susceptible and resistance plants, suggesting that these candidate genes are not responsible for apple scab resistance in ‘HB2’ at the *Rvi12* locus.

Rvi12_Cd3 and *Rvi12_Cd4* showed homology to TGA-like transcription factors which have previously been shown to play a role in disease resistance (Zhou et al, 2000; Zhang et al, 2003). However, these candidates lacked the essential DNA binding bZIP domain (Xiang et al, 1997; Zhou et al, 2000) which are characteristic of TGA transcription factors. Based on previous studies, the DOG1 domain detected in *Rvi12_Cd3* and *Rvi12_Cd4* has been shown to be involved in the control of seed dormancy (Bentsink et al, 2006), but there was no evidence for possible role of the DOG1 domain containing proteins in plant disease resistance.

The presence of a signal peptide and transmembrane motif in *Rvi12_Cd5*, suggests its localization to plasma membrane with the extracellular LRR domain and intracellular protein kinase domain. *Rvi12_Cd5* showed homology to previously well-characterized receptor like kinases, FLS2 and Xa21. Comparison of conserved domains among *Rvi12_Cd5*, FLS2 and Xa21 rice blight resistance protein showed presence of same structural domains including LRR motifs, ATP binding sites and protein kinase-C (PKc) domains essential for pathogen recognition and downstream signaling. The expression of *Xa21* gene was observed even in the absence of pathogen (mock treated resistant plants) and leaves collected immediately after inoculation (Century et al, 1999), much like *Rvi12_Cd5* in our experiment. The presence of structural domains known to be involved in disease resistance and over-expression of *Rvi12_Cd5* in the apple scab resistant variety ‘HB2’ makes it a strong candidate for *Rvi12* based apple scab resistance.

Quantitative real-time PCR expression analysis detected overexpression of three putative candidates including the two coding for TGA-like proteins and one probable LRR receptor like serine/threonine kinase in leaf tissue of the resistant ‘Hansen’s baccata#2’ genotype compared to leaves of the susceptible ‘Gala’ genotype. Further functional characterization of these three putative candidates will reveal which confers resistance at the locus and if the TGA-like proteins that do not contain the bZIP domain are able to provide apple scab disease resistance. A low level of expression of all three putative candidate genes was observed even in leaves of the susceptible ‘Gala’ genotype. Since resistance at other scab resistance loci has been shown to be conferred by genes only expressed in resistant genotypes and not in susceptible genotypes, it would be essential to check if there is any difference in sequences of the candidate genes between the resistant allele and the susceptible allele. Because of the unavailability of susceptible BAC clones covering the *Rvi12* interval, differences between resistant and susceptible genotypes in this study could not be performed directly from BAC sequencing. So further studies on, Sanger sequencing of qRT-PCR

products and candidate genes are required, to check for differences between resistant and susceptible alleles.

4.6. Concluding remarks

This study is the first report suggesting a possible role for an LRR receptor like serine/threonine protein kinase genes in apple scab resistance. However, complementation studies to produce apple plants expressing *Rvi12_Cd3*, *Rvi12_Cd4* and *Rvi12_Cd5* candidate genes individually and in combination would need to be performed to confirm the role of these candidate genes in scab resistance and conclusively demonstrate *Rvi12_Cd5* as the agent of resistance at the *Rvi12* locus. Despite this, the novel markers developed and mapped in this study can be used for extremely efficient marker assisted selection of the seedlings containing the *Rvi12* resistance locus in apple breeding programs, where it can be pyramided with other scab resistance genes for durable apple scab resistance.

Acknowledgements

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Supplementary Table S4.1 Sequences of the BAC-end primers used for screening of the 'HB2' BAC library.

Primer name	Sequence (5'-3')
SNP_23599-F	CCTTGTTGTCTGAGTATGTTGTGG
SNP_23599-R	TAATCCAAGCAACGATCACG
69B16-FSP-F	GCAAGACTTCCGAGACAAGG
69B16-FSP-R	AATTTCAATTGGGCAATTTGG
32N11-RSP-F2	GAAACCGAACGTAGAATGAGC
32N11-RSP-R2	CCAATTTTGGATTTTGGGAAGC
45E3-FSP-F	GGTGATCTGGTGCATCTCG
45E3-FSP-R	TGAACGAGCACTGAAGAAGC
SSR-56H15-RSP-F	TACACACACACACACACACA
SSR-56H15-RSP-R	CGCATCTCTCTCAAATGGCAA
SNP_24482-F	TTTTGTTGGGGATTCTTTGC
SNP_24482-R	GCTTCCAATTCTTCGTACCC

Supplementary Table S4.2 Predicted genes in the *Rvi12* gene mapping interval along with conserved domains, homologous proteins, query cover, E-value and percentage identity to known sequences.

Predicted Genes	Conserved Domains	Homologous proteins	Max Score	Total score	Query cover	E-Value	Identity	Putative candidate genes
HB2_CDS1	CUB superfamily	PREDICTED: nucleolar protein 58-like [Pyrus x bretschneideri]	432	432	100%	6,00E-147	94%	
		PREDICTED: uncharacterized protein LOC103452935 [Malus domestica]	429	429	100%	1,00E-145	94%	
HB2_CDS2	No putative domains	PREDICTED: uncharacterized protein LOC103405167 [Malus domestica]						
		PREDICTED: uncharacterized protein LOC103933713 [Pyrus x bretschneideri]	64.7	64.7	29%	3,00E-09	53%	
		PREDICTED: uncharacterized protein LOC103957011 [Pyrus x bretschneideri]	63.9	63.9	29%	5,00E-09	50%	
HB2_CDS3	No putative domains	FAD-dependent oxidoreductase [Kangiella geojedonensis]	35.0	35.0	58%	4.9	36%	
HB2_CDS4	No putative domains	No significant similarity found						
HB2_CDS5	Membrane FADS-like superfamily	PREDICTED: omega-3 fatty acid desaturase, chloroplastic-like [Malus domestica]	851	851	99%	0.0	99%	
		PREDICTED: omega-3 fatty acid desaturase, chloroplastic-like [Pyrus x bretschneideri]	817	817	99%	0.0	98%	
		PREDICTED: omega-3 fatty acid desaturase, chloroplastic-like [Pyrus x bretschneideri]	769	769	99%	0.0	92%	
HB2_CDS6	RdX superfamily	PREDICTED: selT-like protein [Malus domestica]	414	414	99%	4,00E-144	99%	
		PREDICTED: selT-like protein [Pyrus x bretschneideri]	406	406	99%	4,00E-141	97%	
		PREDICTED: selT-like protein [Prunus mume]	338	338	99%	4,00E-114	80%	

HB2_CDS7	No putative conserved domains	PREDICTED: LOW QUALITY PROTEIN: uncharacterized protein LOC103430801 [Malus domestica]	856	856	92%	0.0	80%
		PREDICTED: uncharacterized protein LOC103930534 [Pyrus x bretschneideri]	846	846	92%	0.0	79%
		PREDICTED: uncharacterized protein LOC103427620 isoform X1 [Malus domestica]	766	766	91%	0.0	72%
		PREDICTED: uncharacterized protein LOC103427620 isoform X2 [Malus domestica]	760	760	91%	0.0	71%
HB2_CDS8	No putative conserved domains	hypothetical protein [Malus domestica]	108	108	85%	1,00E-25	53%
		putative COBL7 (COBRA-LIKE 7) [Malus domestica]	103	156	69%	2,00E-22	81%
		T4.12 [Malus x robusta]	95.5	95.5	45%	5,00E-21	78%
		predicted protein [Malus domestica]	59.3	164	62%	3,00E-15	93%
		hypothetical protein COK_1569 [Mannheimia haemolytica serotype A2 str. BOVINE]	50.1	50.1	18%	9,00E-06	96%
		PREDICTED: exonuclease 3'-5' domain-containing protein 1-like [Malus domestica]	49.3	49.3	23%	5,00E-05	70%
		T1.8 [Malus x robusta]	47.0	47.0	19%	8,00E-04	92%
		PREDICTED: uncharacterized protein LOC103425572 [Malus domestica]	45.1	45.1	21%	0.005	71%
		PREDICTED: probable polygalacturonase At3g15720 [Malus domestica]	42.4	42.4	43%	0.048	48%
PREDICTED: uncharacterized protein At2g41620-like [Malus domestica]	42.4	42.4	15%	0.058	90%		
HB2_CDS9		putative COBL7 (COBRA-LIKE 7) [Malus domestica]	79.0	218	83%	4,00E-14	87%
		hypothetical protein [Malus domestica]	67.8	271	69%	1,00E-10	97%
HB2_CDS10	rad50 domian	T4.14 [Malus x robusta]	436	436	99%	8,00E-153	97%
		hypothetical protein [Cecembia lonarensis]	407	407	90%	1,00E-141	99%
		predicted protein [Malus domestica]	402	402	92%	1,00E-136	94%
		putative COBL7 (COBRA-LIKE 7) [Malus domestica]	417	417	92%	2,00E-134	98%

		PREDICTED: disease resistance protein At4g27190-like [Malus domestica]	79.0	79.0	29%	2,00E-13	61%	
		PREDICTED: serine/threonine-protein kinase HT1-like [Malus domestica]	61.2	61.2	14%	1,00E-07	88%	
HB2_CDS11	BAR superfamily	putative RNA-directed DNA polymerase (Reverse transcriptase) [Malus domestica]	1164	1164	99%	0.0	92%	
	RT like superfamily	PREDICTED: uncharacterized protein LOC103417687 [Malus domestica]	1108	1108	99%	0.0	87%	
		TIR-NBS-LRR type disease resistance protein [Arachis hypogaea]	723	723	99%	0.0	57%	<i>Rvi12_Cd1</i>
		T1.2 [Malus x robusta]	605	742	66%	0.0	85%	
HB2_CDS12	EEP superfamily	T4.15 [Malus x robusta]	655	655	96%	0.0	99%	
		PREDICTED: uncharacterized protein LOC103417687 [Malus domestica]	420	420	68%	2,00E-135	93%	
		PREDICTED: uncharacterized protein LOC103439082 [Malus domestica]	378	428	88%	1,00E-124	79%	
		TIR-NBS-LRR type disease resistance protein [Arachis hypogaea]	322	322	97%	4,00E-95	54%	<i>Rvi12_Cd2</i>
HB2_CDS13	WD40 family	PREDICTED: uncharacterized protein LOC103930534 [Pyrus x bretschneideri]	777	777	90%	0.0	96%	
		PREDICTED: LOW QUALITY PROTEIN: uncharacterized protein LOC103430801 [Malus domestica]	773	773	90%	0.0	96%	
		PREDICTED: uncharacterized protein LOC103427620 isoform X2 [Malus domestica]	735	735	90%	0.0	91%	
		PREDICTED: uncharacterized protein LOC103427620 isoform X1 [Malus domestica]	735	735	90%	0.0	91%	
		Transducin family protein / WD-40 repeat family protein, putative isoform 5 [Theobroma cacao]	514	514	90%	5,00E-170	64%	
		Transducin family protein / WD-40 repeat family protein, putative isoform 4 [Theobroma cacao]	514	514	90%	1,00E-169	64%	
		Transducin family protein / WD-40 repeat family protein, putative isoform 3 [Theobroma cacao]	513	513	90%	3,00E-168	64%	

		Transducin family protein / WD-40 repeat family protein, putative isoform 1 [Theobroma cacao]	513	513	90%	5,00E-168	64%	
HB2_CDS14	No putative domains	PREDICTED: uncharacterized protein LOC103930534 [Pyrus x bretschneideri]	330	330	82%	1,00E-101	87%	
		PREDICTED: uncharacterized protein LOC103427620 isoform X1 [Malus domestica]	320	320	82%	6,00E-98	83%	
		PREDICTED: uncharacterized protein LOC103427620 isoform X2 [Malus domestica]	320	320	82%	6,00E-98	83%	
		PREDICTED: uncharacterized protein LOC103966318 isoform X2 [Pyrus x bretschneideri]	315	315	82%	6,00E-96	82%	
HB2_CDS15	ACAD superfamily	PREDICTED: acyl-coenzyme A oxidase 4, peroxisomal-like [Malus domestica]	445	445	88%	7,00E-152	73%	
		PREDICTED: acyl-coenzyme A oxidase 4, peroxisomal [Malus domestica]	431	431	88%	5,00E-147	73%	
		hypothetical protein PRUPE_ppa005916mg [Prunus persica]	430	430	88%	9,00E-146	70%	
		PREDICTED: acyl-coenzyme A oxidase 4, peroxisomal [Fragaria vesca subsp. vesca]	410	410	86%	3,00E-138	68%	
HB2_CDS16	No putative domains	PREDICTED: uncharacterized protein LOC103940210 [Pyrus x bretschneideri]	99.4	171	53%	9,00E-34	84%	
		PREDICTED: transcription factor TGA4-like [Malus domestica]	127	189	55%	9,00E-33	78%	<i>Rvi12_Cd3</i>
		PREDICTED: transcription factor TGA4-like [Pyrus x bretschneideri]	127	174	48%	5,00E-32	79%	
		hypothetical protein PRUPE_ppa027068mg [Prunus persica]	123	176	55%	2,00E-30	73%	
		PREDICTED: transcription factor TGA7-like [Malus domestica]	124	196	53%	2,00E-30	86%	
		PREDICTED: transcription factor TGA4-like [Prunus mume]	121	173	55%	7,00E-30	72%	
		PREDICTED: transcription factor TGA4-like [Pyrus x bretschneideri]	121	171	49%	1,00E-29	79%	
		PREDICTED: transcription factor TGA2-like [Pyrus x bretschneideri]	119	119	40%	4,00E-29	75%	

		PREDICTED: transcription factor TGA4-like [Malus domestica]	114	165	50%	3,00E-27	65%	
		PREDICTED: transcription factor HBP-1b(c38)-like [Fragaria vesca subsp. vesca]	110	149	51%	5,00E-26	75%	
		PREDICTED: transcription factor HBP-1b(c38) [Cucumis melo]	89.4	118	48%	3,00E-18	47%	
		PREDICTED: transcription factor TGA4-like [Cucumis sativus]	88.6	118	48%	4,00E-18	49%	
HB2_CDS17	SPX superfamily	PREDICTED: SPX domain-containing protein 1-like [Pyrus x bretschneideri]	36.6	69.7	31%	6,00E-04	74%	
		PREDICTED: SPX domain-containing protein 1-like [Malus domestica]	36.2	69.3	31%	6,00E-04	74%	
		unknown [Lotus japonicus]	35.0	62.0	29%	0.097	65%	
		IDS4-like protein [Medicago truncatula]	35.4	60.8	31%	0.24	65%	
		IDS-4-like protein [Castanea sativa]	36.2	36.2	17%	6.1	65%	
HB2_CDS18	No putative domains	PREDICTED: transcription factor TGA7-like [Malus domestica]	242	242	61%	2,00E-75	88%	
		PREDICTED: transcription factor TGA2-like [Pyrus x bretschneideri]	145	145	33%	6,00E-39	92%	<i>Rvi12_Cd4</i>
		PREDICTED: transcription factor TGA4-like [Pyrus x bretschneideri]	130	130	33%	4,00E-33	81%	
		PREDICTED: transcription factor TGA4-like [Malus domestica]	130	130	33%	6,00E-33	80%	
		PREDICTED: transcription factor TGA4-like [Prunus mume]	119	119	33%	1,00E-28	77%	
		hypothetical protein PRUPE_ppa027068mg [Prunus persica]	110	110	32%	2,00E-25	75%	
		PREDICTED: transcription factor HBP-1b(c38)-like [Fragaria vesca subsp. vesca]	96.3	96.3	28%	2,00E-20	71%	
HB2_CDS19	PHF5 superfamily	BnaC04g40780D [Brassica napus]	228	228	99%	9,00E-74	98%	
		PREDICTED: PHD finger-like domain-containing protein 5B [Brassica oleracea var. oleracea]	227	227	99%	2,00E-73	98%	
		BnaA03g13610D [Brassica napus]	224	224	99%	1,00E-72	97%	
		Os04g0663300 [Oryza sativa Japonica Group]	223	223	99%	2,00E-72	100%	

		PHD finger-like domain-containing protein 5A [<i>Zea mays</i>]	223	223	99%	2,00E-72	99%	
		splicing factor 3b-like protein [<i>Arabidopsis thaliana</i>]	223	223	99%	2,00E-72	99%	
		PREDICTED: PHD finger-like domain-containing protein 5B [<i>Brassica rapa</i>]	222	222	99%	4,00E-72	98%	
		Pre-mRNA-splicing factor ini1 [<i>Ricinus communis</i>]	222	222	99%	5,00E-72	99%	
		Pre-mRNA-splicing factor ini1 [<i>Medicago truncatula</i>]	221	221	99%	8,00E-72	99%	
		PREDICTED: PHD finger-like domain-containing protein 5B [<i>Brassica rapa</i>]	221	221	99%	1,00E-71	97%	
HB2_CDS20	LRR receptor like Serine Threonine Protein kinase	PREDICTED: probable LRR receptor-like serine/threonine-protein kinase At3g47570 [<i>Malus domestica</i>]	1849	1849	99%	0.0	92%	<i>Rvi12_Cd5</i>
		PREDICTED: probable LRR receptor-like serine/threonine-protein kinase At3g47570 [<i>Pyrus x bretschneideri</i>]	1724	1724	99%	0.0	86%	
		PREDICTED: probable LRR receptor-like serine/threonine-protein kinase At3g47570 isoform X1 [<i>Pyrus x bretschneideri</i>]	1722	1722	99%	0.0	87%	
		PREDICTED: probable LRR receptor-like serine/threonine-protein kinase At3g47570 [<i>Pyrus x bretschneideri</i>]	1712	1712	99%	0.0	86%	
		PREDICTED: probable LRR receptor-like serine/threonine-protein kinase At3g47570 isoform X2 [<i>Pyrus x bretschneideri</i>]	1708	1708	97%	0.0	86%	
		PREDICTED: probable LRR receptor-like serine/threonine-protein kinase At3g47570 [<i>Pyrus x bretschneideri</i>]	1702	1702	97%	0.0	86%	
		PREDICTED: probable LRR receptor-like serine/threonine-protein kinase At3g47570 [<i>Pyrus x bretschneideri</i>]	1702	1702	99%	0.0	85%	
		PREDICTED: probable LRR receptor-like serine/threonine-protein kinase At3g47570 [<i>Pyrus x bretschneideri</i>]	1697	1697	99%	0.0	86%	
		PREDICTED: probable LRR receptor-like serine/threonine-protein kinase At3g47570 [<i>Pyrus x bretschneideri</i>]	1679	1679	99%	0.0	85%	
		PREDICTED: probable LRR receptor-like serine/threonine-protein kinase At3g47570 [<i>Malus domestica</i>]	1587	1587	86%	0.0	93%	
		PREDICTED: probable LRR receptor-like serine/threonine-protein kinase At3g47570 [<i>Pyrus x bretschneideri</i>]	1580	1580	99%	0.0	82%	

4.7. References

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Chapter 5

Final Remarks and Conclusion

This study was performed to genetically characterize and identify candidate genes for the *Rvi12* scab resistance locus from *M. baccata* ‘Hansen’s baccata #2’ to increase the repertoire of scab resistance genes available for developing new apple varieties with durable scab resistance.

Thorough phenotypic analysis of resistance reactions in a ‘Gala’ × ‘Hansen’s baccata #2’ mapping population, resistance reactions ranging from class 0 to class 3B, as well as the occurrence of the stellate chlorosis phenotype were observed in the segregating progeny. The observed phenotypic segregation ratio of 3:1 between resistant and susceptible seedlings, points to the presence and segregation of two unlinked scab resistance genes in ‘Hansen’s baccata #2’. The *Rvi12* resistance gene was responsible for the resistance reactions from class 0 to class 3B and it was mapped to LG12. The additional scab resistance gene from ‘Hansen’s baccata #2’ conferred the stellate chlorosis-type resistance reactions. The discovery of two unlinked genes segregating in the mapping progeny derived from ‘Hansen’s baccata #2’ could explain the reason for contradictory map positions of *Rvi12* in the reports of Hemmat et al, (2003) and Erdin et al, (2006). Erdin et al, (2006) suggested that an off-type of the ‘Hansen’s baccata #2’ genotype may have been used in the study of Hemmat et al, (2003) and could have been the cause of the different map positions of *Rvi12* in those two studies. From the results obtained from the experiments presented in this thesis, it could be concluded that both the mapping studies of Hemmat et al, (2003) and Erdin et al, (2006) could be correct with their map positions for the resistance gene because of the presence of two different resistance genes in ‘Hansen’s baccata #2’.

In the previous studies of Hemmat et al, (2003), Erdin et al, (2006) and in this study, a mixed conidial inoculum from local orchards was used for inoculation and phenotyping of segregating populations. Because of the unknown composition of the specific isolates and/or races of *V.*

inaequalis present in the different inocula, a certain degree of variability in resistance reactions is possible. It is also difficult to understand the spectrum of resistance of specific resistance genes present in the host plant by using a mixed inoculum. The presence or absence of specific isolates of *V. inaequalis* in the different inocula used for phenotyping can lead to different resistance reactions based on the R-genes and Avr genes interactions in the *Malus-V. inaequalis* 'pathosystem' and thus lead to mapping of completely different resistance genes between studies. Therefore, multiple inoculations with different well characterized reference isolates of *V. inaequalis* would be the best way to get a clear understanding of the specific interaction of R-genes from *Malus* germplasm and their cognate Avr genes from *V. inaequalis* populations. Such an approach would also help with the identification and mapping of novel scab resistance genes and permit a greater understanding of the nature of resistance from such novel sources, as broad- or narrow-spectrum resistance. Based on the new system of nomenclature (Bus et al, 2011) of the differential interaction between *Malus* – *Venturia inaequalis*, 'Hansen's baccata #2' was placed as a temporary differential host 12 because of the uncertain nature of monogenic inheritance of scab resistance. From this study, it is clear that scab resistance of 'Hansen's baccata #2' is not monogenic and is controlled by at least two unlinked genes but the mapping of the second resistance gene was not performed yet. Once the second scab resistance gene from 'Hansen's baccata #2' has been characterized genetically, it would be possible to identify progeny plants carrying just the *Rvi12* resistance from LG12. The progeny plant with *Rvi12* based monogenic scab resistance could then be placed as the new differential host 12.

Five novel SSR markers and 25 novel SNPs closely linked to the *Rvi12* locus were developed in this investigation. The development of novel markers in the specific genetic interval was made possible by the availability of the 'Golden Delicious' reference genome sequence. The use of a large mapping population containing 1285 seedlings and the reference genome guided the design of novel markers and permitted the fine mapping of the *Rvi12* locus from the previously reported genetic interval of 21 cM to less than 1 cM, corresponding to a physical interval of 882 kb of the

‘Golden Delicious’ genome sequence. The strategy of directed marker design from the *Rvi12* mapping interval by using the ‘Golden Delicious’ reference genome sequence worked well and helped with the reduction in time required for novel marker development and efficient fine mapping of the *Rvi12* locus.

A large insert BAC library of ‘Hansen’s baccata #2’ was developed and used for chromosome walking and identification of contiguous BAC clones covering the *Rvi12* mapping interval. The position of *Rvi12* was narrowed down to a single resistant BAC clone by designing novel SSR and SNP markers from BAC-end sequences and from the *Rvi12* interval of the ‘Golden Delicious’ sequence. During the chromosome walking analyses, no susceptible BAC clone covering the *Rvi12* interval was identified. Using PacBio single-molecule real-time (SMRT) sequencing, a single BAC clone containing the *Rvi12* resistance locus was sequenced in its entirety and assembled into a single sequence contig. The PacBio long read sequencing technology was chosen over Illumina or Sanger sequencing because of its superior performance in sequencing complex repetitive genomic regions (Huddleston et al, 2014). The hierarchical genome assembly process (HGAP) assembly results from the BAC clone assembly provided the entire BAC clone sequence in a 118 kb contig without requiring time consuming gap filling steps. Such gap filling steps are often necessary when using short-read sequencing or low coverage Sanger sequencing, since multiple contigs are generally derived from such approaches, and these contigs are then scaffolded, leaving embedded gaps within the assembled sequence. The PacBio sequencing and assembly of the BAC clone that contained a number of possible candidate resistance genes produced a high quality, single contiguous sequence providing the best possible platform for further characterization of the gene candidates. In the BAC contig, the *Rvi12* was contained in a physical interval of 62.3 kb flanked by two mapped markers SNP_32N11-RSP and SCAR-6F11-FSP. These markers were very closely linked to the *Rvi12* locus with just three recombination events between them in a population of 1285 seedlings. The flanking markers developed from the BAC-end sequences and the novel markers

developed using the ‘Golden Delicious’ genome sequence for fine mapping are very tightly linked to the *Rvi12* locus, making them suitable for precise selection of seedlings containing *Rvi12* resistant alleles in marker assisted breeding programs. However, with the available BAC clone sequence, it would be possible to develop additional markers even closer to the resistance locus.

Gene prediction from the BAC clone sequence between the *Rvi12* flanking markers predicted 20 genes in *Rvi12* interval. Comparison of the predicted gene sequences with known protein sequences identified five putative candidate genes that showed homology to genes known to be involved in resistance (TIR-NBS-LRR gene, TGA like transcription factors and an LRR receptor like serine threonine protein kinase). Further analysis of conserved domains of proteins coded by the putative candidate genes revealed the absence of important protein domains such as TIR-NBS-LRR and bZIP domains essential for disease resistance in four out of the five putative candidates. Thus, a single gene encoding a probable LRR receptor like serine/threonine kinase containing all the essential domains for disease resistance was the strongest candidate for *Rvi12*-based apple scab resistance. Quantitative real-time PCR expression analysis detected overexpression of three putative candidates including the two coding for TGA-like proteins and one probable LRR receptor like serine/threonine kinase in leaf tissue of the resistant ‘Hansen’s baccata#2’ genotype compared to leaves of the susceptible ‘Gala’ genotype. Further functional characterization of these three putative candidates will reveal which of the candidate genes confer scab resistance at the *Rvi12* locus. A low level of expression of all three putative candidate genes was observed even in leaves of the susceptible ‘Gala’ genotype. Since resistance at other scab resistance loci has been shown to be conferred by genes expressed only in resistant and not in susceptible genotypes, it would be essential to check if there is any difference in sequences of the candidate genes between the resistant and the susceptible alleles of ‘Hansen’s baccata #2’ and ‘Gala’. Because of the unavailability of susceptible BAC clones covering the *Rvi12* interval, differences between resistant and susceptible genotypes could not be identified in this study through the direct sequencing of

BAC clones. An alternative approach would be to use PCR amplification and Sanger sequencing of the candidate genes from the genomic DNA of ‘Gala’ and ‘Hansen’s baccata #2’, however, due to constraints of time, it was not possible to perform these experiments in the context of this thesis study. Along with identifying functional changes in the gene sequences or promoters of the candidate resistance genes, complementation studies would also need to be performed to confirm the role of these candidates in apple scab resistance.

The markers developed and the candidate genes identified for *Rvi12* during the course of this thesis project provide the first resources for developing apple varieties with *Rvi12* scab resistance and pyramiding of *Rvi12* with other known scab resistance genes. There are different methods that can be used to develop the scab resistant varieties with *Rvi12* scab resistance. Marker assisted pseudo-testcross breeding is one of the methods that can be used to introduce the *Rvi12* gene from wild germplasm to cultivated varieties. The method of pseudo-test crossing was largely used to develop cultivars with *Rvi6* resistance from *M. floribunda* ‘821’. The wild varieties of apple, including ‘Hansen’s baccata #2’ produce small fruits with unacceptable fruit quality. So while introducing the resistance gene through pseudo-testcrossing, because of linkage drag, undesirable characters including low fruit quality are inherited along with the scab resistance. Thus it requires multiple generations of pseudo-testcrossing with different cultivars to develop the scab resistant cultivar with acceptable fruit quality. Because of the long vegetative phase of apple, it takes long time to generate a resistant cultivar with a single major resistance gene itself and to develop apple varieties with pyramided scab resistance it takes many decades of breeding.

An alternative and faster approach for developing cultivars with scab resistance would be to perform transgenesis or cisgenesis with the identified *Rvi12* candidate genes and other known scab resistance genes. Previously, transgenic ‘Gala’ plants were developed containing *Rvi6* and *Rvi15* scab resistance genes and these transgenic genotypes were shown to be resistant to *V. inaequalis*. This method of developing scab resistance is relatively quick compared to the marker assisted

breeding approach. The ethical issues and product acceptability by the consumer of genetically modified transgenic apple varieties are the main hurdles to the commercialization. To date, it is not possible to cultivate transgenic apple lines in many countries of the world, due to the regulatory limitations and a lack of consumer acceptance of such transgenic products.

Cisgenic plants are developed through genetic modification using the natural gene sequences from a crossable, sexually compatible gene pool. Cisgenic ‘Gala’ lines with *Rvi6* scab resistance gene were previously developed and characterized to be scab resistant. It is now possible to generate cisgenic apple lines with other available candidate genes for *Rvi6*, *Rvi15*, *Rvi18*, *Rvi1* from previous studies and from the candidates for *Rvi12* from this study. Transgenic or cisgenic lines can be developed not only with single major resistance genes but also with a gene cassette containing combination of scab resistance genes. The use of this approach could drastically reduce the time required for generation of apple lines containing pyramided scab resistance genes avoiding the problems of linkage drag.

The candidate genes identified for scab resistance to date, code for different classes of resistance proteins such as receptor like protein for *Rvi6*, a TIR-NBS-LRR protein for *Rvi15*, a TIR-NBS-LRR and serine threonine phosphatase 2A for *Rvi1* and a lectin-like receptor for *Rvi18*. This study identified a strong candidate gene coding for a receptor like kinase protein for *Rvi12* based scab resistance. Because of the diversity in the classes of resistance proteins, they can localize to different regions of the cell and detect different avirulence proteins from the pathogen and respond to them through different downstream signaling pathways. Thus, the identification of cognate *Avr* genes recognized by the candidate scab resistance genes and their downstream signaling for disease resistance is essential for selecting the right combination of resistance genes to develop durable resistance in a single genotype.

In conclusion, the *Rvi12* locus was fine mapped, and closely linked markers were developed which can be used to select scab resistant varieties through marker assisted selection. Candidate genes for *Rvi12* were also identified and characterized, and these can be used to generate scab resistant apple varieties in a reduced time-frame through the use of transgenic or cisgenic technology. Further knowledge gained from *Malus-Venturia* interactions and studies on downstream signaling events of scab resistance genes will help to make the decisions surrounding the combination of R-genes selected for pyramiding and thus the development of durable scab resistance. Through the increase in number of identified scab resistance genes and the current active research on studies of downstream signaling events involved in scab resistance, sustainable apple production with durable scab resistant apple cultivars may become a reality in the near future.

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Appendix

Predicted CDS sequences of putative candidate genes for *Rvi12* scab resistance

>*Rvi12_Cd1*

ATGTGGGATTCATGGCTAGTTGTATCCGAAAAGTAGCAAAAGAGGTATTAGGAGAGTCC
AAGGGCTTTGCCCCACACCAAAGGAATCTTGGTGGTGGAAATGAGGAGGTACAAACAAAG
GTGAAGGCTAAGAAGGAATGTTGTAAAGCCTTATACAAGGATAGGACTGATGAAAATGGT
GAAAGGTATAGAAAAGCGAAAACAAGAGGGCAAGAAAAGCTGTGAGAGAAGCTAAGTTAGCG
GCTTACGACGATATGTATAAACGACTAGATACCAAAGAAGGAGAGTTGGATATCTATAAA
CTAGCTAGAGCAAGGGAAAAGAAGACAAGGGACCTAAACCAAGTGAGGTGCATCAAGGAT
GAGGATGGAAAGTTCTTGTACAGAGAATGCGGTAAAGACAGATGGAGAGGTTATTTT
CATAATCTTTTCAATGAAGGACATGAAAGGAGTGCCTCTTTAGGGGAGTTGAGTAACTCA
GAAGAGTGTAGAACTACTCTTTTTATCGTCAATCCGGAAGGAAGAAGTGGTTGTAGCT
TTGAAGAAGATGAAGCATAGAAAAGCAATAGGCCAGACGATATACCAATCGAAGTGTGG
AAAGTTTTGGGAGAGACAGGTATAACATGGCTCACTGACCTTTTCAATAGGATTTTGA
ACGAAGAAGATGCCAAATGAGTGGCGAACGAGCACTTTGGTGCCTATCTACAAGAATAAG
GGCGACGTACAAAATGTCATGAACATAGGGGTATTAAGCTAATGAGTCATACAATGAAG
CTCTGGGAGAGAGTCATTGAGCATAGATTGAGGCAAGAGACACGGGTTTCGGACAACCAA
TTCGGGTTTCATGCCAGGGCGCTCAACCATGGAGGCAATCTATCTCTTACGAAGATTGATG
GAAAGATATAGAGATGGGAAAAGGATTTACACATGGTCTTTATAGATTTGGAGAAAAGCG
TACGATAGGGTCCCAAGAGACATCTTTGGAGGATTTTAGAGAAGAAAGGAGTACGAGTA
GCATATATCCAAGCTATAAAGGATATGTATGAAGGAGCAAAGACTGCCGTAAGAAGTCA
GAAGGACAACTGAAAGCTTTCCATAACTGTAGGATTACATCAAGGCTCATCTTAAGT
CCTTACCTTTTTGTGTTGGTAATGGATGAGTTAACAGGACATATCAAGATGATATTCCT
TGGTGTACGCTTTTCGACAGCATATAGTGTGATAGATGAAACTCAGGAAGGAGTAAAT
GCAAAGCTTAACCTTTGGAGAGAAGTGTGGAATCTAAAGGCTTTCGCTAAGCCGATCA
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AGGATCGGTGATCAAGAAATACCAAAGAGTGATCGTTTTCGTTACCTAGGATCTATCTTG
CAAAAAACGGAGAATTAGATGGAGATCTCAACCATAGAATACAAGCTGGATGGATGAAG
TGGAAAGAGTGCATCCGGCGTGTGTGTGACCGCCGATGCCACTGAAGCTCAAGGGAAAA
TTTTATAGGACGGCAATAAGGCCGGCGATGCTGTATGGCACAGAATGTTGGGCGGTGAAG
CATCAACACGTACACAAAATGGGTGTAGCGGAGATGAGGATGCTTCGTTGGATGTGTGGG
CACACGAGAAAGGATAAGATTAGGAATGAGGATATCCGGGGTAAAGTAGGAGTAGCCGAA
ATTGAAGGAAAGATGAGAGAAAATCGGTTACGGTGGTTTGGACATGTGCAAAGAAGGCCT
ACTGACGCTCCGATTAGAAGATGCGACTATGGGACAGAGGTTTCAGGGCCGAAGGGGTAGA
GGAAGACCTAGGAAAACCTTGGAAAGAGACTCTAAGAAAAGACTAG

>*Rvi12_Cd2*

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GGCCCGAGCACAGTGATAAGTGAGCAAGGGTCGCTGTATCTCCATCGGCACCCGGATGCA
GTGTTAAATGAGCAAGGGGGCCATAGAACTTCTTTTTCGAACGACTCCACTCAAAGTTGT
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ATTAGACGGGGGAGGGTGAAGAAGTTAGGACAGAAGGGTAGAGTTCAAGAGAGCAAATG
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GCAAAGGATCTAGAAAACCTCAGGGTTTTAAACTTTGGTATTCGGGCACACATAGAACGAGA
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TCAAAAACACAAATAGATTTTCTTCTAATGAGGAAAGGGGATCGTATAACTTGTAAAGGAT
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CATATCAAAGAGAGACAAAAGAACAAGACTTGGAAAGTGCCCAAGGACTAG

>*Rvi12_Cd3*

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AACCGTCCCGCAGCAACCGGTATATTTTTCTCGTACTTCCAACGAAAGGAGCCTGCTTTGG
ATCGCCGGGTACAAACCGGTATCGTATTCGGCTCGTGACCGAGTCGGTGCCCGATTTG
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GCGCTCAACGACAAGCTCGCCAAGATTCACAAGAGCGTGGCGGCGTCGCCGTTTCATGGAT
GCAGTGGGCGGCGAGTGGGTTTTTGACGGCGGTGAGGTAGTTTCAGCTAAAGATTCCGGAG
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GAGTCCAATTAG

>Rvi12_Cd4

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ACCCCCCCCCCTCGGCTGTTCCCTCTCTAATGGCGTCTACCTGCACACACCTCTCCGT
CTGAGCCTCAACTCCTCTGGTTTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
GAAAAAACAAAATGCATGAACCTTGCTCTCGCAGTATCCTCACCCATTAGCATTACTTGC
CCACGAAGCAAATCAAAAACCAAAGCCCAAAGAAATCCCCACCTTTTGAATCCCACC
TCCTATTTAATCCCACCATCTTTCTGTTTTTCTCGCCATTAAAGCCCCAGAAAGCTTT
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GAGGCATTCTACGAGGGCTGGCCGGTCCGGCAAGAACAATTCCTCGATGAGCTCTTATCG
GCCAGCAGCAGACGATCGACGAGGCCGAGATGAAGACCTCCGAGATTTGGTGTCTCGG
GTTCTCTTCCATTACCAGCAGTATTACGACGAGAAATCGCGATTTGGACAGAGGGAAGTT
TAA

>Rvi12_Cd5

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ACCTTGCTTGGAAACGAATCTGATCACCTGGCTTTGCTAGACTTCAAGAAAAGAATAACT
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CCATTCCCTTCCATTGGAAATCTCACTCGTCTAATGAAATAAGCTTGGGAATCAACGAG
TTTCATGGAGAAATTCCTCAAGAATTTGGTCTGACTACGTACCCTAGAAAGTCTCAACCTG
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AAAGCTCAAGAAGGCTAA

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