UNIVERSITÀ DEGLI STUDI DI UDINE
hic sunt futura

www.uniud.it

Corso di dottorato di ricerca in:

"Agricultural Sciences and Biotechnology"

in convenzione con (Research and Innovation Centre - Fondazione Edmund Mach)

Ciclo (34°)

Titolo della tesi

"Functional characterization of *Rvi12_Cd5* against apple scab via transgenesis and cisgenesis approaches"

Dottorando **Ayesha Yousaf**

Supervisore Dr. **Mickael Malnoy**
Co-supervisore Dr. **Paolo Baldi**
Dr. **Andrea Patocchi**

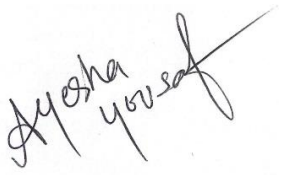
Anno (2022)

* Da completare solo nel caso di convenzione in esser

Declaration

This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly with due reference to the literature and acknowledgement of collaborative research and discussions. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma.

Signature



Date 02-03-2022

Dedication

This thesis is wholeheartedly dedicated to my parents, ***Asia Yousaf*** *and* ***Muhammad Yousaf Jahangir****, especially to my endearing mother, who have always been my source of inspiration and gave me strength of staying on my feet and not giving up on achieving high goals of life.*

To my ***siblings****, who have brought my hopes to the destination.*

Acknowledgment

I would like to thank the following people for helping me during my PhD studies:

I owe a profound sense of gratitude and indebtedness to my Research Supervisor, ***Dr. Mickael Arnaud Malnoy****, for his priceless support, immense patience, constant encouragement and necessary advice. His positive criticism and pragmatic approach to research pushed me forward in deepening my knowledge regarding the subject and carrying out this thesis.*

I pay special regards to co-supervisor, ***Dr****.* ***Paolo Baldi****, for his valuable guidance in outlining my research, constructive discussion, timely assistance in sorting out technical problems in the lab or greenhouse and statistical data analysis guidelines.*

I am also grateful to external co-supervisor ***Dr. Andrea Patocchi****, for his valuable suggestions, willingness to impart his knowledge and optimism to make me feel confident in my abilities to carry out the research work.*

I am also grateful to ***Dr. Lorenza Dalla Costa****, for giving me his valuable time to teach me the Real-Time PCR and statistical data analysis using.*

I greatly appreciate ***Stefano Piazza*** *for his countless advice, positive criticism and encouragement to improve upon my Research Work.*

I would also appreciate ***Valeria Gualandri*** *for her help during preparation and management of all scab trails****.***

I would also like to acknowledge the support of my dear friends, lab mates and colleagues at ***Fondazione Edmund Mach****.*

Ayesha Yousaf

Table of Contents

List of Abbreviations

EcPs	Extracellular Proteins
RGAs	Resistance Gene Analogs
NBS	Nucleotide-Binding Site
GO	Gene Ontology
PEST	Pro–Glu–Ser–Thr Signature
EC	Enzyme Commission Number
KEGG	Kyoto Encyclopedia of Genes and Genomes
KASP	Kompetitive Allele Specific PCR
HIDRAS	High Quality Disease Resistant Apples for Sustainable Agriculture
NBTs	New Plant-Breeding Techniques
RAPD	Random Amplified Polymorphic DNA
BSA	Bulk Segregant Analysis
GSA	Genome Sequence Archive
HB2	Hansen's Baccata #2
MAB	Marker-Assisted Breeding
TM	Transmembrane
ROS	Reactive Oxygen Species (ROS)
H_2O_2	Hydrogen Peroxide
O_2^-	Superoxide
LRR	Leucine-Rich Repeat (LRR)
LRRKm	LRR Receptor-Like Protein Kinase
PR Proteins	Pathogenesis-Related Proteins
TFs	Transcription Factors

EIL2	EIN_3-Like Proteins
DPI	Days Post-Inoculation
ml^-	Per Milliliters
mm	Millimetre
µL	Microlitre
h	Hours
HcrVf	Homologues of *Cladosporium fulvum* Resistance Genes of the Vf Region
HR	Hypersensitive Response
Chl	Chlorosis
N	Necrosis
SN	Stellate Necrosis
SC	Stellate Chlorosis
mr	Melanized Ring
Sh	Subcuticular Hyphae
UV	Ultraviolet
PAMP	Pathogen Associated Molecular Patterns
PTI	AMP-Triggered Immunity
ETI	Effector Triggered Immunity
ISR	Induced Systemic Resistance
GfG	Gene-for-Gene
cf	*Cladosporium fulvum*
MT3	Metallothionein 3-Like Proteins
LOX	Lipoxygenase
LTPs	Lipid-Transfer Proteins
EDS1	Enhanced Disease Susceptibility1 Protein

QTL	Quantitative Trait Locus
TIR-NBS-LRR	Toll-Interleukin Receptor Domain Nucleotide Binding Site–Leucine-Rich Repeat
RPLs	Receptor-Like Proteins
RLKs	Receptor-Like Kinases
ECS	Endocytosis Cell Signaling
NLS	Nuclear Localization Signals
MAP	Mitogen-Activated Protein
SSRs	Simple Sequence Repeats
AFLPs	Amplified Fragment Length Polymorphisms
SNP	Single-Nucleotide Polymorphism
SCAR	Sequence Characterized Amplified Region
MAS	Marker-Assisted Selection
GSA	Gene-Set Analysis
LRR-RLP	Leucine-Rich Repeats Receptor-Like Protein

Summary

The *Rvi12* (*Vb)* region, originating from the Siberian crab apple *Malus. baccata* 'Hansen's baccata #2' (HB2), confers apple scab resistance to the fungal pathogen *Venturia inaequalis*. Previously, gene prediction and *in silico* characterization identified a single putative candidate resistance gene, named as *Rvi12_Cd5* at *Rvi12 (Vb)* locus. The constitutive expression of *Rvi12_Cd5* in' HB2', together with its structural similarity to known resistance genes, makes it the most likely candidate for *Rvi12* scab resistance in apple. In this study, the cloning and functional characterization of *Rvi12_Cd5* was performed to confirm its functional role in *Rvi12* based scab resistance. The gene was cloned with 35S promoter and inserted into the susceptible apple cultivar 'Gala' via *agrobacterium*-mediated transformation. Inoculated transgenic 'Gala' lines, overexpressing *Rvi12_Cd5* were able to induce high to partial resistance against mixed inoculum of *V. inaequalis* with range of resistance symptoms (class 0-3b without class 1) strongly suggesting that the candidate gene *Rvi12_Cd5* is the functional *Rvi12 (Vb)* gene. This is the third cloned gene for apple scab resistance to date. In an overview of *Rvi12_Cd5* natural promoter sequence *in-silico* analysis revealed transcription factor binding sites (TFBSs) corresponding to six major plant defense related transcription factors such as AP2/ERF, bHLH, TGA/bZIP, MYB, NAC, and WRKY. To generate cisgenic lines, *Rvi12_Cd5* under the control of its own promoter was transferred to scab susceptible apple cultivar 'Gala'. A vector designed with three molecular mechanisms for cisgene expression, kanamycin resistance for selection of transgenic apple and T-DNA excision system by heat inducible site specific FLP/FRT recombinase system respectively, was used in *Agrobacterium*-mediated transformation. The candidate gene *Rvi12_Cd5* was able to induce high to partial resistance against *V. inaequalis* under its own promoter. After transformation, all transformed lines were analyzed to assess copy number, integration site, mRNA expression level and resistance to apple scab. All of these lines induced low mRNA expression of *Rvi12_Cd5* compared with the natural expression of 'Hansen's baccata # 2' except for one cisgenic apple line. Even a low expression of *Rvi12_Cd5* was sufficient to induce plant reaction and reduce fungal growth compared with the scab-susceptible 'Gala' except two transformed lines. The site specific FLP/FRT recombinase system was efficace to eliminate completely the T-DNA cassette in five independent apple cisgenic lines carrying a single insertion of *Rvi12_Cd5*. These lines need another layer of validation for T-DNA insertion sites and exogenous DNA removal by PCR and sequencing.

Chapter 1

General Introduction

1.1 Apples: Important fruit crop

For over the centuries, Rosaceae family had gained substantial recognition for its genus *Malus* (apple trees) for its nutritional values and flavor. *Malus x domestica* Borkh, the cultivated apple, is an interspecific hybrid species with allopolyploid origins (Cornille *et al*., 2012; Harris *et al*., 2002; Phipps *et al*., 1991; Robinson *et al*., 2001; Velasco *et al*., 2010). According to morphological and genetic data, *M. sieversii* (Lodeb.) Roem was believed to be the main progenitor species which hybridized with both European and Asian species throughout its domestication, and contributed to the *M. domestica* gene pool (Brown, 2012; Janick *et al*., 1996). *M. sylvestris*, a wild European crabapple, has been recognized as the second most important contributor to the apple genome (Cornille *et al*., 2012; Sun *et al*., 2020). Thus, there are thousands of apple cultivars, yet only a small percentage of them are used commercially. Some of these, like 'Delicious' and 'Gala', are produced all over the world, while others are grown nearly exclusively in one place. Others, such as 'Granny Smith', 'Jonagold', 'McIntosh', 'Cox's Orange', 'Fuji, 'Jonathan', are grown on a large scale around the world (Turechek, 2004).

In 2020, around 86.4 million tons of apples were produced on 4.62 million hectares in over 96 countries (Fig. 1) (FAOSTAT, 2021), placing apple production at the third position (Statista (2021).

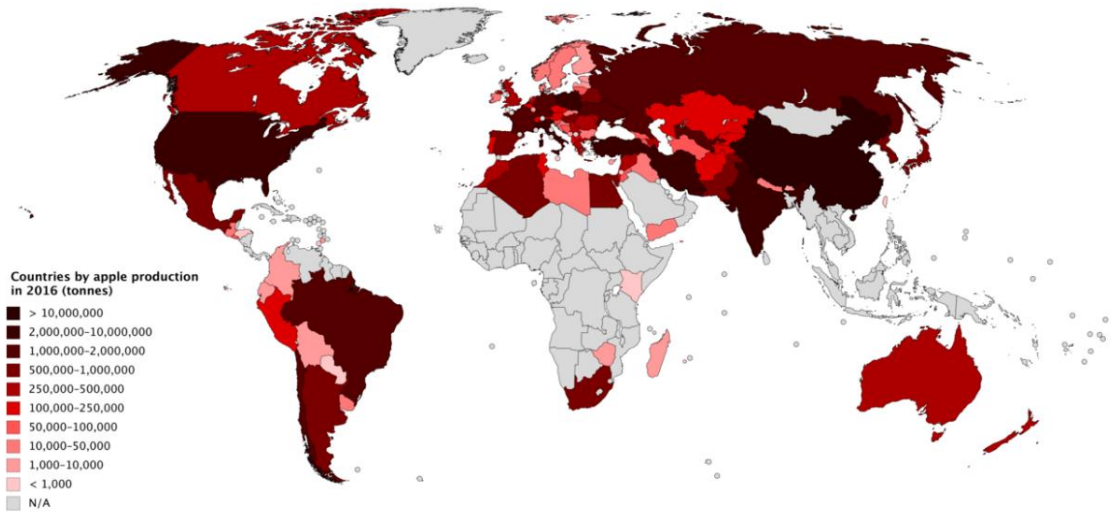


Figure 1: Choropleth map showing countries by apple production in tonnes, (FAO, 2020)

Apple is one of the most common and popular fruit crop, due to its richness in dietary fiber, vitamin C, folic acid, flavonoids and polyphenols, and other beneficial effects on human health (National nutrient database for standard reference, 2021). The epidemiological studies have linked the bioavailability and absorption of major apple phytochemicals which act as strong antioxidants, such as procyanidins, catechin, epicatechin, phloridzin, chlorogenic acid, and the quercetin glycosides, to a lower risk of coronary heart disease, stroke, hypertension, asthma, obesity and type 2 diabetes (Bondonno *et al*., 2017; Boyer and Liu, 2004; Hyson, 2011; Jensen *et al*., 2015; Sandoval-Ramírez *et al*., 2020).

Apart from the health benefits, a variety of other quality characteristics of apple fruit, such as appearance, flavor, composition, texture, and shelf life, are important to the consumers to buy a specific cultivar. For the farmers, also other characteristics of an apple cultivar are important, e.g. productivity, tree architecture, plant vigor, and resilience to abiotic and biotic stresses (Janick, 2006; Kenis *et al*., 2008).

1.2 Apple Scab: Comprehensive perspective

The fungal pathogen *V. inaequalis* Cooke (Wint.) has a significant negative impact on apple productivity (almost) worldwide, as infected fruits are unmarketable (Bowen *et al*., 2010; MacHardy, 1996a). Apple scab affects the majority of commercial apple varieties (Gessler *et al*., 2006a; Holb, 2007). All components of the tree, including leaves, petioles, flowers, fruits, pedicels, and new shoots, can be infected by *V. inaequalis* (Vaillancourt and Hartman, 2005). *V. inaequalis* has a wide geographic range and can be found in practically every temperate regions with chilly, damp weather in Spring where apples are commercially cultivated (Bowen *et al*., 2010; MacHardy, 1996).

1.2.1 Taxonomy, pathogen evolution and host range

On the basis of both morphological and molecular criteria, *V. inaequalis* a hemi-biotrophic fungus, belongs to the family Venturiacee and division ascomycetes (Lumbsch and Huhndorf, 2007). According to coalescence analysis of migration models, *V. inaequalis* originated in Central Asia, which is also the birthplace of multiple *Malus* species (spp). The disease most likely followed the spread of domesticated apples that accompanied people as they travelled to other lands (Gladieux *et al*., 2010).

For nearly a century, *V. inaequalis* has been studied in depth, uncovering its unusual parasitic strategy such as no host cell penetration occurs, yet fungal biomass accumulates in the subcuticular region prior to sporulation (Bowen *et al*., 2011; MacHardy *et al*., 2001). The topology of the *Venturia* species phylogram closely resembles that of the host genera, implying a close co-evolutionary link

between pathogen and their fruit tree hosts (Boone *et al*., 1957). The species limits within a host genus, on the other hand, are less defined; for example, several *Malus* species can hybridize, allowing gene flow between them. The resulting diversity within host genera is expected to contribute to the variability within *Venturia* species (Gladieux *et al*., 2010).

1.2.2 Life cycle, susceptibility symptoms and epidemiology

Apple scab symptoms are easily visible on the leaves and fruits of scab susceptible trees. The symptoms begin as little circular lesions on the adaxial side of the leaf, which are pale green in color. By rupturing cuticula, asexual spore (conidia) and conidiophores development causes the circular lesions which grow in size and turn velvety dark brown (Fig. 2). When the environment is suitable, the pathogen colonizes additional immature tissues, resulting in secondary infections (Vaillancourt and Hartman, 2005).

Figure 2: Scab symptom on apple leaf, showing velvety brown lesions.

Following a phase of saprobic development after leaf abscission (Fig. 3), *V. inaequalis* overwinters mostly as pseudothecia (sexual fruiting bodies) that emerge in apple leaf litter following a short phase (maximum of four weeks) after leaf abscission (i.e. necrotrophic phase). Because the fungus is self-sterile, opposite mating types are required for successful mating (Keitt and Palmiter, 1937). Without the involvement of the teleomorph (sexual stage), some overwintering can occur as conidial pustules on shoots and bud scales (Holb, 2006). Ascospores (sexual spores) produced by

rainwater from pseudothecia commence infection in the spring and early summer. This discharge is timed to coincide with the budburst and unfurling of the host's leaves (MacHardy, 1996a). Early in the growth season, when leaves and fruit are young and vulnerable, the risk of infection is greatest (Xu and Robinson, 2005).

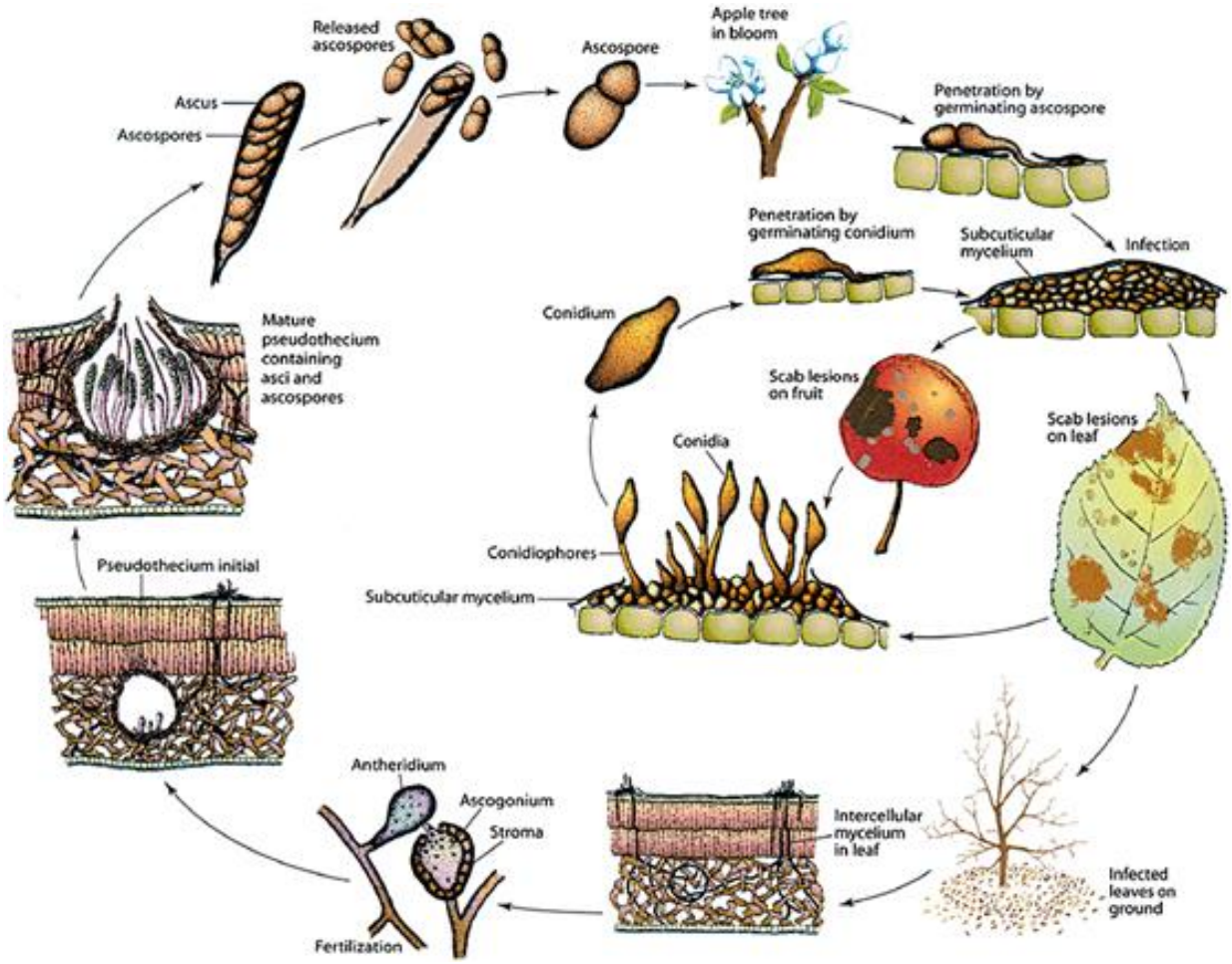


Figure 3: The life cycle of V. inaequalis. Subcuticular mycelium = stroma. (Agrios, 2005).

Ascospore-derived germ tubes penetrate the cuticle either directly or through an appressorium (Keitt and Jones, 1926; Smereka *et al*., 1988), forming multilayer, pseudo-parenchymatous structures known as stromata, which are thought to get nutrition from the subcuticular space (Fig. 4B & 4C). Scab is the name given to the disease by the stromata and the conidia they produce, which cause the characteristic leaf and fruit lesions. Conidia are distributed from lesions by wind and rain, allowing secondary infection to occur during the fruit development period. These spores, which are produced asexually, are responsible for the rise in disease. Older lesions turn dark brown, become corky and impede further growth of the fruit in that area. Eventually, they cause necrosis of the epidermis cells

and the fruit will show cracks (Fig. 4A). In this way, the fruit is more susceptible to infections by secondary pathogens (Jha *et al*., 2009a; Turechek, 2004).

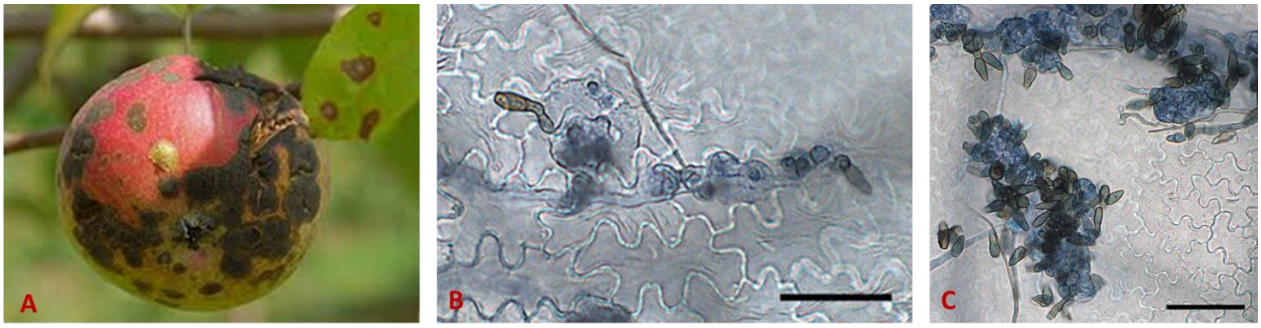


Figure 4: (A) Typical scab symptoms on an apple fruit caused by V. inaequalis (B) initial growth stage of stromata during V. inaequalis leaf infection at 5 days post-inoculation (dpi); and (C) extensive sporulating stromata at 10 dpi; Scale bar: 50 µm (Bowen et al., 2009).

1.3 *Malus-Venturia* interaction based phenotypic features

Different apple cultivars have varying degrees of scab resistance, resulting in a wide range of leaf symptoms that can be linked to various resistance reactions types. These symptoms require a well-defined classification system (Gessler *et al*., 2006). Mostly, resistance reactions are scored 3 to 21 days post inoculation (dpi). To categorize the seedlings' resistance response in greenhouse conditions, various categorization methods have been created. Hough *et al*. developed the first classification system (1944). Based on macroscopic criteria, they classified five types of symptoms: class 1 (pin-point symptoms), class 2 (chlorotic lesions without sporulation), class 3 (few restricted sporulating lesions), class M (intermediate between class 2 and class 3), and class 4 (abundant sporulation). Subsequently, several improvements were made. Chevalier *et al* (1991)'s modified classification system is still the most frequently used today. This scale was originally developed for crosses between a commercial cultivar and a *Rvi6 (Vf)* resistant genotype. Based on micro- and macroscopically resistance reactions six classes were proposed (Tab. 1).

Table 1 The six scab reaction classes (Chevalier *et al*., 1991).

Class 0	No disease symptoms
Class 1	Pin-points Pits
Class 2	Chlorotic lesions
Class 3a	Chlorotic and necrotic lesions, sometimes with light (0-1%) sporulation
Class 3b	Chlorotic and necrotic lesions with obvious (1-50%) sporulation
Class 4	Severe sporulation (>50%) without clear necrotic and chlorotic lesions

Class 0 comprises plant-pathogen interactions that do not involve any macroscopic symptoms (Fig. 5A). Due to the programmed cell death of the cells surrounding the conidia penetration site, Class 1 was defined as a hypersensitive response (HR) or pin point pits (Fig. 5B). These are small (diameter of less than 1 mm), dark depressions in the leaf surface that are the result of a HR of the host to the pathogen attack (Chevalier *et al*., 1991). At the epicenter of the response zone, the epidermal cells collapse, creating the small pit. The response goes beyond the initially affected cell into the pallisade parenchyma and neighboring epidermal cells, often affecting a large number of cells, presumably involving cell-to-cell signaling (Bus *et al*., 2005a). Chlorosis (Chl) and/or very minor necrosis (N) on the leaves without sporulation were classified as Class 2 (Fig 5C). Resistance reactions such as chlorosis or necrosis that have a weak, sparse sporulation were classified as Class 3a (Fig. 5D). The leaf surface of Class 3b had been highly coiled and crinkled after pathogen growth, with extensive sporulation in specific parts of the leaf (Fig. 5E). Complete susceptibility was defined as profuse sporulation on the entire leaf surface and no trace of leaf response in Class 4 (Fig. 5F). Plants harboring the Class 0 – 3b reactions are regarded fully resistant to scab. Plants classed as 3b have a poor resistance response, and there is a considerable risk of misclassification (Tartarini *et al*., 1999b). However, only class 4 plants are considered susceptible (Bus *et al*., 2005a). The resistance reactions displayed by each resistant variety, as well as the durability of the resistance, differ depending on the resistance gene (R-gene) the variety carries. Another two resistance reactions phenotypes were classified as stellate necrosis (SN) and stellate chlorosis (SC) (Fig. 5 G & H).

Twenty apple scab resistant gene has been identified till date, and each gene induces characteristics symptoms following infection (Bus *et al*., 2011a; Clark *et al*., 2014a; Soriano *et al*., 2014a). *Rvi2 (Vh2)* or *Rvi8 (Vh8)* genes, for example, causes SN four to six days after inoculation. Due to the late onset of the resistance response, only minimal subcuticular development is possible. The infection site produces very fine mycelial structures, which are different form distinctive stellate necrotic lesions (Bus *et al*., 2005b, 2005a). When an HR is involved, the response is frequently elicited right after the cuticle is pierced. The pin-point pits are apparent 2–3 days after infection in the case of the *Rvi5 (Vm)* gene. When other R genes are implicated, such as *Rvi15 (Vr2)* (Schouten *et al*., 2014), the reaction may take 10–11 days to manifest (Galli *et al*, 2010a). *Rvi4* (*Vh4)* elicited the classic HR (Bus *et al*., 2005a), while *Rvi12* (*Vb;* Padmarasu *et al*., 2018) and *Rvi11* (*Vbj;* Bus *et al*., 2011a) elicited flecks from yellow to necrosis and necrosis with sparse sporulation similar to *Rvi6* (*Vf)* resistance (classes 2 to 3a).

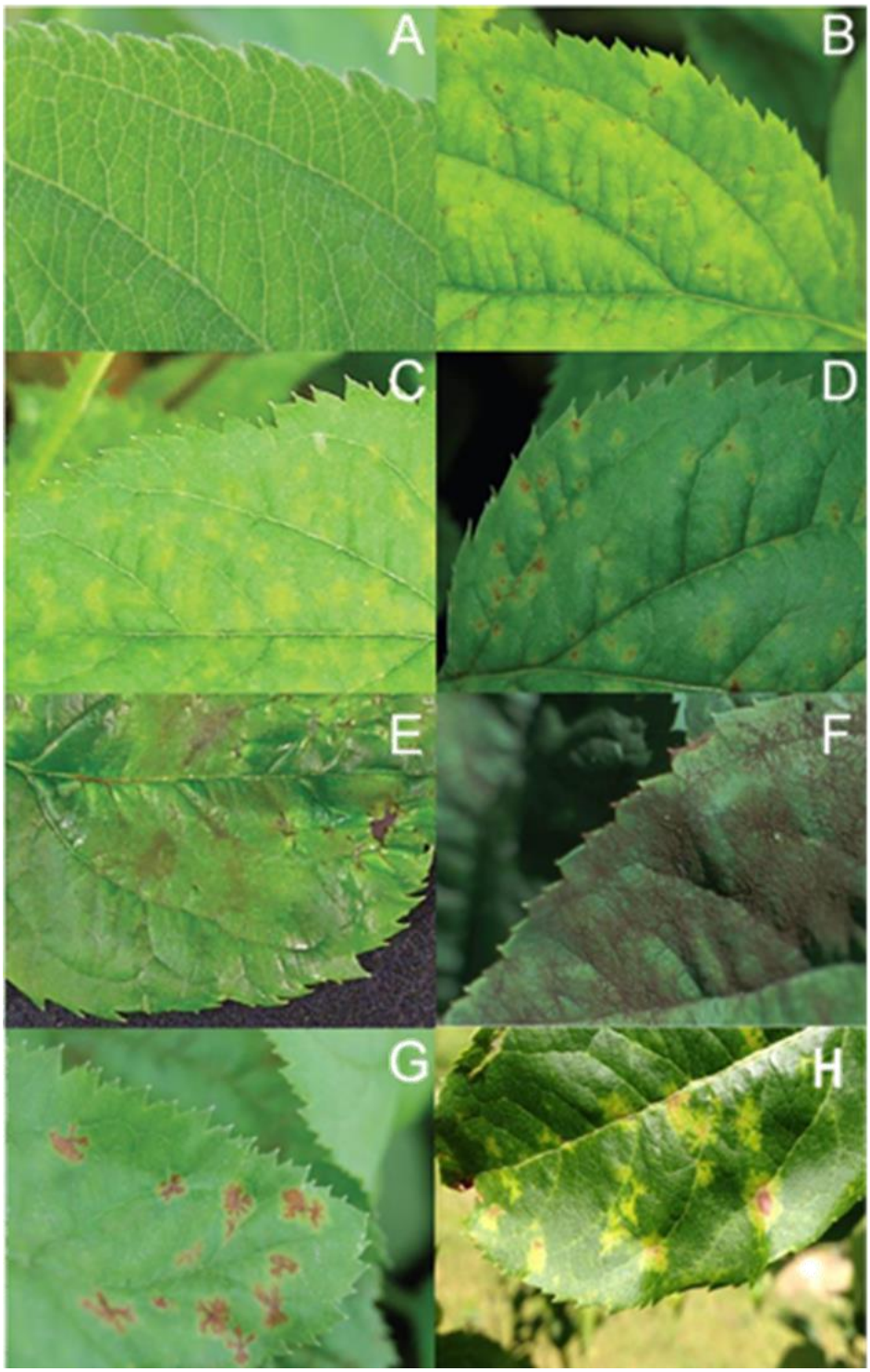


Figure 5: Resistance and susceptibility reactions of apple leaves to Venturia inaequalis inoculation. (A) Class 0 or no symptoms; (B) Class 1 - 'pin point pits'; (C) Class 2 – chlorosis and very small necrosis; (D). Class 3A – chlorosis and necrosis with sparse sporulation; (E) Class 3B – chlorosis and necrosis with restricted sporulation; (F) Class 4 – complete susceptibility with only sporulation; (G) SN – stellate necrosis; (H) SC – stellate chlorosis. Images from A-G were adapted from Gessler et al, (2006a) *and the image of stellate chlorosis reaction (H was obtained from Patocchi A (ACW, Wädenswil, Switzerland).*

1.4 *Malus-Venturia* interaction based cytological features

Susceptibility is frequently connected with a pathogen's uncontrolled growth on a host. Large spreading lesions caused by *V. inaequalis* have been reported on fruit and leaves in the field, however it is unclear whether these are the result of a single or several infection sites (Beresford *et al*., 2004). The cytology of the *V. inaequalis*-apple interaction was first described by Wis (1938). When free moisture is present on the leaf surface, conidia and ascospores germinate, most typically from the apical end of the spore. The development of adhesive mucilage, which is made up of proteins and carbohydrates (β-galactose and *N*-acetylglucosaminyl residues), helps the spore and germ tube clinging to the cuticle. A rather simple appressorium differentiates most of the time which is required for penetration (Fig. 6) (Bowen *et al*., 2011) and a melanin ring can be found at the base of the penetration peg (Steiner and Oerke, 2007).

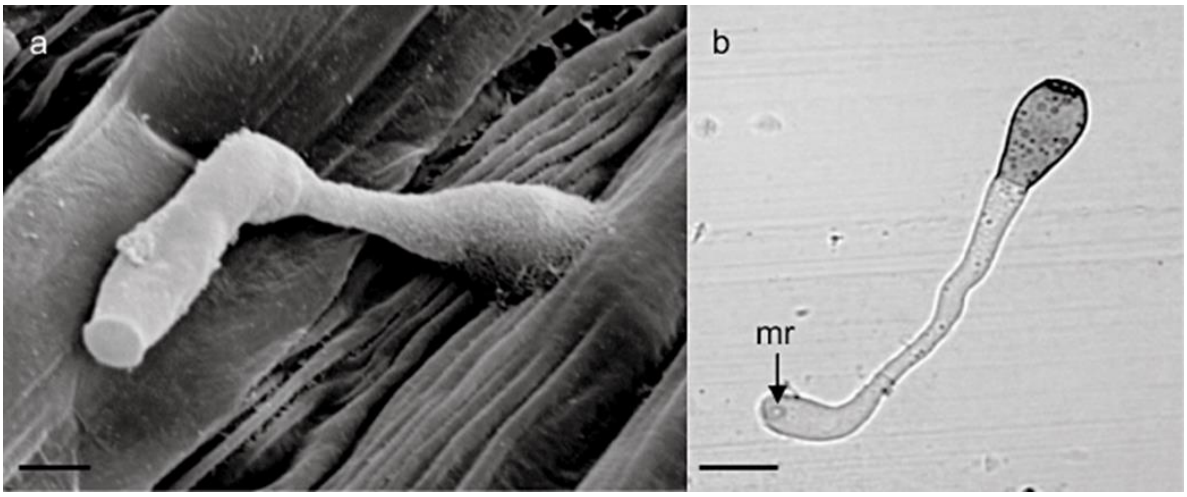


Figure 6: (a) A spore of V. inaequalis 9h post-inoculation, with germination from the apical end of the conidium and formation of an appressorium adhring to the leaf surface. Scale bar represents 5µm. (b) A germinated conidium and appressorium of V. inaequalis adhring to a slide coated in apple wax, 24h post-inoculation; mr, melanized ring at the base of the penetration peg beneath the appressorium. Scale bar represents 10µm (Bowen et al., 2011).

Mechanical pressure isn't required for penetration (Smereka *et al*., 1988), but localized enzymatic hydrolysis of the cuticle is thought to help (Koller and Parker, 1989). The extracellular enzyme cutinase, which is produced by germinating conidia and penetration, can be prevented by plant cutinase inhibitors (Koller *et al*., 1991; Koller and Parker, 1989). Furthermore, esterase-like activity has been seen in conidia germination and appressoria (Nicholson *et al*., 1972). The cuticle appears to thicken dramatically in diseased places, up to 10 times thicker than usual. It's unclear whether this is due to enzyme action or mucilage secretion (Kucheryava *et al*., 2008).

The infected peg transforms into subcuticular runner hyphae once the cuticle is penetrated, which are significantly larger in diameter than surface mycelia. Stromata form at regular intervals from these subcuticular hyphae. Stromata are made up of laterally dividing cells with diameters of up

to 10 µm, which distinguishes them from tubular hyphae seen on the plant surface, agar medium, or in the environment. The stroma's topmost cells increase, resulting in the formation of conidiophores. During the biotrophic phase of growth, *V. inaequalis* appears to have evolved to colonize the subcuticular apoplastic space. This could explain why, despite the production of callose papillae in the incompatible interaction colonization is not restricted (Kucheryava *et al*., 2008).

Apart from the cuticle breach, there is no visible harm to the host during stroma formation. Only after sporulation the epidermal cells beneath the stroma lose their plastids and cytoplasm, which is accompanied by increased vacuolation, eventually leading to necrosis, which is presumably caused by partial cell wall disintegration late in the infection cycle (Valsangiacomo and Gessler, 1988). In *V. inaequalis* growing *in vitro*, cellulase, β-glucosidase, pectinase, endo- and exo-polygalacturonase activities were identified. However, if these enzymes are only produced late in infection, after the host cell membrane has been degraded, they are unlikely to play a significant role in nutrient absorption during infection establishment (Kollar, 1998; MacHardy, 1996; Valsangiacomo and Gessler, 1988). *V. inaequalis*, like other biotrophic phytopathogens, has been shown to influence hormone levels at infection sites, particularly cytokinins, in order to aid in its biotrophic nourishment. The β-glucosidase activity releases active cytokinin from cytokinin-O-glucoside stored in the plant or fungus in this scenario, resulting in the creation of a cytokinin sink at the invasion site. The buildup of cytokinin leads to the translocation of nutrients to the fungus at this site (Cooper and Ashby, 1998).

HR is a mechanism used by plants to prevent the spread of infection by microbial pathogens. During an incompatible reaction with *V. inaequalis*, hypersensitive response (HR) is activated by the plant. Defense response is frequently elicited right after the cuticle is pierced by *V. inaequalis*. The epidermal cells collapse near the HR response zone's core, forming the little pit. Under ultraviolet (UV) light, these cells auto-fluoresce, making the reaction visible. These cells, also, go through ultrastructural changes such cell wall apposition and alterations in cellular organelles; the extent of the ultrastructural changes in epidermal and palisade mesophyll cells differs depending on which R gene is involved (Chevalier *et al*., 1991).

V. inaequalis forms very tiny mycelial structures on resistant hosts with a SN resistance reaction, which often show autofluorescence under UV light (Bus *et al*., 2005a, 2010). When compared to HR and SN reactions, the Chl resistance reaction has less autofluorescence under UV light and includes a wide range of response intensity. These distinctions in resistance phenotypic expression (HR, SN, and Chl) could be due to variances in defense signaling cascades and resistance reactions generated by various R gene-mediated recognition events. They may also represent the pathogenicity role of their cognate effectors, possibly via inhibition of resistance chemical

components. Some incompatible interactions have previously been classed as compatible due to the pathogen's generation of spores. In certain circumstances, however, sporulation is still preceded by a resistance response that is unable to control the infection but slows pathogen dissemination and sporulation rate (Bus, 2006).

1.5 Apple scab resistance mechanisms

Apple scab resistance is one of the most well characterized plant–pathogen interactions in woody plant species. Scab resistance in apples can be either found pre-existed or induced. Barriers against the pathogen, such as cuticle thickness, papillae, play a crucial part in preexisting resistance (Belete & Boyraz, 2017). Biochemical barriers such as phenolics, flavonols, melanoproteins, polygalacturonases-inhibition proteins, and others have also been identified to play a role in defense against fungal attack (Gusberti *et al*., 2013). Apple cultivars carrying *Rvi6* gene, generally have higher total phenol contents, as well as greater amounts of particular phenolic molecules, as compared with susceptible cultivars, even as these levels vary over the course of the season (Petkovsek *et al*., 2009). Another example of a phenolic compound that is present in higher amounts in older leaves and in resistant apple cultivars is chlorogenic acid (Petkovsek *et al*., 2009). Phlorizin is also the most abundant phenolic glycoside in apples and inhibits *V. inaequalis* growth (Gosch *et al*., 2009). It's mostly found in the cuticle, therefore it could affect the fungus's most crucial stage of the infection following inoculation, i.e. the germination and penetration into the subcuticular space. Phlorizin is converted to phloretin by *V. inaequalis*. This chemical also possesses antifungal properties (MacHardy, 1996). From several researches, Gessler *et al*. (2006) conclude that resistance is caused by a local accumulation and transformation activated by an elicitor, rather than a constitutive presence of phenols. When the apple is infected with *V. inaequalis*, flavanols accumulate in the area around the scab sores (Treutter, 2006). Malusfuran and dibenzofuran derivatives are formed in response to fungal infection and inhibit the germination and growth of *V. inaequalis* (Jha *et al*., 2009).

Certain apple cultivars produce reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide gas in response to fungal infection (O_2^-). This is referred to as an 'oxidative burst. These molecules can be transformed into more reactive molecules like the free oxygen radicals HO_2 and OH. ROS are associated to pin-point symptoms because they play a role in the hypersensitive response. This oxidative burst is most common in cultivars with specific main R genes, although it can also be linked to pathogen associated molecular patterns (PAMP) recognition. Following a pathogen attack, the plant accumulates proteins that protect it from ROS. ROS can also function as signal molecules for further defense mechanisms (Lamb & Dixon, 1997; Torres et al., 2006). Not only can ROS harm pathogens, but they also harm plant cells. Certain apple cultivars develop

defensive proteins to counteract this. Increased expressions of catalase, ascorbate peroxidase, polyphenol oxidase, glutathione S-transferase, and reductase were detected in the transgenic 'Gala' (*HcrVf2*) altered after infection. These enzymes are likely involved in the start of an oxidative burst as well as the detoxification of ROS (Paris *et al*., 2009).

In induced resistance, the pathogens are recognized and killed by the plants' advanced innate immune system. PAMP- triggered immunity (PTI) is induced in the resistant cultivars when the plant recognizes PAMP (Jones, 2006; Liu *et al*., 2013; Mazzotta and Kemmerling, 2011) and activates genes to secrete various anti-pathogen compounds such as Chitinases, Cysteine Protease inhibitors, *β*-1, 3-glucanase, ribonuclease-like PR10, ferrochelatase, ADP-ribosylation factor, which hinder the pathogen colonization. Induced systemic acquired resistance (SAR) by salicyclic acid in apple was observed during infection by *V. inaequalis* (Malnoy *et al*., 2007). The presence of phenolic compounds has been attributed to the autofluorescence observed under ultraviolet light during the incompatible reaction in the apple-*Venturia* pathosystem (Clark et al., 2014; Galli et al., 2010). For example, phytoalexins, amino acids, flavonoids, callose, and phenolic acids accumulate in apples during resistance response (Dai et al., 1996; Nicholson & Hammerschmidt, 1992).

To inhibit the host immune response, fungal infections have been found to release numerous types of effector molecules. Effectors are tiny signaling molecules that alter the immunological responses of the host to favor the pathogen. The host plant recognizes some of these effectors, which causes effector-triggering immunity (ETI). This is a gene for gene (GFG) interaction in which the pathogen's avirulence *(Avr)* genes are recognized by the host resistance genes *(R)*, resulting in the activation of plant defense mechanisms (Flor, 1971). According to this concept, Broggini *et al,* (2011) proposed that there should be an avirulence gene in *V. inaequalis* for every scab resistance gene reported in apple. The terminology *Rvik* (R for resistance gene; *vi* for *V. inaequalis*; *k* for differential host) has been assigned to resistance genes, while *AvrRvik* has been assigned to comparable *Avr* genes. To date, 20 scab resistance genes have been identified in apples, yet, just a few avirulence genes in *V. inaequalis* have been identified and characterized (Bus *et al*., 2011a).

Following the infection, the expression of some signal transduction genes changes. When plant receptors detect a pathogen, a cascade of signal transduction occurs, resulting in the expression of defense reactions that is locally and temporally regulated. After pathogen challenge, plants carrying the *Rvi6* gene accumulated several types of activators, kinases, phosphatases, and 'G-proteins' (i.e. guanine nucleotide-binding proteins that convey chemical signals from outside a cell into the inside of the cell) (Paris *et al*., 2009). In the cultivars 'Golden Delicious,' 'Gala,' and 'Florina,' four potential leucine-rich repeat (LRR) receptor-like protein kinases (LRPKm) were discovered. Two genes appear

to be transiently up-regulated in 'Florina' and in transgenic *Rvi6* 'Gala', 24 hours after *V. inaequalis* inoculation, but not in the untransformed, susceptible 'Gala' or 'Golden Delicious'. The LRPKm proteins were found in the plasma membranes of epidermal cells in resistant genotypes following pathogen challenge at the cytological level (Cova *et al*., 2010).

The expression of genes involved in primary metabolism, fundamental signal transduction, basic intracellular traffic, protein destination, and storage is frequently downregulated after infection. The expression of genes involved in chlorophyll production is known to be downregulated in various plant-pathogen interactions. This inhibition of chlorophyll synthesis could explain why certain scab-resistant apple varieties develop chlorotic lesions (Paris *et al*., 2009). When a plant is stressed, the catabolic, energy-supplying glycolysis pathway is often up-regulated in order to preserve equilibrium (Plaxton, 1996). Indeed, the energy supply is required to engage the resistance mechanisms that respond to stress (Block *et al*., 2005; Umeda *et al*., 1994).

A variety of Pathogenesis related proteins (PR proteins) have been shown to play a function in apple scab defense. Many of the PR proteins that are activated in susceptible apple cultivars are present on a constant basis in resistant cultivars (Degenhardt *et al*., 2005; Gau *et al*., 2004; Paris *et al*., 2009). For instance, Gau et al., (2004) studied about the apoplastic protein buildup in *Rvi6*-resistant cv. 'Remo' and the susceptible cv. 'Elstar' and found that only the resistant cultivar constitutively accumulated these proteins. The apoplast of the resistant cv. 'Remo' also had increased quantities of thaumatin-like protein (PR-5), osmotin-like proteins, and a PR-1 protein (Gau *et al*., 2004). In another study (Paris *et al* 2009) inoculating 'Gala' carrying *Rvi6* (*Hcrvf2*), found an enhanced expression of a number of Mal d 1 proteins of the ribonuclease type (PR-10).

An important phase in a plant's reaction to pathogen identification is transcriptional reprogramming. Different families of plant-specific transcription factors (such as WRKY, AP2, MYB, and NAM) have been implicated in the regulation of genes in response to pathogen assaults, elicitors, and wounding. WRKY factors, for example, bind to pathogen response elements in the promoter sequences of defense associated genes called W boxes (TTGACC/T) (Paris et al., 2009; Zhang & Wang, 2005). In apple, a putative AP2 domain-containing protein, an AP2 transcription factor/ethylene-response element and an EIN3-like protein (EIL2) have been detected in elevated concentrations after infection by *V. inaequalis* (Paris *et al*., 2009).

1.6 Principle sources of apple scab resistance

1.6.1 Ontogenic resistance

Plants have developed a complex defense system against pathogens. Apple plants possess various sources of natural defense mechanisms which can be either constitutive or inductive against *V. inaequalis*. One of the natural defense system of apple plants against *V. inaequalis* includes ontogenic resistance. *V. inaequalis* lesions on apple leaves typically spread to a maximum radius of 1 cm from the point of infection before stopping to expand (MacHardy, 1996). This is due to ontogenic resistance, which develops as the leaves and fruit age and is finished by the time the leaves are fully expanded (Gessler and Stumm, 1984). Ontogenic resistance in apples is not race specific and is characterized by delayed disease symptoms (i.e. much longer incubation time) and lower scab incidence in older leaves compared to younger leaves (Li and Xu, 2002).

Infections are mainly formed on unfolding leaves and the internodes between them (Valsangiacomo and Gessler, 1988), with ontogenic resistance developing quicker on the ventral than on the dorsal sides of the leaves throughout their growth phase (Keitt and Jones, 1926). Ontogenic resistance develops more slowly in the greenhouse than in the field, owing to differences in plant tissue maturation rates under differing environmental circumstances (Szkolnik, 1969). Conidial germination and appressorial differentiation rates in *V. inaequalis* are unaffected by leaf age, whereas stroma formation and sporulation rates and amounts decrease as host tissue ages (Gessler and Stumm, 1984). The source of this resistance is unknown, however it has been demonstrated that it becomes latent late in the growing season as leaves begin to fade and dormant lesions reappear, even on the oldest leaves (Gianfranceschi, 1996). This senescence may jeopardize the plant's ability to elicit a resistance response. All apple cultivars exhibit ontogenic resistance, which appears to have never been overcome by the disease. Even if the pathogen can establish sufficient biomass to allow successful sexual crossover throughout the winter, it has no selective drivers to overcome ontogenic resistance (MacHardy *et al*., 2001).

Ontogenic resistance is most likely owing to a mix of defense mechanisms. To begin with, the leaf's constitutive (defense) qualities might vary during ontogenesis. Furthermore, some defense-related proteins are known to be present in larger concentrations in older leaves than in younger leaves in some apple cultivars. This is true for Mal d 1,-1,3-glucanase, chitinase, and type III endochitinase (Paris *et al*., 2009). The nature of ontogenic resistance in *Malus* could be revealed by learning more about the *Cladosporium fulvum*–tomato interaction. *Cf-9B* gives resistance to *C. fulvum* to adult tomato plants, but not to seedlings, despite the fact that it is expressed in immature plants (Panter *et*

al., 2002). This suggests that *Cf-9B*'s homologous effector, *Avr9B*, may be targeting a protein that is guarded by *Cf-9B* and whose accumulation occurs only in mature plants (Wulff *et al*., 2009).

The results of a transcriptomic study permitted to hypothesize that ontogenic resistance in apple may be due to fungal growth inhibition, either due to metal ion sequestration and inhibition of pathogens' enzymatic activity by a metallothionein 3-like protein (MT3), low mineral diffusion between the cell and the sub-cuticular space by a peroxidase 3 (PX3), secondary substances produced by lipoxygenase (LOX), or by the direct action of specific enzymes such as lipid transfer protein LTP. (Gusberti et al., 2013)

1.6.2 Monogenic resistance

Monogenic resistance is the so-called 'race-specific resistance' whereby an R gene in the host has a specific interaction with an avirulence gene of the pathogen. Monogenic race specific resistance usually produced typical resistance reactions with a qualitative effect like no sporulation, necrotic spots or complete lack of symptoms (Lindhout, 2002). Aiming at a reduction of the chemical footprint of apple production, including its consequences on health, the environment and the economy, the deployment of resistance genes from wild *Malus* species in apple breeding has been attempted for a long time. Many researchers are specifically interested in the R genes, which determine the host's participation in and contribution to the gene-for-gene interaction between *Malus* and *V. inaequalis*. This is due to the relative simplicity of their genetic analysis and deployment in breeding.

Using various type of markers over the years, 20 scab resistance genes have been mapped onto 11 out of the 17 linkage groups of the apple (Fig. 7; Tab. 2). Many closely related markers such as SSR (Simple-sequence repeats), AFLP (Amplified Fragment Length Polymorphism), SCAR (sequence characterized amplified regions) and SNP (Single Nucleotide Polymorphism) have been identified and successfully employed for marker assisted selection (MAS) in apple breeding projects (Patocchi *et al*., 2009).

Until now, six scab resistance genes, i.e. *Rvi6* (Patocchi *et al*., 1999; Xu and Korban, 2002a), *Rvi15* (Galli *et al*., 2010a), *Rvi18* (Soriano *et al*., 2014a), *Rvi12* (Padmarasu *et al*., 2018, 2014) *Rvi1* (Cova *et al*., 2015c) and *Rvi5* (Cova *et al*., 2015a) were fine-mapped, and candidate resistance genes for *Rvi6* (Patocchi *et al*., 1999; Vinatzer *et al*., 1998; Xu and Korban, 2004), *Rvi15* (Galli *et al*., 2010b), *Rvi12* (Padmarasu *et al*., 2018), *Rvi18* (Soriano *et al*., 2014a) and *Rvi1* (Cova *et al*., 2015c) have been identified. Out of these only *Rvi6* (Belfanti *et al*., 2004) and Rvi15 (Schouten *et al*., 2014) have been cloned so far.

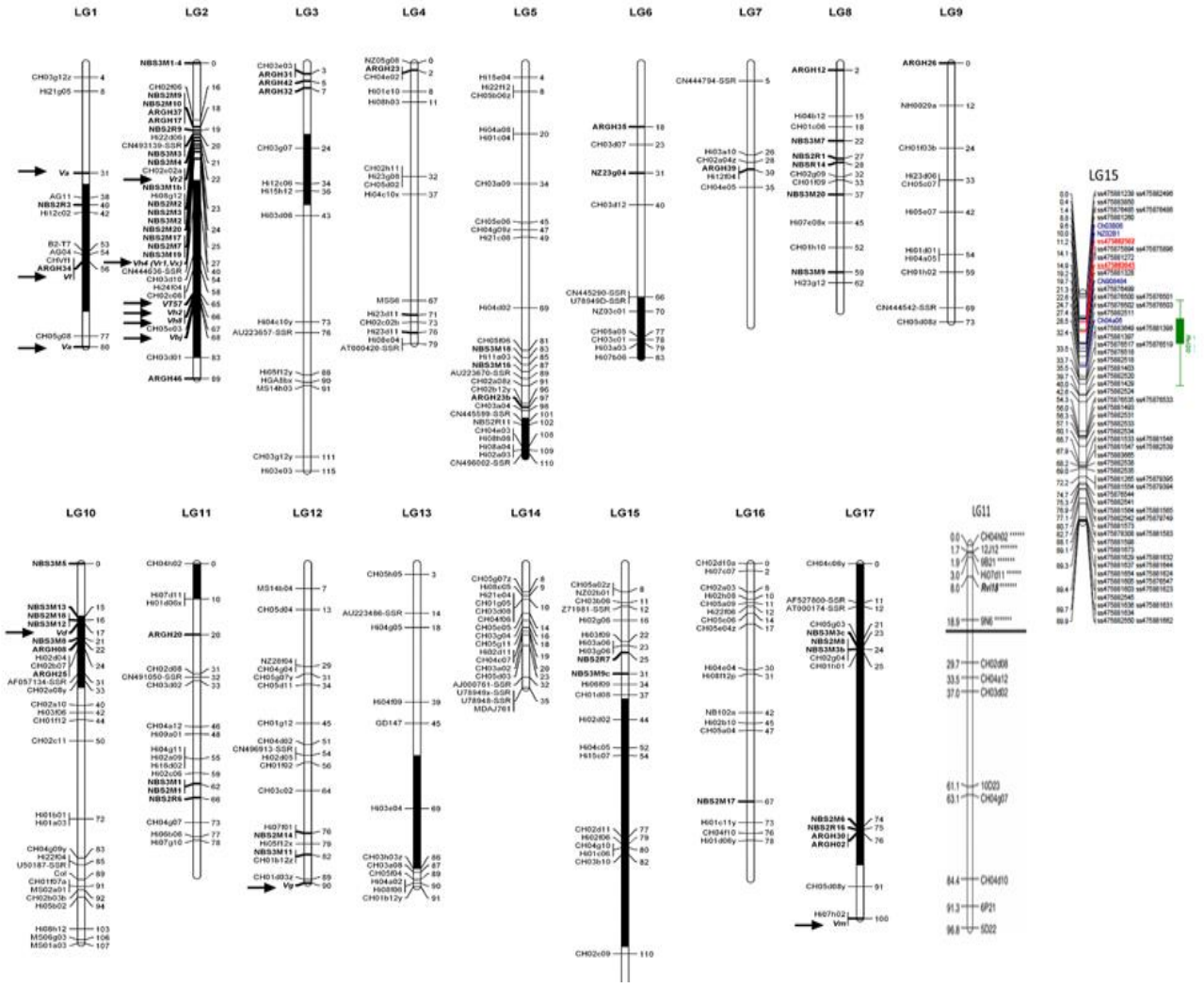


Figure 7: Genetic map positions of the twenty apple scab resistance genes identified on the apple linkage map (Bus et al., 2011a; Clark et al., 2014b; Soriano et al., 2014b).

Rvi1 (Vg) gene was discovered on the Golden Delicious cultivar's genome (Bénaouf and Parisi, 2000) and is responsible for the necrosis in resistant progenies. *Rvi1 was* mapped at the bottom of LG 12 (Durel *et al*., 2004, 2000). Two SSR markers were discovered flanking the *Rvi1* gene, namely Vg12-SSR and Vg15-SSR (Cova *et al*., 2015c). The region has been covered by BAC clones of roughly 110 kb in size. This region contains four putative TIR–NBS–LRR (TNL) genes and one serine/threonine protein phosphatase 2A gene. The putative *Rvi1* locus is made up of TIR–NBS–LRR (TNL) genes (Cova *et al*., 2015c). Despite the fact that most strains in Europe have overcome resistance due to this gene (Parisi *et al*., 2004), it has showed resistance against Race 7 isolates.

Rvi2 (Vh2) has been identified from accession 'TSR34T15' originating from R12740-7A (Russian seedling). *Rvi2* was mapped at lower part of LG2. Markers CH02b10 and OPL19 are bracketing the resistance locus. Resistant segregants carrying *Rvi2* showed stellate necrotic reactions (Bus *et al*., 2005b).

Table 2: Different scab resistance genes and their corresponding avirulence genes (Bénaouf and Parisi, 2000; Bowen et al., 2011; Broggini et al., 2011; Bus et al., 2011a; Clark et al., 2014b; Gennaro, 2012; Jha et al., 2009; Khajuria et al., 2018a; MacHardy, 1996b).

S. no.	Differential host/source accession	Previous name (R locus)	New name (R Locus)	LG	Resistance reaction	Corresponding avirulence gene
1.	Royal Gala	–	–	–	Susceptible	–
2.	Golden Delicious	*Vg*	*Rvi1*	LG 12 (Distal end)	Class 2	*AvrRvi1*[c]
3.	TSR34T15	*Vh2*	*Rvi2*	LG2 (Distal end)	Class 2	*AvrRvi2*
4.	Geneva[a]	*Vh3.4*[b]	*Rvi3*	LG4	Class 2	*AvrRvi3*
5.	TSR33T239	Vh4 = Vx = Vr1	*Rvi4*	LG2 (Distal end)	Class 1	*AvrRvi4*
6.	9-AR2T196	*Vm*	*Rvi5*	LG17 (Distal end)	Class 1	*AvrRvi5*
7.	Priscilla	*Vf*	*Rvi6*	LG1 (Distal end)	Class 0 to 3b	*AvrRvi6*
8.	*Malus × floribunda* 821[a]	*Vfh*	*Rvi7*	LG8	Class 1	*AvrRvi7*
9.	4B5	*Vh8*	*Rvi8*	LG2 (Distal end)	Class 2	*AvrRvi8*
10	K2,	*Vdg*	*Rvi9*	LG2	Class 2	*AvrRvi9*
11.	A723–6[a]	*Va*	*Rvi10*	LG1	Class 1	*AvrRvi10*
12.	*A722–7*	*Vbj*	*Rvi11*	LG2 (Distal end)	Class 0 to 3b	*AvrRvi11*
13.	Hansen's baccata #2	*Vb*	*Rvi12*	LG12 (Distal end)	Class 0 to 3b	*AvrRvi12*
14.	Durello di Forli	*Vd*	*Rvi13*	LG10 (Proximal end)	Class 2	*AvrRvi13*
15.	Dülmener Rosenapfel[a]	*Vdr1*	*Rvi14*	LG6 (Proximal end)	Class 2	*AvrRvi14*
16.	GMAL 2473	*Vr2*	*Rvi15*	LG2 (Proximal end)	Class 1	*AvrRvi15*
17.	MIS op 93.051 G07[a]	*Vmis*	*Rvi16*	LG3	Class 1	*AvrRvi16*
18.	Antonovka APF22[a]	*Va1*	*Rvi17*	LG1	Class 2	*AvrRvi17*
19.	1980-015-025[a]	*V25*	*Rvi18*	LG11	Class 1	*AvrRvi18*
20.	Honeycrisp[a]	–	*Rvi19*	LG1	All classes of resistance reactions	*AvrRvi19*
21.	Honeycrisp[a]	–	*Rvi20*	LG15	All classes of resistance reactions	*AvrRvi20*

Class 0: no symptoms; Class 1: pit type hypersensitive response like reactions; Class 2: irregular edged chlorotic lesions with slight necrotic center with no sporulation; Class 3a: chlorotic and necrotic lesions with rare sporulation; Class 3b: prominent sporulation with chlorotic and necrotic lesions.

a) A temporary differential host until a monogenic nature of resistance gene is confirmed or a monogenic cultivar is generated

b) *Vh3.4* may be the true *Rvi3* but not confirmed

c) Avirulence gene has been identified

Rvi3 has been identified in 'Geneva' (Jefferson, 1970). This gene is flanked by SSR markers Hi08e04 and CH05d02 and has been mapped in LG4 (Bus *et al*., 2011a). The interactions of 'Geneva' with several *V. inaequalis* isolates revealed that in this genotypes there are at least five loci controlling resistance. Further precise mapping permitted to identify a 5 cM area at the distal end of the LG4 that

contained five genes. This region corresponds to ~ 2.2 Mbp according to the 'Golden Delicious' sequence data (Bastiaanse *et al*., 2016). In *Rvi*3 region, nine candidate NBS-LRR genes were predicted, five of which were linked to resistance loci; three genes *(Rvh3.1, Rvh3.2, and Rvh3.3)* were under dominant genetic control, while two genes *(Rvh3.4 and Rvh3.5)* were under recessive genetic control. Bastiaanse *et al*., (2016) anticipated that the best candidate for the *Rvi3* gene may be *Rvh3.4*.

Rvi4 (Vx=Vr1) has been identified as *Rvi2* in accession 'TSR33T239' also originating from R12740-7A (Russian seedling). It was found to be linked to SCAR markers S22 and OPB_{10}, microsatellite marker CH02c02a. *Rvi4* has been mapped on the upper end of LG2 and induces HR (Bus *et al*., 2005b).

Rvi5 (Vm) was found in *M. micromalus* and *M. atrosanguinea* 804 (Dayton, 1970). The first molecular marker for *Rvi5,* mapped at about 6 CM was OPB12 (Cheng *et al*., 1998). Patocchi *et al*, (2005) applying a genome scanning approach (GSA), mapped the gene to distal end of LG 17 and found the SSR marker Hi07h02, which is tightly linked to *Rvi5*. In a follow up study, Cova *et al*. (2015) stated the positional cloning of *Rvi5*. SSR, SCAR, and SNP markers tightly associated to *Rvi5* were developed. *Rvi5* was mapped between the SSR markers FMACH Vm2 and FMACH Vm4 (Cova *et al*., 2015b). The BAC clone was sequenced, spanning the *Rvi5* region and candidate R-genes of the TNL-LRR type were found (Bandara *et al*., 2015). *Rvi5* induces a hypersensitive response visible as small pin points pits. *V. inaequalis* race 5 has overcome this resistance (Patocchi *et al*., 2020a; Williams, 1968). The virulent strain, on the other hand, has not spread and thus has a restricted presence in apple-growing areas (Patocchi *et al*., 2020a).

Rvi6 (Vf) was the first scab resistance gene discovered. It was found in the wild apple, *Malus floribunda* 821 (Crandall, 1926). In apple, this gene is the most studied and the first being cloned (Belfanti *et al* 2006). *Rvi6* induces resistance reactions going from class 0 to class 3b. Unfortunately this resistance has been overcome in Europe and USA (Papp *et al*., 2020b; Patocchi *et al*., 2020a). *Rvi6*-associated molecular markers have been produced and employed in a variety of breeding projects. The first marker connected with this gene was *Pgm-1,* an isozyme marker genetically linked to *Rvi6*. This marker was found at a distance of 8 cM from the *Rvi6* gene (Manganaris *et al*., 1994). The first DNA-based marker associated to *Rvi6* was the RAPD marker OPD20-600 (Yang and Kruger, 1994) and many more followed (Koller *et al*., 1994) (Tartarini, 1996). Three of these RAPD markers, such as M18-CAPS, AL07-SCAR, and AM19-SCAR, have been converted into more reliable and reproducible markers (Gianfranceschi, 1996; Tartarini *et al*., 1999a) . Vinatzer *et al*., (2004) developed the currently most used marker in breeding, SSR CH-Vf1.

M18 and AL07, two molecular markers associated to *Rvi6*, were placed at 0.2 and 1.1 cM on either side of *Rvi6* (Patocchi *et al*., 1999; Vinatzer *et al*., 1998) and were estimated to have a maximal physical distance of 870 kb (Patocchi *et al*., 1999) . Using a BAC library of cultivar Florina, a chromosome walking was started from the flanking markers AM19 (a marker closer to *Rvi6* than AL07) and M18 using a BAC library of Florina (Vinatzer *et al*. 1999). Performing nine chromosomal walking steps, thirteen BAC clones spanning the Rvi6 containing region between M18 and AM19 were isolated. Following the molecular analysis of this interval a cluster of four genes *(HcrVf1, HcrVf2, HcrVf3, and HcrVf4)* was found (Vinatzer *et al*., 2001; Xu *et al*., 2002). These genes encode a protein similar to the *Cf* genes found in tomatoes. *HcrVf3*, called *Vfa3* by Xu *et al*, (2002), was found to be a pseudogene.

HcVf2 gene was transferred into susceptible cultivars 'Elstar' and 'Gala' and found to confer scab resistance (Belfanti *et al*., 2004). In a follow up study *Vfa1* (equal to *HcrVf1*), *Vfa2* (equal to *HcrVf2*, and *Vfa4* (equal to *HcrVf4*) have been systematically introduced into 'Galaxy' and 'McIntosh' cultivars (Malnoy *et al*., 2008). Plants holding *Vfa1, Vfa2*, and their progeny were shown to be moderately resistant to scab, whereas those containing *Vfa4* were found to be susceptible.

Rvi7 was found in *M. floribunda 821* during a screening of apple genotypes with an isolate virulent to *Rvi6*. The gene after scab infection induces pinpoint pitting symptoms. Unfortunately, also this resistance is overcome. Race 7 isolates were able to infect *M. floribunda* and other cultivars carrying *Rvi6* (Roberts and Crute, 1994). *Rvi7(Vfh)* was mapped on LG 8 and found to be associated to SSR marker CH01e12 (Caffier *et al*., 2010; Parisi *et al*., 2004b)

Rvi8 (Vh8) was discovered in *M. sieversii* accession W193B (Bus *et al*., 2005a). It was mapped in the lower part of LG2, it's closely linked to the *Rvi2* gene. *Rvi8* was also shown to be compatible with race 8 of the *V. inaequalis* isolate NZ188B.2. Another study discovered that the marker OPL19 SCAR near CH03d01, is linked to *Rvi2 and Rvi8*. However, more research is needed to determine if they are members of the same cluster or represent different alleles (Bus *et al*., 2005a; Hemmat *et al*., 2002). Upon scab infection genotypes carrying *Rvi8* develop a stellate necrosis response (Bus *et al*., 2011a).

Rvi9 (Vdg) has been identified in the accession no. K2 induces stellate necrosis symptoms (Caffier *et al*., 2015a).

Rvi10 (Va) from accession no. A723 has been mapped on LG1 and found to beassociated to marker AG11. Rvi10 induces HR (Caffier *et al*., 2015b).

Rvi11(Vbj) was discovered in *M. baccata* Jackii (Dayton, 1968) and it is mapped on distal end of LG2. The first molecular marker linked to *Rvi11* was developed by Gygax *et al,* (2004). From a hybrid between A722-7 and Golden Delicious, they discovered three RAPD markers linked to *Rvi11*. K08-SCAR, T06-SCAR, and Z13-SCAR were transformed from RAPD markers to SCAR markers. In another investigation, three SSR markers for *Rvi11* were identifed: CH02c06, CH05e03, and CH03d01(Gygax *et al*., 2004). 17 SNP markers associated to *Rvi11* have been identified and mapped (Jänsch *et al*., 2015). *Rvi11* induces stellate necrosis/chlorosis. So far, no *V. inaequalis* isolates has been able to overcome this gene (Patocchi *et al*., 2020a).

Rvi12 (vb) was found in the Siberian crab apple 'Hansen's baccata #2' (Dayton, 1968). The resistance gene induces chlorosis (class 2) or chlorosis with minimal sporulation (class 3a/3b, Erdin *et al*., 2006). Its first molecular marker $UBC22_{0700}$, allowed tentatively mapping *Rvi12* gene on LG1 (Hemmat *et al*., 2003). Later, SSR markers were used to pinpoint the exact position of this gene (Erdin *et al*., 2006a). *Rvi12* was mapped between the SSR markers Hi02d05 and Hi07f01 on the LG12. SSR and SNP markers linked to this gene have recently been developed (Padmarasu *et al*., 2014). *Rvi12* was fine-mapped inside a 958-kb region in the Golden Delicious genome (Padmarasu *et al*., 2014). Eighteen SNPs linked to the *Rvi12* gene, were reported in Hansen's baccata #2. Putative candidate genes with similarity to known disease resistance genes or genes implicated in disease resistance were found using gene prediction and *in-silico* analysis. The most likely candidate gene for *Rvi12* resistance was an LRR receptor-like serine/threonine kinase, (Padmarasu *et al*., 2018). So far, there a no reports on resistance breakdown of Rvi12 (Patocchi *et al*. 2020).

Rvi13 (Vd) has been identified in 'Durello di Forli' and it is mapped to the proximal end of LG10. The SSR markers CH02b07 and CH04f03 were developed to map this gene which shows stellate necrosis in the hosts bearing it after infection with *V.inaequalis* (Caffier *et al*., 2015b; Laurens *et al*., 2004; Parisi *et al*., 2004; Tartarini *et al*., 2004).

Rvi14 (Vdr1) was discovered from 'Dülmener Rosenapfel' and has been mapped to proximal end of LG6. Typical symptoms of chlorosis can be seen if the host having this gene encounter *V.inaqualis's* attack. SSR marker HB09 has been developed for this gene (Mansy, 2017; Soufflet-Freslon *et al*., 2008).

Rvi15 (*Vr*2) has been identified in the accession GMAL 2473 (Patocchi, 2003; Patocchi *et al*., 2004). The progeny of a cross between Idared and GMAL 2473 was used to map it on LG2 between the SSRs CH02c02a and CH02f06 (Galli *et al*., 2010a; Patocchi *et al*., 2004). ARGH17 and ARGH37, two NBS domain-derived molecular markers, were mapped within 1.5 cM (Galli *et al*., 2010a). Jansch *et al*., (2015) developed nine SNPs tightly associated to *Rvi15*. Later, ten further markers were

generated within a 1.8 cM region around this gene (Galli *et al*., 2010b). The ARGH17 and GmTNL1 markers were discovered to be relatively close to the gene, with a 0.5 cM space between them and used as starting point for a positional cloning of the gene. As a result of this effort, three putative toll interleukin1 receptor-NBS-LRR resistance genes namely *Rvi15-A*, *Rvi15-B*, and *Rvi15-C* were identified. Based on the functional characterization of this gene, Schouten *et al*, (2014) concluded that *Rvi15-C* is responsible for resistance against apple scab. Therefore, this gene was considered as the *Rvi15* gene.

Rvi16 (Vmis) has been found in the accession MIS op 93.051 G07 and mapped on LG3. The two SSR markers Hi03d06 and AU223657 are associated to *Rvi16* (Bus *et al*., 2011a). This gene induces an HR response.

Rvi17 (*Va1*) was mapped in an Antonovka APF22 progeny. *Rvi17* was mapped within 1 cM of *Rvi6* on LG1, and has a specific CH-Vf1 SSR marker allele of 139 bp linked to it. This gene induces chlorotic resistance reaction (Bus *et al*., 2011b; Dunemann and Egerer, 2010).

Rvi18 (V25) was mapped on the LG11 of the apple selection 1980-015-025 (Soriano *et al*., 2014a). For this gene, various molecular markers such as SSR, DArT, AFLP, and SNP markers have been established (Soriano *et al*., 2014b). The gene is flanked by the 9NT DArT marker and the Hi07d11 SSR markers. Performing a positional mapping Soriano *et al* (2014b) identified lectin-like receptor kinase (LRK) as a candidate gene *Rvi18*. The gene has a broad spectrum of action. The gene induces HR (pin point pits).

The apple scab resistant cultivar Honeycrisp was found to carry two resistance genes *Rvi19* and *Rvi20* (Clark *et al*, 2012, 2014a).

Rvi19 was mapped on LG1 (Clark *et al*., 2014a) in the same region as the *Rvi6* gene. SSR markers and SNPs linked to *Rvi19* have been developed (Clark *et al*., 2014a). *Rvi19* and SNP ss475882285 were shown to co-segregate.

Rvi20 was mapped on LG 15 (Clark *et al*., 2014a), and the SNP markers ss475883045 and ss475882502 are tightly associated to the gene.

Gene for Gene Model

The gene-for-gene model is based on a molecular mechanism in which the product of the pathogen's avirulence gene (*Avr*) (e.g. an effector) is recognized by the product of the host's dominant resistance gene (*R*) (Flor, 1971). The defense response is triggered by the contact between the Avr molecule and the R protein. This is referred to as an incompatible interaction since the pathogen's development is stopped and generally, no sporulation occurs. On the contrary, a compatible

interaction occur when the pathogen is not explicitly identified by the plant but is able to develop in the plant and sporulate (Fig. 8).

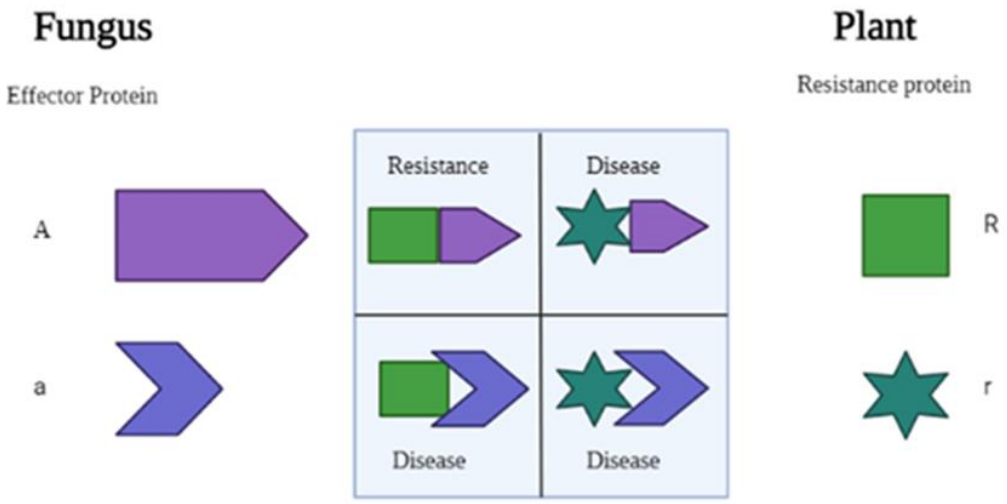


Figure 8: The gene-for-gene model is based on a molecular mechanism in which the product of the pathogen's avirulence gene (Avr) (e.g. an effector) is recognized by the product of the host's dominant resistance gene (R). When the pathogen is recognized, a defense reaction is elicited, and disease does not develop. This is called an incompatible interaction. On the contrary, when the pathogen is not recognized, disease progress unabated. This is called a compatible interaction.

One of the first gene-for-gene exchanges, occurred between *Malus* and *V. inaequalis*. However, straightforward interactions involving only two genes are uncommon (Bus *et al*., 2005b). The 'guard hypothesis' was proposed more recently (Peyraud *et al*., 2017) (Fig. 9). The notion is that the pathogen effectors are not directly recognized, but the R protein recognizes the alteration of the host target protein. As a result, the R protein 'guards' the Avr protein's target. The advantage of this method is that any changes in the Avr protein do not prevent the 'guard' R protein from recognizing it, and hence do not affect the virulence action (DeYoung and Innes, 2006).

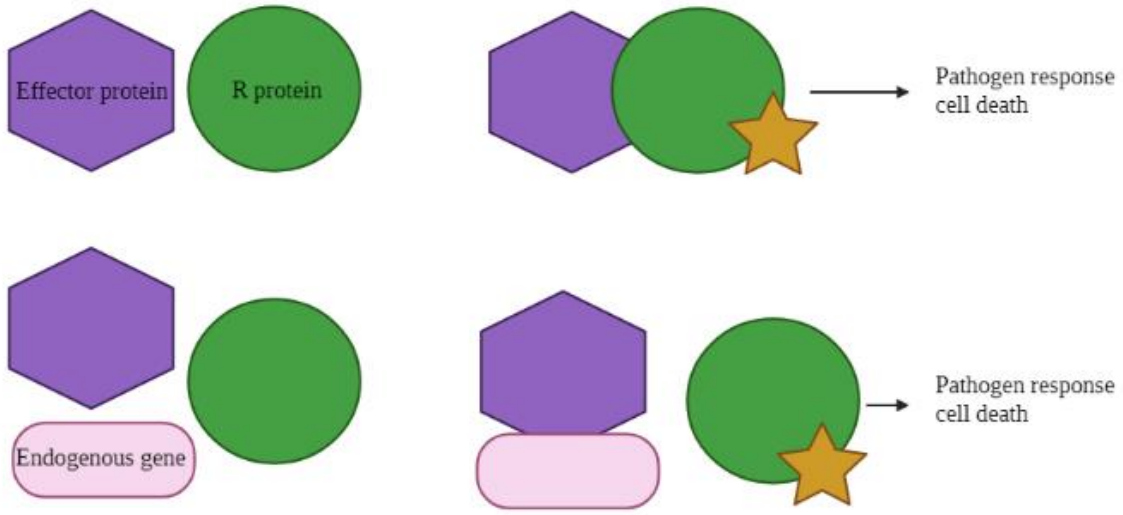


Figure 9: Plant R genes can mediate recognition of pathogen virulence factors via two primary recognition pathways. At the top: a classic gene-for-gene interaction in which is a R protein is activated (marked by a star) when pathogen effector molecules are directly recognized. This causes the plant to activate pathogen-response signaling cascades, which frequently result in programmed cell death. At the bottom is an alternative, indirect hypothesis known as the guard hypothesis, in which plant R proteins connect with and 'guard' host proteins that are common disease targets. When pathogen effectors bind with and modify the target host protein, the corresponding R protein recognizes the change, is activated, and the plant's pathogen response is triggered (Bomblies and Weigel, 2007).

1.6.3 Polygenic resistance

Polygenic resistance is not associated with a typical mechanism of resistance, but only refers to the number of genes involved in resistance. These genes are designated ‘quantitative trait loci’ (QTLs) (Lindhout, 2002). QTLs generally show low level resistance but they may also act on a broad spectrum of pathogen population (Pilet-Nayel *et al*., 2017). In both glasshouse (Calenge *et al*., 2004a) and field (Liebhard *et al*., 2003) trials, a number of scab resistance QTLs have been found (Tab. 3). Some of these overlap or a close to the loci of some scab R genes, such as LGs 1 and 2 (Calenge *et al*., 2004a; Dunemann and Egerer, 2010; Durel *et al*., 2003; Soriano *et al*., 2009). Other QTLs, such as those found on LG11 and LG17, do not overlap with known major apple scab resistance genes, with LG17 serving as a 'hotspot' for broad-spectrum scab resistance QTLs.

QTL-based polygenic resistance has been found in cultivars such as ‘Antonovka Monasir’, ‘Discovery’, ‘Fiesta’, and others (Quamme *et al*., 2003). In the progenies of ‘Discovery’ and ‘Antonovka Monasir’, Quamme *et al*., (2003) discovered stable polygenic resistance. Using the two

mono-conidial isolates of race 6, four QTLs were found in the ‘Prima x Fiesta’ progeny. One of the four QTLs was mapped on LG1 near the *Rvi6* gene. LGs 11 and 17 were home to the other three QTLs (Durel *et al*., 2003). In another study, scab resistance in the cross between apple cvs. ‘Fiesta’ (syn. Red Pippin) and ‘Discovery’ was assessed in the field for three years, this allowing the identification of eight scab-resistance QTLs (Liebhard *et al*., 2003). Six of the eight QTLs were for leaf scab, whereas two were linked to fruit scab. On LG17, a QTL promoting high levels of resistance was discovered in the progeny of the cross ‘Prima x Fiesta’ (Durel *et al*., 2003).

The ‘Discovery' ‘TN10-8' integrated linkage map was used to find 24 QTLs and one major gene for scab resistance. On LG1, LG2, and LG 17, broad spectrum QTLs have been discovered in the cultivar Discovery (Calenge *et al*., 2004b). In this study also isolate-specific QTLs on LGs 12, 13, and 15 were found. *Rvi*15 and *Rvi*8 were found to be co-localized with the QTL on LG2. Major QTLs on LGs 1, 2, 3, 9, 13, and 17 have also been found in the frame of the European DARE (Durable Apple Resistance in Europe) project (DARE project 2002). However, these QTLs clustered mainly on LGs 1, 2, and 11. Three QTLs on LGs 6, 11, and 17 were found in in F1 progeny derived from the cross between ‘Gala’ and ‘Dülmener Rosenapfel’. One QTL was found on LG6, co-localized with *Rvi14* and other two QTLs against apple scab have been found on LG11 and LG17 (Soufflet-Freslon *et al*., 2008).

Apple genotypes with two broad-spectrum scab resistance QTLs, F11 and F17 were investigated to verify if degradation of apple scab resistance occurs in the field (Caffier *et al*., 2014). It was shown that quantitative resistance is susceptible to erosion or complete failure. Pathogens can overcome quantitative resistance by gradually restoring their reproduction rate, colonization capacity, or both (Bourget *et al*., 2015). As a result, QTLs must target the pathogen traits whose restoration will be difficult to occur.

QTLs linked to apple scab resistance can be exploited in a variety of breeding strategies. After pyramiding three QTLs in a cultivar, the efficacy of resistance against *V. inaequalis* was improved (Laloi *et al*., 2017). To find the required combination, however, a comprehensive examination of QTLs is required. For example, The QTLs that decrease spore germination or QTLs that increase latent duration, can be combined for more lasting resistance (Bourget *et al*., 2015).

Table 3: QTLs associated with the resistance against V. inaequalis in apple (Khajuria et al., 2018)

S. no.	Location	No. of QTLs	Identified from cross	Molecular marker identified near QTL	Reference	Remarks
1.	LG1	1	Cross progeny of 'Prima × Fiesta' 'Discovery' × 'TN10–8 progeny	CH-Vf1	(Durel *et al*., 2003) (Calenge *et al*., 2003)	Located close to the *Rvi6*
2.	LG2	1	'Discovery' × 'TN10–8 progeny	CH05e03 (SSR) E33M49–15	(Calenge *et al*., 2004)	Broad spectrum Co localized with *Rvi15* and *Rvi8*
3.	LG5		'Discovery' × 'TN10–8 progeny	–	(Calenge *et al*., 2004)	Isolate specific (two isolates)
4.	LG6	1	Progeny of cross Gala' and 'Dulmener Rosenapfel'	HB09-SSR_DR	(Soufflet-Freslon *et al*., 2008)	Co-localized with *Rvi14*
5.	LG7	1	Cross of Fiesta and Discovery	CH04e03	(Liebhard *et al*., 2003)	
6.	LG10	1	Cross of Fiesta and Discovery	–	(Liebhard *et al*., 2003)	
7.	LG11	1	Cross of Fiesta and Discovery progeny of cross Gala' and 'Dulmener Rosenapfel'	CH04h02_Ga	(Liebhard *et al*., 2003) (Durel *et al*., 2003) (Soufflet-Freslon *et al*., 2008)	Broad-spectrum QTL Same as detected by (Durel et al., 2003) and (Liebhard et al., 2003)
8.	LG12	1 1	Cross of Fiesta and Discovery 'Discovery' × 'TN10-8 progeny	*Rvi*1	(Liebhard *et al*., 2003) (Calenge *et al*., 2004)	Isolate specific
9.	LG13	1	'Discovery' × 'TN10-8 progeny	E32AM58-23	(Calenge *et al*., 2004)	Isolate specific
10.	LG15	1 1	Cross of Fiesta and Discovery 'Discovery' × 'TN10-8 progeny	E32AM59-1	(Liebhard *et al*., 2003) (Calenge *et al*., 2004)	Isolate specific
11.	LG17	1	Cross of Fiesta and Discovery 'Discovery' × 'TN10-8 progeny Cross of Prima and Discovery Progeny of cross Gala' and 'Dulmener Rosenapfel'	E32AM48–17 CH01h01_Ga CH01h01_DR	(Liebhard *et al*., 2003) (Calenge *et al*., 2004) (Durel *et al*., 2003) (Soufflet-Freslon *et al*., 2008)	Highly scab resistant Broad spectrum Same as detected by the (Durel et al., 2003) and (Liebhard et al., 2003)

1.7 Apple scab control practices

1.7.1 Cultural control

The initial technique for preventing apple scab damage was focused on cultural practices aimed at reducing primary ascospore infection (Vaillancourt and Hartman, 2000). To prevent the development of ascospores, falling leaves should be removed and disposed, but this proved impractical in large commercial orchards (Holb, 2006). Regular tree pruning is also recommended to support a better circulation of air in the canopy allowing a faster drying of the leaves (Burchill *et al*.,

1965; Hetherington, 2010; Holb, 2008). The problem with using these cultural practices to control apple scab is that they are not sufficient to control scab.

1.7.2 Chemical control

Chemical fungicide application is used to control apple scab, with the goal of preventing spore germination and fungal hyphae growth. Several fungicides have been used to prevent and control apple scab, including fixed copper, Bordeaux combinations, copper soaps, sulfur, mineral or neem oils, and mycobutanol (Jamar *et al*., 2008). To manage the disease in the early days, chemical fungicides were applied once every 5-7 days at the start of the season and once every 7-10 days in the late season. For a single growth season, up to 30 treatments are necessary. Chemical fungicide application has been decreased to 15-20 rounds per growing season in recent years, and these are applied on a timely basis, based on knowledge obtained about the pathogen's requirements for successful infection (Vaillancourt and Hartman, 2000).

Fungicides are used to prevent ascospore infection from the bud break stage and afterwards during periods when the environment is conducive to fungal infection. Despite the fact that chemical fungicides are successful in controlling the infection, the pathogen may evolve and acquire resistance (Ayer *et al*., 2019; Chapman *et al*., 2011; Frederick *et al*., 2014). Resistance to dodine, methyl benzimidazole carbamate (MBC), demethylation inhibitors (DMIs), and Quinoline outside inhibitor (QoI) fungicides has been observed in several regions (Köller, 1994; Köller *et al*., 2004; Köller and Wilcox, 2001). Chemical fungicides are expensive, and the propensity of *Venturia* to develop resistance to them, as well as the non-sustainable nature of chemical application, has prompted researchers to look for alternate ways to control the disease.

Pesticide levels are usually monitored in agricultural areas, while they are rarely checked in public areas, but there is a recent study conducted in four valleys of South Tyrol, where 71 public playgrounds located near to commercial apple and wine orchards. Grass samples from the chosen playgrounds were collected and analyzed using conventional gas chromatography and mass spectrometry for 315 pesticide residues. The pesticide contamination was found in over slightly less than half of the playgrounds (45%) and a quarter (24%) had several pesticides. Eleven of the 12 chemicals found are categorized as endocrine disruptors, with the insecticide phosmet and the fungicide fluazinam exhibiting the highest quantities (0.069 and 0.26 mg kg1, respectively).

1.7.3 Conventional breeding

Pesticide-free plant genetically resistant to diseases is an intriguing alternative to pesticides. The development of scab-resistant cultivars may be the most effective method. Creating effective

strategies to reduce the inoculum size of pathogen populations by using natural defenses of resistant cultivars, would be a comprehensive approach to improve disease control (Didelot *et al*., 2016).

At the end of the nineteenth century, the first attempts to make scab-resistant cultivars were made (Aderhold, 1902). The wild relative of apple, *M. floribunda*, was discovered to be the initial source of scab resistance. Crandall at the University of Illinois made the first attempt, crossing numerous crab apples with commercial apple cultivars (1926). Many years later, the use of small fruited wild crab apple accessions in breeding was further promoted (Hough, 1944). Hough observed two scab-resistant seedlings among this material's survivors (1944b). Further research led to the discovery of the *Vf* resistance gene (*V* for *Venturia*; *f* for *floribanda*) (Williams *et al*., 1996) and now called as *Rvi6*. In 1945, Shay and Hough launched a breeding effort aimed at discovering scab resistance sources and creating scab resistant cultivars. This partnership, known as the PRI breeding program (Purdue University, Rutgers University, and Illinois University), was the first official attempt to develop a breeding program. Twenty-five *Malus* species and selections were found as having high levels of genetic resistance to apple scab (Dayton, 1968; MacHardy, 1996a; Shay and Hough, 1952). Despite the identification of numerous significant scab resistance genes, only the *Rvi6* resistance gene was widely exploited in apple scab resistant breeding programs during the 20th century. Selections with *Rvi6* resistance were utilized exclusively for scab resistance all across the world, resulting into more than 80 scab-resistant cultivars which have been created so far (Gessler, 2012; Crosby *et al*., 1992).

Since apples are very heterozygous (Velasco *et al*., 2010), the result of a cross between two cultivars is unpredictable. The number of suitable parents is reduced due to incompatibility and the limited number of S alleles available in commercial apple cultivars (Sakurai *et al*., 2000). Apple breeding is further complicated by the long generation cycles, making it a slow procedure. When a trait (e.g. disease resistance) has to be transferred from wild apple species, it takes a lot longer. To remove the majority of the undesired alleles from the wild apple, five or more generation cycles are needed due to genetic drag. The introgression of the dominant gene for scab resistance from wild *M. floribunda* (*HcrVf*), which took 80 years, is one example (Joshi *et al*., 2009).

1.7.4 Marker assisted breeding

Plant pathologists and breeders have long advocated for the pyramiding of key resistance genes into single cultivar and the use of polygenic resistance in breeding programs to prevent or postpone disease resistance breakdown and achieve long-term resistance (Pedersen and Leath, 1988; Poland *et al*., 2009).

Scab resistance pyramiding is challenging and time-consuming using traditional double pseudo-test cross breeding and phenotypic selection (Ibanez and Dandekar, 2007; Joshi *et al*., 2009). Because different scab R genes produce similar resistance responses or one scab R gene can mask the symptoms of another one, phenotypic selection of seedlings with multiple scab R genes is usually impossible. The creation of molecular markers intimately related to each of the scab resistance genes is required for marker assisted selection (MAS) of seedlings expressing multiple scab resistance genes (Bus *et al*., 2009; Patocchi *et al*., 2009; Vanderzande *et al*., 2018). Thus, without waiting for the phenotypic character, progenies lacking scab-resistant genes can be rejected right away. Another significant advantage of MAS is that it can identify the homozygous allele of a resistant gene (Baumgartner *et al*., 2015). The MAS is more reliable than phenotypic indicators in identifying scab resistance (Tartarini *et al*., 1999a). As a result, genetic markers exist for the majority of resistance genes (Baumgartner *et al*., 2016; Jänsch *et al*., 2015). In Europe, a project on molecular marker-based resistance is being developed with the goal of developing long-lasting scab resistant lines (Lespinasse *et al*., 2000). HiDRAS (High-quality Disease Resistant Apples for Sustainable Agriculture) is a project that uses marker-based tactics to improve scab resistance and fruit quality (Gygax, 2004). The multinational Fruit Breedomics research has identified SNPs linked to scab resistance. Disease resistance cultivars and molecular markers-based approaches are the focus of International RosBREED 2.

However, against *V. inaequalis*, there is always the risk that pyramid resistance can be overcome (Caffier *et al*., 2015a). To decrease pathogen adaptability to hosts, it is necessary to understand the ecological and evolutionary dynamics of pathogen populations, as well as the genetics of resistance genes (Sapoukhina *et al*., 2009; Zhan *et al*., 2014).

1.7.5 High speed breeding: Transgenesis and cisgenesis

Targeted introgression of scab-resistant genes into susceptible cultivars is not conceivable in standard breeding operations (Jacobsen and Schouten 2008). The existence of a large number of alleles at each site, as well as heterozygosity, makes introgression more difficult in apples. Furthermore, many cultivars with scab resistant genes may not be similar to commercial cultivars since they may have other undesirable traits (Gessler *et al*., 2006a). More than three decades ago, genetic engineering such as direct transformation methods (Gene gun) and indirect methods (*Agrobacterium tumefaciens*-mediated transformation) have been the primary methods for gene transfer in plants (Altpeter *et al*., 2005; Gelvin, 2003) Transgenic approaches have been mainly implemented for the production of crops with new resistance genes against diseases (Parisi *et al*., 2016). There have been reports of transgenic cultivars being used to obtain scab resistance in

susceptible cultivars within 1 year (Bolar *et al*., 2001; Flachowsky *et al*., 2010). 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 (Lucht, 2015). This problem can be addressed by one of the new plant breeding techniques (NPBTs) developed more recently, such as "cisgenesis", transferring resistance genes along their native promoters isolated from a resistant cultivar to commercial scab susceptible cultivar (cisgenes) of apple (Schouten *et al*., 2006a). Cisgenic refers to a plant that has had gene introduced from the same species among cultivars (Joshi *et al*., 2011), although, GM legislation is also applied to cisgenic plants (Laaninen, 2016).

Regarding generation of transgenic or cisgenic apple, some benchmarks have been met (Belfanti *et al*., 2004; Krens *et al*., 2015; Malnoy *et al*., 2008; Schouten *et al*., 2014). These include cloning and characterization of resistance genes, promoters, and other regulatory elements. *Rvi6* and *Rvi15*, two scab resistance genes, have been cloned and functionally described (Belfanti *et al*., 2004; Schouten *et al*., 2014, Malnoy *et al*. 2008). Many other genes have been fine-mapped, and this information can be utilized to clone them. In addition, native scab resistance promoters of *Rvi6* in apples have been identified (Malnoy *et al*., 2008; Silfverberg-Dilworth *et al*., 2005a) and *Rvi6* was expressed under control of its native promoter into susceptible cultivars, resulting in resistance to the *V. inaequalis* race 6. Using a native promoter and the *Vfa2* gene, highly scab-resistant lines of the cultivars Elstar and Gala were created (Szankowski *et al*., 2009). All of them, however, are transgenic due to the presence of the marker gene and additional regulatory sequences (Joshi *et al*, 2010).

By converting *Rvi6* with the chemically inducible R/RsSystem, the first truly cisgenic apple was established in the Gala cultivar (Vanblaere *et al*., 2011). This was an *Agrobacterium*-mediated transformation in which selectable markers and other foreign sequences were removed later. Seven distinct cultivars 'Brookfield Baigent' ('Royal Gala' mutant), 'Kanzi', 'Mariri Red' ('Braeburn' mutant), 'Mitchgla' ('Gala' mutant), 'Novajo' ('Jonagold' mutant), 'Pinova' and 'Red Jonaprince' ('Jonagold' mutant) were transformed with the *Rvi6* gene using the *Flp/*FRT recombinase system (Würdig *et al*., 2015). Sixteen of the 22 transgenic lines produced were resistant to *V. inaequalis* strain 104. There was no difference in transcription levels between cisgenic *Rvi6* transplanted cultivars and conventionally bred *Rvi6* cultivars (Chizzali *et al*., 2016). In addition, another investigation showed that *Rvi6* transformed cisgenic apple cultivars were more resistant to scab than non-transgenic apple cultivars (Krens *et al*., 2015). *Rvi15* was also employed in the development of

cisgenic apple cultivars (Schouten *et al*., 2014). Cloning of other genes will also aid in the development of more enduring cisgenic apple cultivars. Consumers consider cisgenics to be more natural than transgenics and have a favorable attitude toward cisgenic crops (Belfanti *et al*., 2004b; Cardi, 2016b; Khajuria *et al*., 2018b; Malnoy *et al*., 2008; Schouten *et al*., 2014). 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 (Patocchi *et al*., 2020b).

Governments such as the United States, Australia, and Canada are more enthusiastic about cisgenic crops and are attempting to differentiate them from genetically engineered plants, although the European Union (EU) and other countries remain undecided (Hunter, 2014; Lusser and Davies, 2013). In contrast to EU level regulation concerning the use of NPBTs, the world wide scientific community favor evaluation of plants obtained by NPBTs by focusing on the genetic modifications made to the plant itself and on the final products, in order to analyze that if the induced genomic modifications are within the normal genetic variability of the species (Sprink et al., 2016). For example, when it comes to risk assessment of genetically modified plants, one of the main concern is about translation of new protein and its possible off target effects, but cisgenesis removes that risk in the final product which contains all the components from the same species, and thus do not produce a new or unfamiliar protein and therefore do not provide different outcomes compared to traditional breeding (Podevin *et al*., 2012). In addition, molecular characterization of cisgenic plants to check the absence of heterologous DNA and no additional environmental and food safety risk assessment might ease up GM lesiglation in EU (Schouten *et al*., 2006b).

1.7.6 Advanced biotechnological tools for genetic improvement

Other than cisgenesis, NPBTs also includes site specific mutagenesis or genome editing (Zinc Finger Nuclease (ZFN) technique, Meganuclease (MN) technique, Transcription Activator-Like Effector Nuclease (TALEN) technique, the Clustered Regularly Interspaced Short Palindromic Repeats CRISPR/Cas9, Oligonucleotide-Directed Mutagenesis (ODM)) (Limera *et al*., 2017; Lusser *et al*., 2011). Genome editing allow to edit, delete and replace a specific DNA sequence at a target site for accurate crop improvement (Arora and Narula, 2017). ZFN and TALE are the nucleases which recognize specific target DNA sequences of one TALE or three ZF nucleotides and then provides a nuclease that disrupts DNA adjacent to the recognition sites (Gaj *et al*., 2013). However, most famous genome editing technique CRISPR/Cas9 works through guide RNAs (gRNAs) and Cas9 (endonuclease). The Cas9 produces a double strand breaks adjacent to the gRNA annealing location,

resulting in target specific mutagenesis (Cong *et al*, 2013). The CRISPR/Cas9 ribonucleoproteins (RNPs) were delivered directly to apple protoplast to produce DNA free genome edited apple plants for fire blight disease resistance (Malnoy *et al*., 2016). In other study, the fire blight resistance was also improved in apple cultivars using CRISPR/Cas9-FLP/FRT based gene editing system (Pompili *et al*., 2020). Similar targeted mutations can be produced by transformation of designed site-specific nucleases into the susceptible apple cultivar to develop scab resistance.

Another NBPT technique is intragenesis, where intragene called as hybrid gene comes from same species or sexually compatible species, derived by different promoter and terminator (Rommens, 2007). Intragenic plants achieved by *Agrobacterium*-mediated transformation, does not lead to accidental transfer of vector sequences due to plant-derived transfer DNA borders sequences from the sexually compatible DNA pool (Rommens, 2004). Thus, these plants do not contain any foreign DNA like cisgenic plants. Recently, an apple scab resistance gene *Rvi6* was transferred successfully from apple to pear. Thus, the functionality of *Rvi6* gene was analyzed against another scab specie *V. pyrina* (Perchepied *et al*., 2021). The *Rvi6* gene from apple was expressed constitutively in transgenic pear clones, which were compared to a similar transgenic apple clone harboring the same construct. All transgenic pear clones tested showed significant defense reactions and extremely minimal sporulation after being inoculated with *V. pyrina* in the greenhouse which concluded successful intergeneric transfer of a resistance gene among Rosaceae, with a resistance gene functioning against pear scab pathogen (Perchepied *et al*., 2021).

Virus-induced gene silencing (VIGS) is another advance biotechnological tool to check gene activity by its suppression. It is performed by cloning a cDNA fragment of plant gene into genome of RNA-virus and then transfecting the plant with this DNA construct using Agrobacterium. During viral replication, the double-stranded RNA (dsRNAs) molecules are degraded into small interfering RNA (siRNA) molecules by the plant Dicer-like enzymes to activate the siRNA silencing pathway, which ultimately leads to a knockout or knockdown phenotype for the gene of interest (Burch-Smith *et al*., 2004; Purkayastha and Dasgupta, 2009). Apple latent spherical virus (ALSV) vectors can induce both VIGS and overexpression of gene in apple efficiently (Yamagishi *et al*., 2014). RNA mediated interference (RNAi) is similar to VIGS, but it is heritable in nature while VIGS is transient in nature. In this technique, a DNA construct either produces single or double stranded RNA complementary to the gene of interest is introduced into plant cell where it degrades partial or full transcript from the gene of interest (Small, 2007). Thus, RNAi Strategies can be utilized for resistance against plant fungal pathogens (Gebremichael *et al*., 2021).

Another alternative NBPTs includes trans-grafting techniques such as RNAi technology on rootstocks for producing non-GM grafted scions or GM rootstock, agro-infiltration techniques for rapid transfer of gene, Oligonucleotide-Directed Mutagenesis **(**ODM) to produce new mutation by replacing one or few base pairs and RNA-dependent DNA methylation (RdDM) can be used faster gene transfer, precise genetic improvement by gain or loss of gene function (Limera *et al*., 2017; Lusser *et al*., 2011). However GM plants produced by all these NPBTs techniques falls under EU legislation (Hartung and Schiemann, 2014; Sprink *et al*., 2016).

1.8 Detailed description of the *Rvi12* (*Vb*) resistance

The Siberian crab apple *M. baccata* 'Hansen's baccata #2' (HB2) is resistant in nearly all the locations monitored by the VINQUEST initiative (Patocchi *et al*. 2009, 2021), making this source of resistance attractive for use in durable resistance breeding. The *Rvi12* scab resistance locus which was previously referred to as *Vb* was identified from HB2 (Dayton and Williams, 1968). From the testcross studies of Dayton and Williams (1969), *Rvi12* (Bus *et al*. 2011), 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. The *Rvi12* locus was mapped to LG1 of HB2, 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 *V. 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) 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) and *Rvi12* was mapped to LG12 of the genome. The results obtained from the two mapping studies suggested 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 HB2.

The next step was followed by Padmarasu *et al* (2014) to fine-map the *Rvi12* scab resistance locus. In this study, the scab resistance gene *Rvi12* from HB2' was confirmed as mapping to apple LG12 in the cross 'Gala' × 'Hansen's baccata #2'(Padmarasu *et al*., 2014). 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 HB2. 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 HB2 conferred the stellate chlorosis-type resistance reactions. The discovery of two unlinked genes segregating in the mapping progeny derived from HB2 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 HB2 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 of Padmarasu *et al* (2014), 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 HB2 (Padmarasu *et al*., 2014).

Based on the new system of nomenclature (Bus *et al*, 2011) of the differential interaction between *Malus – V. inaequalis*, HB2 was placed as a temporary differential host 12 because of the uncertain nature of monogenic inheritance of scab resistance. Padmarasu & colleagues (2014) predicted that scab resistance of HB2 was not monogenic and could be 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 HB2 will be 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.

Then, a large insert BAC library of HB2 was developed (Padmarasu *et al* 2018) 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. During the chromosome walking analyses, no susceptible BAC clone covering the *Rvi12* interval was identified. Furthermore, a single BAC clone containing the *Rvi12* resistance locus was sequenced in its entirety and assembled into a single sequence contig (Padmarasu *et al*., 2018).

Gene prediction from the BAC clone sequence between the *Rvi12* flanking markers predicted 20 genes by using FGENESH 2.6 with 82 exons from the 62,249 kb sequence covering the entire *Rvi12* interval. Comparison of the predicted gene sequences with known protein sequences identified five putative candidate genes (*Rvi12_Cd1, Rvi12_Cd2, Rvi12_Cd3, Rvi12_Cd4, Rvi12_Cd5*) 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). 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) (Padmarasu *et al*., 2018).

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.

Quantitative real-time PCR expression analysis detected overexpression of three putative candidates including the two (*Rvi12_Cd3, Rvi12_Cd4*) coding for TGA-like proteins and one (*Rvi12_Cd5*) probable LRR receptor like serine/threonine kinase in leaf tissue of the resistant 'HB2' genotype compared to leaves of the susceptible 'Gala' genotype (Padmarasu *et al*., 2018). A low level of expression of all three putative candidate genes was observed even in leaves of the susceptible 'Gala' genotype. *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 (Padmarasu *et al*., 2018).

In conclusion, the 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. The presence of a signal peptide and transmembrane motif in *Rvi12_Cd5* (Fig. 10), suggests its localization to plasma membrane with the extracellular LRR domain and intracellular protein kinase domain.

Figure 10:: 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 (Padmarasu et al., 2018).

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 experiment performed by Padmarsu *et al* (2018). 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. Thus, a single gene *Rvi12_Cd5* 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 (Padmarsu *et al*., 2018).

1.9 Bibliography

(GMO), E.P. on G.M.O., 2012. Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis. *EFSA J.* 10, 2561.

Aderhold, R., 1902. Ein Beitrag zur Frage der Empfänglichkeit der Apfelsorten für Fusicladium dendriticum Wallr. Fuck. und deren Beziehungen zum Wetter.

Agrios, G., 2005. *Plant Pathology*--Part 2 316.

Akhond, M.A.Y., Machray, G.C., 2009. Biotech crops: technologies, achievements and prospects. *Euphytica* 166, 47–59.

Aldwinckle, H.S., Malnoy, M., 2009. Plant regeneration and transformation in the Rosaceae.

Altpeter, F., Baisakh, N., Beachy, R., Bock, R., Capell, T., Christou, P., Daniell, H., Datta, K., Datta, S., Dix, P.J., 2005. Particle bombardment and the genetic enhancement of crops: myths and realities. *Mol. Breed.* 15, 305–327.

Aman Beshir, J., Kebede, M., 2021. *In silico* analysis of promoter regions and regulatory elements (motifs and CpG islands) of the genes encoding for alcohol production in Saccharomyces cerevisiaea S288C and Schizosaccharomyces pombe 972h. *J. Genet. Eng. Biotechnol.* 19, 1–14.

Andersen, E.J., Ali, S., Byamukama, E., Yen, Y., Nepal, M.P., 2018. Disease resistance mechanisms in plants. *Genes (Basel).* 9, 339.

Arora, L., Narula, A., 2017. Gene editing and crop improvement using CRISPR-Cas9 system. *Front. Plant Sci.* 8, 1932.

Ayer, K.M., Villani, S.M., Choi, M.-W., Cox, K.D., 2019. Characterization of the *VisdhC* and *VisdhD* genes in *Venturia inaequalis*, and sensitivity to fluxapyroxad, pydiflumetofen, inpyrfluxam, and benzovindiflupyr. *Plant Dis.* 103, 1092–1100.

Belete, T., & Boyraz, N. (2017). Critical review on apple scab (*Venturia inaequalis*) biology, epidemiology, economic importance, management and defense response to causal agent. *J Plant Physiol Pathol*, *5*.

Block, M. De, Verduyn, C., Brouwer, D. De, & Cornelissen, M. (2005). Poly (ADP-ribose) polymerase in plants affects energy homeostasis, cell death and stress tolerance. *The Plant Journal*, *41*(1), 95–106.

Bakker, J.J., 1999. *19e Rassenlijst voor kleinfruit*. NFO.

Bandara, N.L., Cova, V., Tartarini, S., Gessler, C., Patocchi, A., Cestaro, A., Troggio, M., Velasco, R., Komjanc, M., 2015. Isolation of *Rvi5* (*vm*) locus from Malus × domestica 'Murray.' In *III International Symposium on Molecular Markers in Horticulture 1100* (pp. 21-24).

Barbieri, M., Belfanti, E., Tartarini, S., Vinatzer, B.A., Sansavini, S., Silfverberg-Dilworth, E., Gianfranceschi, L., Hermann, D., Patocchi, A., Gessler, C., 2003a. Progress of map-based cloning of the *Vf*-resistance gene and functional verification: preliminary results from expression studies in transformed apple. *HortScience*, *38*(3), 329-331329.

Baumgartner, I.O., Kellerhals, M., Costa, F., Dondini, L., Pagliarani, G., Gregori, R., Tartarini, S., Leumann, L., Laurens, F., Patocchi, A., 2016. Development of SNP-based assays for disease resistance and fruit quality traits in apple (Malus × domestica Borkh.) and validation in breeding pilot studies. *Tree genetics & genomes*, *12*(3), 1-21.

Baumgartner, I.O., Patocchi, A., Frey, J.E., Peil, A., Kellerhals, M., 2015. Breeding elite lines of apple carrying pyramided homozygous resistance genes against apple scab and resistance against powdery mildew and fire blight. *Plant Mol. Biol. Report*. 33, 1573–1583.

Beckerman, J.L., Sundin, G.W., Rosenberger, D.A., 2015. Do some IPM concepts contribute to the development of fungicide resistance? Lessons learned from the apple scab pathosystem in the United States. *Pest Manag. Sci*. 71, 331–342.

Belfanti, E., Barbieri, M., Tartarini, S., Vinatzer, B., Gennari, F., Paris, R., Sansavini, S., Silfverberg-Dilworth, E., Patocchi, A., Hermann, D., Gianfranceschi, L., Gessler, C., 2004. Gala apple transformed with the putative scab resistance gene *HcrVf2*. *Acta Hortic*, *663*(1), 453-456.

Belfanti, E., Silfverberg-Dilworth, E., Tartarini, S., Patocchi, A., Barbieri, M., Zhu, J., ... & Sansavini, S. (2004). The *HcrVf2* gene from a wild apple confers scab resistance to a transgenic cultivated variety. *Proceedings of the National Academy of Sciences*, *101*(3), 886-890.

Bénaouf, G., & Parisi, L. (2000). Genetics of host-pathogen relationships between *Venturia inaequalis* races 6 and 7 and Malus species. *Phytopathology*, *90*(3), 236-242.

Beresford, R.M., Henshall, W.R., Palmer, J.W., 2004. A new apple scab risk model that integrates ascospore release infection risk and susceptible leaf area. *New Zeal. Plant Prot*. 57, 20–24.

Bolar, J.P., Norelli, J.L., Harman, G.E., Brown, S.K., Aldwinckle, H.S., 2001. Synergistic activity of endochitinase and exochitinase from Trichoderma atroviride (T. harzianum) against thee pathogenic fungus (*Venturia inaequalis*) in transgenic apple plants. *Transgenic Res 10*. https://doi.org/10.1023/A:1013036732691

Bomblies, K., Weigel, D., 2007. Hybrid necrosis: autoimmunity as a potential gene-flow barrier in plant species. *Nat. Rev. Genet*. 8, 382–393.

Bondonno, N.P., Bondonno, C.P., Ward, N.C., Hodgson, J.M., Croft, K.D., 2017. The cardiovascular health benefits of apples: Whole fruit vs. isolated compounds. *Trends Food Sci. Technol*. 69, 243–256. https://doi.org/10.1016/J.TIFS.2017.04.012

Boone, D.M., Kline, D.M., Keitt, G.W., 1957. *Venturia inaequalis* (Cke.) Wint. XIII. Pathogenicity of induced biochemical mutants. *Am. J. Bot*. 791–796.

Bourget, R., Chaumont, L., Durel, C., Sapoukhina, N., 2015. Sustainable deployment of QTLs conferring quantitative resistance to crops: first lessons from a stochastic model. *New Phytol*. 206, 1163–1171.

Bowen, J.K., Mesarich, C.H., Bus, V.G.M., Beresford, R.M., Plummer, K.M., Templeton, M.D., 2011. *Venturia inaequalis*: The causal agent of apple scab. *Mol. Plant Pathol*. 12, 105–122. https://doi.org/10.1111/J.1364-3703.2010.00656.X

Bowen, J.K., Mesarich, C.H., Rees-George, J., Cui, W., Fitzgerald, A., Win, J., Plummer, K.M., Templeton, M.D., 2009. Candidate effector gene identification in the ascomycete fungal phytopathogen *Venturia inaequalis* by expressed sequence tag analysis. *Mol. Plant Pathol*. 10, 431–448. https://doi.org/10.1111/J.1364-3703.2009.00543.X

Boyer, J., Liu, R.H., 2004. Nutrition Journal Apple phytochemicals and their health benefits.

Broggini, G.A.L., Bus, V.G.M., Parravicini, G., Kumar, S., Groenwold, R., Gessler, C., 2011. Genetic mapping of 14 avirulence genes in an EU-B04×1639 progeny of *Venturia inaequalis*. *Fungal Genet. Biol*. 48, 166–176. https://doi.org/10.1016/J.FGB.2010.09.001

Brown, S., 2012. Apple. Fruit Breed. 329–367. https://doi.org/10.1007/978-1-4419-0763-9_10

Burch-Smith, T.M., Anderson, J.C., Martin, G.B., Dinesh-Kumar, S.P., 2004. Applications and advantages of virus-induced gene silencing for gene function studies in plants. *Plant J*. 39, 734–746.

Burchill, R.T., Hutton, K.E., Crosse, J.E., Garrett, C.M.E., 1965. Inhibition of the perfect stage of

Venturia inaequalis (Cooke) Wint., by urea. *Nature* 205, 520–521.

Bus, V., Laurens, F.N.D., Van De Weg, W.E., Rusholme, R.L., Rikkerink, E.H.A., Gardiner, S.E., Bassett, H.C.M., Kodde, L.P., Plummer, K.M., 2005a. The *Vh8* locus of a new gene-for-gene interaction between *Venturia inaequalis* and the wild apple *Malus sieversii* is closely linked to the *Vh2* locus in *Malus pumila* R12740-7A. *New Phytol*. 166, 1035–1049. https://doi.org/10.1111/J.1469-8137.2005.01395.X

Bus, V., Rikkerink, E., Aldwinckle, H.S., Caffier, V., Durel, C.E., Gardiner, S., Gessler, C., Groenwold, R., Laurens, F., Cam, B. le, 2009. A proposal for the nomenclature of *Venturia inaequalis* races. *Acta Hortic*. 739–746.

Bus, V., Rikkerink, E.H.A., Van de Weg, W.E., Rusholme, R.L., Gardiner, S.E., Bassett, H.C.M., Kodde, L.P., Parisi, L., Laurens, F.N.D., Meulenbroek, E.J., 2005b. The *Vh2* and *Vh4* scab resistance genes in two differential hosts derived from Russian apple R12740-7A map to the same linkage group of apple. *Mol. Breed*. 15, 103–116.

Bus, V.G.M., Bassett, H.C.M., Bowatte, D., Chagné, D., Ranatunga, C.A., Ulluwishewa, D., Wiedow, C., Gardiner, S.E., 2010. Genome mapping of an apple scab, a powdery mildew and a woolly apple aphid resistance gene from open-pollinated Mildew Immune Selection. *Tree Genet. genomes* 6, 477–487.

Bus, V.G.M., Rikkerink, E.H.A., Caffier, V., Durel, C.-E., Plummer, K.M., 2011a. Revision of the Nomenclature of the Differential Host-Pathogen Interactions of *Venturia inaequalis* and *Malus* . Annu. Rev. *Phytopathol*. 49, 391–413. https://doi.org/10.1146/annurev-phyto-072910-095339

Bastiaanse, H., Bassett, H. C., Kirk, C., Gardiner, S. E., Deng, C., Groenworld, R., ... & Bus, V. G. (2016). Scab resistance in 'G eneva'apple is conditioned by a resistance gene cluster with complex genetic control. *Molecular plant pathology*, *17*(2), 159-172.

Caffier, V., Didelot, F., Pumo, B., Causeur, D., Durel, C.E., Parisi, L., 2010. Aggressiveness of eight *Venturia inaequalis* isolates virulent or avirulent to the major resistance gene *Rvi6* on a non-*Rvi6* apple cultivar. *Plant Pathol*. 59, 1072–1080. https://doi.org/10.1111/J.1365-3059.2010.02345.X

Caffier, V., Lasserre-Zuber, P., Giraud, M., Lascostes, M., Stievenard, R., Lemarquand, A., van de Weg, E., Expert, P., Denancé, C., Didelot, F., Le Cam, B., Durel, C.E., 2014. Erosion of quantitative host resistance in the apple × *Venturia inaequalis* pathosystem. *Infect. Genet. Evol*. 27, 481–489. https://doi.org/10.1016/J.MEEGID.2014.02.003

Caffier, V., Patocchi, A., Expert, P., Bellanger, M.-N., Durel, C.-E., Hilber-Bodmer, M., Broggini,

G.A.L., Groenwold, R., Bus, V.G.M., 2015b. Virulence Characterization of *Venturia inaequalis* Reference Isolates on the Differential Set of *Malus* Hosts. https://doi.org/10.1094/PDIS-07-14-0708-RE 99, 370–375. https://doi.org/10.1094/PDIS-07-14-0708-RE

Calenge, F., Faure, A., Goerre, M., Gebhardt, C., Van De Weg, W.E., Parisi, L., Durel, C.E., 2004b. Quantitative Trait Loci (QTL) analysis reveals both broad-spectrum and isolate-specific QTL for scab resistance in an apple progeny challenged with eight isolates of *Venturia inaequalis*. *Phytopathology* 94, 370–379. https://doi.org/10.1094/Phyto.2004.94.4.370

Cardi, T., 2016. Cisgenesis and genome editing: combining concepts and efforts for a smarter use of genetic resources in crop breeding. *Plant Breed*135, 139–147. https://doi.org/10.1111/pbr.12345

Chapman, K.S., Sundin, G.W., Beckerman, J.L., 2011. Identification of resistance to multiple fungicides in field populations of *Venturia inaequalis*. *Plant Dis.* 95, 921–926.

Cheng, F.S., Weeden, N.F., Brown, S.K., Aldwinckle, H.S., Gardiner, S.E., Bus, V.G., 1998. Development of a DNA marker for *(Vm),* a gene conferring resistance to apple scab. Genome. https://doi.org/10.1139/g98-020

Chevalier, M., Lespinasse, Y., Renaudin, S., 1991. A microscopic study of the different classes of symptoms coded by the *Vf* gene in apple for resistance to scab (*Venturia inaequalis*). *Plant Pathol* 40. https://doi.org/10.1111/j.1365-3059.1991.tb02374.x

Chizzali, C., Gusberti, M., Schouten, H.J., Gessler, C., Broggini, G.A., 2016. Cisgenic *Rvi6* scab-resistant apple lines show no differences in *Rvi6* transcription when compared with conventionally bred cultivars. *Planta* 243. https://doi.org/10.1007/s00425-015-2432-z

Clark, M., Bradeen, J., Luby, J., & Bedford, D. (2012, September). Apple Cultivar' Honeycrisp' exhibits genetic resistance to apple scab. *In Hortscience* (Vol. 47, No. 9, pp. S312-S313).

Clark, M.D., Bus, V.G.M., Luby, J.J., Bradeen, J.M., 2014a. Characterization of the defence response to *Venturia inaequalis* in 'Honeycrisp'apple, its ancestors, and progeny. *Eur. J. plant Pathol.* 140, 69–81.

Clark, M.D., Bus, V.G.M., Luby, J.J., Bradeen, J.M., 2014b. Clark MD, Luby JJ, Bradeen JM, Bus VGM (2014) Identification of candidate genes at *Rvi19* and *Rvi20*, two apple scab resistance loci in the 'Honeycrisp' apple (Malus × domestica). *In Plant and Animal Genome XXII Conference. Plant and Animal Genome.*

Cooper, S.J., Ashby, A.M., 1998. Comparison of cytokinin and cytokinin-O-glucoside cleaving β-

glucosidase productionin vitro by *Venturia inaequalis* and other phytopathogenic fungi with differing modes of nutritionin planta. *Physiol. Mol. Plant Pathol*. 53, 61–72.

Cornille, A., Gladieux, P., Smulders, M.J., Roldán-Ruiz, I., Laurens, F., Cam, B., Nersesyan, A., Clavel, J., Olonova, M., Feugey, L., Gabrielyan, I., Zhang, X.G., Tenaillon, M.I., Giraud, T., 2012. New insight into the history of domesticated apple: secondary contribution of the European wild apple to the genome of cultivated varieties. *PLoS Genet 8*. https://doi.org/10.1371/journal.pgen.1002703

Cova, V., Bandara, N.L., Liang, W., Tartarini, S., Patocchi, A., Troggio, M., Velasco, R., Komjanc, M., 2015a. Fine mapping of the *Rvi5 (Vm)* apple scab resistance locus in the 'Murray'apple genotype. *Mol. Breed*. 35, 1–12.

Cova, V., Lasserre-Zuber, P., Piazza, S., Cestaro, A., Velasco, R., Durel, C.E., Malnoy, M., 2015c. High-resolution genetic and physical map of the *Rvi1 (Vg)* apple scab resistance locus. *Mol. Breed*. https://doi.org/10.1007/s11032-015-0245-1

Cova, V., Paris, R., Passerotti, S., Zini, E., Gessler, C., Pertot, I., Loi, N., Musetti, R., & Komjanc, M. (2010). Mapping and functional analysis of four apple receptor-like protein kinases related to *LRPKm1* in *HcrVf2*-transgenic and wild-type apple plants. *Tree Genetics & Genomes*, *6*(3), 389–403.

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., ... & Marraffini, L. A. a.(2013) Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science*, *339*(6121), 819-823.

Chevalier, M., Lespinasse, Y., & Renaudin, S. (1991). A microscopic study of the different classes of symptoms coded by the *Vf* gene in apple for resistance to scab (*Venturia inaequalis*). *Plant Pathology*, *40*(2), 249-256.

Crandall, C.S., 1926. Apple breeding at the University of Illinois. *Illinois Agric Exp Stn bull* 275.

Crosby, J.A., Janick, J., Pecknold, P.C., Korban, S.S., O'Connon, P.A., Ries, S.M., Goffreda, J., Voordeckers, A., 1992. Breeding apples for scab resistance: 1945-1990. *Fruit Var J* 46.

Dayton, D.F., 1970. Prima-an early fall red apple with resistance to apple scab. *Fruit Var. Hortic. Dig*. 24, 20–22.

Dayton, D.F., 1968. Independent genes in *Malus* for resistance to *Venturia inaequalis*., in: *Proc. Amer. Soc. Hort. Sci*. pp. 89–94.

DeYoung, B.J., Innes, R.W., 2006. Plant NBS-LRR proteins in pathogen sensing and host defense.

Nat. Immunol. 7, 1243–1249.

Didelot, F., Caffier, V., Orain, G., Lemarquand, A., Parisi, L., 2016. Sustainable management of scab control through the integration of apple resistant cultivars in a low-fungicide input system. *Agric. Ecosyst. Environ*. 217, 41–48.

Dunemann, F., Egerer, J., 2010. A major resistance gene from Russian apple "Antonovka" conferring field immunity against apple scab is closely linked to the *Vf* locus. *Tree Genet. Genomes* 6, 627–633. https://doi.org/10.1007/S11295-010-0278-X

Durel, C.E., Calenge, F., Parisi, L., Weg, W.E., Kodde, L.P., Liebhard, R., Gessler, C., Thiermann, M., Dunemann, F., Gennari, F., Tartarini, S., 2004. An overview of the position and robustness of scab resistance QTLs and major genes by aligning genetic maps of five apple progenies. *Acta Hortic* 663. https://doi.org/10.17660/ActaHortic.2004.663.17

Durel, C.E., Parisi, L., Laurens, F., Van de Weg, W.E., Liebhard, R., Jourjon, M.F., 2003. Genetic dissection of partial resistance to race 6 of Venturia inaequalis in apple. *Genome* 46, 224–234.

Durel, C.E., Weg, E., Venisse, J.S., Parisi, L., 2000. Localisation of a major gene for scab resistance on the European genetic map of the Prima × Fiesta cross. *OILB/WPRS Bull* 23.

Dai, G. H., Nicole, M., Andary, C., Martinez, C., Bresson, E., Boher, B., Daniel, J. F., & Geiger, J. P. (1996). Flavonoids accumulate in cell walls, middle lamellae and calloserich papillae during an incompatible interaction between *Xanthomonas campestris pv. malvacearum* and cotton. *Physiol Mol Plant Pathol*, *49*. https://doi.org/10.1006/pmpp.1996.0055

Degenhardt, J., Al-Masri, A. N., Kürkcüoglu, S., Szankowski, I., & Gau, A. E. (2005). Characterization by suppression subtractive hybridization of transcripts that are differentially expressed in leaves of apple scab-resistant and susceptible cultivars of *Malus domestica*. *Molecular Genetics and Genomics 2005 273:4*, *273*(4), 326–335. https://doi.org/10.1007/S00438-005-1136-7

Erdin, N., Tartarini, S., Broggini, G.A.L., Gennari, F., Sansavini, S., Gessler, C., Patocchi, A., 2006. Mapping of the apple scab-resistance gene *Vb*. *Genome*. https://doi.org/10.1139/G06-096

Flachowsky, H., Szankowski, I., Fischer, T.C., Richter, K., Peil, A., Höfer, M., Dörschel, C., Schmoock, S., Gau, A.E., Halbwirth, H., Hanke, M.V., 2010. Transgenic apple plants overexpressing the Lc gene of maize show an altered growth habit and increased resistance to apple scab and fire blight. *Planta*. https://doi.org/10.1007/s00425-009-1074-4

Flor, H.H., 1971. Current status of the gene for gene concept. *Annu Rev Phytopathol* 9. https://doi.org/10.1146/annurev.py.09.090171.001423

FAOSTAT, F., 2021. Statistical data. Food and Agriculture Organization of the United Nations, Rome.

Frederick, Z.A., Villani, S.M., Cooley, D.R., Biggs, A.R., Raes, J.J., Cox, K.D., 2014. Prevalence of quinone outside inhibitor resistance and the stability of qualitative QoI resistance in *Venturia inaequalis*. *Plant Dis* 98, 1122–1130.

Gaj, T., Gersbach, C.A., Barbas III, C.F., 2013. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol*. 31, 397–405.

Galli, P., Broggini, G.A.L., Gessler, C., Patocchi, A., 2010a. Phenotypic characterization of the *Rvi15* (*Vr2*) apple scab resistance. JPP 92.

Galli, P., Potacchi, A., Broggini, G.A.L., Gessler, C., Potachi, A., 2010b. The *Rvi15* (*Vr2*) scab resistance locus contains three TIR NBS LRR genes. *Mol Plant-Microbe Interact* 23. https://doi.org/10.1094/MPMI-23-5-0608

Gebremichael, D.E., Haile, Z.M., Negrini, F., Sabbadini, S., Capriotti, L., Mezzetti, B., Baraldi, E., 2021. RNA interference strategies for future management of plant pathogenic fungi: prospects and challenges. *Plants* 10, 650.

Gelvin, S.B., 2003. *Agrobacterium*-mediated plant transformation: the biology behind the "gene-jockeying" tool. *Microbiol. Mol. Biol. Rev*. 67, 16–37.

Gennaro, D., 2012. Gene annotation and structural analysis of the *AvrRvi1* region in *Venturia inaequalis*. https://doi.org/10.3929/ethz-a-007619052

Gessler, C., Patocchi, A., Sansavini, S., Tartarini, S., Gianfranceschi, L., 2006. *Venturia inaequalis* resistance in apple. *CRC. Crit. Rev. Plant Sci*. https://doi.org/10.1080/07352680601015975

Gessler, C., Stumm, D., 1984. Infection and Stroma Formation by *Venturia inaequalis* on Apple Leaves with Different Degrees of Susceptibility to Scab. *J. Phytopathol*. 110, 119–126. https://doi.org/10.1111/J.1439-0434.1984.TB03399.X

Gessler, C., I.P., 2012. *Vf* scab resistance of *Malus*. *Trees* 26, 95–108. https://doi.org/10.1007/s00468-011-0618-y

Gladieux, P., Zhang, X., Róldan-ruiz, I., Caffier, V., Leroy, T., Devaux, M., Van Glabeke, S., Coart,

E.L.S., Le Cam, B., 2010. Evolution of the population structure of *Venturia inaequalis*, the apple scab fungus, associated with the domestication of its host. *Mol. Ecol*. 19, 658–674.

Gusberti, M., Gessler, C., Broggini, G.A.L., 2013. RNA-Seq Analysis Reveals Candidate Genes for Ontogenic Resistance in *Malus-Venturia* Pathosystem. *PLoS One* 8, e78457. https://doi.org/10.1371/JOURNAL.PONE.0078457

Gygax, M., Gianfranceschi, L., Liebhard, R., Kellerhals, M., Gessler, C., Patocchi, A., 2004. Molecular markers linked to the apple scab resistance gene *Vbj* derived from *Malus baccata* jackii. *Theor. Appl. Genet*. https://doi.org/10.1007/s00122-004-1803-9

Gianfranceschi, L., Koller, B., Seglias, N., Kellerhals, M., & Gessler, C. (1996). Molecular selection in apple for resistance to scab caused by *Venturia inaequalis*. *Theoretical and Applied Genetics*, *93*(1), 199-204.

Gosch, C., Halbwirth, H., Kuhn, J., Miosic, S., & Stich, K. (2009). Biosynthesis of phloridzin in apple (*Malus domestica* Borkh.). *Plant Science*, *176*(2), 223–231.

Gau, A. E., Koutb, M., Piotrowski, M., & Kloppstech, K. (2004). Accumulation of pathogenesis-related proteins in the apoplast of a susceptible cultivar of apple (*Malus domestica* cv. Elstar) after infection by *Venturia inaequalis* and constitutive expression of PR genes in the resistant cultivar Remo. *Eur J Plant Pathol*, *7*. https://doi.org/10.1023/B:EJPP.0000041552.98673.e3

Harris, S.A., Robinson, J.P., Juniper, B.E., 2002. Genetic clues to the origin of the apple. Trends Genet. 18, 426–430.

Hartung, F., Schiemann, J., 2014. Precise plant breeding using new genome editing techniques: opportunities, safety and regulation in the EU. *Plant J*. 78, 742–752.

Hemmat, M., Brown, S.K., Aldwinckle, H.S., Weeden, N.F., 2003. Identification and mapping of markers for resistance to apple scab from "Antonovka" and "Hansen's baccata #2." *Acta Hortic 622*. https://doi.org/10.17660/ActaHortic.2003.622.13

Hemmat, M., Brown, S.K., Weeden, N.F., 2002. Tagging and mapping scab resistance genes from R12740-7A apple. *J Am Soc Hortic Sci* 127.

Hetherington, S., 2010. An IPM manual for Australia's apple and pear industry. *Horticultural Australia*.

Holb, I.J., 2008. Timing of first and final sprays against apple scab combined with leaf removal and pruning in organic apple production. *Crop Prot*. 27, 814–822.

Holb, I.J., 2007. Classification of apple cultivar reactions to scab in integrated and organic production systems. *Can. J. plant Pathol*. 29, 251–260.

Holb, I.J., 2006. Effect of six sanitation treatments on leaf litter density, ascospore production of *Venturia inaequali*s and scab incidence in integrated and organic apple orchards. E*ur. J. Plant Pathol*. 115, 293–307.

Hough, L.F., 1944a. A survey of scab resistance of foliage on seedling in selected apple progenies. *Proc Am Soc Hortic Sci* 44.

Hough, L.F., 1944b. A survey of the scab resistance of the foliage on seedlings in selected apple progenies, in: *Proc. Amer. Soc. Hort. Sci*. pp. 260–272.

Hunter, P., 2014. "Genetically Modified Lite" placates public but not activists: new technologies to manipulate plant genomes could help to overcome public concerns about GM crops. *EMBO Rep* 15.

Hyson, D.A., 2011. A comprehensive review of apples and apple components and their relationship to human health 1,2. https://doi.org/10.3945/an.111.000513

Hemmat, M., Brown, S. K., Aldwinckle, H. S., Weeden, N. F., & Mehlenbacher, S. A. (2003). Identification and mapping of markers for resistance to apple scab from 'Antonovka' and 'Hansen's baccata# 2'. *Acta horticulturae*.

Ibanez, A.M., Dandekar, A.M., 2007. I. 10 Apple. *Manag. Ed. H Lörz* 241.

Jamar, L., Lefrancq, B., Fassotte, C., Lateur, M., 2008. A during-infection spray strategy using sulphur compounds, copper, silicon and a new formulation of potassium bicarbonate for primary scab control in organic apple production. *Eur. J. plant Pathol*. 122, 481–493.

Janick, J., Cummins, J.N., Brown, S.K., Hemmat, M., 1996. Apples In: Janick, J. and Moore, JN (eds) Fruit Breeding, Vol. 1, Tree and Tropical Fruits.

Janick, J., & Paris, H. S. (2006). The cucurbit images (1515–1518) of the Villa Farnesina, Rome. *Annals of Botany*, *97*(2), 165-176.

Jänsch, M., Broggini, G.A.L., Weger, J., Bus, V.G.M., Gardiner, S.E., Bassett, H., Patocchi, A., 2015. Identification of SNPs linked to eight apple disease resistance loci. *Mol. Breed*. 35, 1–21. https://doi.org/10.1007/S11032-015-0242-4

Jefferson, R.M., 1970. History, progeny, and location of crabapples of documented authentic origin.

US Department of Agriculture, Washington, DC. https://doi.org/10.5962/bhl.title.58654

Jensen, E. N., Buch-Andersen, T., Ravn-Haren, G., & Dragsted, L. O. (2009). Mini-review: The effects of apples on plasma cholesterol levels and cardiovascular risk–a review of the evidence. *The Journal of Horticultural Science and Biotechnology*, *84*(6), 34-41.

Jha, G., Thakur, K., Thakur, P., 2009. The *venturia* apple pathosystem: Pathogenicity mechanisms and plant defense responses. *J. Biomed. Biotechnol*. https://doi.org/10.1155/2009/680160

Joshi, S., Soriano, J.M., Schaart, J., Broggini, G.A.L., Szankowski, I., Jacobsen, E., Krens, F., Schouten, H.J., 2009. Approaches for development of cisgenic apples. *Towar. durable Resist. to apple scab using cisgenes* 9.

Joshi, S. G. (2010). *Towards durabale resistance to apple scab using cisgenes*. Wageningen University and Research.

Joshi, S.G., Schaart, J.G., Groenwold, R., Jacobsen, E., Schouten, H.J., Krens, F.A., 2011. Functional analysis and expression profiling of *HcrVf1* and *HcrVf2* for development of scab resistant cisgenic and intragenic apples. *Plant Mol Biol 75*. https://doi.org/10.1007/s11103-011-9749-1

Jones, J. D. G. Dangl,. JL (2006). *The plant immune system. nature*, *444*, 323-329.

Keitt, G.W., Jones, L.K., 1926. *Studies of the epidemiology and control of apple scab*.

Keitt, G.W., Palmiter, D.H., 1937. Heterothallism in *Venturia inaequalis*. *Science* (80-.). 85, 498.

Kenis, K., Keulemans, J., Davey, M.W., 2008. Identification and stability of QTLs for fruit quality traits in apple. *Tree Genet. Genomes* 2008 44 4, 647–661. https://doi.org/10.1007/S11295-008-0140-6

Khajuria, Y.P., Kaul, S., Wani, A.A., Dhar, M.K., 2018b. Genetics of resistance in apple against *Venturia inaequalis* (Wint.) Cke. *Tree Genet. Genomes*. https://doi.org/10.1007/s11295-018-1226-4

Kollar, A., 1998. Characterization of an endopolygalacturonase produced by the apple scab fungus, *Venturia inaequalis*. *Mycol. Res*. 102, 313–319.

Koller, B., Gianfranceschi, L., Seglias, N., Mcdermott, J., Gessler, C., 1994. DNA markers linked to *Malus floribunda* 821 scab resistance. *Plant Mol Biol 26*. https://doi.org/10.1007/BF00013746

Köller, W., 1994. Chemical control of apple scab-status quo and future. *Nor. J. Agric. Sci.*

Koller, W., Parker, D.M., 1989. Purification and characterization of cutinase from *Venturia*

inaequalis. Phytopathology.

Koller, W., Parker, D.M., Becker, C.M., 1991. Role of cutinase in the penetration of apple leaves by *Venturia inaequalis*. *Phytopathology 81*. https://doi.org/10.1094/Phyto-81-1375

Köller, W., Parker, D.M., Turechek, W.W., Avila-Adame, C., Cronshaw, K., 2004. A two-phase resistance response of *Venturia inaequalis* populations to the QoI fungicides kresoxim-methyl and trifloxystrobin. *Plant Dis*. 88, 537–544.

Köller, W., Wilcox, W.F., 2001. Evidence for the predisposition of fungicide-resistant isolates of *Venturia inaequalis* to a preferential selection for resistance to other fungicides. *Phytopathology 91*, 776–781.

Krens, F.A., Schaart, J.G., van der Burgh, A.M., TinnenbroekCapel, I.E.M., Groenwold, R., Kodde, L.P., Broggini, G.A.L., Gessler, C., Schouten, H.J., 2015. Cisgenic apple trees; development, characterization, and performance. *Front. Plant Sci*. https://doi.org/10.3389/fpls.2015.00286

Kucheryava, N., Bowen, J.K., Sutherland, P.W., Conolly, J.J., Mesarich, C.H., Rikkerink, E.H.A., Kemen, E., Plummer, K.M., Hahn, M., Templeton, M.D., 2008. Two novel *Venturia inaequalis* genes induced upon morphogenetic differentiation during infection and in vitro growth on cellophane. *Fungal Genet. Biol*. 45, 1329–1339.

Laaninen, T., 2016. New plant-breeding techniques: Applicability of GM rules (Briefing)(Brussels: European Parliamentary Research Service (EPRS)). Available World Wide Web http//www. Eur. Eur. eu/RegData/etudes/BRIE/2016/582018/EPRS_BRI 582018_EN. pdf.

Laloi, G., Vergne, E., Durel, C.-E., Le Cam, B., Caffier, V., 2017. Efficiency of pyramiding of three quantitative resistance loci to apple scab. *Plant Pathol*. 66, 412–422.

Laurens, F., Durel, C.E., Lascostes, M., 2004. Molecular characterization of French local apple cultivars using SSRs. Acta Hortic 663. https://doi.org/10.17660/*ActaHortic*.2004.663.114

Lespinasse, Y., Durel, C.E., Parisi, L., Laurens, F., Chevalier, M., Pinet, C., 2000. An European project: D.A.R.E-Durable Apple Resistance in Europe (FAIR 5 CT97-3898). Durable resistance of apple to scab and powdery mildew: one step more towards an environmental friendly orchard. IOBC/WPRS Bull 23.

Li, B., Xu, X., 2002. Infection and development of apple scab (*Venturia inaequalis*) on old leaves. J. *Phytopathol.* 150, 687–691. https://doi.org/10.1046/J.1439-0434.2002.00824.X

Liebhard, R., Koller, B., Patocchi, A., Kellerhals, M., Pfammatter, W., Jermini, M., Gessler, C., 2003.

Mapping quantitative field resistance against apple scab in a “Fiesta” x “Discovery” progeny. *Phytopathology*. https://doi.org/10.1094/PHYTO.2003.93.4.493

Limera, C., Sabbadini, S., Sweet, J.B., Mezzetti, B., 2017. New biotechnological tools for the genetic improvement of major woody fruit species. *Front. Plant Sci*. 8, 1418.

Lindhout, P., 2002. The perspectives of polygenic resistance in breeding for durable disease resistance. *Euphytica* 124, 217–226.

Lucht, J., 2015. Public acceptance of plant biotechnology and GM crops. *Viruses 7*, 4254–4281. https://doi.org/10.3390/v7082819

Lumbsch, H.T., Huhndorf, S.M., 2007. Outline of ascomycota–2007. *Myconet* 13, 1–58.

Lusser, M., Davies, H. V, 2013. Comparative regulatory approaches for groups of new plant breeding techniques. *New Biotechnol* 30. https://doi.org/10.1016/j.nbt.2013.02.004

Lusser, M., Parisi, C., Plan, D., Rodríguez-Cerezo, E., 2011. New plant breeding techniques: state-of-the-art and prospects for commercial development. Publications office of the European Union Luxembourg.

Liebhard, R., Koller, B., Patocchi, A., Kellerhals, M., Pfammatter, W., Jermini, M., & Gessler, C. (2003). Mapping quantitative field resistance against apple scab in a ‘Fiesta’×‘Discovery’progeny. *Phytopathology*, 93(4), 493-501.

Lamb, C., & Dixon, R. A. (1997). The oxidative burst in plant disease resistance. *Annual Review of Plant Biology*, *48*(1), 251–275.

Liu, W., Liu, J., Ning, Y., Ding, B., Wang, X., Wang, Z., & Wang, G. L. (2013). Recent progress in understanding PAMP and effector triggered immunity against the rice blast fungus *Magnaporthe oryzae*. *Mol Plant*, *6*. https://doi.org/10.1093/mp/sst015

MacHardy, 1996a. Apple scab: biology, epidemiology, and management. *American Phytopathological Society (APS Press).*

MacHardy, 1996b. Pathogen development and host tissue reaction. *Apple scab biology, epidemiology, and management. APS press, St. Paul.*

MacHardy, W.E., Gadoury, D.M., Gessler, C., 2001. Parasitic and biological fitness of *Venturia inaequalis*: relationship to disease management strategies. *Plant Dis*. 85, 1036–1051.

Malnoy, M., Xu, M., Borejsza-Wysocka, E., Korban, S. S., & Aldwinckle, H. S. (2008). Two

receptor-like genes, *Vfa1* and *Vfa2*, confer resistance to the fungal pathogen *Venturia inaequalis* inciting apple scab disease. *Molecular plant-microbe interactions*, 21(4), 448-458.

Malnoy, M., Viola, R., Jung, M.H., Koo, O.J., Kim, S., Kim, J.S., Velasco, R., Kanchiswamy, C.N., 2016. DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. *Front. Plant Sci*. 7. https://doi.org/10.3389/fpls.2016.01904

Malnoy, M., Jin, Q., Borejsza-Wysocka, E. E., He, S. Y., & Aldwinckle, H. S. (2007). Overexpression of the apple MpNPR1 gene confers increased disease resistance in Malus× domestica. *Molecular Plant-Microbe Interactions*, *20*(12), 1568-1580.

Manganaris, A.G., Alston, F.H., Weeden, N.F., Aldwinckle, H.S., Gustafson, H.L., Brown, S.K., 1994. Isozyme locus *Pgm-1* is tightly linked to a gene (*Vf*) for scab resistance in apple. *J Am Soc Hortic Sci* 119.

Mansy, S., 2017. Occurrence of *Venturia inaequalis* races in Poland able to overcome specific scab resistance genes. *Eur J Plant Pathol* 147. https://doi.org/10.1007/s10658-016-1003-x

Mazzotta, S., & Kemmerling, B. (2011). Pattern recognition in plant innate immunity. *J Plant Pathol*, *93*.

Mikulic Petkovsek, M., Stampar, F., & Veberic, R. (2009). Seasonal changes in phenolic compounds in the leaves of scab-resistant and susceptible apple cultivars. *Canadian Journal of Plant Science*, *89*(4), 745–753.

National nutrient database for standard reference, U., 2021. FoodData Central [WWW Document]. URL https://fdc.nal.usda.gov/fdc-app.html#/?query=apple (accessed 8.2.21).

Nicholson, R.L., Kuc, J., Williams, E.B., 1972. Histochemical demonstration of transitory esterase activity in *Venturia inaequalis*. *Phytopathology* 62. https://doi.org/10.1094/Phyto-62-1242

Nicholson, R. L., & Hammerschmidt, R. (1992). Phenolic compounds and their role in disease resistance. *Annu Rev Phytopathol*, *30*. https://doi.org/10.1146/annurev.py.30.090192.002101

Padmarasu, S., Sargent, D.J., Jaensch, M., Kellerhals, M., Tartarini, S., Velasco, R., Troggio, M., Patocchi, A., 2014. Fine-mapping of the apple scab resistance locus *Rvi12* (*Vb*) derived from 'Hansen's baccata #2.' *Mol. Breed*. https://doi.org/10.1007/s11032-014-0167-3

Padmarasu, S., Sargent, D.J., Patocchi, A., Troggio, M., Baldi, P., Linsmith, G., Poles, L., Jänsch, M., Kellerhals, M., Tartarini, S., Velasco, R., 2018. Identification of a leucine-rich repeat receptor-like serine/threonine-protein kinase as a candidate gene for *Rvi12* (*Vb*)-based apple scab

resistance. *Mol. Breed*. 38. https://doi.org/10.1007/S11032-018-0825-Y

Panter, S.N., Hammond-Kosack, K.E., Harrison, K., Jones, J.D.G., Jones, D.A., 2002. Developmental control of promoter activity is not responsible for mature onset of *Cf-9B*-mediated resistance to leaf mold in tomato. *Mol. plant-microbe Interact*. 15, 1099–1107.

Papp, D., Singh, J., Gadoury, D., Khan, A., 2020. New North American isolates of *Venturia inaequalis* can overcome apple scab resistance of malus floribunda 821. *Plant Dis*. 104, 649–655. https://doi.org/10.1094/PDIS-10-19-2082-RE

Paris, R., Cova, V., Pagliarani, G., Tartarini, S., Komjanc, M., Sansavini, S., 2009. Expression profiling in *HcrVf2*-transformed apple plants in response to *Venturia inaequalis*. https://doi.org/10.1007/s11295-008-0177-6

Parisi, C., Tillie, P., Rodríguez-Cerezo, E., 2016. The global pipeline of GM crops out to 2020. Nat. *Biotechnol*. 34, 31–36.

Parisi, L., Fouillet, V., Schouten, H.J., Groenwold, R., Laurens, F., Didelot, F., Evans, K., Fischer, C., Gennari, F., Kemp, H., Lateur, M., Patocchi, A., Thissen, J., Tsipouridis, C., 2004a. Variability of the pathogenicity of *Venturia inaequalis* in Europe. *Acta Hortic*. 663, 107–114. https://doi.org/10.17660/ACTAHORTIC.2004.663.13

Patocchi, A., Gianfranceschi, L., & Gessler, C. (1999). Towards the map-based cloning of *Vf*: fine and physical mapping of the *Vf* region. *Theoretical and applied genetics*, 99(6), 1012-1017.

Patocchi, A. (2000). Map-based cloning of resistance gene homologues in the *Vf*-region of the apple (Malus sp.) (Doctoral dissertation, ETH Zurich).

Patocchi, A., Bigler, B., Koller, B., Liebhard, R., Kellerhals, M., & Gessler, C. (2003). Mapping of *Vr2*, a third apple scab resistance gene of Russian seedling (R12740-7A). In Plant & Animal Genomes XI Conference, Poster (p. P540).

Patocchi, A., Bigler, B., Koller, B., Kellerhals, M., & Gessler, C. (2004). *Vr2*: a new apple scab resistance gene. *Theoretical and Applied Genetics*, 109(5), 1087-1092.

Patocchi, A., Frei, A., Frey, J. E., & Kellerhals, M. (2009). Towards improvement of marker assisted selection of apple scab resistant cultivars: *Venturia inaequalis* virulence surveys and standardization of molecular marker alleles associated with resistance genes. *Molecular Breeding*, 24(4), 337-347.

Patocchi, A., Wehrli, A., Dubuis, P. H., Auwerkerken, A., Leida, C., Cipriani, G., ... & Bus, V. G.

(2020). Ten years of VINQUEST: First insight for breeding new apple cultivars with durable apple scab resistance. *Plant disease*, 104(8), 2074-2081.

Perchepied, L., Chevreau, E., Ravon, E., Gaillard, S., Pelletier, S., Bahut, M., ... & Vergne, E. (2021). Successful intergeneric transfer of a major apple scab resistance gene (*Rvi6*) from apple to pear and precise comparison of the downstream molecular mechanisms of this resistance in both species. *BMC genomics*, 22(1), 1-18.

Pedersen, W.L., Leath, S., 1988. Pyramiding major genes for resistance to maintain residual effects. *Annu*. *Rev*. *Phytopathol*. 26, 369–378.

Peyraud, R., Dubiella, U., Barbacci, A., Genin, S., Raffaele, S., Roby, D., 2017. Advances on plant–pathogen interactions from molecular toward systems biology perspectives. **Plant J**. 90, 720–737.

Phipps, J.B., Robertson, K.R., Rohrer, J.R., Smith, P.G., 1991. Origins and evolution of subfam. Maloideae (Rosaceae). *Syst Bot* 16. https://doi.org/10.2307/2419283

Pilet-Nayel, M.-L., Moury, B., Caffier, V., Montarry, J., Kerlan, M.-C., Fournet, S., Durel, C.-E., Delourme, R., 2017. Quantitative Resistance to Plant Pathogens in Pyramiding Strategies for Durable Crop Protection. *Front. Plant Sci*. 0, 1838. https://doi.org/10.3389/FPLS.2017.01838

Podevin, N., Devos, Y., Davies, H.V., Nielsen, K.M., 2012. Transgenic or not? No simple answer! New biotechnology-based plant breeding techniques and the regulatory landscape. *EMBO Rep*. 13, 1057–1061.

Poland, J.A., Balint-Kurti, P.J., Wisser, R.J., Pratt, R.C., Nelson, R.J., 2009. Shades of gray: the world of quantitative disease resistance. *Trends Plant Sci*. 14, 21–29.

Pompili, V., Dalla Costa, L., Piazza, S., Pindo, M., & Malnoy, M. (2020). Reduced fire blight susceptibility in apple cultivars using a high-efficiency CRISPR/Cas9-FLP/FRT-based gene editing system. *Plant biotechnology journal*, 18(3), 845-858.

Purkayastha, A., Dasgupta, I., 2009. Virus-induced gene silencing: a versatile tool for discovery of gene functions in plants. P*lant Physiol. Biochem*. 47, 967–976.

Plaxton, W. C. (1996). The organization and regulation of plant glycolysis. *Annual Review of Plant Biology*, *47*(1), 185–214.

Quamme, H.A., Hampson, C.R., Hall, J.W., Sholberg, P.L., Bedford, K.E., Randall, P., 2003. Inheritance of apple scab resistance from polygenic sources based on greenhouse and field

evaluation. *Acta Hortic* 622. https://doi.org/10.17660/ActaHortic.2003.622.29

Roberts, A.L., Crute, I.R., 1994. A scab resistance from Malus floribunda 821 (*Vf*) is rendered ineffective by isolates of *Venturia inaequalis* is from *Malus floribunda*. *Nor J Agric Sci* 17.

Robinson, J.P., Harris, S.A., Juniper, B.E., 2001. Taxonomy of the genus Malus Mill.(Rosaceae) with emphasis on the cultivated apple, Malus domestica Borkh. Plant Syst. Evol. 226, 35–58.

Rommens, C.M., 2007. Intragenic crop improvement: Combining the benefits of traditional breeding and genetic engineering. *J. Agric. Food Chem*. 55, 4281–4288.

Rommens, C.M., 2004. All-native DNA transformation: a new approach to plant genetic engineering. *Trends Plant Sci*. 9, 457–464.

Sakurai, K., Brown, S.K., Weeden, N., 2000. Self-incompatibility alleles of apple cultivars and advanced selections. *HortScience 35*, 116–119.

Sandoval-Ramírez, B.A., Catalán, Ú., Calderón-Pérez, L., Companys, J., Pla-Pagà, L., Ludwig, I.A., Romero, M.P., Solà, R., 2020. The effects and associations of whole-apple intake on diverse cardiovascular risk factors. *A narrative review*. https://doi.org/10.1080/10408398.2019.1709801 60, 3862–3875. https://doi.org/10.1080/10408398.2019.1709801

Sapoukhina, N., Durel, C.E., Cam, B., 2009. Spatial deployment of gene-for-gene resistance governs evolution and spread of pathogen populations. *Theor Ecol 2*. https://doi.org/10.1007/s12080-009-0045-5

Schouten, H.J., Brinkhuis, J., Burgh, A.M., Schaart, J.G., Groenwold, R., Broggini, G.A.L., Gessler, C., 2014. Cloning and functional characterization of the *Rvi15* (*Vr2*) gene for apple scab resistance. *Tree Genet Genomes* 10. https://doi.org/10.1007/s11295-013-0678-9

Schouten, H.J., Krens, F.A., Jacobsen, E., 2006a. Do cisgenic plants warrant less stringent oversight? *Nat. Biotechnol*. 24, 753.

Schouten, H.J., Krens, F.A., Jacobsen, E., 2006b. Cisgenic plants are similar to traditionally bred plants: international regulations for genetically modified organisms should be altered to exempt cisgenesis. *EMBO Rep*. 7, 750–753. https://doi.org/10.1038/sj.embor.7400769

Shay, J.R., Hough, L.F., 1952. Evaluation of apple scab resistance in selections of *Malus*. *Am. J. Bot*. 288–297.

Silfverberg-Dilworth, E., Besse, S., Paris, R., Belfanti, E., Tartarini, S., Sansavini, S., Patocchi, A.,

Gessler, C., 2005. Identification of functional apple scab resistance gene promoters. *Theor. Appl. Genet*. 110, 1119–1126. https://doi.org/10.1007/s00122-005-1940-9

Small, I., 2007. RNAi for revealing and engineering plant gene functions. *Curr. Opin. Biotechnol.* 18, 148–153.

Smereka, K.J., Kausch, A.P., MacHardy, W.E., 1988. Intracellular junctional structures in germinating ascopores of *Venturia inaequalis*. *Protoplasma* 142, 1–4.

Soriano, J.M., Joshi, S.G., Van Kaauwen, M., Noordijk, Y., Groenwold, R., Henken, B., Van De Weg, W.E., Schouten, H.J., 2009. Identification and mapping of the novel apple scab resistance gene *Vd3*. *Tree Genet. Genomes* 5, 475–482. https://doi.org/10.1007/S11295-009-0201-5

Soriano, J.M., Madduri, M., Schaart, J.G., van der Burgh, A., van Kaauwen, M.P.W., Tomic, L., Groenwold, R., Velasco, R., van de Weg, E., Schouten, H.J., 2014a. Fine mapping of the gene *Rvi18* (*V25*) for broad-spectrum resistance to apple scab, and development of a linked SSR marker suitable for marker-assisted breeding. *Mol. Breed*. 34, 2021–2032.

Soufflet-Freslon, V., Gianfranceschi, L., Patocchi, A., Durel, C.E., 2008. Inheritance studies of apple scab resistance and identification of *Rvi14*, a new major gene that acts together with other broad-spectrum QTL. *Genome 51*, 657–667. https://doi.org/10.1139/G08-046

Sprink, T., Eriksson, D., Schiemann, J., Hartung, F., 2016. Regulatory hurdles for genome editing: process-vs. product-based approaches in different regulatory contexts. *Plant Cell Rep. 35*, 1493–1506.

Statista, 2021. • Fruit: world production by type 2019 | Statista [WWW Document]. URL https://www.statista.com/statistics/264001/worldwide-production-of-fruit-by-variety/ (accessed 8.1.21).

Steiner, U., Oerke, E.-C., 2007. Localized melanization of appressoria is required for pathogenicity of Venturia inaequalis. *Phytopathology 97*, 1222–1230.

Sun, X., Jiao, C., Schwaninger, H., Chao, C.T., Ma, Y., Duan, N., Khan, A., Ban, S., Xu, K., Cheng, L., Zhong, G.-Y., Fei, Z., 2020. Phased diploid genome assemblies and pan-genomes provide insights into the genetic history of apple domestication. *Nat. Genet*. 2020 5212 52, 1423–1432. https://doi.org/10.1038/s41588-020-00723-9

Szankowski, I., Waidmann, S., Degenhardt, J., Patocchi, A., Paris, R., Silfverberg-Dilworth, E., Broggini, G., Gessler, C., 2009. Highly scab-resistant transgenic apple lines achieved by

introgression of *HcrVf2* controlled by different native promoter lengths. *Tree Genet. Genomes 5*, 349–358. https://doi.org/10.1007/s11295-008-0191-8

Szkolnik, M., 1969. 534 Vol. 53, No. 7--Plant disease reporter--july 1969 maturation and discharge of ascospores of *Venturia inaequalis*. *Plant Dis. Report. 53*, 534.

Tartarini, S., 1996. RAPD markers linked to the *Vf* gene for scab resistance in apple. Appl Genet 92. https://doi.org/10.1007/BF00221891

Tartarini, S., Gennari, F., Pratesi, D., Palazetti, C., Sansavini, S., 2004. Characterization and genetic mapping of a major scab resistance gene from the old Italian apple cultivar 'Durello di Forl'ı. *Acta Hortic* 663. https://doi.org/10.17660/ActaHortic.2004.663.16

Tartarini, S., Gianfranceschi, L., Sansavini, S., Gessler, C., 1999a. Development of reliable PCR markers for the selection of the *Vf* gene conferring scab resistance in apple. *Plant Breed* 118. https://doi.org/10.1046/j.1439-0523.1999.118002183.x

Tartarini, S., Sansavini, S., Vinatzer, B., Gennari, F., Domizi, C., 1999b. Efficiency of marker assisted selection (MAS) for the *Vf* scab resistance gene, in: Eucarpia Symposium on Fruit Breeding and *Genetics* 538. pp. 549–552.

Turechek, W.W., 2004. Apple Diseases and their Management. *Dis. Fruits Veg*. Vol. I 1–108. https://doi.org/10.1007/1-4020-2606-4_1

Torres, M. A., Jones, J. D., & Dangl, J. L. (2006). Reactive oxygen species signaling in response to pathogens. *Plant physiology*, *141*(2), 373-378.Vaillancourt, L.J., Hartman, J.R., 2005. Apple scab. The Plant Health Instructor DOI: 10.1094. PHI-I-2000-1005-01 Updated.

Treutter, D. (2006). Significance of flavonoids in plant resistance: a review. *Environmental Chemistry Letters*, *4*(3), 147–157.

Umeda, M., Hara, C., Matsubayashi, Y., Li, H.-H., Liu, Q., Tadokoro, F., Aotsuka, S., & Uchimiya, H. (1994). Expressed sequence tags from cultured cells of rice (*Oryza sativa* L.) under stressed conditions: analysis of transcripts of genes engaged in ATP-generating pathways. *Plant Molecular Biology*, *25*(3), 469–478.

Vaillancourt, L.J., Hartman, J.R., 2000. Apple scab. *Plant Heal. Instr*.

Valsangiacomo, C., Gessler, C., 1988. Role of the cuticular membrane in ontogenic and *Vf*-resistance of apple leaves against *Venturia inaequalis*. *Phytopathology* 78, 1066–1069.

Vanblaere, T., Szankowski, I., Schaart, J., Schouten, H., Flachowsky, H., Broggini, G.A.L., Gessler, C., 2011. The development of a cisgenic apple plant. *J. Biotechnol*. https://doi.org/10.1016/j.jbiotec.2011.05.013

Vanderzande, S., Piaskowski, J.L., Luo, F., Edge Garza, D.A., Klipfel, J., Schaller, A., Martin, S., Peace, C., 2018. Crossing the Finish Line: How to Develop Diagnostic DNA Tests as Breeding Tools after QTL Discovery. *J. Hortic*. 05. https://doi.org/10.4172/2376-0354.1000228

Velasco, R., Zharkikh, A., Affourtit, J., Dhingra, A., Cestaro, A., Kalyanaraman, A., Fontana, P., Bhatnagar, S.K., Troggio, M., Pruss, D., 2010. The genome of the domesticated apple (Malus× domestica Borkh.). *Nat. Genet*. 42, 833–839.

Vinatzer, B.A., Patocchi, A., Gianfranceschi, L., Tartarini, S., Zhang, H.B., Gessler, C., Sansavini, S., 2001. Apple contains receptor-like genes homologous to the Cladosporium fulvum resistance gene family of tomato with a cluster of genes cosegregating with *Vf* apple scab resistance. *Mol. Plant-Microbe Interact*. https://doi.org/10.1094/MPMI.2001.14.4.508

Vinatzer, B.A., Patocchi, A., Tartarini, S., Gianfranceschi, L., Sansavini, S., Gessler, C., 2004. Isolation of two microsatellite markers from BAC clones of the *Vf* scab resistance region and molecular characterization of scab-resistant accessions in *Malus* germplasm. *Mol Breed 123*.

Vinatzer, B.A., Zhang, H.B., Sansavini, S., 1998. Construction and characterization of a bacterial artificial chromosome library of apple. *Theor Appl Genet* 97. https://doi.org/10.1007/s001220051008

Williams, E. T. (1968). A new physiological race of *Venturia inaequalis*, incitant of apple scab. *Plant Dis. Rep*., 52, 799-801.

Williams, E.B., Dayton, D.F., Shay, J.R., 1996. Allelic genes in *Malus* for resistance to *Venturia inaequalis*. Proc Am Soc Hortic Sci 88.

Wis, K.N., 1938. A cytological study of host-parasite relations of *Venturia inaequalis* on apple leaves'. *J. Agric. Res*. 56.

Wulff, B.B.H., Chakrabarti, A., Jones, D.A., 2009. Recognitional specificity and evolution in the tomato–Cladosporium fulvum pathosystem. *Mol. plant-microbe Interact*. 22, 1191–1202.

Würdig, J., Flachowsky, H., Saß, A., Peil, A., Hanke, M., 2015. Improving resistance of different apple cultivars using the *Rvi6* scab resistance gene in a cisgenic approach based on the Flp/FRT recombinase system. *Mol Breed 35*. https://doi.org/10.1007/s11032-015-0291-8

Xu, M.L., Korban, S.S., 2004. Somatic variation plays a key role in the evolution of the *Vf* gene family residing in the *Vf* locus that confers resistance to apple scab disease. *Mol Phylogenet Evol* 32. https://doi.org/10.1016/j.ympev.2003.12.004

Xu, M.L., Korban, S.S., 2002. A cluster of four receptor-like genes resides in the *Vf* locus that confers resistance to apple scab disease. *Genetics* 162.

Xu, X.M., Robinson, J., 2005. Modelling the effects of wetness duration and fruit maturity on infection of apple fruits of Cox's Orange Pippin and two clones of Gala by *Venturia inaequalis*. *Plant Pathol*. 54, 347–356.

Yamagishi, N., Kishigami, R., Yoshikawa, N., 2014. Reduced generation time of apple seedlings to within a year by means of a plant virus vector: a new plant-breeding technique with no transmission of genetic modification to the next generation. *Plant Biotechnol*. J. 12, 60–68.

Yang, H., Korban, S.S., 1996. Screening apples for OPD20/600 using sequencespecific primers. *Theor Appl Genet* 92. https://doi.org/10.1007/BF00223383

Yang, H., Kruger, J., 1994. Identification of an RAPD marker linked to the *Vf* gene for scab resistance in apples. *Plant Breed* 12. https://doi.org/10.1111/j.1439-0523.1994.tb00691.x

Zhang, Y., & Wang, L. (2005). The WRKY transcription factor superfamily: its origin in eukaryotes and expansion in plants. *BMC Evolutionary Biology*, *5*(1), 1–12.

Zhan, J., Thrall, P.H., Burdon, J.J., 2014. Achieving sustainable plant disease management through evolutionary principles. *Trends Plant Sci* 19. https://doi.org/10.1016/j.tplants.2014.04.010

Chapter 2

Aims of the thesis

Apple scab is a serious disease, caused by the fungus, *V. inaequalis*. The development of durable apple scab resistant cultivars is one of the long-term aims of the apple breeding community. This can be accomplished by creating transgenic or cisgenic apple lines with scab resistance genes and pyramiding of major functionally characterized scab resistance genes into susceptible apple cultivar for long lasting disease resistance.

For developing apple scab resistant varieties with *Rvi12* scab resistance and pyramiding of *Rvi12* with other known scab resistance genes, further functional characterization of *Rvi12_Cd5* putative candidate gene is needed to confirm its role in *Rvi12* resistance. A faster approach for developing cultivars with scab resistance would be to perform transgenesis or cisgenesis with the identified *Rvi12_Cd5* candidate gene (Padmarasu *et al*., 2018). Previously, transgenic 'Gala' plants were developed containing *Rvi6* (Belfanti *et al*., 2004; Malnoy *et al*., 2008) and *Rvi15* (Schouten *et al*., 2014) scab resistance genes and these transgenic genotypes were shown to be resistant to *V. inaequalis*. Transgenesis or cisgenesis based methods of developing scab resistance is relatively quick compared to the marker assisted breeding approach (Krens *et al*., 2015; Jacobsen and Schouten, 2008; Joshi *et al*., 2009; Schaart *et al*., 2016; Szankowski *et al*., 2009). Cisgenic 'Gala' lines with *Rvi6* scab resistance gene were previously developed and characterized to be scab resistant (Vanblaere *et al*., 2011). It is now possible to generate cisgenic apple lines with putative *Rvi12_Cd5* candidate gene (Padmarasu *et al*., 2018). In addition, molecular studies gained from *Malus-Venturia* interactions and knowledge on downstream signaling events of *Rvi12_Cd5* will help to make the decisions surrounding the combination of R-genes selected for pyramiding and thus the development of durable scab resistance (Bowen *et al*., 2011).

The main prerequisite for cisgenesis is to have availability of functionally characterized resistance genes which can be achieved by transgenesis. In this study, cloning and functional characterization of one of the scab resistance genes *Rvi12_Cd5* is being performed to prove that the candidate gene is responsible for *Rvi12* locus resistance from 'Hansen's baccata #2' and then leading to the development of cisgenic apple cultivars carrying *Rvi12* scab resistance.

In chapter 3, we aim to overexpress the candidate gene *Rvi12_Cd5* into susceptible apple cultivar 'Gala' and induce kanamycin resistance for selection of transgenic apple. The purpose of overexpressing the *Rvi12_Cd5* into susceptible 'Gala' via stable and fast gene transfer technique is to investigate the functional role of *Rvi12_Cd5*. This chapter deals with gene functional study of *Rvi12_Cd5* by relating phenotypic and genotypic characteristics of transgenic apple.

Chapter 4 deals with investigation of cis-regulatory elements of *Rvi12_Cd5* native promoter by *in-silico* analysis in order to understand possible key role of promoter for enhancing the expression

quantity or quality of *Rvi12_Cd5* against apple scab. The following analysis also helps to predict importance of native promoter sequence length for *Rvi12_Cd5* expression.

In chapter 4, we also aim to express *Rvi12_Cd5* under its own native promoter, induce antibiotic resistance for selection of successful transformants and produce cisgenic apple by FLP/FRT based exogenous DNA removal. The motive of natural *Rvi12_Cd5* expression is to inquire the gene expression quality or quantity for scab resistance under native promoter activity. The other important factor about production of cisgenic apple containing *Rvi12_Cd5* gene, aims to keep the original genetic makeup of plant with increased scab resistance with minimum inclusion of foreign DNA.

2.1 Final Objectives

The final objectives of this thesis work were thus to,

- Cloning and functional characterization of apple scab resistant gene *Rvi12_Cd5* derived from 'Hansen's baccata #2'
- Sequencing and *in-silico* analysis of putative native promoter region of candidate gene *Rvi12_Cd5*
- "Development and molecular characterization of cisgenic apple lines expressing scab resistant gene *Rvi12_Cd5*"

2.2 Bibliography

Belfanti, E., Silfverberg-Dilworth, E., Tartarini, S., Patocchi, A., Barbieri, M., Zhu, J., Vinatzer, B. A., Gianfranceschi, L., Gessler, C., & Sansavini, S. (2004). The *HcrVf2* gene from a wild apple confers scab resistance to a transgenic cultivated variety (Vol. 101, Issue 3). www.pnas.orgcgidoi10.1073pnas.0304808101

Bowen, J.K., Mesarich, C.H., Bus, V.G.M., Beresford, R.M., Plummer, K.M., Templeton, M.D., 2011. *Venturia inaequalis*: The causal agent of apple scab. *Mol. Plant Pathol*. 12, 105–122. https://doi.org/10.1111/J.1364-3703.2010.00656.X

Jacobsen, E., & Schouten, H. J. (2008). Cisgenesis, a new tool for traditional plant breeding, should be exempted from the regulation on genetically modified organisms in a step by step approach. *Potato Res*, *51*. https://doi.org/10.1007/s11540-008-9097-y

Joshi, S. G., Soriano, J. M., Kortstee, A., Schaart, J. G., Krens, F. A., Jacobsen, E., & Schouten, H. J. (2009). Development of cisgenic apples with durable resistance to apple scab. *Acta Horticulturae*. https://doi.org/10.17660/ActaHortic.2009.839.53

Krens, F. A., Schaart, J. G., van der Burgh, A. M., TinnenbroekCapel, I. E. M., Groenwold, R., Kodde, L. P., Broggini, G. A. L., Gessler, C., & Schouten, H. J. (2015). Cisgenic apple trees; development, characterization, and performance. *Frontiers in Plant Science*. https://doi.org/10.3389/fpls.2015.00286

Malnoy, M., Xu, M., Borejsza-Wysocka, E., Korban, S. S., & Aldwinckle, H. S. (2008). Two receptor-like genes, *Vfa1* and *Vfa2*, confer resistance to the fungal pathogen *Venturia inaequalis* inciting apple scab disease. *Mol Plant-Microbe Interact*, *21*. https://doi.org/10.1094/MPMI-21-4-0448

Padmarasu, S., Sargent, D. J., Patocchi, A., Troggio, M., Baldi, P., Linsmith, G., Poles, L., Jänsch, M., Kellerhals, M., Tartarini, S., & Velasco, R. (2018). Identification of a leucine-rich repeat receptor-like serine/threonine-protein kinase as a candidate gene for *Rvi12 (Vb)*-based apple scab resistance. *Molecular Breeding*, *38*(6). https://doi.org/10.1007/S11032-018-0825-Y

Schaart, J. G., van de Wiel, C. C. M., Lotz, L. A. P., & Smulders, M. J. M. (2016). Opportunities for products of new plant breeding techniques. *Trends in Plant Science*, *21*(5), 438–449.

Schouten, H. J., Brinkhuis, J., Burgh, A. M., Schaart, J. G., Groenwold, R., Broggini, G. A. L., & Gessler, C. (2014). Cloning and functional characterization of the *Rvi15* (*Vr2*) gene for apple

scab resistance. *Tree Genet Genomes*, *10*. https://doi.org/10.1007/s11295-013-0678-9

Szankowski, I., Waidmann, S., Degenhardt, J., Patocchi, A., Paris, R., Silfverberg-Dilworth, E., Broggini, G., & Gessler, C. (2009). Highly scab-resistant transgenic apple lines achieved by introgression of *HcrVf2* controlled by different native promoter lengths. *Tree Genet Genomes*, *5*. https://doi.org/10.1007/s11295-008-0191-8

Vanblaere, T., Szankowski, I., Schaart, J., Schouten, H., Flachowsky, H., Broggini, G. A. L., & Gessler, C. (2011). The development of a cisgenic apple plant. *Journal of Biotechnology*. https://doi.org/10.1016/j.jbiotec.2011.05.013

Chapter 3

Cloning and functional characterization of apple scab resistant gene *Rvi12 (Vb)_Cd5* derived from 'Hansen's baccata #2'

3.1 Abstract

The *Rvi12* (*Vb)* region, originating from the Siberian crab apple *Malus. baccata* 'Hansen's baccata #2' (HB2), contains a major gene conferring resistance to the fungal pathogen *Venturia inaequalis*, the causal agent of apple scab disease. Previously, a single BAC clone spanning the *Rvi12* locus was identified and sequenced, followed by the fine mapping of the resistance gene. The gene prediction and *in silico* characterization identified a single putative candidate resistance gene, named as *Rvi12_Cd5*, belonging to the LRR receptor-like serine/threonine-protein kinase family. The constitutive expression of *Rvi12_Cd5* in HB2, together with its structural similarity to known resistance genes, makes it the most likely candidate for *Rvi12* scab resistance in apple. In this study, the cloning and functional characterization of *Rvi12_Cd5* was performed to confirm its role in *Rvi12* based scab resistance. The gene was cloned with 35S promoter, marker gene *nptII*, terminator and introns into a vector D301P9OU10-35S-PK123, and inserted into the susceptible apple cultivar 'Gala' via *Agrobacterium*-mediated transformation. Inoculated transgenic 'Gala' lines, overexpressing *Rvi12_Cd5* were able to induce high to partial resistance against mixed inoculum of *V. inaequalis* with range of resistance symptoms (class 0-3b without class 1) strongly suggesting that the candidate gene *Rvi12_Cd5* is the functional *Rvi12 (Vb) gene*. This is the third cloned gene for apple scab resistance to date.

Key words: Transgenic apple. *Venturia inaequalis*. LLR-TrD-Kinase domains. Functional genetics.

3.2 Introduction

Apple scab, caused by the Apoplastic fungal pathogen *Venturia inaequalis* Cooke (Wint.), is a common apple disease, particularly in humid conditions. It is one of the most endemic and destructive diseases of apple (*Malus* × *domestica*) worldwide. Currently, the disease is managed by applying up to 15-20 fungicides per year in commercial orchards (MacHardy, 1996b; Vaillancourt and Hartman, 2000). When cultivating susceptible cultivars in places with significant precipitation and disease pressure, 20 to 30 applications may be required (Manktelow *et al*., 1996). The propensity of *V. inaequalis* to develop resistance to pesticdes, as well as the non-sustainable nature of chemical application, has prompted researchers to look for alternative ways to control the disease (Ayer *et al*. 2019; Chapman *et al*. 2011; Frederick *et al*. 2014). In a recent study, the effect of number and concentration of pesticides on the environmental site features near apple orchards in South Tyrol (Italy) was investigated. Many of the discovered residues were endocrine active chemicals, and according to EU regulators, some of them (thiacloprid, bupirimate, captan, folpet) are "suspected human carcinogens". As a result, there should be more effective control of pesticide application regulations to reduce human health risks (Linhart *et al*., 2019; Linhart *et al*., 2021). The use of resistant genes (R-genes) present in small-fruited wild species of apple is an alternative to chemical pesticides (Williams and Kuc, 1969). Several breeding projects have worked on introgression of apple scab resistance into apple cultivars, especially for introgression of the *Vf* resistance (Gessler and Pertot, 2012). These attempts were initiated when Crandall's cross between a susceptible cultivar and the resistant crab apple *Malus floribunda* 821 was first built a century ago, in 1914 (Crandall, 1926; Gessler and Pertot, 2012). A putatively resistant and a susceptible sibling from this progeny were crossed, and showed a 1:1 segregation for resistance, indicating monogenic resistance by *Vf* (Hough, 1944a; Williams, 1966). Due to the resistant parent *M. floribunda* 821's low fruit quality, it took around five generations to remove the vast majority of the undesirable alleles from *M. floribunda* while preserving the desired *Vf*-gene for scab resistance (Janick, 2006). *Vf c*ultivars with acceptable fruit quality were introduced to the market about 80 years after the first cross. Unfortunately, most of the scab-resistant cultivars released to date carry only one gene, *Rvi6* (*Vf*), and its value is questionable since emergence of pathogen avirulence gene *avrRvi6* which occur all over Europe (Bengtsson et al., 2000; Lefrancq and Lateur, 2009; Parisi et al., 2004a) and have also been observed in the U.S.A. (Beckerman *et al*., 2015; Papp *et al*., 2020b).

Many loci for resistance to apple scab have been discovered in *Malus*, both major genes and QTLs (Bus *et al*., 2011b; Calenge *et al*., 2004a; Gessler *et al*., 2006b; Khajuria *et al*., 2018a). When conventional breeding is applied, the introduction of two or more R genes for durable resistance may

take decades due to the lengthy juvenile period, self-incompatibility, and the impossibility of precisely reproduce the heterozygous state of desired cultivar (Janick *et al*., 1996). The solution is to use genetic manipulation to introduce a combination of R genes into elite cultivars without simultaneously introducing undesirable alleles (Borejsza-Wysocka *et al*., 2010; Calenge *et al*., 2004a; Gessler *et al*., 2006b; Schouten *et al*., 2006a). Modern fast-breeding technologies allow to rapidly transfer R genes into elite germplasm (Schaart *et al*., 2016) such as "cisgenesis", which is the process of adding natural genes from crossable donor plants without adding foreign genes. This method eliminates the introduction of undesirable alleles, whereas traditional breeding removes these alleles gradually over generations (Schouten *et al*., 2007, 2006a). Cisgenesis also permits high-grade cultivars to keep their demonstrated fruit quality and other desirable features (Krens *et al*, 2015; Joshi *et al*., 2009; Vanblaere *et al*., 2011). A requirement of cisgenesis is the availability of isolated functional genes (Bolar *et al*., 2001; Flachowsky *et al*., 2011a, 2010).

The majority of plant disease R genes discovered so far are members of the nucleotide-binding site leucine-rich repeat containing proteins (NBS-LRR) family, which includes both Toll/interleukin1 domain-containing (TIR-NBS-LRR) genes (Dinesh-Kumar *et al*., 1995; Gassmann *et al*., 1999; Lawrence *et al*., 1995) and coiled-coil (CC-NBS-LRR) genes (Meyers *et al*., 1999; Mindrinos *et al*., 1994). R genes also include receptor-like kinases (RLKs) (Gómez-Gómez and Boller, 2000; Song *et al*., 1995), receptor-like proteins (RLPs) (Dixon *et al*., 1996; Jones *et al*., 1994; Parniske *et al*., 1997; Wulff *et al*., 2004), and kinase-like genes (Martin *et al*., 1993). To date, 20 significant scab resistance genes in apple have been identified in several cultivars or wild relatives (Bus *et al*., 2011b; Clark *et al*., 2014a; Soriano *et al*., 2014a). Out of them, six scab resistance genes *Rvi6* (Patocchi *et al*., 1999; Xu and Korban, 2002a), *Rvi15* (Galli *et al*., 2010a), *Rvi18* (Soriano *et al*., 2014a), *Rvi12* (Padmarasu *et al*., 2018, 2014), *Rvi1* (Cova *et al*., 2015c) and *Rvi5* (Cova *et al*., 2015a) were fine-mapped, and candidate resistance genes for *Rvi6* (Patocchi *et al*., 1999; Vinatzer *et al*., 1998; Xu and Korban, 2004), *Rvi15* (Galli *et al*., 2010b), *Rvi12* (Padmarasu *et al*., 2018), *Rvi18* (Soriano *et al*., 2014a) and *Rvi1* (Cova *et al*., 2015c) have been identified, while only two scab resistance genes *Rvi6* and *Rvi15* have been cloned and functionally described so far (Belfanti *et al*., 2004; Schouten *et al*., 2014, Malnoy *et al*. 2008; Silfverberg-Dilworth *et al*., 2005b). The *Rvi6 (Vf)* scab resistance locus contained a cluster of four *Cladosporium fulvum*-like resistance genes named *HcrVf1* to *HcrVf4* (Xu and Korban, 2002a). *HcrVf2*, referring to the homology to *C. fulvum* resistance genes in tomato as RLPs with extracellular leucine rich repeats, a single transmembrane domain and a short cytoplasmic carboxyl terminal domain, was obtained using map-based cloning (Boris A Vinatzer *et al*., 2001). Scab-resistant transgenic apple plants were developed by *Agrobacterium tumefacien* mediated transfor-

mation using *HcrVf2* in two independent studies (Belfanti *et al*., 2004; Malnoy *et al*., 2008) and cisgenic 'Gala' plants containing *HcrVf2* were developed and functionally characterized (Jansch *et al*., 2014; Joshi *et al*., 2011; Vanblaere *et al*., 2014, 2011). *Rvi15 (Vr2)* locus contained three TIR-NBS-LRR protein coding candidate genes named *Vr2-A*, *Vr2-B*, and *Vr2-C* (Galli *et al*., 2010b). *Rvi15-C* was confirmed to be responsible for apple scab resistance based on its functional characterization. As a result, this gene was identified as the *Rvi15* gene (Galli *et al*., 2010a, 2010b; Schouten *et al*., 2014) which belongs to the Toll and mammalian interleukin-1 receptor protein nucleotide-binding site leucine-rich repeat structure resistance gene family (Schouten *et al*., 2014).

For over a decade, *Rvi12* resistance from the Siberian crab apple *M. baccata* 'Hansen's baccata #2' (HB2) has been observed to be resistant or rarely overcome by *V. inaequalis*, in the scab test orchards around the globe monitored by the VINQUEST initiative project (Patocchi *et al*., 2020, 2009), making this source of resistance attractive for use in durable resistance breeding. From the testcross studies of Dayton and Williams (1996), *Rvi12 (Vb)* (Bus *et al*., 2011a), it was found to be a novel resistance source which is not allelic to *Rvi6* from *M. floribunda* '821'. The first mapping study of *Rvi12* to apple linkage group 1 (LG1) of HB2, was conducted by Hemmat and colleagues (2003). Subsequent mapping of *Rvi12* to LG12 of HB2 was conducted by Erdin *et al*, (2006). Chlorosis (class 2; Chl) or chlorosis with minimal sporulation (class 3a/3b) was reported as the 'HB2' response to *V. inaequalis* inoculation, and infection did not elicit a hypersensitive response (class 1; HR) like the *Rvi5* and *Rvi15* resistance genes (Chevalier *et al*., 1991; Erdin *et al*., 2006; Hemmat *et al*., 2003). After 8 years, to solve the contradictory map positions of *Rvi12* obtained in two individual studies mentioned earlier, the next step was followed by Padmarasu *et al* (2014) which confirmed map position of *Rvi12* to LG12 in the cross 'Gala' × 'HB2' in an interval between single sequence repeat (SSR) markers. In this study, Padmarasu *et al* (2014) characterized resistance reactions such as no symptoms (Class 0) and chlorosis without or with sporulation (class 2, 3a/3b) in large population of plants of the 'Gala'×'HB2' progeny. Further, by using extended large segregating population, the *Rvi12* scab resistance gene was fine mapped to an interval spanning 882 kb of the 'Golden Delicious' genome sequence, a genetic location flanked by single nucleotide polymorphism (SNP) markers (Padmarasu *et al*., 2014). Starting from these markers, utilizing chromosome walking of a HB2 bacterial artificial chromosome (BAC)-library; a single BAC clone spanning the *Rvi12* interval was identified (Padmarasu *et al*., 2018). Following Pacific Biosciences (PacBio) RS II sequencing and the use of the hierarchical genome assembly process (HGAP) of the 6F11 BAC clone sequence, the *Rvi12* resistance locus was localized to a 62.3-kb genomic region (Padmarasu *et al*., 2018). Gene prediction from the BAC clone sequence between the *Rvi12* flanking markers predicted and *in-silico* characterization identified five putative candidate genes (*Rvi12_Cd1, Rvi12_Cd2, Rvi12_Cd3, Rvi12_Cd4,*

Rvi12_Cd5) 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 containing all the essential domains for disease resistance as the most likely candidate gene (*Rvi12_Cd5)* for *Rvi12* resistance (Padmarasu *et al*., 2018). 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 (Padmarasu *et al*., 2018).

To take a step forward to enhance cloned and functionally characterized apple scab resistance genes profile, we attempted the cloning of the *Rvi12_Cd5* candidate gene, the insertion into the susceptible cultivar 'Gala' using *A. tumefaciens*-mediated transformation, and testing of the resulting plants for resistance to apple scab.

3.3 Experimental procedures

3.3.1 Cloning of *Rvi12_Cd5* including 35S promoter

A candidate gene sequence named as *Rvi12_Cd5* (3352 bp) from 6F11 BAC contig, coding for a LRR receptor-like serine/threonine-protein kinase family and predicted to be responsible for *Rvi12* based scab resistance in wild germplasm '*HB2'* (Padmarasu *et al*., 2018) was used as gene of insert for the construct. The binary vector used to transform *M. domestica* apple cv. 'Gala' was designed and assembled (DNA Cloning Service, Hamburg, Germany). The nucleotide sequence of candidate gene *Rvi12_Cd5* (Padmarasu *et al*., 2018) was used as reference for the oligosynthesis of the gene, followed by cloning into a binary vector D301P9OU10-35S-PK123 (Fig. 1). The T-DNA cassette flanked by the left and right borders (Fig. 1; grey boxes), integrated with two well defined molecular mechanisms respectively, for antibiotic resistance and over expression of *Rvi12_Cd5*. The antibiotic resistance system (Fig. 1; red boxes) was characterized by the *Neomycin phosphotransferase II* gene (*nptII*), controlled by the *Arabidopsis thaliana Ubiquitin-10* promoter (*Pro-Ubq10At*), which conferred kanamycin resistance to apple transformants allowing the antibiotic-assisted selection following transformation. The *Rvi12_Cd5* over expression system (Fig. 1; red boxes) was characterized through the control of the *Cauliflower Mosaic Virus 35S* promoter (*35S-Pro*).

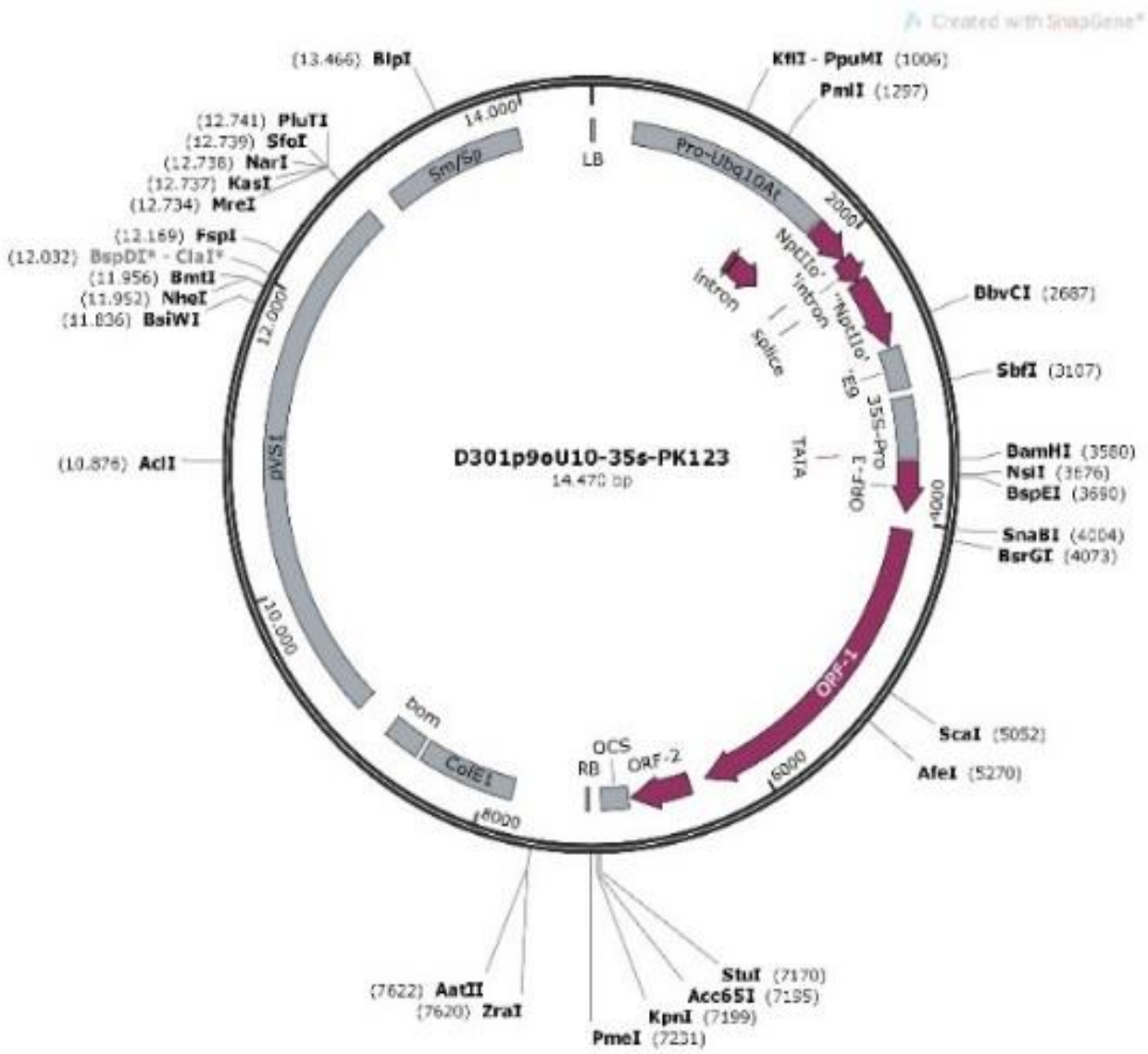


Figure 1: Construction map of D301P9OU10-35S-PK123 binary vector used for apple cv. 'Gala' transformation. Binary vector was produced by cloning a 72.31 bps T-DNA cassette into a 72.39 bps p9 vector backbone. The T-DNA cassette contains process of over-expression of Rvi12_Cd5 gene (3353 bps) under 35S promoter and kanamycin resistance system for plant selection after the apple transformation via Agrobacterium tumefaciens. Colicin E1 and pVS1 origins of replication (ColE1 and pVS1); streptomycin/spectino-mycin resistance genes (Sm/Sp); left and right borders (LB and RB); Cauliflower Mosaic Virus 35S promoter (35S-P); Neomycin phosphotransferase II (NptII); E9 terminator (E9); Arabidopsis thaliana Ubiquitin-10 promoter (Pro-Ubq10At); Terminator element as octopine synthase gene (OCS).

3.3.2 *Agrobacterium* mediated transformation of *Rvi12_Cd5* to susceptible 'Gala'

By electroporation, 100 ng of purified binary vector with the inserted *Rvi12_Cd5* gene was transferred to 50 µl aliquot of *A. tumefaciens* strain EHA105 competent cells (Chetty *et al*., 2012; Hood *et al*., 1993), carrying the helper plasmid pCH32. *A. tumefaciens* mediated transformation of apple was performed using the protocol described by Szankowski *et al*. (2003) and Vanblaere *et al*, (2011) with minor modifications (Fig. 2). Baby jars containing susceptible cv. 'Gala Galaxy' plantlets in a shoot propagation medium (Pessina et al., 2016) were maintained in a growth chamber at 24 ±

1°C with a 16/8-h light/dark period (100 mmol/m2/s). The top four youngest leaves of 3-4 weeks old *in-vitro* propagated shoots of Gala were used as explants. For inoculation, 250 µl *A. tumefaciens* (Tab. 1; bacterial colonies harboring D301P9OU10-35S-PK123 construct confirmed by PCR using primers Rvi12_Cd5_F, Rvi12_Cd5_R) from a glycerol stock, was grown overnight at 28°C and shook at 250 rpm in 5 ml Luria broth (LB) media (2 g/l Tryptone, 1 g/l NaCl and 2 g/l yeast extract), supplemented with antibiotics (5 µl rifampicin for *Agrobacterium* and 5 µl spectinomycin for plasmid). The 100-150 µl of *A. tumefaciens* growth solution was spread on 2-3 solid LB broth media plates supplemented with same antibiotics as mentioned before and incubated at 28°C for 3 days. A 50 ml falcon tube was used to collect the bacterial growth from plates by adding MS medium (Murashige and Skoog, 1962) containing 100 µM acetosyringone to an OD of approximately 0.6. The solution was further incubated at room temperature, shaking at 50 rpm for 3-4 hours. Meanwhile, the leaves of 'Gala' were cut into 2-3 mm strips without apex and basal part and infected with *A. tumefaciens* suspension filled in petri plates. The 450 and 850 explants for two transformations respectively (Tab. 2), were gently shaken at ± 50 rpm for 30-45 minutes and co-cultivated upside down during three days at 25°C in the dark on co-culture medium containing 4.4 g/l MS salts and vitamins (ready to use from Duchefa, Netherlands), 30 g/l sorbitol, 0.1 g/l Myo-Inositol, 3.1 µM 6-Benzylaminopurine (BAP), 2.6 µM 1-Naphthalene acetic acid (NAA), 22 µM Thidiazuron (TDZ) (after autoclaving) and 7.5 g/l plant agar; pH (5.6–5.8). After co-cultivation, the explants were washed twice in a 50 ml falcon tube filled with 40 ml Milli-Q water (autoclaved) and once with liquid MS medium supplemented with 300 mg/l Ticarcillin for 15 min on a shaker with ~200 rpm (2x 100 explants in the tube and the tube is horizontal on the shaker).

The explants were transferred to selective regeneration medium containing 4.4 g/l MS salts and vitamins, 30 g/l Sorbitol, 0.1 g/l Myo-Inositol, 3.1 µM BAP, 2.6 µM NAA, 22 µM TDZ (after autoclaving), 50 mg/l Meropenem/Cefotaxime dissolved in DMSO to eliminate *A. tumefaciens*, 50 mg/l kanamycin for selection of transformants and 7.5 g/l plant agar; pH (5.6–5.8). The explants were cultured for 3/4 weeks in the dark, then under photoperiod of 16/8 h and sub-cultured on fresh selective regeneration medium at 2 week intervals. When the callus emerged from the explants and shoot-like structures started to emerge from the calli, regenerants (obtained after 6–7 months from co-culture with *agrobacterium*) were transferred to proliferation medium containing 4.4 g/l MS salts and vitamins 0.096 g/l FeEDDHA, 30 g Sucrose, 3.1 µM BAP and 7.5 g/l plant agar; pH (5.6–5.8), for several weeks for elongation of shoots.

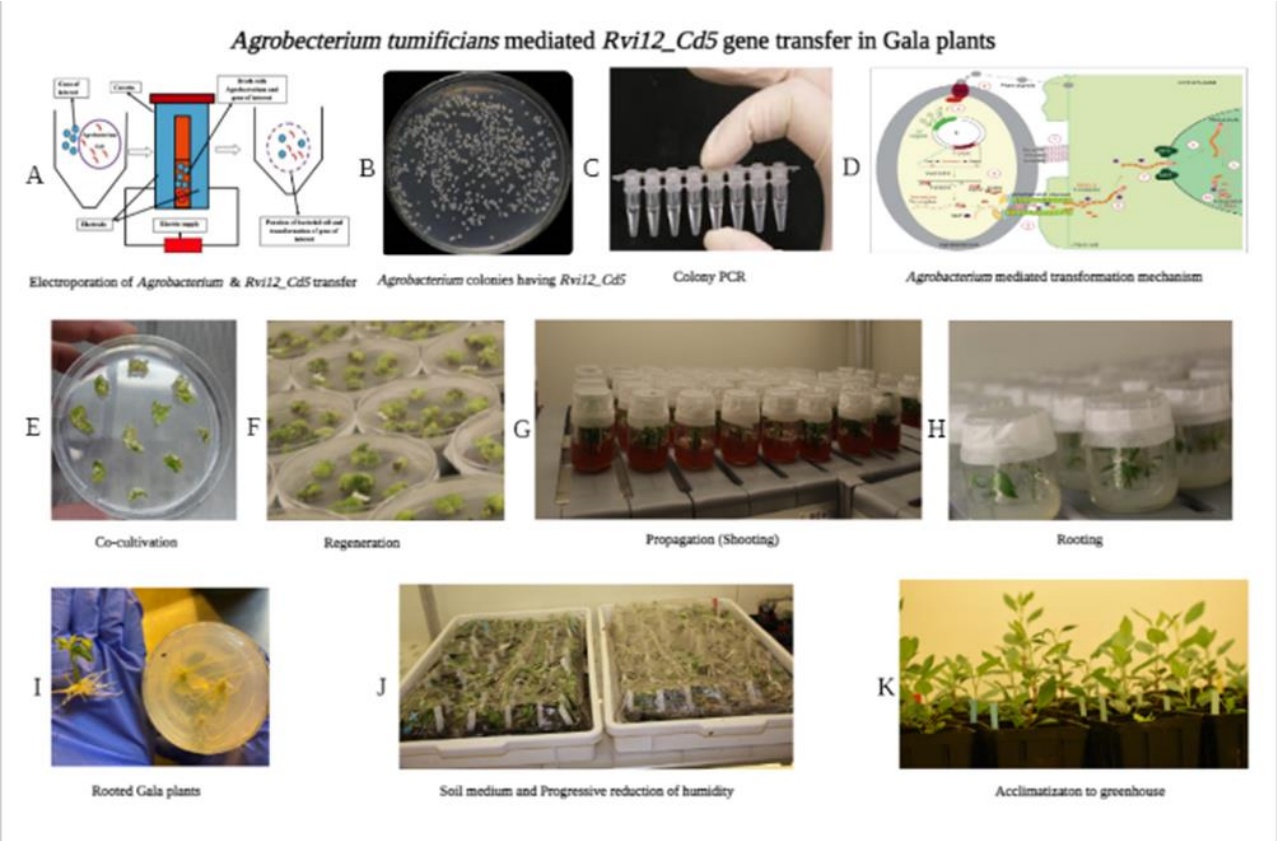


Figure 2: Agrobacterium mediated transformation of cv. 'Gala' with Rvi12_Cd5 was successful and yielded several independent transgenic lines, which were multiplied in-vitro. Transformation steps; (A) Electroporation, (B) Agrobacterium colony production, (C) Colony PCR, (D) Molecular mechanism of agrobacterium mediated transformation, (E) Co-cultivation, (F) Regeneration, (G) Propagation, (H) Rooting, (I) Root growth, (J) Soil medium and progressive reduction of humidity, (K), Acclimatization to greenhouse.

3.3.3 Identification of transgenic 'Gala' lines

For each plant from putative transformants and untransformed control, genomic DNA from 100 mg leaf tissue was extracted using the Nucleospin® Plant II, Mini kit (Macherey–Nagel, Düren, Germany) by following the manufacturer's instruction, quantified by the NanoDrop 8000 Spectrophotometer (Thermo Scientific Inc, MA, USA), diluted to 50 ng/l. Then, 1.5 µl DNA was used for polymerase chain reactions (PCRs) employing the thermocycle-3000 (Biometra), the GoTaq Green Master Mix 2X (Promega, Fitchburg, MA) and primers (Tab. 1; 35 S_F, PK123_R1, PK123_R2) to amplify *Rvi12_Cd5* gene. The thermal protocol used was as follows: 2 min at 95 °C followed by 35 cycles of denaturation, annealing and extension of 30 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C, respectively, with a final extension of 5 min at 72 °C. PCR product (10 µl) was electrophoresed on a 1% agarose gel at 120 V, bands were visualized over UV light following staining with GelRed (Biotium, CA, USA) and compared to the 1 kb Plus DNA ladder (1 kb plus DNA Ladder, Invitrogen-Thermo Fisher Scientific).

Table 1: Sequences of primers and probes used for the PCR-based screening of apple transformants, the quantification of NPTII copy number by Taqman real-time PCRs, RT-qPCR for expression quantity of Rvi12_Cd5 and Colony PCR.

Primer and probe name	Sequence (5'-3')	Amplicon length (bps)	Annealing temperature (°C)
Identification of transformants			
C_26_35S	F: 5′GCTATCGTTCAAGATGCCTCT3′	1572	58
PK123_R1	R: 5′CAATGGAAGGGAATGGAGCC3′†		
PK123_R2	R: 5′CGAGCTCATTTGGTAGGC3′†		
Transgene copy number			
MdTOPO6	F: 5′TGTGGAAGGAGATCAAAGCGCA3′§	196	58
	R: 5′CGCGTTGCTTCTTTGCTGCA3′§		
MdTOPO6_probe	FAM-5′-ACATGCCAACAGGAACAATCACA-3′-TAMRA§		
NPTII	F: 5′CTTGCCGAATATCATGGTGGAA3′§	100	58
	R: 5′GGTAGCCAACGCTATGTCCTGA3′§		
NPTII_probe	FAM-5′-TTCTGGATTCATCGACTGTGGC-3′-TAMRA§		
Expression analysis			
MdACT_F	F: 5′TGACCGAATGAGCAAGGAAATTACT3′$	230	
MdACT_R	R: 5′TACTCAGCTTTGGCAATCCACATC3′$		60
Rvi12_Cd5_F2	F: 5′GAGTCTTAAACTTGCCCGC3′†	253	
Rvi12_Cd5_R2	R: 5′CCTGTGAAATTATTCATACAAC3′†		
Colony PCR			
Rvi12_Cd5_F	F: 5′ATGATGGAGCATTCACGTACTATTCG3′†	3352	58
Rvi12_Cd5_R	R: 5′TTAGCCTTCTTGAGCTTTGAGGTACG3′†		

† (Padmarasu *et al*., 2018) - *Primers for quantification of transgene expression levels*

§ (Dalla Costa *et al*., 2019) - *Primers for determination of CN number of transgene*

$(Perini *et al*., 2014)- *Primers for quantification of endogenous gene expression levels*

3.3.4 Transgene copy number

The quantification of *nptII* copy number (CN) for calculating the T-DNA insertion in positive transformants, was performed according to TaqMan real-time PCR method developed by Dalla Costa *et al* (2019) for apple, and specific primers and probes for the endogenous gene *MdTOPO6* and for the marker gene *nptII* were used (Tab. 1). The reaction was performed in a 96-well plate on a C1000 thermal cycler (Bio-Rad, Hercules, USA) equipped with CFX96 real-time PCR detection system (Bio-Rad, Hercules, USA). The real-time PCR singleplex reaction was carried out in a 10 µl final volume containing 1 × SsoAdvanced Universal Probes Supermix (Bio-Rad, Hercules, USA), genomic DNA, diluted up to 15-20 ng/l, 0.3 µM primers (Sigma, Haverhill, UK) and a 0.2 µM specific Taqman probe (Tab. 1) (Sigma, Haverhill, UK). The thermal protocol was as follows: polymerase activation for 3 min at 95 °C followed by 40 cycles of denaturation of 10 s at 95 °C, annealing of 5 s

at 58 °C and 5 s at 60 °C and an elongation of 30 s at 72 °C. Four points were used to build the transgene and endogenous gene standard curves, starting with 10^6 molecules of a plasmid pGEM-T easy (Promega, Madison, Wisconsin, USA) calibrator (cloned with fragments of *nptII*: *MdTOPO6)* at serial dilution of 1:5. The *nptII* CN per cell was determined for each sample using the formula: (*nptII* total copies/*MdTOPO6* total copies) × 2. On the basis of the standard curves obtained, the total copies of *nptII* and *MdTOPO6* were determined as the mean values of the quantification cycles (C_q) of two technical replicates.

3.3.5 Rooting and acclimatization of transgenic Gala lines to greenhouse

After determination of integrated transgene CN, the elongated *in-vitro* shoots of selected transgenic 'Gala' lines from the shoot propagation medium, were transferred to rooting medium supplemented with 2.2 g/l MS salts and vitamins, 15 g/l Sucrose, 0.5 ml/l Myo-Inositol, 0.2 mg/l indole-3-butyric acid (IBA) and 7.5 g/l plant agar; pH (5.6–5.8) (Pessina et al., 2016), maintained in a growth chamber in the dark for 2 weeks and subsequently under 16/8-h light/dark conditions, until complete root formation. Rooted plants were acclimatized in soil ('Terriccio Vegetal Radic' - TerComposti S.p.a., Brescia, Italy) and, by progressively reducing humidity for 3 weeks. Well-acclimatized plants were maintained at greenhouse conditions (24 ± 1°C, 16/8-h light/dark period, relative humidity of 70% ±5%).

3.3.6 RNA extraction and cDNA synthesis

For each plant, RNA extraction from 100 mg frozen leaf tissue of un-inoculated *in-vitro* grown transgenic Gala lines and outdoor grown HB2, was performed using the Spectrum plant total RNA kit (Sigma-aldrich, MO, USA) following the manufacturer's instructions. The RNA samples were run on a 1% (v/w) agarose gel to determine RNA quality. For reverse transcription-polymerase chain reaction (RT-PCR), Invitrogen ™ SuperScript ™ (5X IV VILO ™ Master Mix with 10X ezDNase ™ Enzyme) cDNA Synthesis Kit (Thermo Fisher Scientific Inc., MA, USA) was used for cDNA synthesis and using thermocycle-3000 (Biometra). The thermal protocol for the cDNA synthesis was as follows: 10 min at 25°C, 1h at 42°C and 5 min at 85°C. RNA and cDNA were quantified using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific Inc., MA, USA). Each sample's cDNA was diluted to 50 ng/µl.

3.3.7 Expression analysis

For quantitative RT-PCR (qRT-PCR), primers specific for the gene of interest and housekeeping gene were designed (Tab. 1; MdACT_F, MdACT_R, Rvi12 Cd_5F2, Rvi12 Cd_5R2). The qPCR 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 s, 40 cycles of 95°C for 1 s and 60°C 20 s followed by a cycle at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s 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 *MdACT* was employed as housekeeping gene for normalization of data (Perini *et al*., 2014).

3.3.8 Scab resistance test

The scab disease tests were conducted in the greenhouse. A mixed population of *V. inaequalis* collected in the field was used, composed of fresh and vital conidia. To collect the conidia, apple leaves showing heavy sporulation lesions were rinsed with water in a 50 ml falcon tube and leafs debris was removed by filtration, using a funnel (Trichter) and glass wool (or cotton). Conidia concentration in a "Thomakammer" (depth 0.1 mm) was measured. The artificial inoculum was done on 3-4 top leaves of each plant. The leaves were tagged and plants were inoculated with a suspension of *V. inaequalis* (concentration of $5x10^5$ conidia ml^{-1}) using an atomizer connected to a compressed air supply (Bus *et al*., 2005b; Yepes and Aldwinckle, 1993). Plants were incubated in a mist chamber (16-h photoperiod of 40 μmol m^{-2} s^{-1}, 18 ± 1°C, & 100% relative humidity-RH) for 48 h, followed by 10 days of growth at 60% RH and again in mist chamber (100% RH) for another two days. Then the plants were left in the greenhouse compartment at 60% RH until the 21^{th} day. Macroscopic symptoms (qualitative) were evaluated on all leaves 7, 12, 14 and 21 days post inoculation (dpi) according to the following scale described by Chevalier *et al* (1991), indicative for types of disease: class 0, no macroscopically visible symptoms; class 1, HR pinpoints; class 2, chlorotic lesions with very small necrosis and no sporulation: class 3a, localized chlorotic and necrotic lesions with light sporulation; 3b, chlorotic and necrotic lesions with sparse sporulation; class 4, severe sporulation covering entire leaf. The maximum level of macroscopic symptoms recorded at 21 dpi and their median and confidence limits of 18 to 45 leaves per transformed line were computed to describe the overall response of each line and control (Belfanti *et al*, 2004). Based on symptom classes, the infection severity was also calculated according to the following the scale, indicative for percentage of infection on leaves: class 0 = 0 %, class 1 = 5 %, 2 = 25 %, 3a = 50 %, 3b = 75 % and 4 = 100 %. Mean percentage of scab infection severity and their confidence limit of most infected three to nine per transformed line was tested for the normality and equality of variance with the shapiro-Wilk test followed by Leven's test. Data were subjected to ANOVA followed by Duncan's highest significance difference (HSD) post-hoc test for multiple comparisons. All the analysis were performed using SPSS version 26.

The presence of germinated *V. inaequalis* conidial (sporulation) lesions on the leaf surface of inoculated plants was assessed 21 days dpi by optical microscopy. 2-3 leaf discs were prepared per line and control. The leaves were harvested by using 2ml eppendorf and discolored by incubating for 30 minutes in a solution of 4% acetic acid and 96% ethanol (vol/vol) at 75°C water bath. Discoloration solution was removed and leaf pieces were covered with the coloration solution of 70% Ethanol, 29.9% water and 0.1% Aniline-blue lactophenol (vol/vol) and incubated for 7 minutes in a 75°C in a water bath. The coloration solution was removed and leaf pieces were washed 2-3 times with the wash solution of 70% Ethanol to remove the excess of coloration solution. Then, incubation at room temperature for 0.5-2h in conservation solution 1 of 50% Ethanol and 50% Lactic acid (vol/vol), was performed. In final step, the leaf pieces were mounted on a microscope glass with conservation solution 2 of 100% Lactic acid and protected by with a coverslip (Bruzzese and Hasan, 1983; Gessler and Stumm, 1984; MacHardy, 1996a).

3.4 Results

3.4.1 Generation of Transgenic Gala lines

A total of 1300 'Gala' leaf explants were infected with *A. tumefaciens* containing the binary vector for over expression of candidate gene *Rvi12_Cd5* under *35 S* promoter and antibiotic selection system (Fig. 1). For *Rvi12_Cd5* transfer to susceptible cv. 'Gala', two independent transformation events were carried out (Tab. 2) Respectively, 75 regenerants of 'Gala' were produced approximately 6–7 months after transformation (Fig. 2) and 62 of them were tested by PCR to screen for the integration of T-DNA (primers listed in Tab. 1). A total of 60 'Gala' lines had the amplification of *Rvi12_Cd5* transgene integrated in their genome (Fig. 3**)** and no *A. tumefaciens* contamination, resulting in transformation efficiency of 4.61 % (Tab. 2).

Table 2: Efficiency of Agrobacterium tumefaciens-mediated transformations in 'Gala' apple cultivar.

Cultivar	Transformation	No. of leaf explants infected	No. of regenerants collected	PCR screening		Transformation Efficiency (%)†
				No. of regenerants tested	No. of positive regenerants *(Rvi12_Cd5)*	
Gala	A	450	15	4	4	4.61
Gala	B	850	60	58	56	

†The transformation efficiency was calculated by dividing the number of regenerants positive for *Rvi12_Cd5* by the number of leaf explants infected.

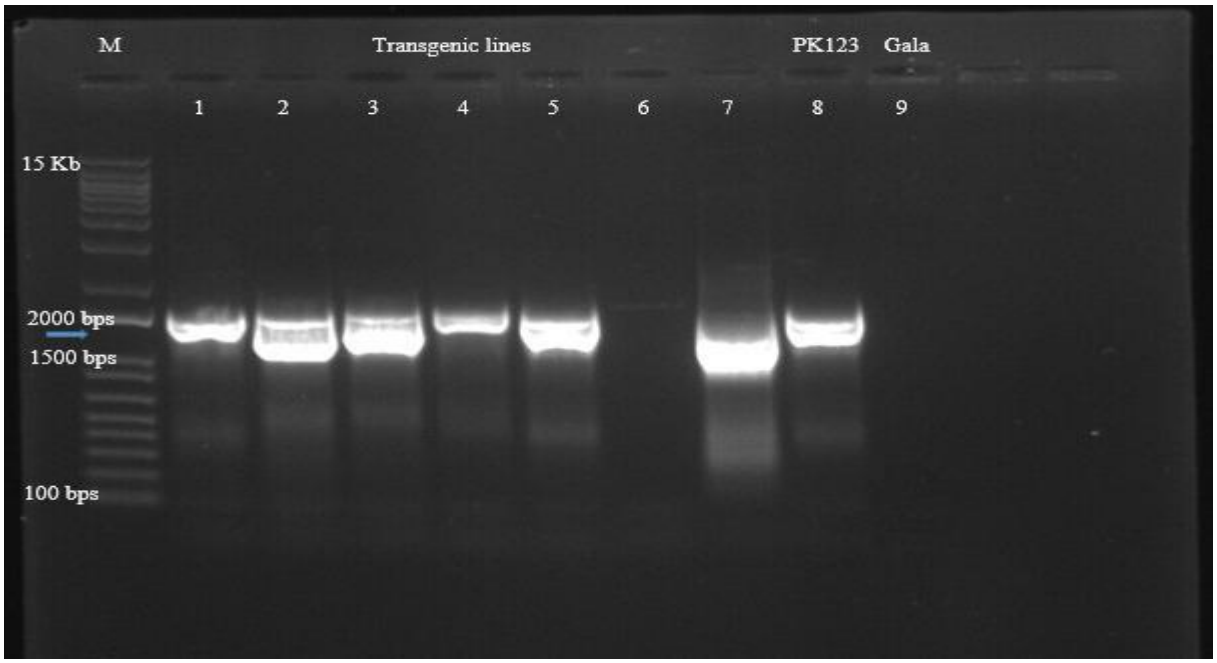


Figure 3: Genomic DNA analysis of transformants, containing Rvi12_Cd5. For investigation of the presence of the T-DNA, a polymerase chain reaction was applied using primers specific for the gene Rvi12_Cd5 (Tab. 1). The independent transformed Gala lines are represented as lane 1-7 while the lane 8 and 9, indicates purified vector PK_123 (Positive control) and *Gala (Negative control) respectively. 'Gala' was the genotype used for transformation, while Hansen's baccata #2 (HB2) is the donor of the Rvi12_Cd5 (Vb) gene. M indicates the 1 kb Plus DNA Ladder (Invitrogen-Thermo Fisher Scientific) for band size estimation. The arrow indicates the band corresponding to the Rvi12_Cd5 gene found in the transgenic lines.*

3.4.2 Molecular characterization of selected Transgenic Gala lines for acclimatization

To address the molecular basis for confirmed positive transformants, 36 out of 60 *in-vitro* transgenic 'Gala' lines evaluated for carrying *Rvi12_Cd5*, were subsequently characterized for T-DNA integration copy number (CN) by quantifying the *nptII* selection marker gene (Tab. 1). *NptII* CN ranged from a minimum of 0.04 to a maximum of 2.4 per cell among lines T_1 to T_{36} (Fig. 4). Due to T-DNA integration CN (*NptII* CN = ± 1 to 2) and successful *in-vitro* root production, 7 lines: T_{10}, T_{18}, T_{20}, T_{25}, T_{29}, T_{30}, and T_{34} were selected and re-screened by RT-qPCR (Tab. 1; Fig. 5) in order to confirm the mRNA expression level of *Rvi12_cd5* after 1 year of micro-propagation. The transcript levels were determined in *in-vitro* transformed lines (uninoculated with *V. inaequalis*) and compared with untransformed *in-vitro* grown 'Gala' plants (Negative control) and outdoor grown 'HB2' (Positive control). As revealed by RT-qPCR, uninoculated transformed 'Gala' lines displayed higher mRNA levels of *Rvi12_Cd5* than 'HB2' while untransformed 'Gala' showed zero expression level (Fig. 5). According to Fig. 5, seven transgenic lines showed a minimum of 26 times and a maximum of 221 times more expression than 'HB2' in 7 transformed 'Gala' lines in comparison with 'HB2' plants. Lines T_{29}, T_{30}, and T_{34} showed higher level of gene expression than T_{10}, T_{18}, T_{20} and T_{25}. These 7 lines were believed to be promising for scab test, thus, acclimatized to greenhouse.

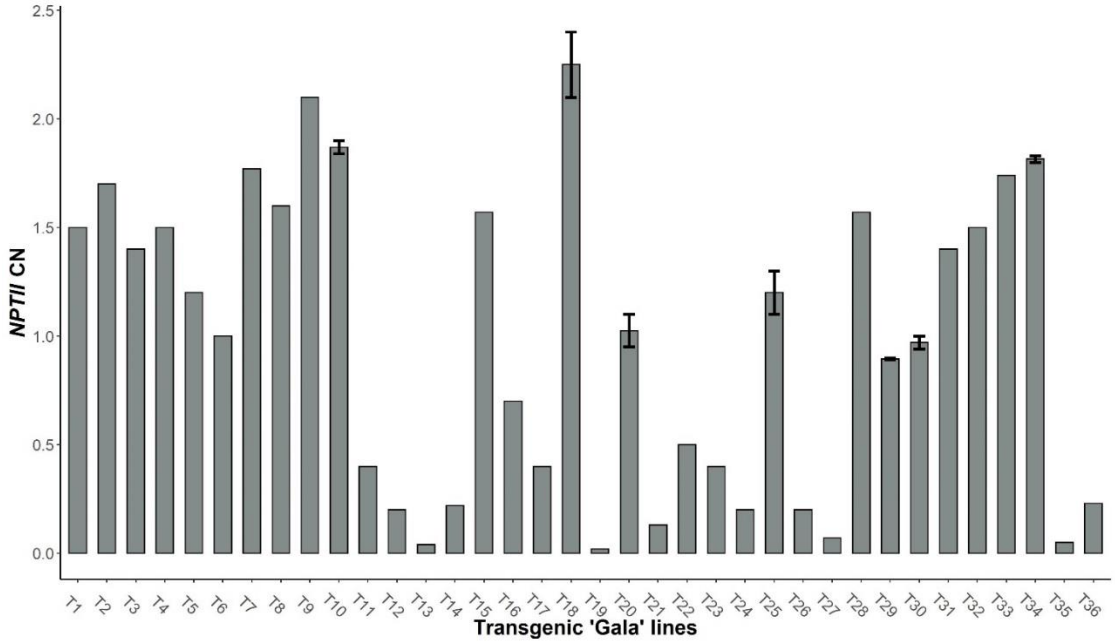


Figure 4: Frequency value scale of quantified nptII CN among transformed 'Gala'. In-vitro 36 transgenic independent lines were analyzed by TaqMan real-time PCR to quantify nptII copy number (CN). The CN of plants: T_5, T_6, T_9, T_{10}, T_{15}, T_{16}, T_{18}, T_{20}, T_{25}, T_{29}, T_{30}, *and* T_{34} *is the mean ± SD of two biological replicates while the other plants were analyzed in a single biological replicate. Primer sequences are listed in Table 1.*

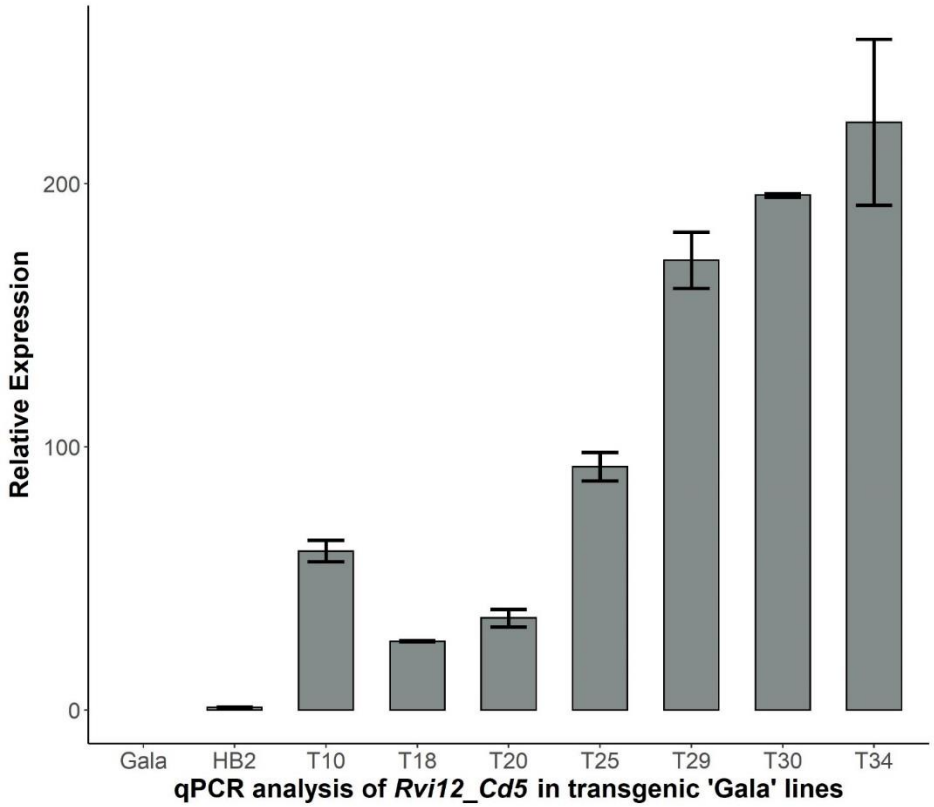


Figure 5: Mean normalized expression quantity of Rvi12_Cd5 in transformed 'Gala' lines compared to controls. In-vitro uninoculated 7 transgenic independent lines, untransformed in-vitro 'Gala' (Negative control) and greenhouse 'HB2' (Positive control), were analyzed by RT-qPCR to quantify mRNA levels of Rvi12_Cd5. The quantity of transcripts in transformed plants: T_{10}, T_{18}, T_{20}, T_{25}, T_{29}, T_{30}, *and* T_{34} *is the mean of the normalized expression values of three technical replicates using the Delta-Delta CT method (Livak and Schmittgen, 2001). Primer sequences are listed in Table 1.*

3.4.3 Disease resistance evaluation

When the acclimatized 6-15 biological replicates of 7 independent transformed 'Gala' lines had grown at least ten leaves, scab disease test was conducted in the greenhouse. The upper 3-4 unfolded young leaves of the transformed 'Gala' lines along with untransformed 'Gala' lines (control) were inoculated with artificial inoculums of *V. inaequalis*. Disease symptoms were recorded for individual plants from each of the transformed lines and control. The maximum level of resistance reactions were assessed macroscopically at 14 dpi for first and at 21 dpi for second and third scab trails respectively. (Fig. 6; Tab. 3) using the scale of Chevalier *et al* (1991). Based on median values, transgenic 'Gala' lines T_{10}, T_{18}, T_{20}, T_{30}, and T_{34} demonstrated high resistance to *V. inaequalis* in contrast to control and did not provide any visible resistance reactions or hardly any sporulation as indicated by the class 0 with no symptom. Lines T_{30}, and T_{34} also exhibited chlorotic lesions with very small necrosis in center on some leaves and this phenotype was classified as class 2 due to no sporulation at all. Other transgenic 'Gala' lines T_{18}, T_{25} and T_{29} showed partial resistance by displaying necrotic lesions with slight chlorosis and light sporulation as class 3a after 12-14 dpi. However, few leaves of lines T_{10}, T_{20}, T_{25} and T_{29} also showed restricted sporulation with or without resistance reaction, classified as class 3b or 4 respectively. Symptoms like class 1 with HR was not observed in any transformed line. The untransformed 'Gala' leaves (control), showed abundant sporulation without any resistance reaction at 10-12 dpi and classified to class 4 with complete susceptibility. In the three scab trials, results of ANOVA ($F_{9,\ 209} = 29.959$, $p < 0.001$), followed by Tukey's HSD test, revealed that the average infection symptoms severity in the transgenic lines treated with *V. inaequalis* were significantly lower than in 'Gala' (control). Among transgenic lines, line T_{30} showed the lowest symptoms percentage (average symptom severity rate 4.166%). This percentage was not significantly different in comparison to that obtained by transgenic line T_{35} (5.21%), T_{18} (11.66%), T_{10} (20%) and T_{20} (23.6%). On the contrary, it was significantly different in comparison to that obtained by T_{29} (35.42%) and T_{25} (50%). The 'Gala' (control) showed the highest symptoms severity (90.00%).

Table 3: Results of three greenhouse inoculation of 'Gala' plants at 14 days and 21 days post inoculation with Venturia inaequalis.

Line or Cultivar	***Rvi12_Cd5* gene presence**	**No. of inoculated plants**	**No. of leaves**	**Symptom[∞]**				**Severity[£]**	
				Confidence limit				**Confidence interval**	
				Med[*]	**L**	**U**	**Max**	**Mean[ł] (±SD)[a]**	**Groups[b]**
T_{10}	+	15	45	0	0	3b	4	20.0±4.60	A, B, C
T_{18}	+	10	30	0	0	0	3a	11.7±3.11	A, B
T_{20}	+	6	18	0	0	3a	3b	23.6±7.14	B, C
T_{25}	+	10	30	3a	3a	3b	4	50.0±5.7	D
T_{29}	+	8	24	3a	0	3b	4	35.4±7.20	C, D
T_{30}	+	6	18	0	0	0	2	4.2±2.26	A
T_{34}	+	8	24	0	0	0	2	5.2±2.12	A
Gala	-	8	24	4	4	4	4	91.7±5.76	E

*Median values of symptoms and their confidence limits were calculated by using the data from the three to four youngest leaves per plant present at the time of each plant's inoculation. Med., median; Max., maximum; L, lower; U, upper.

*Symptoms were assessed by adapting six scab classes after Chevalier *et al*, (1991) with 0 = no macroscopically visible symptom, 1 = hypersensitive pinpoints, 2 = chlorotic areas with or without very small necrosis and no sporulation, 3a = chlorotic and necrotic areas with slight sporulation (weak resistance), 3b = chlorotic and necrotic areas with sparse sporulation (weak susceptibility), and 4 = sporulation covering entire leaf (complete susceptibility). These values represent degrees of resistance, with 4 representing susceptibility. Confidence limit p = 0.01.

[£]Severity values indicates percentage of infection based on symptoms classes with 0 = 0 %, 1 = 5 %, 2 = 25 %, 3a = 50 %, 3b = 75 % and 4 = 100 %. Confidence limit p= 0.05.

[ł]Mean percentage of scab infection severity and their confidence limit were calculated by using the data from three most infected leaves per plant after assigning them symptom classes. SD., ± standard deviation.

[b]Differences between transformed lines were placed in groups based on Duncan HSD at a p value of 0.05.

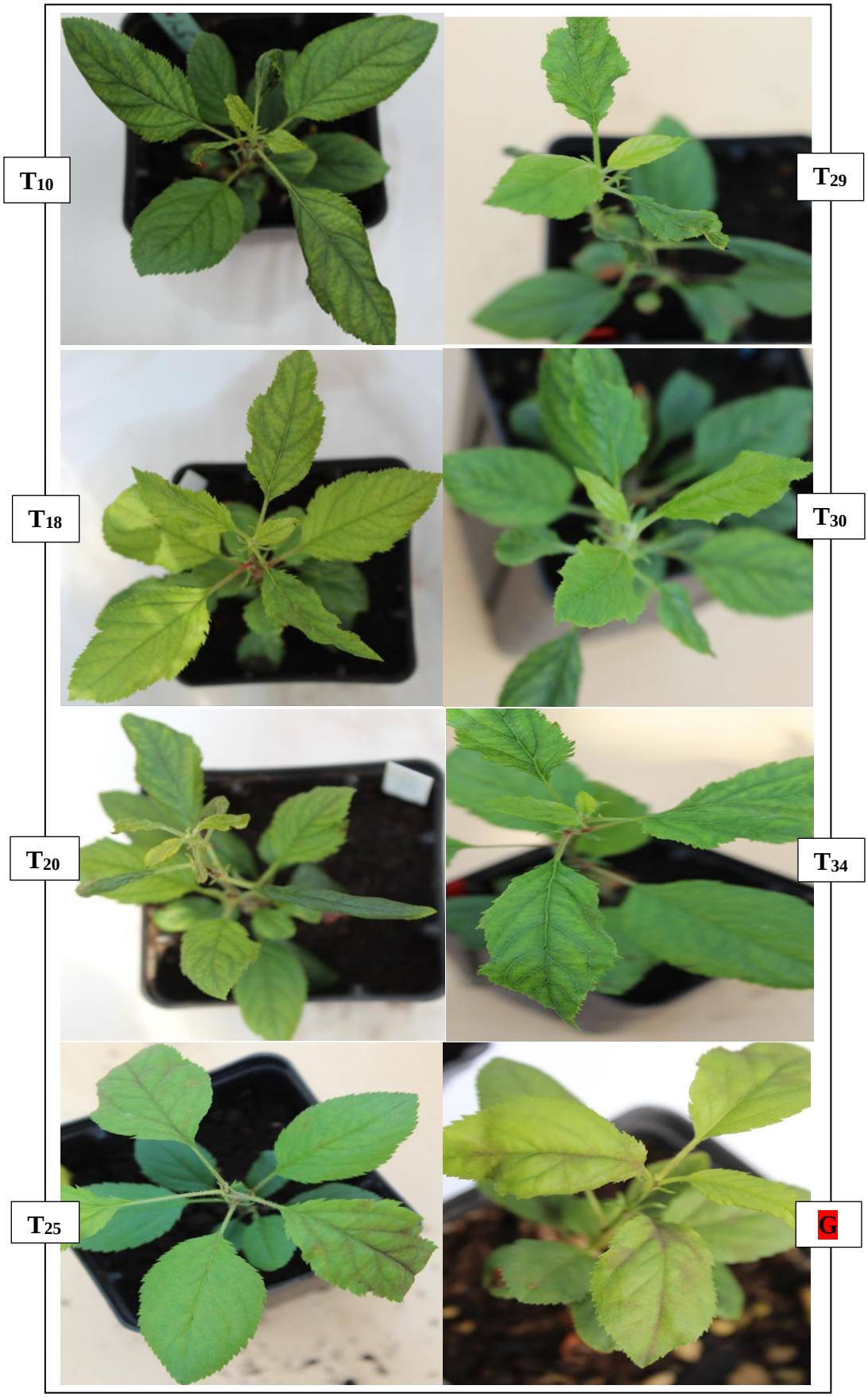


Figure 6: Pictures taken after 14 days post inoculation, showing scab induced phenotypes in transformed 'Gala' lines (T_{10}-T_{34}) with Rvi12_Cd5 and untransformed 'Gala' plants (G).

The presence of germinated *V. inaequalis* conidia was confirmed on the leaves, showing no symptoms, resistance reactions or sporulation from all transgenic lines and sporulation from control (Fig. 7). In most cases, no fungal development beyond that of appressoria and penetration peg formation was observed in transformed leaves showing no symptom at microscopic level (Fig. 7; A: 1, 2). In a few cases, immature collapsed subcuticular hyphae was also observed in clean leaves.

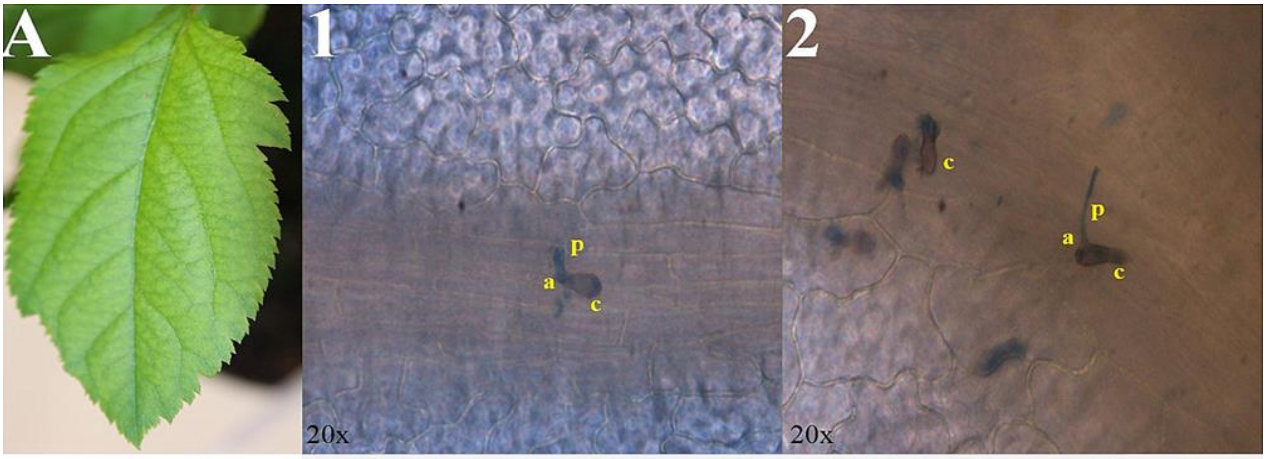


Figure 7: Disease symptoms and cytology of leaves of the transformed 'Gala' lines T_{10}, T_{18}, T_{20}, T_{30}, and T_{34} after inoculation with V. inaequalis, showed symptom class 0 (A); microscopic level (1, 2). Light microscopy of leaf tissues stained with aniline blue at 16 dpi; Bar = 20 µm.

Leaves showing chlorotic lesion with very small necrosis revealed browning of 30-40 epidermal cells (presumably mesophyll cells also) around *V. inaequalis* conidium at microscopic level (Fig. 8; B: 3). The leaf tissue area away from resistance reaction, showed blocked germination of numerous conidia (Fig. 8; B: 4).

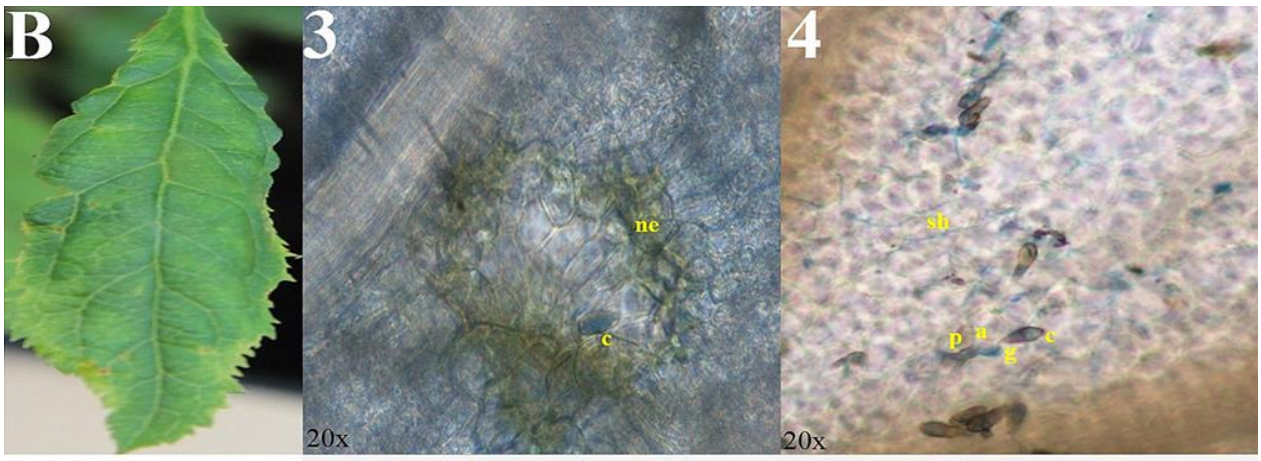


Figure 8: Disease symptoms and cytology of leaves of the transformed 'Gala' lines T_{30}, and T_{34} showed symptom class 2 (B); microscopic level (3, 4). Light microscopy of leaf tissues stained with aniline blue at 16 dpi; Bar = 20 µm.

The leaves showing necrotic lesions with slight chlorosis and light sporulation showed browning of epidermal cells around germinating conidia with advance infection hyphae (Fig. 9; C: 5), or stroma like structure (Fig. 9; C: 6).

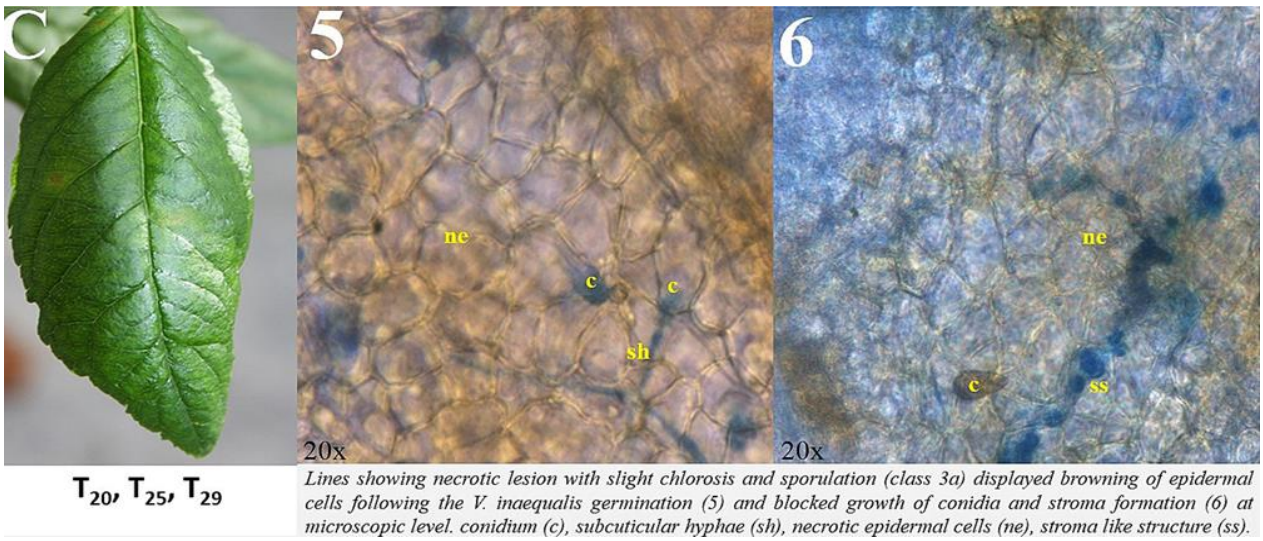


Figure 9: Disease symptoms and cytology of leaves of the transformed 'Gala' lines T_{20}, T_{25}, and T_{29} showed class 3a (C); microscopic level (5, 6). Light microscopy of leaf tissues stained with aniline blue at 16 dpi; Bar = 20 µm.

However, in leaves that showed necrotic lesions with sparse sporulation, browning of more than 40 mesophyll cells was observed around primary subcuticular stroma harboring mixture of aborted or sporulating conidiophores (Fig: 10; D: 7, 8).

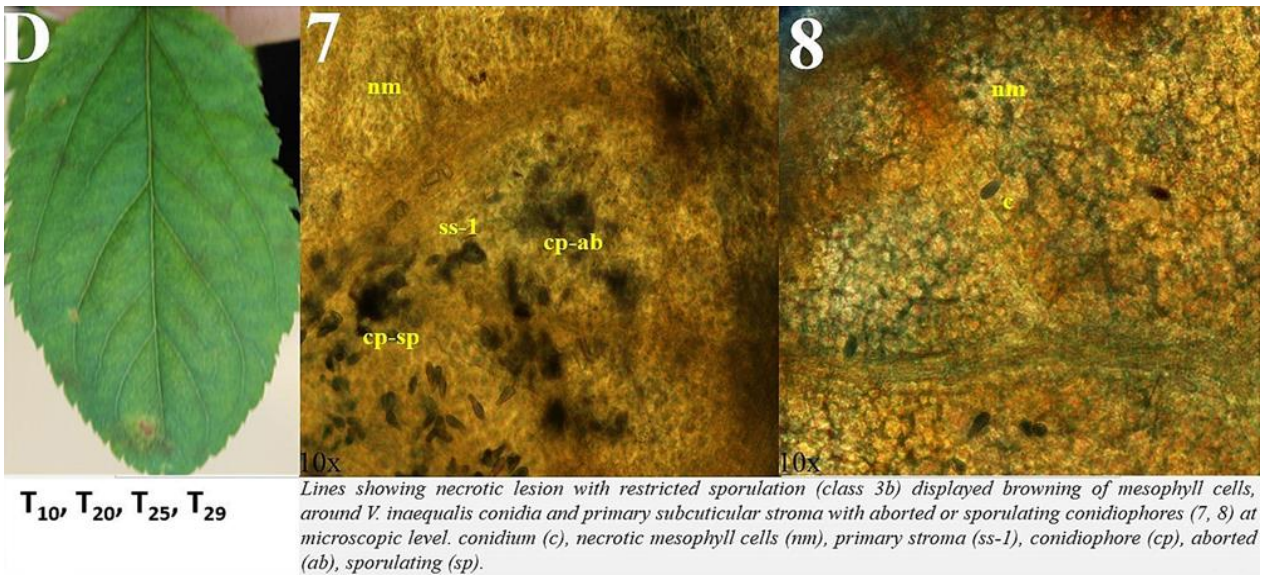


Figure 10: Disease symptoms and cytology of leaves of the transformed 'Gala' lines T_{10}, T_{20}, T_{25}, and T_{29} showed class 3b (D); microscopic level (7, 8). Light microscopy of leaf tissues stained with aniline blue at 16 dpi; Bar = 10 µm.

Cytology of transformed leaves with restricted sporulation, showed isolated or scattered primary and secondary stroma with sporulating conidiophores (Fig. 11; E: 9, 10).

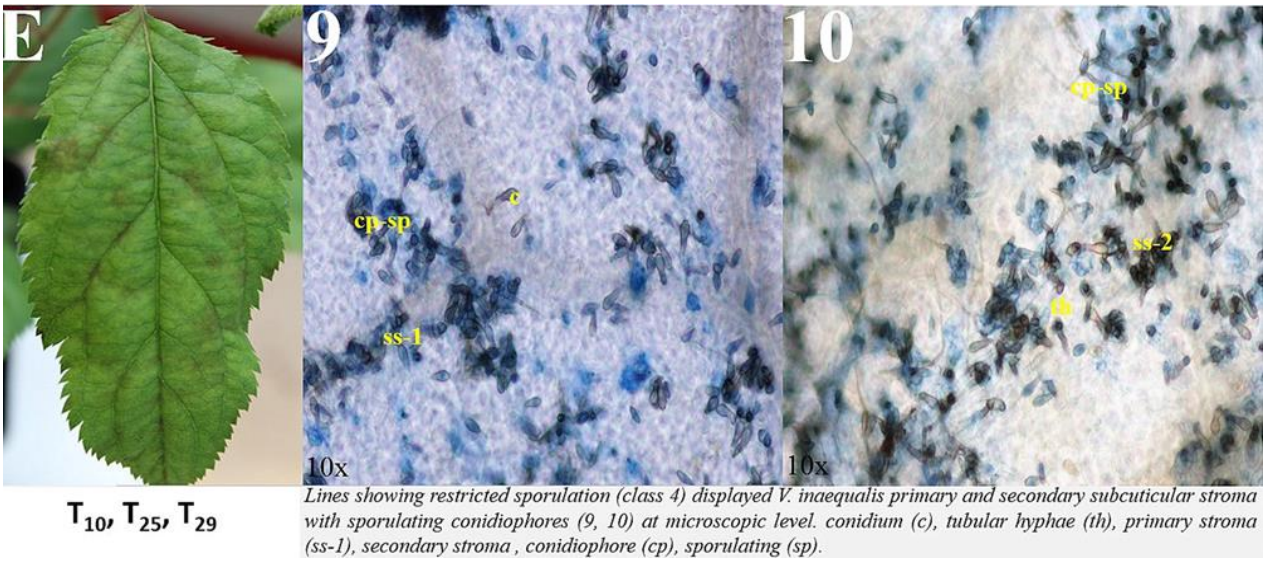


Figure 11: Disease symptoms and cytology of leaves of the transformed 'Gala' lines T_{10}, T_{25}, and T_{29} showed class 4 (E); microscopic level (9, 10). Light microscopy of leaf tissues stained with aniline blue at 16 dpi; Bar = 10 µm.

Whereas, untransformed leaves of control with abundant sporulation, showed homogenous mycelial stroma forming dense subcuticular network under microscope (Fig. 12; F; 11, 12).

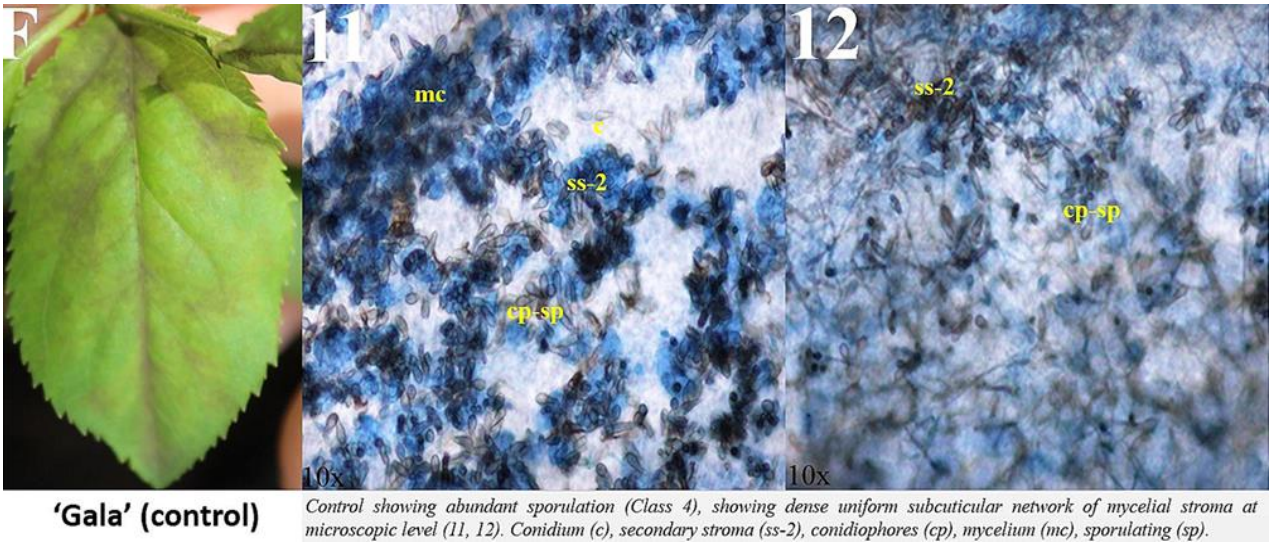


Figure 12: As control, inoculated leaf of untransformed 'Gala' (F); microscopic level (11, 12) is shown. Light microscopy of leaf tissues stained with aniline blue at 16 dpi; Bar = 10 µm.

3.5 Discussion

In this study, we cloned scab resistance candidate gene *Rvi12_ Cd5* in susceptible 'Gala' apple cultivar to perform functional characterization of this gene. The overexpression of *Rvi12_Cd5* in

transgenic plants showed significant resistance compared to control (Fig. 6-12), highlighting the importance of *Rvi12_ Cd5* in the onset of the scab disease. Our data confirmed preliminary results of Padmarasu *et al* (2018) who hypothesized that *Rvi12_Cd5* candidate gene is the most likely gene responsible for *Rvi12* based resistance due its constitutive expression in 'Hansen's baccata 2' (HB2) and presence of all important protein domains in its sequence necessary for defense against *V. inaequalis*.

The transformation of *Rvi12_ Cd5* was carried out by *A. tumefaciens*-mediated method. By utilizing *A. tumefaciens*-mediated transformation of *Rvi12_Cd5* in susceptible apple cultivar 'Gala Galaxy' we were able to get 60 transgenic lines (Tab. 2) with T-DNA insertions into their genome (Fig. 4), indicating the efficiency of the method used in this study. In past, this method has produced stable and high transformation efficiencies in many apple cultivars such as 'Jonagold' (De Bondt *et al*., 1996), 'Royal Gala' (Yao *et al*., 1995), 'Greensleeves' (Maximova *et al*., 1998), 'Orin' (Murata *et al*., 2000), 'Elstar' (Szankowski *et al*., 2000) and 'Pinova' (Flachowsky *et al*., 2007). Many genes such as β-glucuronidase (GUS), antibiotic protein (attacin E) (Malnoy *et al*., 2010), green fluorescent protein (GFP) (Maximova *et al*., 1998), flowering inducing regulators *MdTFL1* (Kotoda *et al*., 2006), *BpMADS4* (Flachowsky *et al*., 2007), phytohormone metabolizer (*iaaM*, *iaaH* or *IPT*) (Li *et al*., 2011) have been used to make transgenic apple plants by using *A. tumefaciens*-mediated gene transfer method. Also, many transgenic lines carrying scab resistant genes such as *Rvi6* (Belfanti *et al*., 2004; Malnoy *et al*., 2008), *Rvi15* (vr2) (Schouten *et al*., 2014) have been obtained by using *A. tumefaciens*-mediated transformation.

Gene expression is regulated at the transcriptional, post-transcriptional, and post-translational levels in plants. Transcriptional regulation plays the most important function in the activation and suppression of expression (Hernandez-Garcia and Finer, 2014). Based on mRNA transcript expression analysis before scab infection, all *in-vitro* transformed 'Gala' lines over expressed the *Rvi12 _Cd5* gene compared to outdoor grown 'HB2' (positive control) while untransformed susceptible 'Gala' (negative control) did not give any expression at all. However, mRNA expression level of *Rvi12_Cd5* was comparable between all transgenic lines. Based on comparable mRNA expression levels in transgenic lines among each other and according to the resistance reaction classes (Tab. 3; median values) assigned to these transgenic lines after scab infection, T_{30} and T_{34} showed high resistance (class 0) with high mRNA expression, T_{25} and T_{29} showed partial resistance (class 3a) with high and medium mRNA expression respectively, T_{10} showed high resistance with medium mRNA expression and T_{18} and T_{20} showed high resistance with low mRNA expression. So it can be concluded that correlation between mRNA and resistance level was either positive in case of T_{29}, T_{30}, T_{34} or no

correlation in case of T_{10}, T_{18}, T_{20}, and T_{25}. The possible explanation for the T_{25} showing partial resistance level with high mRNA expression, could be due to different transcriptional activation leading to different defense response in the host plants (Jada *et al*., 2014; Qu *et al*., 2021) or due to environmental or experimental factors during transformation to inoculation steps. Whereas, in case of the $T_{10,}$ T_{18} and T_{20} showing high resistance with medium or low mRNA expression, the contradictory results could be due to positional effect of the inserted T-DNA which consequently may modify transgene expression (Dean *et al*., 1988; Iglesias *et al*., 1997; Kumar and Fladung, 2001). Though, it can be interesting to check mRNA expression after inoculation, in order to better understand the reason of resistance variance between lines that is probably due to post-inoculation effect. Despite the positive or negative correlation between mRNA expression and resistance reaction level among transgenic lines, all lines showed significantly higher constitutive mRNA expression of *Rvi12_Cd5* compared to 'HB2' and were able to induce high or partial resistance against *V. inaequalis*.

Thirty six transgenic lines were analyzed for T-DNA insertion CN (*nptII* copy number) by RT-qPCR. Twelve of them showed 1 to 2 T-DNA inserts into their genome. Consequently, seven transformants carrying 1-2 T-DNA per cell used for scab infection trail, showed a variation in transgene expression as mentioned above and neither a positive nor a negative correlation was found between copy number and gene expression. Similarly, no correlation was found between T-DNA copy number and *Rvi6* gene expression in studies of Belfanti *et al*, (2004) and Joshi *et al*, (2011).

Previously, thorough phenotypic analysis after using mixed inoculum of *V. inaequalis* for scab test, mapping populations for *Rvi12 (Vb)* locus from 'HB2', responded to *V. inaequalis* by exhibiting chlorosis or chlorosis with minimal sporulation. (Chevalier *et al*., 1991; Erdin *et al*., 2006; Hemmat *et al*., 2003). Later, Padmarasu *et al* (2014) characterized *Rvi12* resistance reactions as no symptoms (Class 0) and chlorosis without or with sporulation (class 2, 3a/3b) against mixed inoculum of *V. inaequalis*, in large mapping population of 'Gala'×'HB2' progeny. In all of these mapping populations for *Rvi12*, infection did not elicit a hypersensitive response (HR) like those of the *Rvi5* or *Rvi15* resistance gene (Bandara *et al*., 2015; Chevalier *et al*., 1991; Erdin *et al*., 2006b; Galli *et al*., 2010a; Hemmat *et al*., 2003; Padmarasu *et al*., 2014). Considering the hypothesis based on *in-silico* gene prediction analysis performed by Padmarasu *et al* (2018), that the *Rvi12_ Cd5* candidate gene derived from 'HB2' might be responsible for *Rvi12 (Vb)* based resistance, we found that overexpression of *Rvi12_ Cd5* was able to induce resistance in transformed susceptible 'Gala' plants and elicited range of resistance reactions (Fig. 7-12; 0, 2, 3a, 3b) against mixed inoculum, similar to range of reactions found in mapping populations of *Rvi12* locus (Erdin *et al*., 2006a; Hemmat *et al*., 2003; Padmarasu *et al*., 2014). Similarly, the diverse responses were also observed in *Rvi6*, *Rvi11* and *Rvi19* and *Rvi20*

(Belfanti *et al*., 2004b; Clark *et al*., 2014; Gygax *et al*., 2004; Malnoy *et al*., 2008), which range from 0 to 3b.

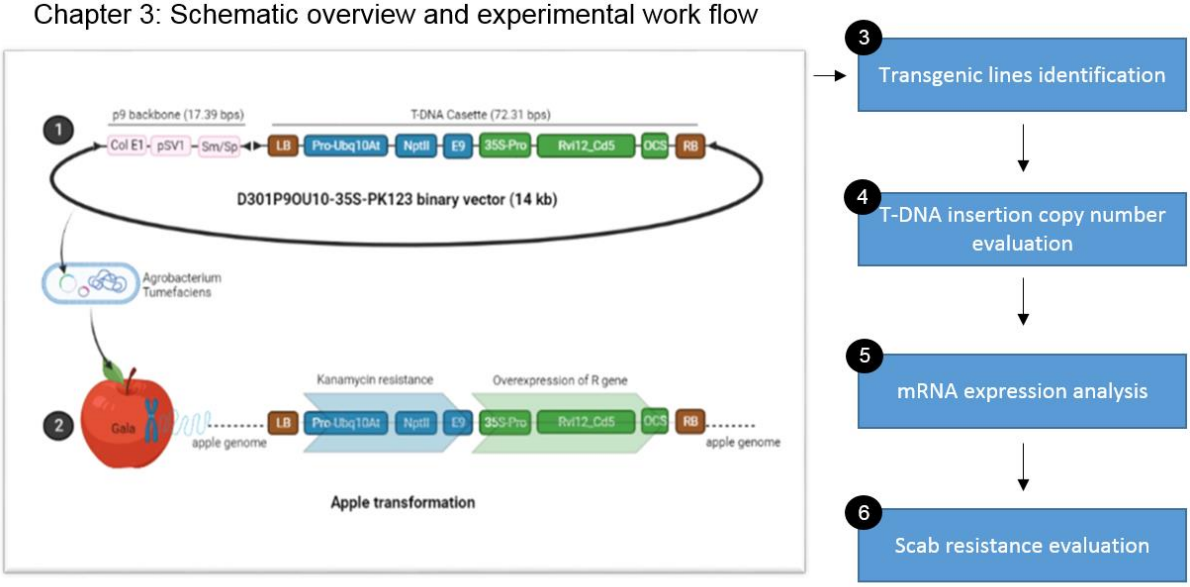
In three independent scab infection trails (Tab. 3), overall reaction was considered by median value (Tab. 3), thus, five lines showed no symptoms (class 0) and two lines showed partial resistance (class 3a), while untransformed susceptible 'Gala' showed sporulation (class 4). T_{30} and T_{34} provided consistent high resistance against mixed inoculum of *V. inaequalis* by showing clean leaves or small chlorotic lesions with very small necrosis (class 2) with no sporulation at all throughout scab infection trails. T_{18} and T_{20} also showed high resistance (class 0) with no sporulation but few leaves showed necrotic lesions with slight or sparse sporulation (3a/3b), despite of that, majority of leaves of these lines were symptomless (class 0), thus, considered as high resistant transgenic plants. Another similar case was observed in line T_{10}, which showed symptomless leaves with no sporulation and classified as high resistant but this line also showed rarely sporulation with or without resistance reaction (3b/4) on few leaves of some biological replicates. Since we used mixed inoculum of *V. inaequalis,* the possible explanation for lines T_{10}, T_{18}, T_{20} could be the presence of aggressive strain of *V. inaequalis* on those leaves triggering the resistance reaction or producing sporulation, however, different strains used in the inoculum are unknown. Other reason could be due to environmental or experimental factors during transformation to inoculation steps (Pang *et al*., 1996). In contrast, T_{25} and T_{29} showed consistent partial resistance with light or sparse sporulation (3a/3b) on majority of their leaves with exception of sporulation (class 4) on few leaves, thus, considered as partial resistant lines. The induction of partial resistance in T_{25} and T_{29} lines could be due to either mutation of the *Rvi12_Cd5* during the different steps of the transformation process or possibly T-DNA insert was mutated in its promoter region, resulting in possible changes of inoculation-induced upregulation, or, alternatively, a mutation in the coding region may have affected translation or functionality of the *Rvi12_Cd5* protein leading to different defense response (Jada *et al*., 2014; Qu *et al*., 2021). Few transformed leaves showing restricted sporulation (class 4), were macroscopically distinctive from leaves of untransformed control showing abundant sporulation (Fig. 7-12). Overall, we were not able to link mRNA expression with resistance level because it did not correlate homogenously as described before. The non-homogenic mRNA expression data among lines indicates the possibility of chimerism (Butaye *et al*., 2005). Similar variation in attacin E gene expression was observed in transgenic apple lines due to chimerism (Flachowsky *et al*., 2008). However, conclusively, it can be said that the mixed inoculum was effective in eliciting incompatible and compatible reactions, which resulted in symptom classes (0, 2, 3a, 3b, 4).

The compatible and incompatible reactions based phenotypes observed in transgenic lines and control were subsequently validated at microscopic level (Fig. 7-12). Microscopic observations are fully in agreement with macroscopic symptoms and lack of visible symptoms was is not due to the pathogen inoculum escapes. Detailed cytology observed in all transgenic lines and control provided strong validation of assigned symptom classes and suggested that *Rvi12_ Cd5* gene might be the functionally active *Rvi12*.

3.6 Concluding remarks

This results presented in this paper provide consistent indications that the *Rvi12_Cd5* gene derived from 'HB2' can induce apple scab resistance in transformed 'Gala' plants and thus reports the cloning and functional characterization of third apple scab resistance gene to date. The induction of range of resistance reactions against *V. inaequalis* in transgenic lines carrying *Rvi12_Cd5*, supports that *Rvi12_Cd5* might be the at least one of the two proposed resistance genes active in 'HB2' (Padmarasu *et al*., 2018). The functional characterization of the *Rvi12_Cd5* gene through transgenic 'Gala' lines provides now the first requirement to produce cisgenic apple lines (Vanblaere *et al*., 2011) and an opportunity to create durable resistance by combining *Rvi12* with other scab resistance genes like *Rvi16* and *Rvi15* in susceptible cultivars (Pedersen and Leath, 1988; Poland *et al*., 2009). The presence of *Rvi12, Rvi6* and *Rvi15* in the same genetic background, can allow studying the mechanisms and downstream reactions of these three resistance genes in extensive detail.

Chapter 3: Schematic overview and experimental work flow



Functional characterization of *Rvi12_Cd5*

3.7 Bibliography

Bandara, N. L., Cova, V., Tartarini, S., Gessler, C., Patocchi, A., Cestaro, A., Troggio, M., Velasco, R., & Komjanc, M. (2015). Isolation of *Rvi5* (*vm*) locus from Malus × domestica 'Murray.' *Acta Hortic*, *1100*. https://doi.org/10.17660/ActaHortic.2015.1100.1

Beckerman, J. L., Sundin, G. W., & Rosenberger, D. A. (2015). Do some IPM concepts contribute to the development of fungicide resistance? Lessons learned from the apple scab pathosystem in the United States. *Pest Management Science*, *71*(3), 331–342.

Belfanti, E., Barbieri, M., Tartarini, S., Vinatzer, B., Gennari, F., Paris, R., Sansavini, S., Silfverberg-Dilworth, E., Patocchi, A., Hermann, D., Gianfranceschi, L., & Gessler, C. (2004). Gala apple transformed with the putative scab resistance gene HcrVf2. *Acta Horticulturae*, *663*, 453–456. https://doi.org/10.17660/ActaHortic.2004.663.78

Belfanti, E., Silfverberg-Dilworth, E., Tartarini, S., Patocchi, A., Barbieri, M., Zhu, J., ... & Sansavini, S. (2004). The *HcrVf2* gene from a wild apple confers scab resistance to a transgenic cultivated variety. *Proceedings of the National Academy of Sciences*, *101*(3), 886-890. https://doi.org/10.1073/pnas.0304808101

Bengtsson, M., Lindhard, H., & Grauslund, J. (2000). Occurrence of races of *Venturia inaequalis* in an apple scab race screening orchard in Denmark. *IOBC Wprs Bulletin*, *23*(12), 225–230.

Bolar, J. P., Norelli, J. L., Harman, G. E., Brown, S. K., & Aldwinckle, H. S. (2001). Synergistic activity of endochitinase and exochitinase from Trichoderma atroviride (T. harzianum) against thee pathogenic fungus (*Venturia inaequalis*) in transgenic apple plants. *Transgenic Res*, *10*. https://doi.org/10.1023/A:1013036732691

Borejsza-Wysocka, E., Norelli, J. L., Aldwinckle, H. S., & Malnoy, M. (2010). Stable expression and phenotypic impact of attacin E transgene in orchard grown apple trees over a 12 year period. *BMC Biotechnology*, *10*(1), 1–9.

Bruzzese, E., & Hasan, S. (1983). A whole leaf clearing and staining technique for host specificity studies of rust fungi. *Plant Pathology*, *32*(3), 335–338.

Bus, V. G. M., Rikkerink, E. H. A., Caffier, V., Durel, C.-E., & Plummer, K. M. (2011a). Revision of the Nomenclature of the Differential Host-Pathogen Interactions of *Venturia inaequalis* and Malus. *Http://Dx.Doi.Org/10.1146/Annurev-Phyto-072910-095339*, *49*, 391–413. https://doi.org/10.1146/ANNUREV-PHYTO-072910-095339

Bus, V. G. M., Rikkerink, E. H. A., Caffier, V., Durel, C.-E., & Plummer, K. M. (2011b). Revision of the Nomenclature of the Differential Host-Pathogen Interactions of *Venturia inaequalis* and *Malus* . *Annual Review of Phytopathology*, *49*(1), 391–413. https://doi.org/10.1146/annurev-phyto-072910-095339

Bus, V., Rikkerink, E. H. A., Van de Weg, W. E., Rusholme, R. L., Gardiner, S. E., Bassett, H. C. M., Kodde, L. P., Parisi, L., Laurens, F. N. D., & Meulenbroek, E. J. (2005). The *Vh2* and *Vh4* scab resistance genes in two differential hosts derived from Russian apple R12740-7A map to the same linkage group of apple. *Molecular Breeding*, *15*(1), 103–116.

Butaye, K. M. J., Cammue, B., Delauré, S. L., & De Bolle, M. F. C. (2005). Approaches to minimize variation of transgene expression in plants. *Molecular Breeding*, *16*(1), 79–91.

Calenge, F., Faure, A., Goerre, M., Gebhardt, C., Van De Weg, W. E., Parisi, L., & Durel, C. E. (2004). Quantitative Trait Loci (QTL) analysis reveals both broad-spectrum and isolate-specific QTL for scab resistance in an apple progeny challenged with eight isolates of *Venturia inaequalis*. *Phytopathology*. https://doi.org/10.1094/PHYTO.2004.94.4.370

Chetty, V. J., Ceballos, N., Garcia, D., Narváez-Vásquez, J., Lopez, W., & Orozco-Cárdenas, M. L. (2012). Evaluation of four *Agrobacterium tumefaciens* strains for the genetic transformation of tomato (*Solanum lycopersicum L*.) cultivar Micro-Tom. *Plant Cell Reports 2012 32:2*, *32*(2), 239–247. https://doi.org/10.1007/S00299-012-1358-1

Chevalier, M., Lespinasse, Y., & Renaudin, S. (1991). A microscopic study of the different classes of symptoms coded by the *Vf* gene in apple for resistance to scab (*Venturia inaequalis*). *Plant Pathol*, *40*. https://doi.org/10.1111/j.1365-3059.1991.tb02374.x

Clark, M. D., Bus, V. G. M., Luby, J. J., & Bradeen, J. M. (2014). Characterization of the defence response to *Venturia inaequalis* in 'Honeycrisp'apple, its ancestors, and progeny. *European Journal of Plant Pathology*, *140*(1), 69–81.

Cova, V., Bandara, N. L., Liang, W., Tartarini, S., Patocchi, A., Troggio, M., Velasco, R., & Komjanc, M. (2015). Fine mapping of the *Rvi5* (*Vm*) apple scab resistance locus in the 'Murray'apple genotype. *Molecular Breeding*, *35*(10), 1–12.

Cova, V., Lasserre-Zuber, P., Piazza, S., Cestaro, A., Velasco, R., Durel, C. E., & Malnoy, M. (2015). High-resolution genetic and physical map of the *Rvi1* (*Vg*) apple scab resistance locus. *Molecular Breeding*. https://doi.org/10.1007/s11032-015-0245-1

Crandall, C. S. (1926). Apple breeding at the University of Illinois. *Illinois Agric Exp Stn Bull*, *275*.

Dalla Costa, L., Bozzoli, M., Pompili, V., Piazza, S., Broggini, G. A. L., Patocchi, A., & Malnoy, M. (2019). Development of a Taqman real-time PCR method to quantify nptII in apple lines obtained with 'established'or 'new breeding'techniques of genetic modification. *European Food Research and Technology*, *245*(3), 643–652.

De Bondt, A., Eggermont, K., Penninckx, I., Goderis, I., & Broekaert, W. F. (1996). *Agrobacterium*-mediated transformation of apple (*Malus x domestica Borkh*.): an assessment of factors affecting regeneration of transgenic plants. *Plant Cell Reports*, *15*(7), 549–554.

Dean, C., Jones, J., Favreau, M., Dunsmuir, P., & Bedbrook, J. (1988). Influence of flanking sequences on variability in expression levels of an introduced gene in transgenic tobacco plants. *Nucleic Acids Research*, *16*(19), 9267–9283.

Dinesh-Kumar, S. P., Whitham, S., Choi, D., Hehl, R., Corr, C., & Baker, B. (1995). Transposon tagging of tobacco mosaic virus resistance gene N: its possible role in the TMV-N-mediated signal transduction pathway. *Proceedings of the National Academy of Sciences*, *92*(10), 4175–4180.

Dixon, M. S., Jones, D. A., Keddie, J. S., Thomas, C. M., Harrison, K., & Jones, J. D. G. (1996). The tomato *Cf-2* disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. *Cell*, *84*(3), 451–459.

Erdin, N., Tartarini, S., Broggini, G. A. L., Gennari, F., Sansavini, S., Gessler, C., & Patocchi, A. (2006a). Mapping of the apple scab-resistance gene *Vb*. *Genome*. https://doi.org/10.1139/G06-096

Flachowsky, H., Le Roux, P. M., Peil, A., Patocchi, A., Richter, K., & Hanke, M. V. (2011). Application of a high-speed breeding technology to apple (*Malus*×*domestica*) based on transgenic early flowering plants and marker-assisted selection. *New Phytologist*, *192*(2), 364–377. https://doi.org/10.1111/J.1469-8137.2011.03813.X

Flachowsky, H., Peil, A., Sopanen, T., Elo, A., & Hanke, V. (2007). Overexpression of BpMADS4 from silver birch (Betula pendula Roth.) induces early-flowering in apple (*Malus*× *domestica* Borkh.). *Plant Breeding*, *126*(2), 137–145.

Flachowsky, H., Riedel, M., Reim, S., & Hanke, M.-V. (2008). Evaluation of the uniformity and stability of T-DNA integration and gene expression in transgenic apple plants. *Electronic Journal of Biotechnology*, *11*(1), 26–40.

Flachowsky, H., Szankowski, I., Fischer, T. C., Richter, K., Peil, A., Höfer, M., Dörschel, C.,

Schmoock, S., Gau, A. E., Halbwirth, H., & Hanke, M. V. (2010). Transgenic apple plants overexpressing the Lc gene of maize show an altered growth habit and increased resistance to apple scab and fire blight. *Planta*. https://doi.org/10.1007/s00425-009-1074-4

Galli, P., Broggini, G. A. L., Gessler, C., & Patocchi, A. (2010). Phenotypic characterization of the *Rvi15* (*vr2*) apple scab resistance. *JPP*, *92*.

Galli, P., Potacchi, A., Broggini, G. A. L., Gessler, C., & Potachi, A. (2010). The *Rvi15* (*Vr2*) scab resistance locus contains three TIR NBS LRR genes. *Mol Plant-Microbe Interact*, *23*. https://doi.org/10.1094/MPMI-23-5-0608

Gassmann, W., Hinsch, M. E., & Staskawicz, B. J. (1999). The Arabidopsis RPS4 bacterial-resistance gene is a member of the TIR-NBS-LRR family of disease-resistance genes. *The Plant Journal*, *20*(3), 265–277.

Gessler, C., Patocchi, A., Sansavini, S., Tartarini, S., & Gianfranceschi, L. (2006). *Venturia inaequalis* resistance in apple. In *Critical Reviews in Plant Sciences*. https://doi.org/10.1080/07352680601015975

Gessler, C., & Stumm, D. (1984). Infection and Stroma Formation by *Venturia inaequalis* on Apple Leaves with Different Degrees of Susceptibility to Scab. *Journal of Phytopathology*, *110*(2), 119–126. https://doi.org/10.1111/J.1439-0434.1984.TB03399.X

Gessler, Cesare, & Pertot, I. (2012). *Vf* scab resistance of Malus. In *Trees - Structure and Function*. https://doi.org/10.1007/s00468-011-0618-y

Gómez-Gómez, L., & Boller, T. (2000). *FLS2*: an LRR receptor–like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Molecular Cell*, *5*(6), 1003–1011.

Gygax, M., Gianfranceschi, L., Liebhard, R., Kellerhals, M., Gessler, C., & Patocchi, A. (2004). Molecular markers linked to the apple scab resistance gene *Vbj* derived from *Malus baccata* jackii. *Theoretical and Applied Genetics*. https://doi.org/10.1007/s00122-004-1803-9

Hemmat, M., Brown, S. K., Aldwinckle, H. S., & Weeden, N. F. (2003). Identification and mapping of markers for resistance to apple scab from "Antonovka" and "Hansen's baccata #2." *Acta Hortic*, *622*. https://doi.org/10.17660/ActaHortic.2003.622.13

Hernandez-Garcia, C. M., & Finer, J. J. (2014). Identification and validation of promoters and cis-acting regulatory elements. *Plant Science*, *217*, 109–119.

Hood, E. E., Gelvin, S. B., Melchers, L. S., & Hoekema, A. (1993). New Agrobacterium helper

plasmids for gene transfer to plants. *Transgenic Research 1993 2:4*, *2*(4), 208–218. https://doi.org/10.1007/BF01977351

Hough, L. F. (1944). A survey of scab resistance of foliage on seedling in selected apple progenies. *Proc Am Soc Hortic Sci*, *44*.

Iglesias, V. A., Moscone, E. A., Papp, I., Neuhuber, F., Michalowski, S., Phelan, T., Spiker, S., Matzke, M., & Matzke, A. J. (1997). Molecular and cytogenetic analyses of stably and unstably expressed transgene loci in tobacco. *The Plant Cell*, *9*(8), 1251–1264.

Jada, B., Soitamo, A. J., Siddiqui, S. A., Murukesan, G., Aro, E.-M., Salakoski, T., & Lehto, K. (2014). Multiple different defense mechanisms are activated in the young transgenic tobacco plants which express the full length genome of the Tobacco mosaic virus, and are resistant against this virus. *PloS One*, *9*(9), e107778.

Janick, J, Cummins, J. N., Brown, S. K., & Hemmat, M. (1996). *Apples In: Janick, J. and Moore, JN (eds) Fruit Breeding, Vol. 1, Tree and Tropical Fruits*. John Wiley & Sons, New York. str.

Janick, J. (2006, August). The origins of horticultural technology and science. In *XXVII International Horticultural Congress-IHC2006: Global Horticulture: Diversity and Harmony, an Introduction to IHC2006 759* (pp. 41-60).

Jansch, M., Paris, R., Amoako-Andoh, F., Keulemans, W., Davey, M. W., Pagliarani, G., Tartarini, S., & Patocchi, A. (2014). A phenotypic, molecular and biochemical characterization of the first cisgenic scab-resistant apple variety 'Gala.' *Plant Mol Biol Rep*, *32*. https://doi.org/10.1007/s11105-013-0682-0

Jones, D. A., Thomas, C. M., Hammond-Kosack, K. E., Balint-Kurti, P. J., & Jones, J. D. (1994). Isolation of the tomato *Cf-9* gene for resistance to Cladosporium fulvum by transposon tagging. *Science*, *266*(5186), 789–793.

Joshi, S. G., Schaart, J. G., Groenwold, R., Jacobsen, E., Schouten, H. J., & Krens, F. A. (2011). Functional analysis and expression profiling of *HcrVf1* and *HcrVf2* for development of scab resistant cisgenic and intragenic apples. *Plant Mol Biol*, *75*. https://doi.org/10.1007/s11103-011-9749-1

Joshi, S. G., Soriano, J. M., Kortstee, A., Schaart, J. G., Krens, F. A., Jacobsen, E., & Schouten, H. J. (2009). Development of cisgenic apples with durable resistance to apple scab. *Acta Horticulturae*. https://doi.org/10.17660/ActaHortic.2009.839.53

Krens, F. A., Schaart, J. G., Van der Burgh, A. M., Tinnenbroek-Capel, I. E., Groenwold, R., Kodde, L. P., ... & Schouten, H. J. (2015). Cisgenic apple trees; development, characterization, and performance. *Frontiers in plant science*, *6*, 286.

Khajuria, Y. P., Kaul, S., Wani, A. A., & Dhar, M. K. (2018). Genetics of resistance in apple against *Venturia inaequalis* (Wint.) Cke. *Tree Genetics and Genomes*, *14*(2). https://doi.org/10.1007/S11295-018-1226-4

Kotoda, N., Iwanami, H., Takahashi, S., & Abe, K. (2006). Antisense expression of *MdTFL1*, a *TFL1*-like gene, reduces the juvenile phase in apple. *Journal of the American Society for Horticultural Science*, *131*(1), 74–81.

Kumar, S., & Fladung, M. (2001). Gene stability in transgenic aspen (Populus). II. Molecular characterization of variable expression of transgene in wild and hybrid aspen. *Planta*, *213*(5), 731–740.

Lawrence, G. J., Finnegan, E. J., Ayliffe, M. A., & Ellis, J. G. (1995). The *L6* gene for flax rust resistance is related to the Arabidopsis bacterial resistance gene *RPS2* and the tobacco viral resistance gene N. *The Plant Cell*, *7*(8), 1195–1206.

Lefrancq, B., & Lateur, M. (2009). Monitoring and occurrence of new races of *Venturia inaequalis* on apple in Belgium. *Communications in Agricultural and Applied Biological Sciences*, *74*(3), 623–631.

Li, J., Komori, S., Sasaki, K., Mimida, N., Matsumoto, S., Wada, M., Soejima, J., Ito, Y., Masuda, T., & Tanaka, N. (2011). Pre-culture before *Agrobacterium* infection to leaf segments and meropenem improves the transformation efficiency of apple (*Malus*× *domestica* Borkh.). *Journal of the Japanese Society for Horticultural Science*, *80*(3), 244–254.

Linhart, C., Panzacchi, S., Belpoggi, F., Clausing, P., Zaller, J. G., & Hertoge, K. (2021). Year-round pesticide contamination of public sites near intensively managed agricultural areas in South Tyrol. *Environmental Sciences Europe*, *33*(1), 1–12.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods*, *25*(4), 402–408.

MacHardy. (1996a). Apple scab: biology, epidemiology, and management. *American Phytopathological Society (APS Press).*

MacHardy. (1996b). Pathogen development and host tissue reaction. *Apple scab biology,*

epidemiology, and management. APS press.

Malnoy, M, Xu, M., Borejsza-Wysocka, E., Korban, S. S., & Aldwinckle, H. S. (2008). Two receptor-like genes, *Vfa1* and *Vfa2*, confer resistance to the fungal pathogen *Venturia inaequalis* inciting apple scab disease. *Mol Plant-Microbe Interact*, *21*. https://doi.org/10.1094/MPMI-21-4-0448

Malnoy, M., Boresjza-Wysocka, E. E., Norelli, J. L., Flaishman, M. A., Gidoni, D., & Aldwinckle, H. S. (2010). Genetic transformation of apple (*Malus x domestica*) without use of a selectable marker gene. *Tree Genetics & Genomes*, *6*(3), 423-433.

Manktelow, D. W. L., Beresford, R. M., Batchelor, T. A., & Walker, J. T. S. (1996). Use patterns and economics of fungicides for disease control in New Zealand apples. *Acta Horticulturae*, *422*, 187–192. https://doi.org/10.17660/ACTAHORTIC.1996.422.31

Martin, G. B., Brommonschenkel, S. H., Chunwongse, J., Frary, A., Ganal, M. W., Spivey, R., Wu, T., Earle, E. D., & Tanksley, S. D. (1993). Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science*, *262*(5138), 1432–1436.

Maximova, S. N., Dandekar, A. M., & Guiltinan, M. J. (1998). Investigation of *Agrobacterium*-mediated transformation of apple using green fluorescent protein: high transient expression and low stable transformation suggest that factors other than T-DNA transfer are rate-limiting. *Plant Molecular Biology*, *37*(3), 549–559.

Meyers, B. C., Dickerman, A. W., Michelmore, R. W., Sivaramakrishnan, S., Sobral, B. W., & Young, N. D. (1999). Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. *The Plant Journal*, *20*(3), 317–332.

Mindrinos, M., Katagiri, F., Yu, G.-L., & Ausubel, F. M. (1994). The *A. thaliana* disease resistance gene *RPS2* encodes a protein containing a nucleotide-binding site and leucine-rich repeats. *Cell*, *78*(6), 1089–1099.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, *15*(3), 473–497.

Murata, M., Haruta, M., Murai, N., Tanikawa, N., Nishimura, M., Homma, S., & Itoh, Y. (2000). Transgenic apple (*Malus*× *domestica*) shoot showing low browning potential. *Journal of Agricultural and Food Chemistry*, *48*(11), 5243–5248.

Padmarasu, S., Sargent, D. J., Jaensch, M., Kellerhals, M., Tartarini, S., Velasco, R., Troggio, M., & Patocchi, A. (2014). Fine-mapping of the apple scab resistance locus *Rvi12 (Vb)* derived from

‘Hansen’s baccata #2.’ *Molecular Breeding*. https://doi.org/10.1007/s11032-014-0167-3

Padmarasu, S., Sargent, D. J., Patocchi, A., Troggio, M., Baldi, P., Linsmith, G., Poles, L., Jänsch, M., Kellerhals, M., Tartarini, S., & Velasco, R. (2018). Identification of a leucine-rich repeat receptor-like serine/threonine-protein kinase as a candidate gene for *Rvi12 (Vb)*-based apple scab resistance. *Molecular Breeding*, *38*(6). https://doi.org/10.1007/S11032-018-0825-Y

Pang, S., Ja, F., Carney, K., Stout, J., Tricoli, D. M., Quemada, H. D., & Gonsalves, D. (1996). Post-transcriptional transgene silencing and consequent tospovirus resistance in transgenic lettuce are affected by transgene dosage and plant development. *The Plant Journal*, *9*(6), 899–909.

Papp, D., Singh, J., Gadoury, D., & Khan, A. (2020). New North American isolates of *venturia inaequalis* can overcome apple scab resistance of malus floribunda 821. *Plant Disease*, *104*(3), 649–655. https://doi.org/10.1094/PDIS-10-19-2082-RE

Parisi, L., Fouillet, V., Schouten, H. J., Groenwold, R., Laurens, F., Didelot, F., Evans, K., Fischer, C., Gennari, F., Kemp, H., Lateur, M., Patocchi, A., Thissen, J., & Tsipouridis, C. (2004). Variability of the pathogenicity of *Venturia inaequalis* in Europe. *Acta Horticulturae*, *663*, 107–114. https://doi.org/10.17660/ACTAHORTIC.2004.663.13

Parniske, M., Hammond-Kosack, K. E., Golstein, C., Thomas, C. M., Jones, D. A., Harrison, K., Wulff, B. B. H., & Jones, J. D. G. (1997). Novel disease resistance specificities result from sequence exchange between tandemly repeated genes at the *Cf-4/9* locus of tomato. *Cell*, *91*(6), 821–832.

Patocchi, A., Frei, A., Frey, J. E., & Kellerhals, M. (2009). Towards improvement of marker assisted selection of apple scab resistant cultivars: *Venturia inaequalis* virulence surveys and standardization of molecular marker alleles associated with resistance genes. *Molecular Breeding*, *24*(4), 337–347. https://doi.org/10.1007/S11032-009-9295-6

Patocchi, A, Gianfranceschi, L., & Gessler, C. (1999). Towards the map-based cloning of *Vf*: fine and physical mapping of the Vf region. *Theor Appl Genet*, *99*. https://doi.org/10.1007/s001220051409

Patocchi, Andrea, Wehrli, A., Dubuis, P. H., Auwerkerken, A., Leida, C., Cipriani, G., Passey, T., Staples, M., Didelot, F., Philion, V., Peil, A., Laszakovits, H., Rühmer, T., Boeck, K., Baniulis, D., Strasser, K., Vávra, R., Guerra, W., Masny, S., … Bus, V. G. M. (2020). Ten Years of VINQUEST: First Insight for Breeding New Apple Cultivars with Durable Apple Scab Resistance. *Plant Disease*, *104*(8), 2074–2081.

Pedersen, W. L., & Leath, S. (1988). Pyramiding major genes for resistance to maintain residual effects. *Annual Review of Phytopathology*, *26*(1), 369–378.

Perini, P., Pasquali, G., Margis-Pinheiro, M., de Oliviera, P. R. D., & Revers, L. F. (2014). Reference genes for transcriptional analysis of flowering and fruit ripening stages in apple (*Malus*× *domestica Borkh*.). *Molecular Breeding*, *34*(3), 829–842.

Pessina, S., Lenzi, L., Perazzolli, M., Campa, M., Dalla Costa, L., Urso, S., Valè, G., Salamini, F., Velasco, R., & Malnoy, M. (2016). Knockdown of *MLO* genes reduces susceptibility to powdery mildew in grapevine. *Horticulture Research*, *3*. https://doi.org/10.1038/hortres.2016.16

Poland, J. A., Balint-Kurti, P. J., Wisser, R. J., Pratt, R. C., & Nelson, R. J. (2009). Shades of gray: the world of quantitative disease resistance. *Trends in Plant Science*, *14*(1), 21–29.

Qu, J., Dry, I., Liu, L., Guo, Z., & Yin, L. (2021). Transcriptional profiling reveals multiple defense responses in downy mildew-resistant transgenic grapevine expressing a TIR-NBS-LRR gene located at the *MrRUN1/MrRPV1* locus. *Horticulture Research*, *8*(1), 1–12.

Schaart, J. G., van de Wiel, C. C. M., Lotz, L. A. P., & Smulders, M. J. M. (2016). Opportunities for products of new plant breeding techniques. *Trends in Plant Science*, *21*(5), 438–449.

Schouten, H J, Brinkhuis, J., Burgh, A. M., Schaart, J. G., Groenwold, R., Broggini, G. A. L., & Gessler, C. (2014). Cloning and functional characterization of the *Rvi15 (Vr2)* gene for apple scab resistance. *Tree Genet Genomes*, *10*. https://doi.org/10.1007/s11295-013-0678-9

Schouten, H J, Soriano, J. M., Joshi, S. G., Kortstee, A. J., Krens, F. A., Schaart, J. G., van der Linden, K., Allan, A. C., Hellens, R. P., & Espley, R. V. (2007). Cisgenesis is a promising approach for fast, acceptable and safe breeding of pip fruit. *XII EUCARPIA Symposium on Fruit Breeding and Genetics 814*, 199–204.

Schouten, Henk J, Krens, F. A., & Jacobsen, E. (2006). Do cisgenic plants warrant less stringent oversight? *Nature Biotechnology*, *24*(7), 753.

Song, W.-Y., Wang, G.-L., Chen, L.-L., Kim, H.-S., Pi, L.-Y., Holsten, T., Gardner, J., Wang, B., Zhai, W.-X., & Zhu, L.-H. (1995). A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. *Science*, *270*(5243), 1804–1806.

Soriano, J. M., Madduri, M., Schaart, J. G., van der Burgh, A., van Kaauwen, M. P. W., Tomic, L., Groenwold, R., Velasco, R., van de Weg, E., & Schouten, H. J. (2014). Fine mapping of the gene *Rvi18 (V25)* for broad-spectrum resistance to apple scab, and development of a linked SSR

marker suitable for marker-assisted breeding. *Molecular Breeding*, *34*(4), 2021–2032.

Szankowski, I., Briviba, K., Fleschhut, J., Schönherr, J., Jacobsen, H. J., & Kiesecker, H. (2003). Transformation of apple (*Malus domestica* Borkh.) with the stilbene synthase gene from grapevine (Vitis vinifera L.) and a *PGIP* gene from kiwi (Actinidia deliciosa). *Plant Cell Reports*, *22*(2), 141–149.

Szankowski, I., Lübke, A., & Jacobsen, H.-J. (2000). Influence of sonication on regeneration and transformation efficiencies in apple. *IV International Symposium on In Vitro Culture and Horticultural Breeding 560*, 505–508.

Vaillancourt, L. J., & Hartman, J. R. (2000). Apple scab. *The Plant Health Instructor*.

Vanblaere, T., Flachowsky, H., Gessler, C., & Broggini, G. A. L. (2014). Molecular characterization of cisgenic lines of apple 'Gala'carrying the *Rvi6* scab resistance gene. *Plant Biotechnology Journal*, *12*(1), 2–9.

Vanblaere, T., Szankowski, I., Schaart, J., Schouten, H., Flachowsky, H., Broggini, G. A. L., & Gessler, C. (2011). The development of a cisgenic apple plant. *Journal of Biotechnology*. https://doi.org/10.1016/j.jbiotec.2011.05.013

Vinatzer, B A, Zhang, H. B., & Sansavini, S. (1998). Construction and characterization of a bacterial artificial chromosome library of apple. *Theor Appl Genet*, *97*. https://doi.org/10.1007/s001220051008

Vinatzer, Boris A, Patocchi, A., Gianfranceschi, L., Tartarini, S., Zhang, H.-B., Gessler, C., & Sansavini, S. (2001). Apple Contains Receptor-like Genes Homologous to the Cladosporium fulvum Resistance Gene Family of Tomato with a Cluster of Genes Cosegregating with *Vf Apple Scab Resistance* (Vol. 14, Issue 4).

Williams, E. B. (1966). Allelic genes in *Malus* for resistance to *Venturia inaequalis*. *Proc. Amer. Soc. Hort. Sci.*, *88*, 52–56.

Williams, E. B., Dayton, D. F., & Shay, J. R. (1996). Allelic genes in Malus for resistance to *Venturia inaequalis*. *Proc Am Soc Hortic Sci*, *88*.

Williams, E. B., & Kuc, J. (1969). Resistance in *Malus* to *Venturia inaequalis*. *Annual Review of Phytopathology*, *7*(1), 223–246.

Wulff, B. B. H., Kruijt, M., Collins, P. L., Thomas, C. M., Ludwig, A. A., De Wit, P. J. G. M., & Jones, J. D. G. (2004). Gene shuffling-generated and natural variants of the tomato resistance

gene *Cf-9* exhibit different auto-necrosis-inducing activities in Nicotiana species. *The Plant Journal*, *40*(6), 942–956.

Xu, M. L., & Korban, S. S. (2002). A cluster of four receptor-like genes resides in the *Vf* locus that confers resistance to apple scab disease. *Genetics*, *162*.

Xu, M. L., & Korban, S. S. (2004). Somatic variation plays a key role in the evolution of the Vf gene family residing in the Vf locus that confers resistance to apple scab disease. *Mol Phylogenet Evol*, *32*. https://doi.org/10.1016/j.ympev.2003.12.004

Yao, J.-L., Cohen, D., Atkinson, R., Richardson, K., & Morris, B. (1995). Regeneration of transgenic plants from the commercial apple cultivar Royal Gala. *Plant Cell Reports*, *14*(7), 407–412.

Yepes, L. M., & Aldwinckle, H. S. (1993). Pathogenesis of *Venturia inaequalis* on shoot-tip cultures and on greenhouse-grown apple cultivars. *Phytopathology*, *11*. https://doi.org/10.1094/Phyto-83-1155

Chapter 4

Development of cisgenic apple lines expressing scab resistant gene *Rvi12_Cd5*

4.1 Abstract

Cisgenesis is a promising approach to accelerate the transfer of scab resistant genes controlled by natural promoter, to apple by genetic engineering, leaving no or minimal exogenous DNA in the final product. It is a step forward to reduce environmental concerns and increase consumer's acceptance. Here we report overview of *Rvi12_Cd5* natural promoter structure, cis- regulatory elements and their functions after expanding promoter sequence length from 900 bps to 2618 bps by Sanger sequencing. A comprehensive *in-silico* analysis of *Rvi12_Cd5* promoter sequence revealed transcription factor binding sites (TFBSs) corresponding to six major plant defense related transcription factors such as AP2/ERF, bHLH, TGA/bZIP, MYB, NAC, and WRKY. The putative cisgenic apple lines were generated by inserting the endogenous apple scab resistance gene *Rvi12_ Cd5* under the control of its own promoter to scab susceptible apple cultivar 'Gala'. A vector designed with three molecular mechanisms for cisgene expression, kanamycin resistance for apple transformats and T-DNA excision system by heat inducible site specific FLP/FRT recombinase system respectively, was used in *Agrobacterium*-mediated transformation. The candidate gene *Rvi12_Cd5* was able to induce high to partial resistance against *V. inaequalis* under its own promoter. This gene is derived from the scab-resistant apple 'Hansen's baccata # 2' (wild Siberian crab apple). After transformation, all transformed lines were analyzed to assess copy number, integration site, mRNA expression level and resistance to apple scab. All of these lines induced low mRNA expression of *Rvi12_Cd5* compared with the natural expression of 'Hansen's baccata # 2', except for one cisgenic apple line. Even a low expression of *Rvi12_Cd5* was sufficient to induce plant reaction and reduce fungal growth compared with the scab-susceptible 'Gala' except two transformed lines. The site specific FLP/FRT recombinase system was efficient to eliminate completely the T-DNA cassette in five independent apple cisgenic lines carrying a single insertion of *Rvi12_Cd5*.

Key words: *Malus x domestica*. Apple scab. FLP/FRT recombination. Cisgenesis.

4.2 Introduction

Apple (*Malus* × *domestica*) orchards are often susceptible to scab disease, caused by *Venturia inaequalis*. Scab is the most important disease in terms of economic cost in apple orchards worldwide (Bowen *et al*., 2011; MacHardy *et al*., 2001). Currently, the scab disease is managed by applying up to 15-30 fungicides per year in commercial orchards (MacHardy, 1996a; Manktelow *et al*., 1996; Vaillancourt and Hartman, 2000). However, the excessive use of chemical fungicides may contaminate the environment and harm human health (Gessler, 2011). Furthermore, prolonged use of chemical control may result in fungicide resistant isolates of *V. inaequalis* (Bonaterra *et al*., 2012). An alternative strategy is to use natural defense mechanism of apple plants against pathogens. The *V. inaequalis*-*Malus* interaction has been known for a long time (Boone *et al*., 1957; Williams and Shay, 1957). The interaction between *V. inaequalis* and *Malus* species occurs in accordance to the gene-for-gene concept (Flor, 1971). By coevolving with *V. inaequalis*, twenty apple scab resistance genes has been identified in *Malus* mapping populations or from wild relatives (Bus *et al*., 2011a; Jha *et al*., 2009b). In this regard, *Rvi6 (Vf)* locus of *M. floribunda* was the first one to successfully undergo the process of introgression in *M. domestica*. The *Rvi6* locus has been widely adopted in resistance breeding programs against apple scab (Crosby *et al*., 1992; Gessler and Pertot, 2012; Boris A Vinatzer *et al*., 2001; Williams and Kuc, 1969; Xu and Korban, 2002b). Unfortunately, most of the scab-resistant cultivars released to date carry *Rvi6* (*Vf*), and its value is questionable since emergence of pathogen avirulence gene *avrRvi6* which occur in Europe and U.S.A (Beckerman et al., 2015; Bengtsson et al., 2000; Lefrancq and Lateur, 2009; Papp et al., 2020a; Parisi et al., 2004a) (Beckerman *et al*., 2015; Papp *et al*., 2020b). Since scab resistance still primarily relies on *Vf*, breeding of other resistant genes are needed to overcome *Rvi6* resistance breakdown.

Traditional breeding programs have allowed development of scab-resistant apple varieties by establishing series of backcrossing's between resistant hybrid and susceptible commercial cultivars (Cusin *et al*., 2017), but it is a painstakingly slow process with a low efficiency rate, because of the long juvenile phase (15-20 years) and high degree of genetic heterozygosity present in this perennial fruit tree (Fenning and Gershenzon, 2002; Gardiner *et al*., 2007; Gessler and Pertot, 2012). When a trait e.g, disease resistance has to be introduced from wild apple species to susceptible one, additional time is required (S. G. Joshi *et al*., 2009). It took several decades to introduce *Rvi6*-resistant cultivars with good fruit quality market by traditional breeding (Bakker *et al*, 1999). Recent biotechnological advances in genomics such as new plant breeding techniques (NBPTs) for woody fruit trees have accelerated the gene transfer from small-fruited wild apple species to susceptible commercial apple cultivars in short period of time (Aldwinckle and Malnoy, 2009; Cusin *et al*., 2017; Flachowsky *et*

al., 2011b; Gessler and Patocchi, 2007; Kumar *et al*., 2010; Laurens *et al*., 2018). One example is the successful transfer of resistance gene *Rvi6 (HcrVf2)* via *Agrobacterium tumefaciens* to susceptible apple cultivars in order to produce transgenic apple scab resistance cultivars within 1 year (Barbieri *et al*., 2003a; Belfanti *et al*., 2004; Malnoy *et al*., 2008; Silfverberg-Dilworth *et al*., 2005a).

However, large-scale implementations of transgenesis in apple or other food crops have been severely hampered by suggested or perceived risks related to genetic modification (GM). A large proportion of the consumers in Europe view GM foods as a risk to both health and the environment (Gaskell *et al*., 2010). The notion of cisgenesis was developed to address some of the primary objections, with the goal of decreasing public worries and biosafety issues as much as feasible (Schouten *et al*., 2007, 2006a). Cisgenesis is the process of adding natural genes including the native promoter, terminator, exons, and introns, from crossable donor plants. No foreign genes, notably antibiotic resistance genes of bacterial origin, should be found in the final product (Schouten *et al*., 2006b, 2006a). In apple, introgression of resistance genes by traditional breeding results in new cultivars with the accompanying linkage drag (co-transfer of DNA sequences that are linked to the gene of interest), while introducing cisgenes by biotechnological methods maintains the original cultivar characteristics (Borejsza-Wysocka *et al*., 2010; Jacobsen and Schouten, 2008). This method eliminates the introduction of undesirable alleles, whereas traditional breeding removes these alleles gradually over generations (Jacobsen and Schouten, 2008; Schaart *et al*., 2016). Cisgenesis also permits high-grade cultivars to keep their demonstrated fruit quality and other desirable features (Krens *et al*, 2015; Joshi *et al*., 2009; Vanblaere *et al*., 2011). Consumers judged such crops to be more acceptable (De Marchi *et al*., 2019; Gaskell *et al*., 2010; Heng *et al*., 2021; Lusk and Sullivan, 2002; Marette *et al*., 2021). The European Food Safety Authority (EFSA Panel on Genetically Modified Organisms ((GMO), 2012) compared the hazards associated with cisgenic, intragenic and conventionally bred plants and concluded that hazards associated with cisgenic plants are similar to the ones associated with conventionally bred plants. The legal regulation of GM plants in Europe (e.g. (EC) 1829/2003) requires molecular characterization, which includes the amount of vector and/or donor nucleic acid remaining in the modified organism, sequence data for flanking regions, level of expression of the specific protein resulting from the genetic modification, and information on the organization of the inserted genetic material at the insertion site, with the goal of determining whether the genetic modification raise any issues regarding the potential for producing new toxins or allergens (EFSA, 2011). Cisgenic scab resistant apple lines may cause less concerns compared with a transgenic product, for example allergenicity caused by the product of the inserted foreign gene (Vanblaere *et al*., 2014).

The concept of cisgenic plants which carry gene expression controlled by its own sequences, initiated by Schouten *et al*., (2006a, 2006b), attracted many researchers who highlighted cisgenesis by discussion and long debate to breed apples in a way that would be acceptable to a wide public (Akhond and Machray, 2009; E Jacobsen, 2008; Houdebine, 2007; Jacobsen and Nataraja, 2008; Lammerts van Bueren *et al*., 2007; Schubert and Williams, 2006). To deal with diseases of vegetative propagated plants which are directly consumed and have quality traits, cisgenesis was found the most interesting approach. The potential of cisgenes has been discussed in apple against apple scab (Gessler *et al*., 2009; Joshi, 2010; Joshi *et al*., 2011; Krens *et al*., 2015; Vanblaere *et al*., 2011, 2014). Three methods to produce cisgenic lines have been designed so far (Schaart *et al*., 2011). The first method is to avoid any foreign selection genes, second method is to use two separate T-DNAs, one for selectable marker genes and the other for gene of interest from apple, followed by segregation of these two T-DNAs in the progeny, and the third method is to use excision of selectable marker genes by inducing site specific recombination (R/Rs) (Schaart *et al*., 2011, 2004). In 2011, the development of three scab resistant cisgenic 'Gala' lines carrying only *Rvi6* (*HcrVf2*) without any foreign genes, were achieved followed by the recombinase-based marker excision (Vanblaere *et al*., 2011). The *Rvi6* controlled by its own promoter induced resistance to apple scab (Barbieri *et al*., 2003b; Belfanti *et al*., 2004; Joshi, 2010; Vanblaere *et al*., 2014). Consequently, these cisgenic lines were characterized molecularly (Vanblaere *et al*., 2014). Later, Krens *et al*, (2015) reported the development of four other cisgenic apple lines carrying *Rvi6* under control of its own promoter by site specific recombination (R/Rs) induced excision of non-apple gene sequences from the T-DNA of the transgenic lines described by Joshi *et al*, (2011). Induction of excision was performed by dexamethasone treatment followed by selection on 5-fluorocytosine (Schaart *et al*., 2004) to produce seven cisgenic lines carrying *Rvi6* (Krens *et al*., 2015; Vanblaere *et al*., 2014). Krens *et al*, (2015) also confirmed durability of scab resistance in cisgenic lines compared to susceptible control over a period of 3 years in the field after propagation, grafting, growing, and planting. In the same year, development of two more cisgenic lines carrying *Rvi6* controlled by its own promoter was reported (Würdig *et al*., 2015). In this case, the exact excision of exogenous DNA was achieved by site specific recombination (Flp/FRT) induced by heat treatment and confirmed by sequencing the previously determined T-DNA integration site. Both cisgenic lines were able to confer full resistant to *V. inaequalis* (Würdig *et al*., 2015). Site specific recombination (Flp/FRT) was also used in cisgenic apple lines which induced resistance against fire blight (Kost *et al*., 2015).

Ability of apple scab resistance genes to induce resistance against *V. inaequalis* depends on gene expression. Which is regulated at the transcriptional, post-transcriptional, and post-translational levels. Transcriptional regulation, which is predominantly regulated by gene promoters, plays the

most important function in the activation and suppression of expression (Aman Beshir and Kebede, 2021). The induction of RNA polymerase activity is mediated by proteins called transcription factors (TFs) that recognize certain DNA motifs (transcription factor binding sites; typically 4 to 30 base pairs long) in the promoter and so initiate transcription (Borneman *et al*., 2007; Lee and Young, 2000; Robertson, 2002; Setlow, 2012). Such motifs usually lies in a core promoter region which comprises the TATA box at -40 bps and CAAT box at -69 to -100 bps upstream of the transcriptional initiation site (TSS) (Lee and Young, 2000; Mantovani, 1999; Molina and Grotewold, 2005; Setlow, 2012). The TATA box is the binding site for the transcription initiation complex (Burley and Roeder, 1996; Lee and Young, 2000). The proximal and distal regions of promoters are located upstream of the core promoter region. Enhancers, silencers, insulators, and motifs are found in the proximal and distal areas of the promoter and contribute to the fine regulation of gene expression at the transcriptional level (Lee and Young, 2000). Gene expression is mainly regulated by the motifs localized upstream of the transcriptional start site (TSS) near core promoter (Hernandez-Garcia and Finer, 2014). The motifs present in the genes associated with defense responses can provide special insights into gene regulation (constitutive or inducible) and plant signaling under stress conditions (Yamaguchi-Shinozaki and Shinozaki, 1994). TFs which corresponds to defense related motifs are grouped into six major families named as AP2/ERF, bHLH, TGA/bZIP, MYB, NAC, and WRKY (Tsuda and Somssich, 2015). To date, no study has been reported on motif analysis and corresponding TFs of apple scab resistance genes, however, many researchers have studied functional length of native promoter, involved in quality expression of scab resistance gene *Rvi6* (*HcrVf2*) and consequently they have used these lengths of promoter to develop cisgenic scab resistant apple plants (Joshi *et al*., 2011; Krens *et al*., 2015; Malnoy *et al*., 2008; Schouten *et al*., 2014; Silfverberg-Dilworth *et al*., 2005c; Iris Szankowski *et al*., 2009; Vanblaere *et al*., 2011; Wurdig *et al*., 2015). Other than *Rvi6* (*HcrVf2*), no other apple scab resistance gene has been used for production of cisgenic apple scab resistant cultivars so far. In our study, we intended to study functional ability of native promoter of *Rvi12_Cd5* by *in-silico* analysis and production of scab resistant cisgenic 'Gala' lines by cloning the construct carrying candidate gene *Rvi12_Cd5* derived from wild germplasm 'Hansen's baccata # 2'(HB2), under its native promote. This confirms the hypothesis that this gene can be expressed efficiently under the native promoter to confer scab resistance in apple transformants, after kanamycin resistance selection and exogenous DNA removal by heat shock inducible Flp/*FRT* recombinase system (Herzog *et al*., 2012; Würdig *et al*., 2013), in order to develop the first cisgenic apple lines carrying *Rvi12_ Cd5* based scab resistance.

4.3 Experimental procedures

4.3.1 Sequencing and *In-silico* analysis of *Rvi12_ Cd5* native promoter

The BAC clone 6F11 of genotype 'HB2' (Padmarasu *et al*., 2018) carrying 903 bps sequence upstream of start codon ATG (transcription starting site) of apple scab resistance gene *Rvi12_Cd5* (3352 bps), was defined as short promoter length (SP). For further extension of promoter length, BAC clone 6F11 was grown overnight into lysogeny broth (LB) media (10 g/l Tryptone, 5 g/l NaCl and 10 g/l yeast extract) supplemented with antibiotics (25 µg/ml chloramophenicol for BAC and 25 µg/ml spectomycin for expression plasmid). DNA from BAC clone was extracted using NucleoSpin plasmid purification mid kit (Macherey–Nagel, Düren, Germany) and electrophoresed on a 1% agarose gel at 120 V. Bands were visualized over UV light following staining with GelRed, (Biotium, CA, USA), compared to the 1 kb Plus DNA ladder (Invitrogen-Thermo Fisher Scientific Inc., MA, USA) and quantified using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific Inc., MA, USA).

1 ng/2 µl of BAC 6F11 plasmid DNA was used as a template for Sanger sequencing (FEM Sequencing Platform Facility) (Tab. 1; Rvi12_seq1, Rvi12_seq2 Rvi12_seq3, Rvi12_seq4, Rvi12_seq5, Rvi12_seq6). The obtained sequences were checked on Brenchling online software (https://www.benchling.com/) to confirm the sequence quality. Overlapping sequences were extracted to finalize the one complete sequence of extended promoter region. The first *in silico* analysis was performed using PlantCARE (Lescot *et al*., 2002), a specific online tool for predicting cis-regulatory elements (motifs) of promoters. An additional analysis was performed online using PlantPAN 3.0 (Chow *et al*., 2019).

4.3.2 Preparation of *Rvi12_Cd5* construct with native promoter

The binary vector carrying sequence of *Rvi12_Cd5* with its native SP was designed and assembled (DNA Cloning Service, Hamburg, Germany). The oligo synthesis of nucleotide sequence of cisgene from reference BAC clone 6F11, was followed by cloning into a binary vector D308pBNpt-MdPK1-3 (Fig. 1). The T-DNA cassette flanked by the left and right borders (Fig. 1: LB, RB), integrated with three well defined molecular mechanisms respectively, for kanamycin resistance system, T-DNA excision (transgene sequence elimination) and cisgene *Rvi12_Cd5* expression. The antibiotic resistance was characterized by the *Neomycin phosphotransferase II* gene (*nptII*), driven by the *Cauliflower Mosaic Virus 35S* promoter, which conferred kanamycin resistance to apple transformants during the antibiotic-assisted selection following the transformation. The T-DNA excision system consisted of the FLP/FRT recombinase of *Saccharomyces cerevisiae* combined to the heat-shock inducible promoter (HSP) of the soya bean gene *Hsp17.5-E* (Czarnecka *et al*., 1989). The system was

designed with the *Flippase* gene (FLP) under the control of the heat-shock inducible promoter and the two *Flippase Recognition Target* sites (FRT) next to the LB and terminator element as octopine synthase gene (OCS) in order to remove the transgenes (with predicted exception of 34 to 96 bps of exogenous DNA) in T-DNA cassette. The cisgene expression system was characterized by expression of *Rvi12_Cd5* controlled by its native SP next to RB.

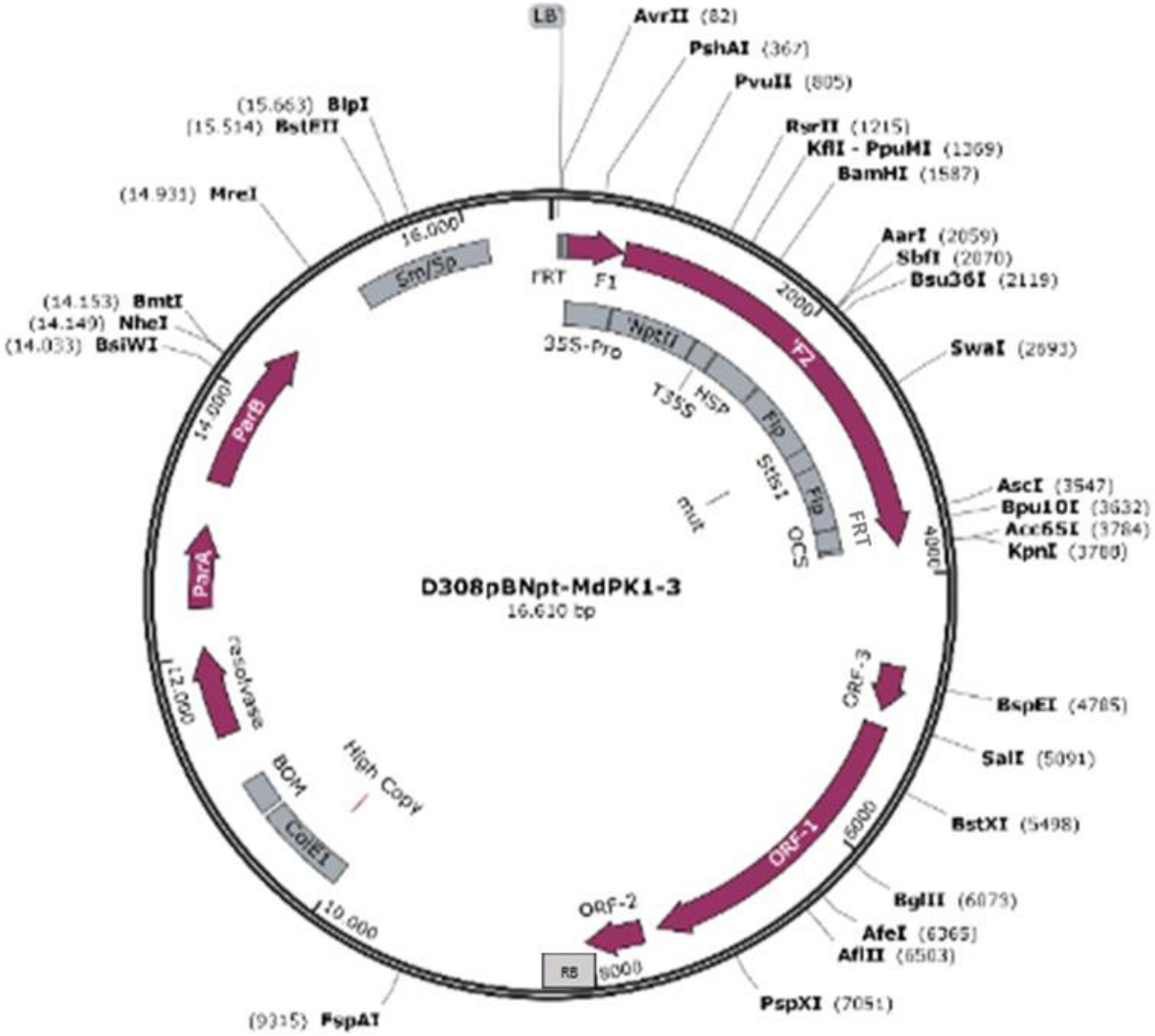


Figure 1: Construction map of the 16 kb binary vector D308pBNpt-MdPK1-3 containing the cisgene Rvi12_Cd5 controlled by its native short promoter (SP) used for apple cv. 'Gala' transformation. Binary vector was produced by cloning a 8 kb T-DNA cassette into a 8 kb pB vector backbone. The segment between the left (LB) and the right border (RB) is transferred into the plant cell, and the exogenous DNA segment between the two Flippase (FLP) Recognition Target sites (FRT) is then removed on heat shock inducible recombinase-mediated deletion, with the exception of 34 to 96 bps of exogenous DNA. (ORF), Open reading frame of apple scab resistance gene Rvi12_Cd5 with SP from the Siberian crab apple Malus. baccata 'Hansen's baccata #2'; (Colicin E1 and BOM), origins of replication and basis of mobility; (Sm/Sp), streptomycin/spectinomycin resistance genes; (LB and RB), left and right borders; (35S-P and 35ST), Cauliflower Mosaic Virus 35S promoter and terminater; (NptII), Neomycin phosphotransferase II functions as kanamycin resistance gene for positive transformation selection; (OCS), Terminator element as octopine synthase gene; (Resolvase), site-specific recombinase; (ParA), the partition ATPase; (ParB) the centromere-binding protein; (Stsl1), Enhancer element; (HSP), Heat shock promoter of the soya bean gene; (FLP), flippase gene; (FRT), Flippase Recognition Target sites.

4.3.3 *Agrobacterium* mediated transformation

By electroporation, the binary vector D308pBNpt-MdPK1-3 (Fig. 1) with the inserted *Rvi12_Cd5* gene along its SP was transferred to the competent cells of *A. tumefaciens* strain EHA105 (Chetty *et al*., 2012; Hood *et al*., 1993), carrying the helper plasmid pCH32. *A. tumefaciens* mediated transformation of apple was performed using the protocol by Szankowski *et al*. (2003) and Vanblaere *et al*, (2014) with minor modifications as described above in chapter 3. The 450 and 900 explants were used for two transformations respectively (Fig 2; Tab. 2).

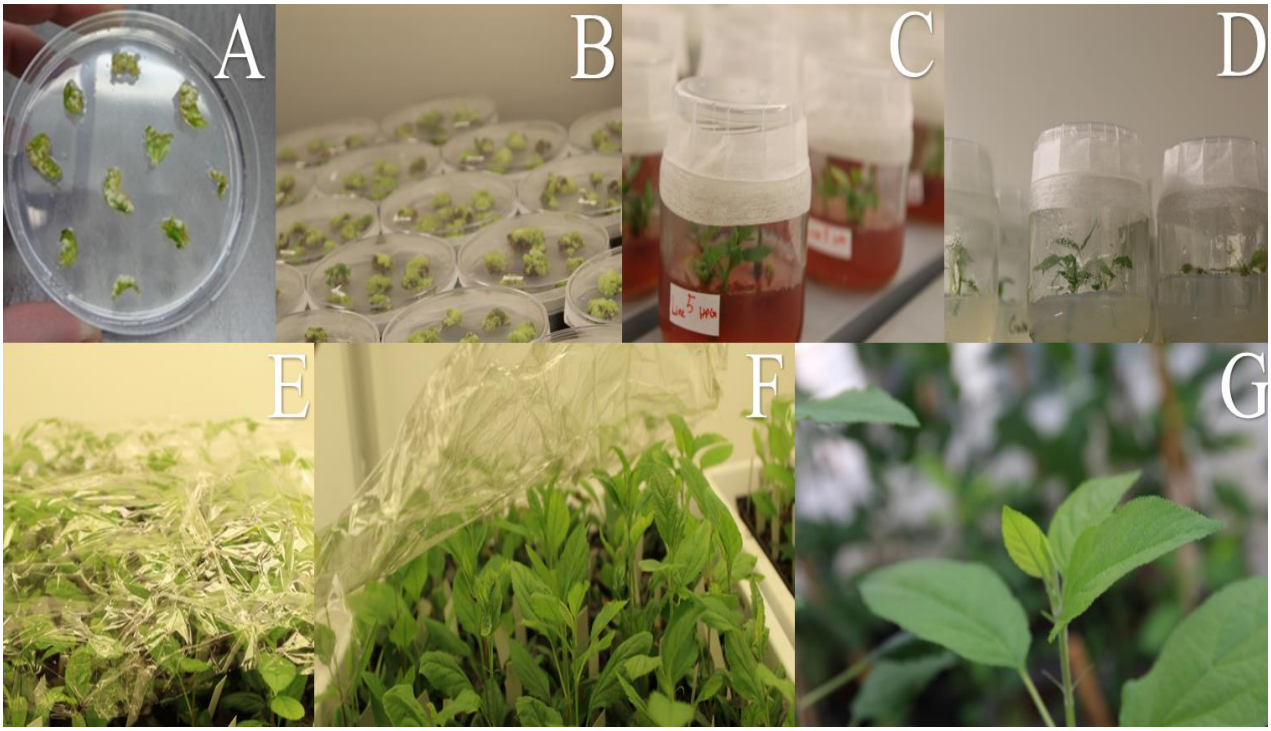


Figure 2: Agrobacterium mediated transformation of cv. 'Gala' with Rvi12_Cd5 along SP yielded several independent transformed lines, which were multiplied in-vitro. Transformation steps; (A) Co-cultivation, (B) Regeneration, (C) Micro-propagation, (D) Rooting, (E) Transfer to soil medium and humidity, (F) progressive reduction of humidity, (G) Acclimatization to greenhouse.

4.3.4 Presence of cisgene and *nptII* copy number determination

For each plant from putative transformants and untransformed control, genomic DNA was extracted from100 mg leaf tissue of plants as described in chapter 3. The 1.5-2 µl DNA was used for polymerase chain reactions (PCRs) employing the thermocycle-3000 (Biometra), 12.5 µl of the GoTaq Green Master Mix 2X (Promega, Fitchburg, MA) and 1 µl of each primer (Tab. 1; SY170612171-093_F, SY170612171-093_R) in the total reaction to amplify *Rvi12_Cd5* gene. The thermal protocol used was as follows: 2 min at 95 °C followed by 35 cycles of denaturation, annealing and extension of 30 s at 95 °C, 30 s at 61 °C and 1 min at 72 °C, respectively, with a final extension of 5 min at 72 °C. Then, the corresponding PCR products were electrophoretically checked on the gel.

The quantification of *nptII* copy number (CN) for calculating the T-DNA insertion in positive transformants, was performed according to TaqMan real-time PCR method developed by Dalla Costa *et al* (2019) for apple, and specific primers and probes for the endogenous gene *MdTOPO6* and for the marker gene *nptII* were used (Tab. 1) as described above in chapter 3.

Table 1: Sequences of primers and probes used for the PCR-based screening of apple transformants, the quantification of nptII copy number by Taqman real-time PCRs, RT- qPCR for expression analysis of Rvi12_Cd5, Colony PCR, BAC amplification, T-DNA in

Primer and probe name	Sequence (5'-3')	Amplicon length (bps)	Annealing temperature (°C)
Promoter sequencing			
Rvi12_seq1	F: 5′CCACAGCTTATAAGGAACGG3′	1718	58
Rvi12_seq2	F: 5′CTTACATTGGATGGCCACAG 3′		
Rvi12_seq3	F: 5′ATGTTATGGCATCATGTTGGC3′		
Rvi12_seq4	F: 5′CTCAATTAGGAGTGAAACTTGG3′		
Rvi12_seq5	F: 5′ ATGTCGATACCAAGAGGCTC 3′		
Rvi12_seq6	F: 5′ TGCAATATCCAGTTTTCCAGC 3′		
Identification of *Rvi12_Cd5* gene in transformants			
SY170612171-093_F			
SY170612171-093_R	F: 5′GGAGTCTTAAACTTGCCCGC3′†	1574	61
	R: 5′CAGAACCAAAACTTCCCGTG3′†		
***NptII* copy number**			
MdTOPO6	F: 5′TGTGGAAGGAGATCAAAGCGCA3′§	196	58
	R: 5′CGCGTTGCTTCTTTGCTGCA3′§		
MdTOPO6_probe	FAM-5′-ACATGCCAACAGGAACAATCACA-3′-TAMRA§		
NPTII	F: 5′CTTGCCGAATATCATGGTGGAA3′§	100	58
	R: 5′GGTAGCCAACGCTATGTCCTGA3′§		
NPTII_probe	FAM-5′-TTCTGGATTCATCGACTGTGGC-3′-TAMRA§		
mRNA expression analysis			
MdACT_F	F: 5′TGACCGAATGAGCAAGGAAATTACT3′$	230	
MdACT_R	R: 5′TACTCAGCTTTGGCAATCCACATC3′$		60
Rvi12_Cd5_F2	F: 5′GAGTCTTAAACTTGCCCGC3′†	253	
Rvi12_Cd5_R2	R: 5′CCTGTGAAATTATTCATACAAC3′†		
Colony PCR			
Rvi12 Amp1_F	F: 5′CGTATCCGATGCGTATTGGG3′†	4255	58
Rvi12 Ampend_R	R: 5′TTAGCCTTCTTGAGCTTTGAG3′†		
T-DNA insertion sites			
GW1	F: 5′GTAATACGACTCACTATAGGGC3′&	400-700	59
35S-P	R: 5′GCTGGGCAATGGAATCCGAG3′		

† **(Padmarasu *et al*., 2018)** - Primers for quantification of transgene expression levels

§ **(Dalla Costa *et al*., 2019)** - Primers for determination of *nptII* CN number of transgene

$ **(Perini *et al*., 2014)**- Primers for quantification of endogenous gene expression levels

& **(Herzog *et al*., 2012)**- Primers for T-DNA insertion sites

4.3.5 Rooting and acclimatization of transformed Gala lines to greenhouse

After determination of integrated transgene CN, the elongated *in-vitro* shoots of selected transgenic 'Gala' lines from the shoot propagation medium, were transferred to rooting medium, thus leading to the acclimatizing of the lines at greenhouse conditions as described above in chapter 3.

4.3.6 Quantitative expression

For each plant, RNA extraction, RNA quality check, cDNA synthesis, RNA & cDNA quantification and qRT-PCR was performed with similar protocols as described in chapter 3.

4.3.7 Scab resistance evaluation

When the acclimatized biological replicates of 15 independent transformed 'Gala' lines had grown at least ten leaves, scab disease test was conducted in the greenhouse. A mixed population of *V. inaequalis* collected in the field was used, composed of fresh and vital conidia. The conidia was checked for concentration & germination rate as described in chapter 3. The upper 3-4 young leaves were tagged and plants were inoculated with a suspension of *V. inaequalis* (concentration of $5x10^5$ conidia ml^{-1} with germination rate of 80-90 % ml^{-1}). The incubation protocol for infection, macroscopic evaluation, statistical analysis and optical microscopy slide preparation were performed similarly as described above for transgenic plants in chapter 3. The symptom classes and infection severity of upper most affected three to nine leaves per transformed line were computed to describe the overall symptom class of each line and control. For microscopic evaluation, 10-27 leaf discs were prepared per symptom class.

4.3.8 Identification of the T-DNA genomic insertion site

Genomic DNA (1 μg extracted from one unheated biological replicate of 40 transformed lines) was subjected to 4 steps of Next Generation Sequencing (NGS) method described by Dalla costa *et al*, (2020). Step 1: Genomic DNA fragmentation was followed by three low-intensity sonication cycles of 30-s with 90-s interval on a Bioruptor® NGS (Diagenode) and purification according to 1.8× AMPure XP Beads protocol (Agencourt). The purified sonicated DNA was subsequently checked on a D1000 ScreenTape (Agilent) to screen the DNA fragmentation between 200 and 1000 bps. The ends of DNA fragments were treated according to the NEBNext® End Repair Module E6050S protocol (New England Biolabs) and the obtained DNA solution was again purified and checked, as mentioned above. Step 2: Adaptors of the Universal GenomeWalker™ 2.0 kit (Takara Bio, Kusatsu, Japan) were ligated to the purified genomic blunt DNA fragments using T4 Ligase (Thermo Scientific, Waltham, MA, USA), according to the manufacturer's instruction. Step 3: PCR was performed using primers (Tab. 1; GW1_F and 35S-P_R) to amplify those fragments containing the junction between the genomic DNA and the LB of T-DNA (Fig. 15). The PCR product was purified with 0.8× AMPure XP Beads and checked on a D1000 ScreenTape to remove and validate the removal of DNA fragments smaller than 200 bps respectively. Step 4: The amplicon library product was sequenced by MiSeq Illumina (PE300) platform at the Sequencing Platform Facility of Fondazione Edmund Mach (San Michele all'Adige, Italy). Bioinformatic analysis was performed by visualizing the sequencing reads on Unipro UGENE Software v1.31.1 (Okonechnikov *et al*., 2012) to identify those containing the 35S-P_R primer sequence next to LB. From the selected reads, all the recognizable vector sequence was removed and the remaining flanking unknown sequence (putative genomic DNA) was

blasted against the apple genome assembly GDDH13 v1.1 (reference genome) (Daccord *et al*., 2017) in order to identify putative T-DNA genomic insertion sites. In addition, the insertion positions were further analyzed to see annotated genes in chromosomes using J browse. Furthermore, Illumina sequences were blasted against plasmid sequence to analyze the putative insertion sequence of LB and FRT site next to LB.

4.3.9 Heat shock induction of the FLP/FRT recombination system

The induction of the FLP/FRT recombination system was based on Dalla Costa *et al*. (2016). From 2 to 6 *in-vitro* transformed plants for each line were incubated at 42 °C for 6-h in a hybridization oven 'hybridizer HB-1000' (UVP, Upland, CA). At the end of the heat-shock inductions, leaves, the vegetative apex (in most of the cases necrotic) and first 1–2 basal internodes of each plant were cut and discarded. The two central nodes of the stem without any leaf were collected and placed horizontally onto a fresh propagation medium to promote the regeneration of new shoots. After 1 month, the first 2-3 leaves of each single regenerated shoots per baby jar (3-9 baby jars for each line) were collected for DNA extraction, Nano-drop quantification and *nptII* copy number by Taqman real time PCR according to the method previously described in chapter 3.

4.3.10 Accuracy of T-DNA insertion sites and FRT-flanked box excision

For the T-DNA insertion site validation, genome-specific forward primers/insertion sites to anneal genomic DNA sequence and reverse primer/35S promoter near to LB are designed to perform PCR followed by DNA extraction of unheated lines. After confirmation of T-DNA insertion sites, the genomic DNA of the *in-vitro* propagated heat-induced clones of lines will be extracted and amplified by PCR with forward primers/chromosomes to anneal genomic DNA sequence near LB and reverse primers/chromosomes to anneal genomic DNA sequence near RB to verify complete removal of exogenous DNA. The corresponding PCR products will be electrophoretically checked, gel purified according to the method previously described and sequenced (FEM Sequencing Platform Facility).

4.4 Results

4.4.1 *In-silico* analysis of *Rvi12_Cd5* native promoter motifs and corresponding TFs

The sequence of promoter was extended from short promoter (SP; 900 bps) to long promoter (LP; 2618 bps) upstream region relative to TSS (ATG start codon) via Sanger sequencing. A total of 1718 bps were added to reach length of LP. PlantCARE (Lescot *et al*., 2002) predicted occurrence of 45 motifs with different positions on upstream region (- strand) and downstream region (+ strand) of promoter sequence relative to TSS. The sequence showed common core promoter elements like

TATA box at -233 bps and analogous CAAT box at -40 bps. Other than these motifs, only defense related motifs were extracted from the data involved in auxin (AUX), abscisic acid (ABA), gibberellin (GA), jasmonic acid (JA), salicylic acid (SA), systemic acquired resistance (SAR), Exogenous methyl jasmonate (MeJA), ethylene (ET) defense related pathway and provide binding sites for defense related transcription factors such as MYB, WRKY and bZIP (Tab. 2). Additional analysis by PlantPAN 3.0 (Chow *et al*., 2019) was performed to predict specific transcription factor binding sites (TFBSs; motifs) which allows binding of defense related transcription factors (TFs). Collection of almost 760 motif matches was obtained and extraction of TFBSs based on 100 % homology to TFs identification (TFID) matrix database among 76 plant species was performed. Overall, six defense related TFBSs, domains and their corresponding TFs; AP2/ERF, bHLH, TGA/bZIP, MYB, NAC, and WRKY were identified on different positions (Fig 3). Due to high number of positions matches of TFBSs on promoter sequence, homologous to other plants, only brief view of localized TFBSs is shown (Fig. 4). Overall, the highest number of TFBSs were found in the *Rvi12_Cd5* promoter sequence for WRKY TF.

Table 2: Prediction of defense related motifs of promoter and their homology to other species, motif specific sequences, positions and their functions by PlantCARE (Lescot et al., 2002). Pathogenesis related (PR), abscisic acid (ABA), gibberellin (GA), jasmonic acid (JA), salicylic acid (SA), systemic acquired resistance (SAR), Exogenous methyl jasmonate (MeJA), auxin (AUX) and ethylene (ET), Gibberellin-dependent alpha amylase (GARC), aspartic acid (Asp), and histidine (His), myeloblastosis oncogene (MYB), Gibberellin (GA)-responsive elements (GARE), MYB binding sites (MBS), ABA-responsive element (ABRE), basic leucine zipper (bZIP).

Site Name	**Specie**	**Motif sequence**	**Position**	**Motif function**
ABRE	*Zea mays*	TACGTG CACGTA	+478 -478	• ABA responsiveness
TCA-element	*Nicotiana Tabacum*	CCATCTTTTT	-1764 -2131	• SA responsiveness
TGA element	*Brassica Oleracae*	AACGAC	-2507	• AUX responsiveness • bZIP binding site • AUX and SA inducible transcription • Induction of SAR by PR-1 • Defense responses to biotrophic pathogens and oxidative cell death • Positively cell death
GARE-Motif TATC-Box	*Brassica Oleracae* *Oryza Sativa*	TCTGGTG TATCCCA	-144 -712 +161 -833	• GA responsivenes

			-1771	
MBS	*Arabiodopsis Thaliana* *Nicotiana Tabacum* *Hordeum Vulgare*	CAACTG CAACAG CAACCA CCGTTG CAACTG TAACTG CAACAG CAACGG	+1021 +144 1045+ 252+ 2544- 162- 712+ 894+ 2422+ 1143- 2482+ 1021+ 1064+ 144+ 1045+ 252+ 162- 712+ -894 +1143 -2422 -2482	• MYB binding site • Integration of AUX, ET signaling pathways • Response regulator involved in His to Asp phosphorylay signal transduction • Photosynthetic capacity optimization by responses to variable environmental and endogenous cues • GARC
CCAAT-Box				
TC-rich repeats	*Nicotiana Tabacum*	CTTTTCTTAAC	-80 -663	• Defense and Stress responsivenes
TGACG-Motif	*Hordeum Vulgare*	TGACG	-2600	• MeJA responsiveness
Wun-Motif	*Brassica Oleracae*	AAATTTCCT	-1791	• WRKY binding site • Wound responsive element • Positively modulates defense related gene expression and disease resistance

				• May regulates early events of leaf senescence

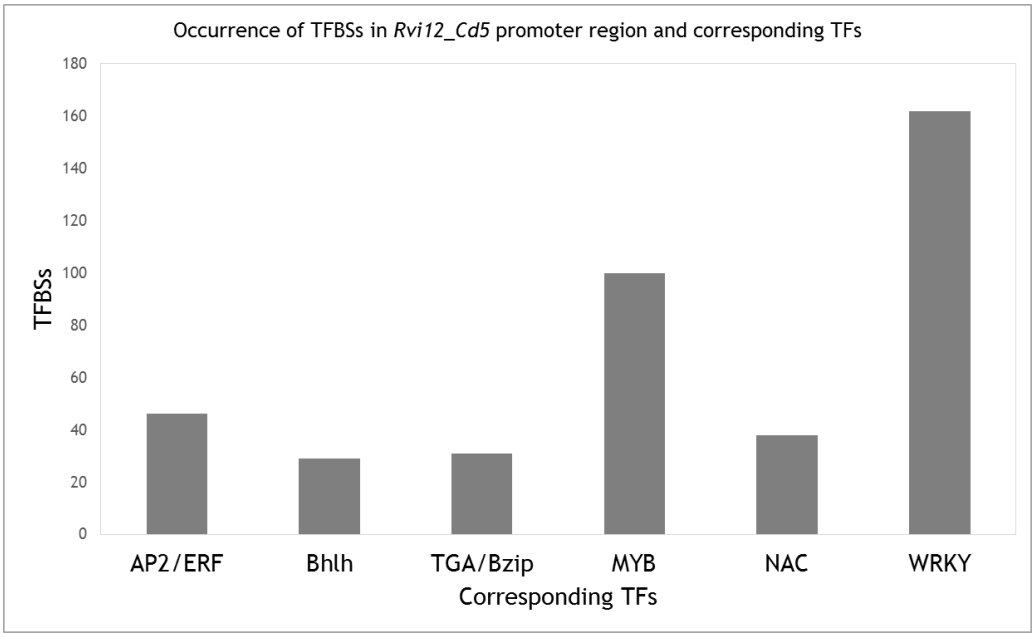


Figure 8: Occurrence of plant defense related TFBSs and co-responding TFs in promoter region of Rvi12_Cd5 based on database collected from PlantPAN 3.0 (Chow et al., 2019).

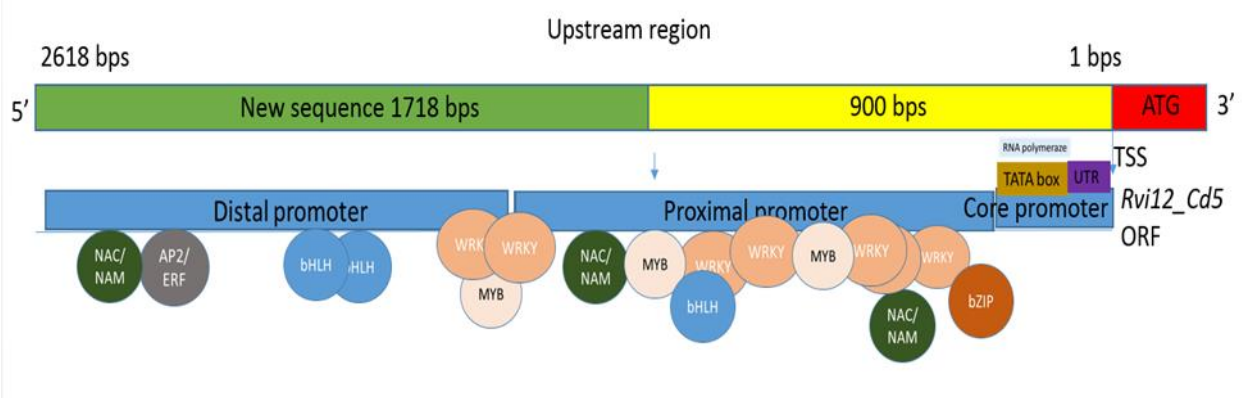


Figure 9: Visualization of average TFBSs and TFs on Rvi12_Cd5 promoter region. Transcription starting site (TSS), base pairs (bps), open reading frame (ORF).

4.4.2 Generation of transformants

Two independent transformation events were performed and a total of 1350 'Gala' leaf explants were infected with *A. tumefaciens* containing the binary vector D308pBNpt-MdPK1-3 (Fig. 1). Sixty regenerants of 'Gala' were collected approximately 7–8 months after culture in selective medium (Fig. 2). All of the regenerants were tested by PCR to screen for the integration of T-DNA (primers listed in Tab. 1). A total of 45 'Gala' lines presents the amplification of *Rvi12_Cd5* gene (Fig. 5) and no *A. tumefaciens* contamination, resulting in transformation efficiency of 3.3% (Tab. 3). All the other 15 'Gala' lines showed no amplification of *Rvi12_Cd5*. Of the 45 transformants generated, 5 lines did not grow well in propagation medium, so only 40 lines were maintained and propagated.

Table 3: Efficiency of Agrobacterium tumefaciens-mediated transformations in 'Gala' apple cultivar.

Cultivar	Transformation	No. of leaf explants infected	No. of regenerants collected	PCR screening		Transformation Efficiency (%)†
				No. of regenerants tested	No. of positive regenerants *(Rvi12_Cd5)*	
Gala	A	450	15	15	15	3.3
Gala	B	900	45	45	30	

†The transformation efficiency was calculated by dividing the number of regenerants positive for *Rvi12_Cd5* by the number of leaf explants infected. (Considering Gala A + Gala B)

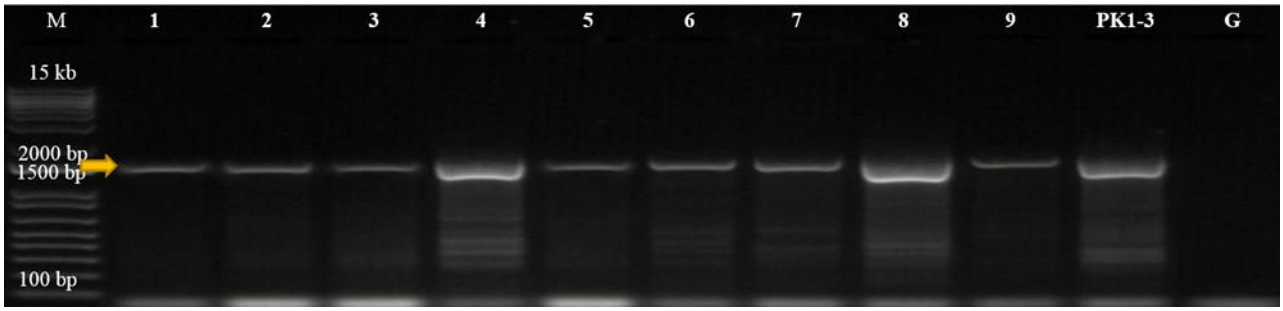


Figure 5: Genomic DNA analysis of transformants, containing Rvi12_Cd5. For investigation of the presence of the Rvi12_Cd5, a polymerase chain reaction was applied using gene specific primers (Tab. 1). The independent transformed Gala lines are represented as lane 1-9 while the lane 9 and 10, indicates purified vector PK_1-3 (Positive control) and Gala (Negative control) respectively. 'Gala' was the genotype used for transformation, while Hansen's baccata #2 (HB2) is the donor of the Rvi12_Cd5 (Vb) gene. M indicates the 1 kb Plus DNA Ladder (Invitrogen-Thermo Fisher Scientific) for band size estimation. The arrow indicates the band corresponding to the Rvi12_Cd5 gene found in the transformants.

4.4.3 *NptII* CN and mRNA expression analysis of transformants

All the 40 transformed lines were characterized for T-DNA integration copy number (CN) by quantifying the *nptII* selection marker gene. *NptII* Copy number ranged from a minimum of 0.1 to a maximum of 5.59 per cell among lines C_1 to C_{40} (Fig. 6).

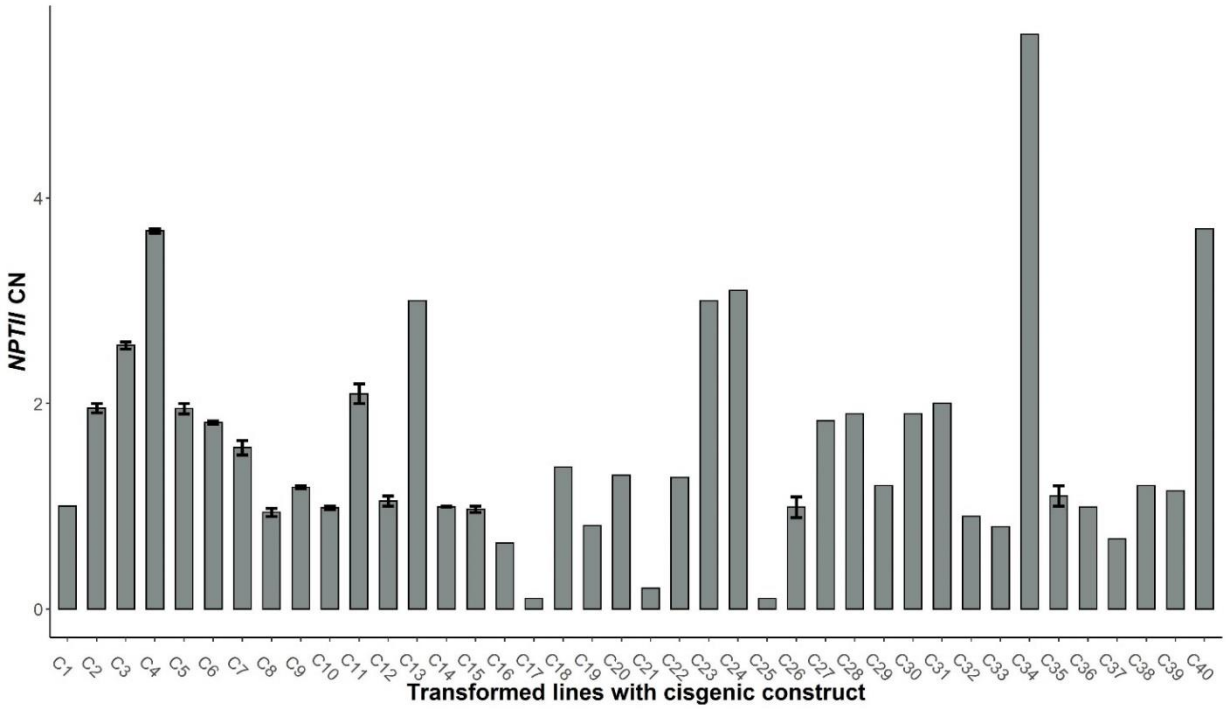


Figure 10: Frequency value scale of quantified nptII CN among transformed 'Gala'. In-vitro 40 transformed independent lines were analyzed by TaqMan real-time PCR to quantify nptII copy number (CN). The CN of plants C_2, C_3, C_4, C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11}, C_{12}, C_{14}, C_{15}, C_{26} and C_{35}: *is the mean ± SD of two biological replicates while the other plants were analyzed in a single biological replicate. Primer sequences are listed in Table 1.*

On the basis of T-DNA integration CN (1-3) and *in-vitro* root production, 15 lines (C_2, C_3, C_4, C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11}, C_{12}, C_{14}, C_{15}, C_{26} and C_{35}) were analyzed for expression analysis of *Rvi12_Cd5* by RT-qPCR (Fig. 7), after 1.5 year of micro-propagation. The transcript levels were determined in *in-vitro* transformed lines and compared with untransformed *in-vitro* 'Gala' plants (Negative control) and greenhouse grown 'HB2' (Positive control). As revealed by RT-qPCR, uninoculated transformed 'Gala' lines displayed lower mRNA levels of *Rvi12_Cd5* than 'HB2' except line C_6 while untransformed 'Gala' showed zero expression level (Fig. 7). There was a wide variation in expression of *Rvi12_Cd5* controlled by native SP. The *Rvi12_Cd5* expression levels were observed in the range of minimum value of 0.02 to maximum value of 1.11, in 15 transformed 'Gala' lines in comparison with resistant control plants (value = 1). Line C_6 showed an expression level slightly higher than 'HB2', C_4 and C_7 showed half expression while other lines showed considerably lower expression (C_2, C_8, C_{14}, C_{15} and C_{26}) than 'HB2'. All 15 lines were acclimatized to greenhouse, thus proceeded for scab test.

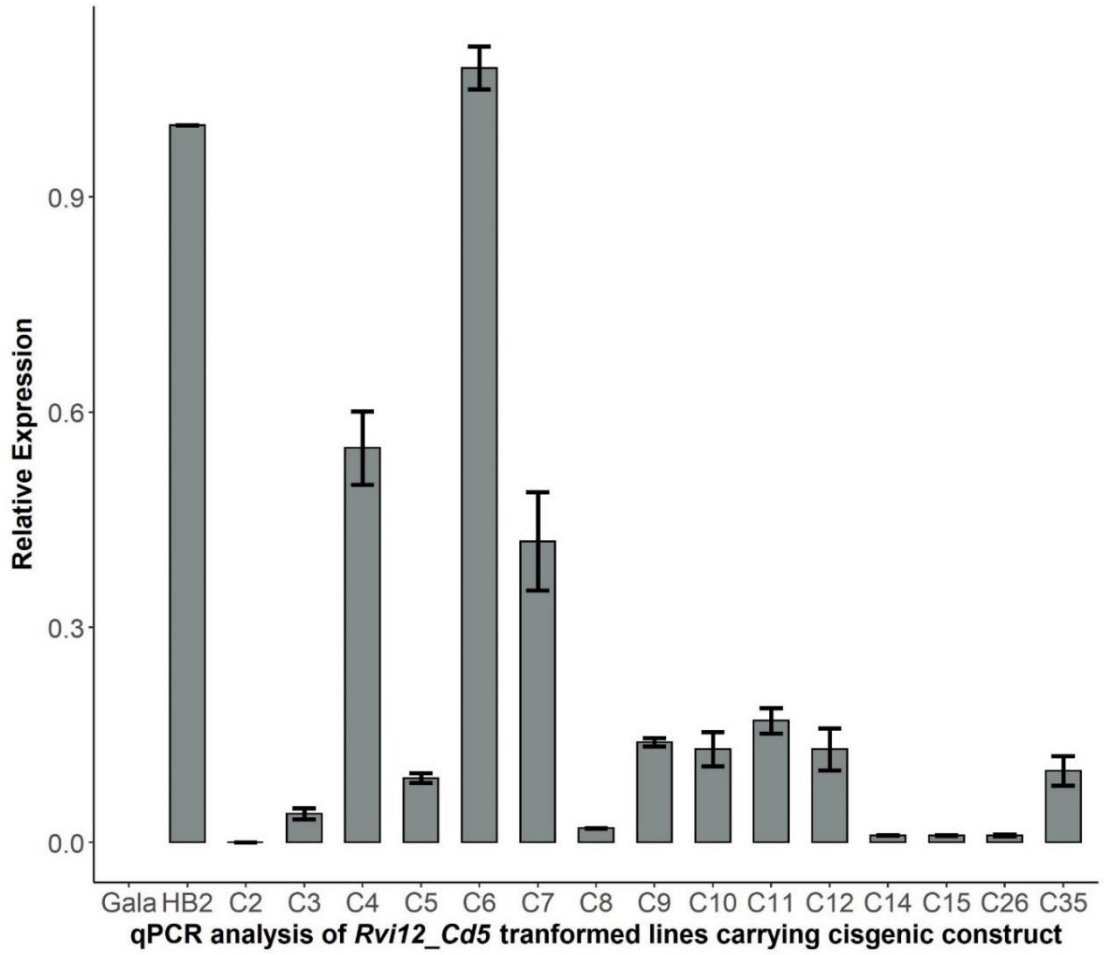


Figure 7: Figure 5: Mean normalized expression quantity of Rvi12_Cd5 in transformed 'Gala' lines compared to controls. In-vitro uninoculated 15 transformed independent lines, untransformed in-vitro 'Gala' (Negative control) and greenhouse 'HB2' (Positive control), were analyzed by RT-qPCR to quantify mRNA/protein levels of Rvi12_Cd5. The quantity of transcripts in transformed plants C_2, C_3, C_4, C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11}, C_{12}, C_{14}, C_{15}, C_{26} and C_{35} is the mean of the normalized expression values of three technical replicates using the Delta-Delta CT method (Livak and Schmittgen, 2001). Primer sequences are listed in Table 1.

4.4.4 Spectrum of resistance to mixed inoculum of *V. inaequalis*

After artificial infection with a mixed inoculum of *V.inaequalis*, plants were analyzed for resistance phenotype development. The upper most affected leaves of 3-6 biological replicates of 15 independent transformed 'Gala' lines along with 7 untransformed 'Gala' plants (control) were macroscopically analyzed. Macroscopic symptoms of 10-18 young leaves were recorded for each individual line at 21 dpi using the scale of Chevalier *et al* (1991) (Fig. 8). All untransformed 'Gala' plants (control) showed high susceptibility to mixed inoculum with distinct abundant sporulation following the scab inoculation. Symptoms started to develop after 7-10 dpi and severity peaked at 21 dpi, with heavy sporulation (expanded blotches) observed on all inoculated young leaves of untransformed 'Gala' (control) and classified to class 4 with complete susceptibility without any resistance reaction. Control showed the highest infection symptoms severity (100.00 %). In contrast, transformed 'Gala' lines expressing *Rvi12_Cd5* showed zero, slight and sparse sporulation with a range of resistance

reactions classified from class 0, 2, 3a, 3b to class 4 on some leaves (Tab. 4). Based on the median values, lines C_6, C_{10} and C_{12} showed response as class 0, lines C_3, C_5, C_7 and C_8 showed class 2 after 10-12 dpi, lines C_4, C_{14}, C_{15} and C_{26} showed class 3a at 14-21 dpi, whereas lines C_2, C_9, C_{11}, and C_{35} showed class 3b at 14-21 dpi. The transformed leaves showing class 4 at 21 dpi, showed a delayed resistance reaction of class 3b around restricted sporulation areas compared to control (dense sporulation) at 22-28 dpi. Overall, symptom class 1 with HR response was not observed in any plant. In two scab infection trials, results of ANOVA ($F_{15,\ 14.026} = 0.000$), followed by Tukey's HSD test (Tab. 4), revealed that the average infection symptoms severity in the transgenic lines inoculated with *V. inaequalis* was significantly lower than 'Gala' (control) except C_{11} and C_{35}. Among transformed lines, line C_6 and C_{10} showed the lowest infection symptoms percentage (average symptom severity rate 8.3 %) and line C_{11} and C_{35} showed highest infection symptoms (average symptom severity rate 76.3 %).

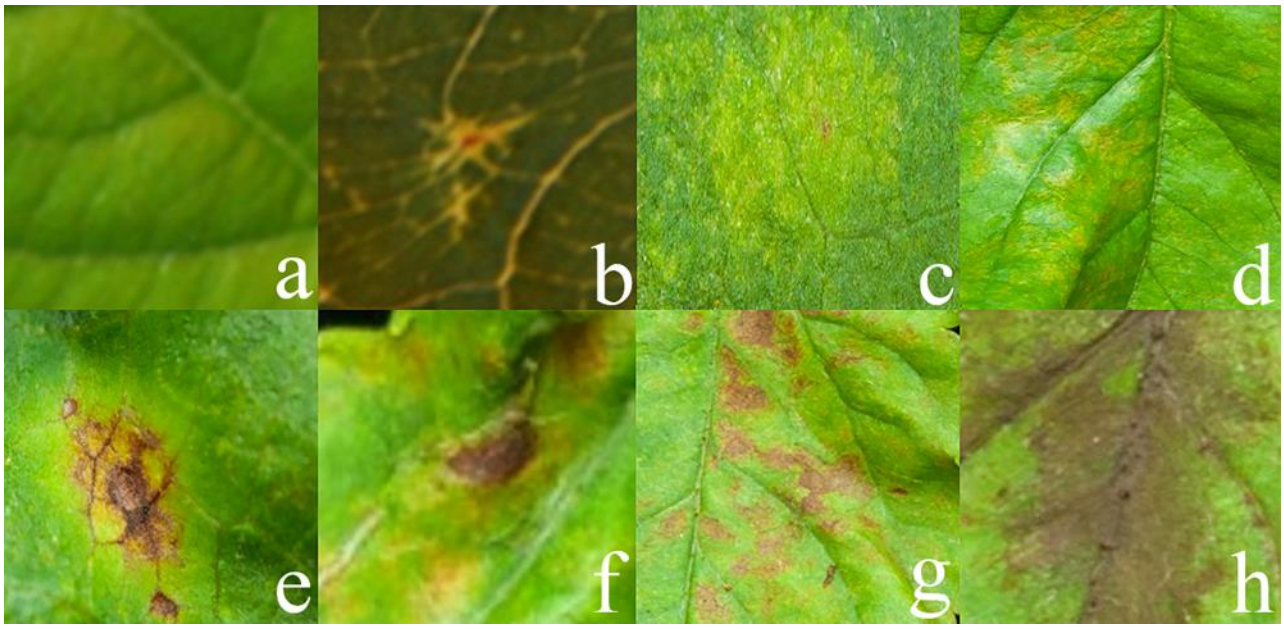


Figure 8: Detailed visualization of symptoms classes of leaves of the transformed apple cultivar 'Gala' and untransformed control after inoculation with V. inaequalis. Class 0 (a), class2 type 1(b), class 2 type 2 (c), class 3a (d, e), class 3b (f, g), and class 4(h).

Line or Cultivar	*Rvi12_Cd5* gene presence	No. of inoculated plants	No. of leaves (3-4 leaves per line)	Symptom[∞] (Test 1+ Test 2)				Severity[£]	
				Confidence interval				Confidence interval	
				Med[*]	L	U	Max	Mean[ł] (±SD)[a]	Groups[b]
C_2	+	6	18	3b	2	3b	3b	51.4±7.7	B, C, D
C_3	+	6	18	2	0	2	3a	16.7±4.04	A, B
C_4	+	5	16	3a	0	3b	3b	39.0±8.52	A, B, C
C_5	+	5	16	2	0	2	3b	25.0±6.5	A, B, C
C_6	+	3	10	0	0	2	2	8.3±4.2	A
C_7	+	3	10	2	0	2	3a	17.5±6.50	A, B
C_8	+	3	10	2	0	3b	3b	27.5±10.83	A, B, C
C_9	+	5	16	3b	3a	3b	4	59.4±7.7	C, D
C_{10}	+	4	12	0	0	0	2	8.3±3.6	A
C_{11}	+	5	16	3b	3b	4	4	75.0±8.23	D, E
C_{12}	+	6	18	0	0	2	3a	34.7±8.4	A, B, C
C_{14}	+	6	18	3a	0	3b	4	45.8±8.4	B, C, D
C_{15}	+	5	16	3a	3a	3b	4	48.7±8.3	B, C, D
C_{26}	+	5	16	3a	2	3b	4	42.2±7.11	A, B, C, D
C_{35}	+	6	18	3b	3a	4	4	76.3±5.14	D, E
Gala	-	7	21	4	4	4	4	100.0±0.0	E

Table 4: Results of two greenhouse inoculation of 'Gala' plants 21days post inoculation with Venturia inaequalis

*Median values of symptoms and their confidence limits were calculated by using the data from the three to four youngest leaves per plant present at the time of each plant's inoculation. Med., median; Max., maximum; L, lower; U, upper.

*Symptoms were assessed by adapting six scab classes after Chevalier *et al*, (1991) with 0 = no macroscopically visible symptom, 1 = hypersensitive pinpoints, 2 = chlorotic areas with or without very small necrosis and no sporulation, 3a = chlorotic and necrotic areas with slight sporulation (weak resistance), 3b = chlorotic and necrotic areas with sparse sporulation (weak susceptibility), and 4 = sporulation covering entire leaf (complete susceptibility). These values represent degrees of resistance, with 4 representing susceptibility. Confidence limit p = 0.01.

[£]Severity values indicates percentage of infection based on symptoms classes with 0 = 0 %, 1 = 5 %, 2 = 25 %, 3a = 50 %, 3b = 75 % and 4 = 100 %. Confidence limit p= 0.05.

[ł]Mean percentage of scab infection severity and their confidence limit were calculated by using the data from three most infected leaves per plant after assigning them symptom classes. SD., ± standard deviation.

[b]Differences between transformed lines were placed in groups based on Duncan HSD at a p value of 0.05.

The microscopic evaluations on inoculated leaves revealed the presence of germinated *V. inaequalis* conidia was confirmed on leaves of both transformed lines and control plants (Fig. 9-14). No fungal growth except germination of conidia, formation of appressoria-like swellings on the leaf surface and penetration pegs was observed in transformed leaves showing class 0 (Fig. 9; A: 1, 2).

Figure 11: Disease symptom and cytology of leaves of the transformed 'Gala' lines C_6, C_{10} & C_{12} after inoculation with V. inaequalis. The leaves showed class 0 symptom (A); microscopic level (1, 2). Light microscopy of leaf tissues stained with aniline blue at 22 dpi; Bar = 20 µm.

Numerous conidia germination, appressorium development and penetration peg were blocked without damaging epidermal cells in the transformed leaves showing class 2 phenotype 1 (Fig. 10; B: 3, 4).

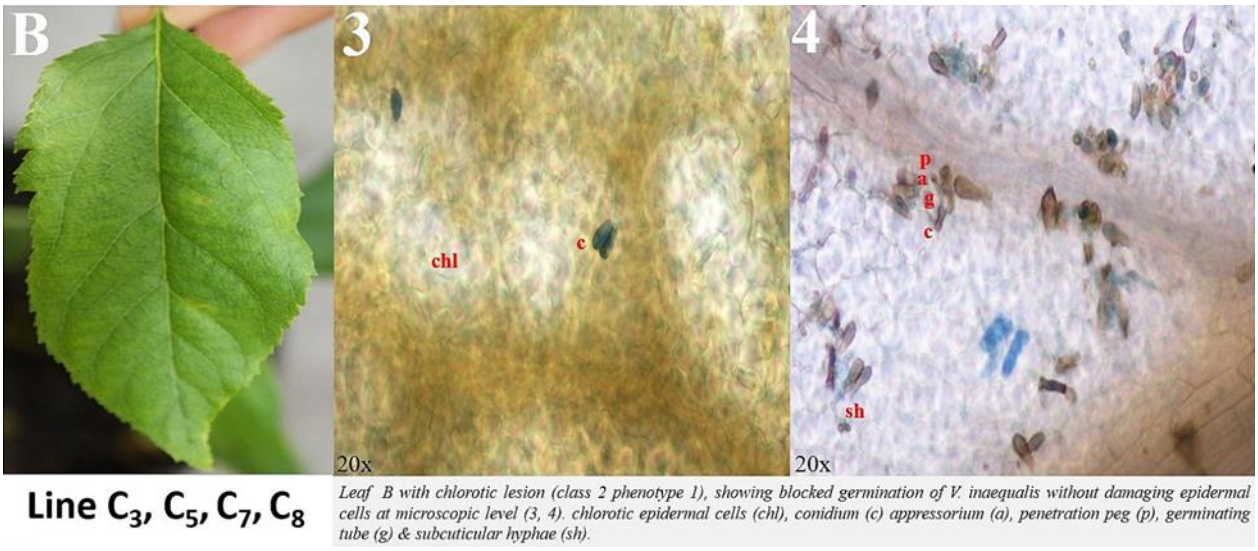


Figure 10: Disease symptom and cytology of leaves of the transformed 'Gala' lines C_3, C_5, C_7 and C_8 after inoculation with V. inaequalis. The leaves showed symptom class 2 phenotype 1(B); microscopic level (3, 4). Light microscopy of leaf tissues stained with aniline blue at 22 dpi; Bar = 20 µm.

The leaves of transformed lines showing class 2 phenotype 2 exhibited browning of epidermal cells (25-30 cells) at penetration point of *V. inaequalis* conidia (Fig. 11, C: 5, 6). In this case, short

and slightly branched subcuticular hyphae was also observed in some cases but further growth was blocked at necrotic spot. The reaction resulted in a complete block of fungal growth and no conidiophore formation and sporulation was observed in leaves showing class 2 (both phenotypes).

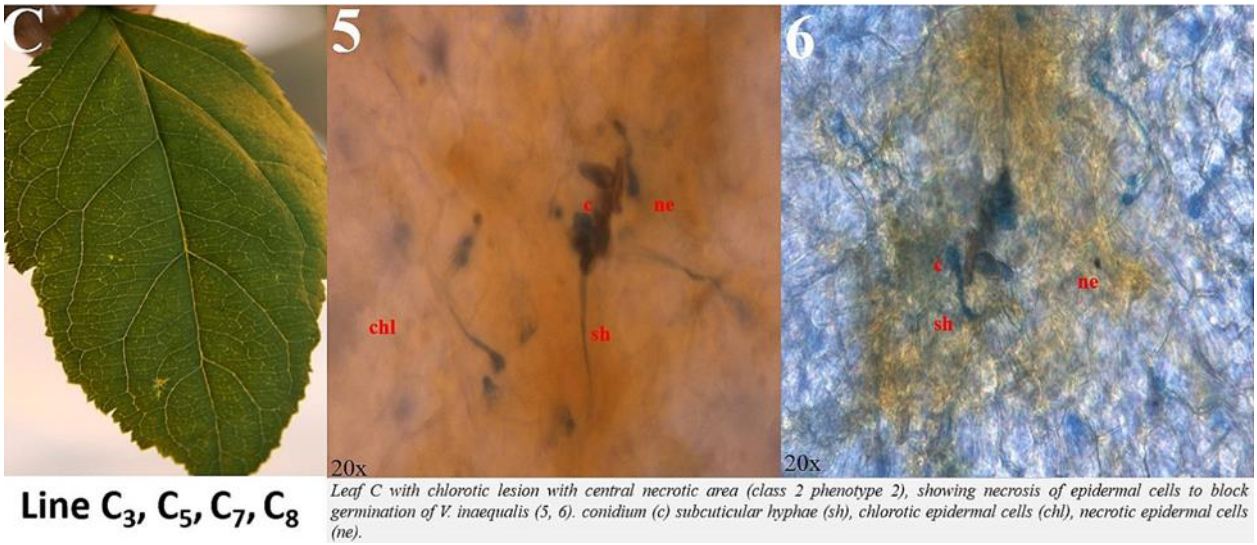


Figure 11: Disease symptom and cytology of leaves of the transformed 'Gala' lines C_3, C_5, C_7 and C_8 after inoculation with V. inaequalis. The leaves showed symptom class 2 phenotype 2 (C); microscopic level (5, 6). Light microscopy of leaf tissues stained with aniline blue at 22 dpi; Bar = 20 µm.

In the transformed leaves showing class 3a, epidermal and presumably mesophyll cells (>40 cells) were affected significantly in localized necrotic area (Fig. 12; D: 7, 8). Subcuticular stroma like structures were seen collapsed in these necrotic areas. Away from these necrotic cells, the healthy part of leaf tissue showed either ceased germination of conidia or primary stroma in initial development state.

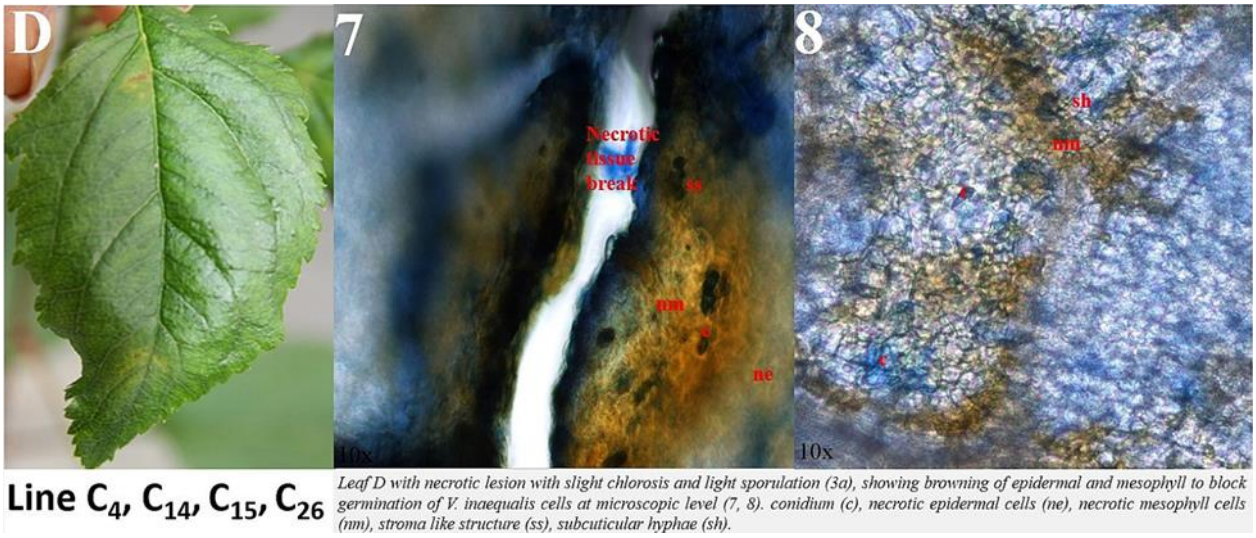


Figure 12: Disease symptom and cytology of leaves of the transformed 'Gala' lines C_4, C_{14}, C_{15}, and C_{26} after inoculation with V. inaequalis. The leaves showed symptom class 3a (D); microscopic level (7, 8). Light microscopy of leaf tissues stained with aniline blue at 22 dpi; Bar = 10 µm.

In case of transformed leaves showing class 3b, a lot of epidermal and mesophyll cells were collapsed in the extensive necrotic area. In this case, developed primary subcuticular stroma structure with the tubular hyphae producing either aborted conidiophores or sporulating conidiophores, was observed (Fig. 13; E: 9, 10) and completely dead cells were found at center of necrotic lesions with abundant sporulation was seen in other part of leaf tissue.

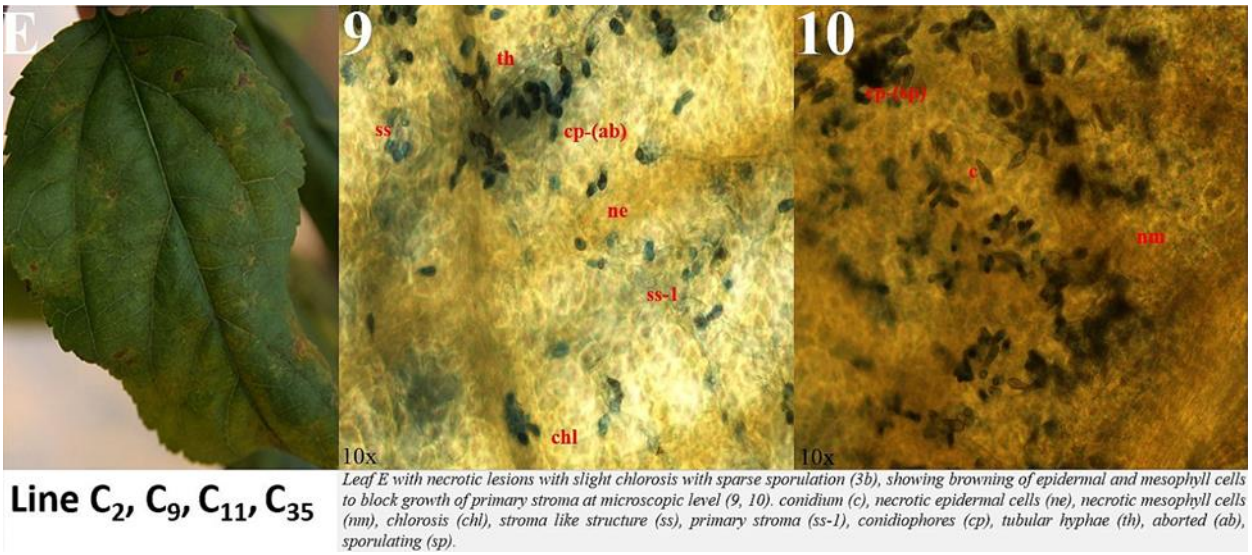


Figure 13: Disease symptom and cytology of leaves of the transformed 'Gala' lines C_2, C_9, C_{11}, and C_{35} after inoculation with V. inaequalis. The leaves showed symptom class 3b (E); microscopic level (9, 10). Light microscopy of leaf tissues stained with aniline blue at 22 dpi; Bar = 10 µm.

In contrast to mycelial growth observed in leaves showing class 3b, dense uniform subcuticular network of mycelial stroma was observed in transformed and untransformed (control) leaves showing class 4. The secondary subcuticular stroma with abundant sporulation of conidiophores was observed along annelids like structure (Fig. 14; F: 11, 12).

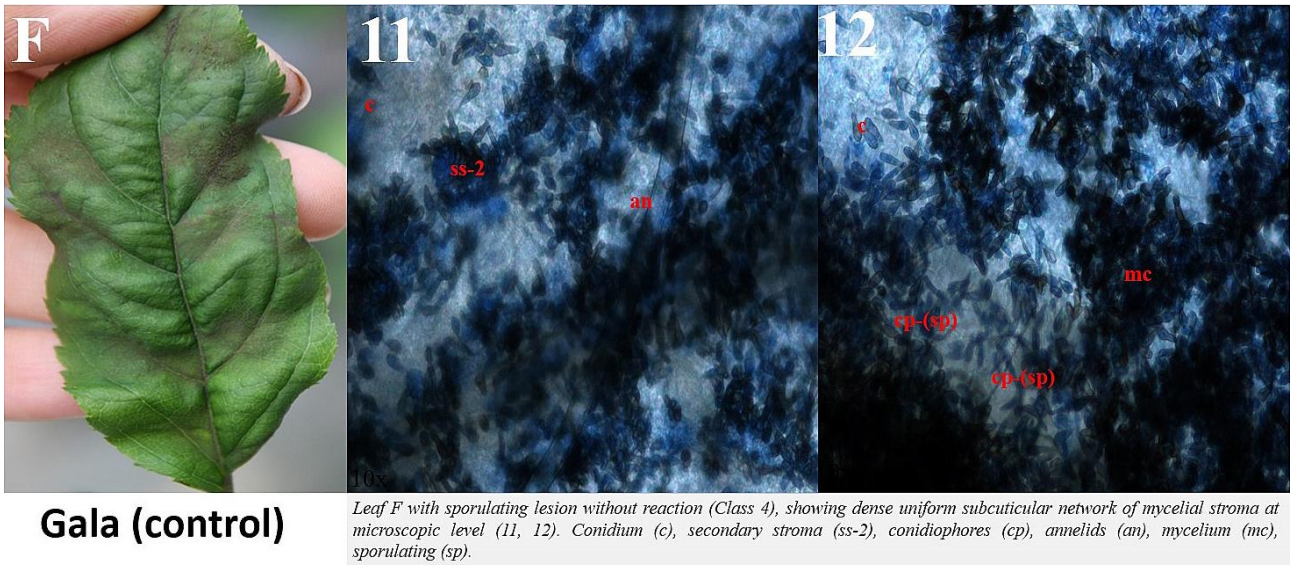


Figure 14: As control, inoculated leaf of untransformed 'Gala' (F) showed symptom class 4; microscopic level (11, 12).. Light microscopy of leaf tissues stained with aniline blue at 16 dpi; Bar = 10 µm.

.

4.4.5 T-DNA integration sites in apple genome

All transformed lines (C_1 to C_{40}) were used to identify the T-DNA insertion sites (Tab. 5) by employing NGS method as described in experimental procedures. The Illumina sequence results (putative genomic DNA sequences of lines), were blasted against the apple genome GDDH13 v1.1 (Daccord *et al*., 2017) and integrations were found in 24 lines (90 to 100 % homology match to reference genome) on 15 different chromosomes (Tab. 5). Some independent lines showed insertion on same chromosome and sometimes even in the same position. Using Jbrowse software (Daccord *et al*., 2017), gene annotation was checked at chromosome positions where T-DNA integrations were found. The data revealed that T-DNA integration points fell in intergenic regions in all lines except two (C_{21} and C_{23}). The Illumina sequences were also blasted against plasmid sequence (Fig. 2) in order to check number of nucleotides of integrated LB and FRT site next to LB (Fig. 15). FRT sites were trimmed in 11 lines while LB was trimmed completely or partially in 24 lines.

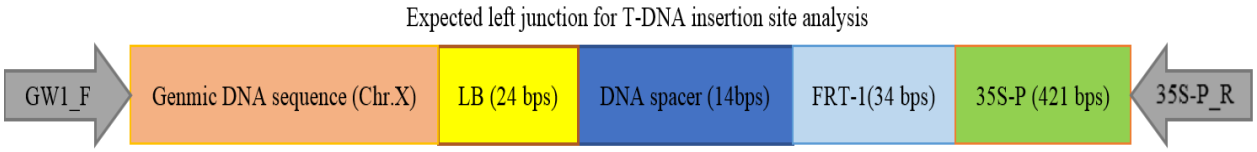


Figure 15: Identification of the T-DNA insertion site in transformed apple lines (C_1-C_{40}). Visualization of the T-DNA insertion sites identified by illumina sequencing on chromosomes by the amplification expected with the combinations of primers GW1_F + 35S-P_R. GenomeWalker adapter 1(GW1); left border (LB); flippase recognition target site one (FRT-1); Cauliflower Mosaic Virus 35S promoter (35S-P); chromosome (Chr).

Table 5: For each plant line, T-DNA insertion point on chromosome was identified using the NGS method. Chromosome positions, gene annotation at insertion site and trimming of total or partial elements (LB, FRT) are shown in table. Chromosome (Chr.); base pairs (bps); not applicable (N/A).

Lines	Chromosome (percentage % of identical matchs)	Chr. position	gene function	T-DNA missing sequence (205 bps)	LB (24 bps)	FRT/FLP site next to LB (34 bps)
C_1	Unknown	N/A	N/A	N/A	N/A	N/A
C_2	Chr.08 (99) Chr.08 (90)	5196917-5197145 5239005-5239048	Intergenic region Intergenic region	-113	-24	-34
C_3	Chr.08 (99) Chr.17 (99)	5196917-5197194 6696106-6696240	Intergenic region Intergenic region	-113	-24	-34
C_4	Unknown	N/A	N/A	N/A	N/A	N/A
C_5	Chr.13 (92) Chr.17 (92)	2068566-2068145 33255052-33254632	Intergenic region	-113	-24	-34
C_6	Unknown	N/A	N/A	N/A	N/A	N/A
C_7	Chr.04 (100) Chr.04 (95)	3799003-3798748 3802575-3802329	Intergenic region UDP-Glycosyltransferase superfamily protein	-3	-3	Inserted
C_8	Chr.04 (99)	3799003-3798710	Intergenic region	-3	-3	Inserted
C_9	Unknown	N/A	N/A	N/A	N/A	N/A
C_{10}	Chr.16 (99)	2012911-2012667	Intergenic region	-2	-2	Inserted
C_{11}	Chr.16 (99)	2012911-2012635	Intergenic region	-2	-2	Inserted
C_{12}	Chr.16 (99)	2012911-2012631	Intergenic region	-2	-2	Inserted
C_{13}	Unknown	N/A	N/A	N/A	N/A	N/A
C_{14}	Unknown	N/A	N/A	N/A	N/A	N/A
C_{15}	Unknown	N/A	N/A	N/A	N/A	N/A
C^{16}	Unknown	N/A	N/A	N/A	N/A	N/A
C_{17}	Unknown	N/A	N/A	N/A	N/A	N/A
C_{18}	Chr.16 (98) Chr.08 (97) Chr.06 (97)	24689064-24688937 12056972-12056845 19668632-19668632	Intergenic region Intergenic region Intergenic region	-2	-2	Inserted
C_{19}	Chr.13 (93) Chr.03 (93)	35556494-35556391 14735471-14735573	Intergenic region Intergenic region	Inserted	Inserted	Inserted
C_{20}	Chr.16 (99)	2012911-2012696	Intergenic region	-2	-2	Inserted
C_{21}	Chr.08 (100)	1324562-1324442	LSD1-like 3	Inserted	Inserted	Inserted
C_{22}	Chr.16 (100)	2012911-2012620	Intergenic region	-2	-2	Inserted
C_{23}	Chr.04 (99)	23993010-23993358	RING/U-box superfamily protein	-46	-24	-7
C_{24}	Unknown	N/A	N/A	N/A	N/A	N/A
C_{25}	Unknown	N/A	N/A	N/A	N/A	N/A
C_{26}	Unknown	N/A	N/A	N/A	N/A	N/A
C_{27}	Chr.05 (94)	44331718-44331977	Intergenic region	-3	-3	Inserted
C_{28}	Chr.15 (99)	17516131-17515904	Intergenic region	-56	-24	-18
C_{29}	Chr.08 (99)	5196917-5197329	Intergenic region	-113	-24	-34
C_{30}	Chr.17 (95) Chr.13 (94)	15236709-15236669 6382824-6382857	Intergenic region Intergenic region	-113	-24	-34
C_{31}	Chr.13 (92)	2068566-2068158	Intergenic region	-113	-24	-34
C_{32}	Unknown	N/A	N/A	N/A	N/A	N/A
C_{33}	Chr.08 (99)	5196917-5197325	Intergenic region	-113	-24	-34
C_{34}	Unknown	N/A	N/A	N/A	N/A	N/A
C_{35}	Chr.16 (99)	2012911-2012637	Intergenic region	-2	-2	Inserted
C_{36}	Chr.16 (99)	2012911-2012618	Intergenic region	-2	-2	Inserted
C_{37}	Chr.05 (96) Chr.12 (94) Chr.00 (93) Chr.02 (93) Chr.14 (93)	33382698-33382670 15646222-15646189 29012090-29011808 18044156-18044126 3347332-3347300	Intergenic region Intergenic region Intergenic region Intergenic region Intergenic region	-101	-24	-34
C_{38}	Chr.16 (100)	2012911-2012627	Intergenic region	-6	-6	Inserted
C_{39}	Chr.16 (99)	2012911-2012620	Intergenic region	-2	-2	Inserted
C_{40}	Chr.11 (100)	9769021-9769191	Intergenic region	-42	-24	-4

4.4.6 Exogenous DNA excision in transformed lines

The transformed lines C_2, C_5, C_8, C_{10}, C_{14}, C_{15}, C_{26} and C_{35} (CN = 1 or 2) were subjected to heat-shock treatment for activation of the FLP/FRT recombination system (Fig. 16) for the removal of exogenous DNA flanked by the two FRT sites (Fig. 2). To check the level of T-DNA elimination, the marker gene *nptII*, was quantified by Taqman real-time PCR (Tab. 1) in shoots (9-27 per line) regenerated from central nodes of heat-induced plantlets. Among 114 plants (heated sub-clones of 8 independent transformed lines), 15 showed 100 % of T-DNA removal, 10 exhibited between 90% and 70% (0.1 - 0.3 CN), while the rest showed removal between 60% and 10% or even less than 10 % (Tab. 6). The plants showing *nptII* CN equal or near to zero were propagated for 4 months.

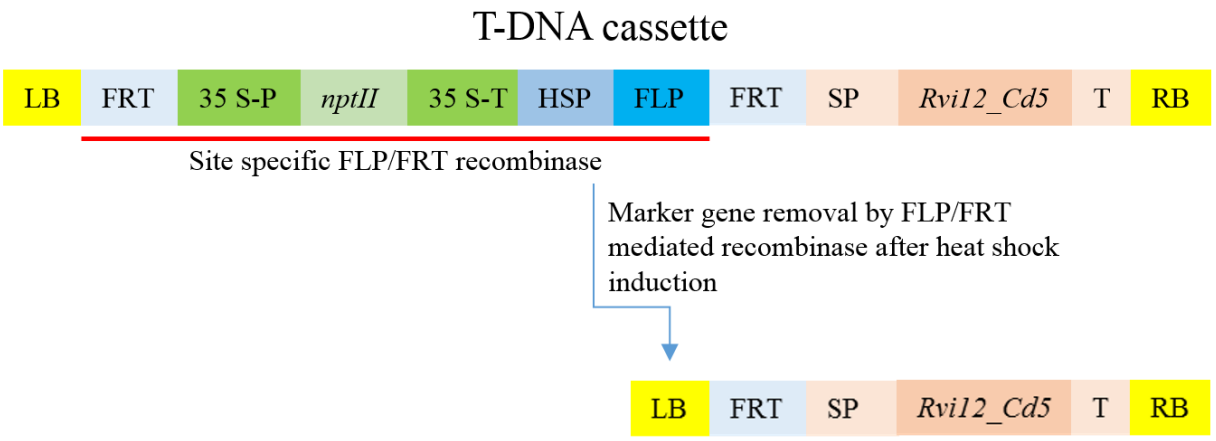


Figure 16: Illustration of expected T-DNA cassette after heat shock inducible site specific FLP/FRT mediated removal of marker gene. Left border (LB), Right border (RB), Cauliflower Mosaic Virus 35S promoter and terminator (35S-P, 35S-T), Heat shock promoter (HSP), Flippase recognition sites (FRT), Flippase (FLP), native short promoter (SP), native terminator (T), Neomycin phosphotransferase II marker gene (nptII).

Table 6: Assessing T-DNA excision in transformed apple lines carrying heat shock inducible FRT/FLP recombinase system in binary vector illustrated in Fig. 1, for removal of marker gene (nptII).

Lines ID	CN before heat heat treatment	FRT site integration Next to LB	No. of propagated plants per line used for heat treatment (8h at 42°C)	No. of propagated plants per line showed CN = 0 after heat treatment	Efficiency % [£]
C_2	2.0	Broken	9	-	0
C_5	1.9	Broken	9	-	0
C_8	0.9	Inserted	21	2	9.0
C_{10}	1.0	Inserted	12	2	16
C_{14}	0.99	N/I	27	8	29
C_{15}	1.0	N/I	9	1	11
C_{26}	0.89	N/I	15	-	0
C_{35}	1.0	Inserted	12	2	16

[£]Number of plants having CN = 0 divided by total number of plants used for heat treatment. Not identified (N/I); Copy number (CN of *nptII*).

4.4.7 On-going analysis: Validation of the T-DNA insertion sites and T-DNA excision

The line C_6 will be checked for T.DNA insertion site analysis. Then, the unheated lines C_6, C_8, C_{10} and C_{12} showing perfect sequence identities (99-100%) on chromosomes will be chosen for PCR based screening by using specific forward primers annealing on the upstream genomic region of target chromosome and with a reverse primer annealing on the promoter 35S used for expression of marker gene (*nptII*), to ensure the accurate physical map of inserted gene on apple genome. Highly resistant lines C_6 and C_{12} will be treated with heat shock treatment for T-DNA removal. Thus, heat induced clones of selective lines C_6, C_8, C_{10} and C_{12} showing complete removal of exogenous T-DNA cassette by real-time PCR will be used for PCR by using forward primers/chromosomes to anneal genomic DNA sequence near LB and reverse primers/chromosomes to anneal genomic DNA sequence near RB. The PCR products will be gel electrophoresed and purified for sequencing.

4.5 Discussion

Regulation of gene expression at the promoter level is mainly controlled by the motifs localized in core promoter upstream of the TSS (Hernandez-Garcia and Finer, 2014), however, upstream of core promoter, proximal and distal regions can contribute to the fine regulation of gene expression

(Lee and Young, 2000). The *Rvi12_Cd5* core promoter showed TATA box at -233 bps and analogous CAAT box at -40 bps for transcription initiation (Breathnach and Chambon, 1981; Smale and Baltimore, 1989; Smith, 2000; Tripathi, 2010), which theoretically indicates that SP 900 bps used in the study should be able to provide quantitative *Rvi12_Cd5* expression whereas, LP 2618 bps may provide quantitative as well as qualitative expression of gene. The size of the active, fully functional promoter for regulation of transcription is directly dependent on the availability and activity of TFs, and the type, number, position, and combination of motifs present in and around the promoter (core, proximal and distal regions) (Lee and Young, 2000; Zou *et al*., 2011). In this regard, *in-silico* analysis predicted many *Rvi12_Cd5* promoter motifs responsive to plant hormones, such as salicylic acid (SA), ethylene (ET), and jasmonic acid (JA) (Tab. 2), which play a central role in the regulation of plant immune responses against pathogen attack (Denancé *et al*., 2013). In addition, six major plant defense related TFs like WRKY, AP2/ERF, bHLH, bZIP, MYB, and NAC (Andersen *et al*., 2018), binding motifs were found in *Rvi12_Cd5* promoter sequence. In the 'HB2' genome, the most abundant TF-encoding genes belonged to the following TF families: bHLH (208 genes), MYB (180 genes), NAC (175 genes), ERF (157 genes), and C2H2 (147 genes) (Chen *et al*., 2019) which resonates with to TFs findings in *Rvi12_Cd5* promoter derived from 'HB2'. The majority of defense related TFs were positioned on the core and proximal region of promoter compared to distal region (Fig. 4). These predicted motif sequences may or may not be functional and practical demonstration of the contribution of the motif to promoter activity is essential. Overall, SP 900 bps seemed to be appropriate choice for *Rvi12_Cd5* expression in this study.

The *A. tumefaciens*-mediated gene transfer has emerged as the most widely used method in plant genetic engineering. Following the *A. tumefaciens*-mediated transformation of vector constructed for production of transformed lines (Fig. 2), *Rvi12_Cd5* controlled by native SP was successfully transferred to 45 'Gala' plants (Tab. 3), with the T-DNA cassette integrated into their genome (Tab. 5), indicating that the method used is efficient. In past, the functional analysis, expression profiling of transformed *Rvi6* (*HcrVf2*) controlled by its own promoter in 'Gala' (Joshi *et al*., 2011), led to development of cisgenic lines carrying *Rvi6* (*HcrVf2*) by *A. tumefaciens*-mediated transformation (Krens *et al*., 2015; Vanblaere *et al*., 2011; Würdig *et al*., 2015). Compared to our transformation efficiency rate of 3.3 %, Vanblaere *et al*, Krens *et al*, and Würdig *et al*, achieved transformation efficiency rate of 0.6 % in 'Gala', 0.5% in 'Gala' and 0.4% in 'Brookfield Baigent' ('Royal Gala' mutant) respectively. In other studies on fire blight, cisgenic 'Gala' lines were obtained by *A. tumefaciens*-mediated transformation, with efficiency rate of 13.8% and 0.7% (Kost *et al*, (2015) and 1.5% (Pompili *et al*., 2020). The possible explanation of different efficiency results by *A. tumefaciens* transformation method achieved in past and in present study could be due to number of factors such as

concentration of *A. tumefaciens*, co-cultivation conditions, pH, attachment of bacteria to wounded plants, induction of virulence gene, and composition of growth media (Chow *et al*., 2016; Kong *et al*., 2000; Li *et al*., 2017).

Fifteen uninoculated *in-vitro* transformed lines were analyzed for mRNA expression level. All lines showed low level compared to 'HB2'except one line C_6 (0.9 fold higher) and mRNA level was comparable between lines. The reason for low transcript expression could be the use of RNA material extracted from *in-vitro* transformed leaves compared to outdoor grown leaves of 'HB2'. Previously, the constitutive expression of *Rvi12_Cd5* was also checked in outdoor grown leaves of 'HB2' (Padmarasu *et al*., 2018). So, there might be environmental factor involved in low mRNA expression. In accordance, Malnoy *et al*, (2008) also observed low mRNA expression in *Rvi6* (*HcrVf2*) transformed *in-vitro* lines before inoculation except one line and observed significant higher mRNA level after greenhouse inoculation. In contrast, Szankowski *et al*, (2009) observed no change in mRNA expression level *in-vitro* and greenhouse plants. There are several studies conducted on lengths of *Rvi6* (*HcrVf2*) native promoter and their functional abilities, such as, Malnoy *et al*, (2008) and Vanblaere *et al.* (2011) observed low *Rvi6* mRNA expression level by using 2 kb length and 242 bps respectively, whereas Szankowski *et al*, (2009) observed high *Rvi6* mRNA expression level by using 288 bps and 799 bps. In contrast, Joshi *et al*, (2011), observed *Rvi6* mRNA expression level was not affected by 288 bps or 2kb. Similar to Joshi *et al*, (2011), Würdig *et al*, (2015) observed the same pattern of *Rvi6* expression levels by using 1315 bps and 536 bps. Considering all these contradictory mRNA expression results by using different length of *Rvi6* native promoter, it is not clear that promoter length might a big factor involved in gene transcription, yet, right motifs combinations and their positions located on promoter length for effective transcription seems more important (Lee and Young, 2000; Zou *et al*., 2011). We also compared mRNA expression level and T-DNA copy number (CN) of transformed lines but no correlation between them was found, similar to observations of Joshi *et al*, (2011) and Vanblaere *et al*, (2014) in their studies.

Phenotypic investigation of fifteen transformed plants after two independent *V. inaequalis* infection trials, revealed consistent range of significant visible resistance reactions (class 0, 2, 3a, 3b) (Chevalier *et al*., 1991) compared to control, suggesting that *Rvi12_Cd5* controlled by its own promoter is capable of conferring resistance to *V. inaequalis* when inserted in susceptible 'Gala' plants. Based on median values (Tab. 4), seven transformed lines (C_3, C_5, C_6, C_7, C_8, C_{10} and C_{12}) showed high apple scab resistance response from class 0 to 2 and eight transformed lines (C_2, C_4, C_9, C_{11}, C_{14}, C_{15}, C_{26}, and C_{35}) showed partial resistance from class 3a to 3b, whereas, untransformed 'Gala' pro-

duced heavy sporulation, assigned as class 4. The induction of apple scab resistance in these transformed lines was observed with low mRNA expression level compared to 'HB2' except C_6. Similarly, Vanblaere *et al*., (2014) also observed apple scab resistance in cisgenic *Rvi6* plants with quite low level of mRNA expression. During phenotypic evaluation, assigning of reaction phenotypes into intermediate classes from 3a to 3b was difficult in some leaves macroscopically. In addition, class 2 was also difficult to decide when resistance reactions were either chlorotic only (phenotype 1) or chlorotic lesion with necrotic center (phenotype 2) without sporulation at all. That's why, class 2 was assigned to two types of phenotypes (Fig. 8). Also six transformed lines (C_9, C_{11}, C_{14}, C_{15}, C_{26} and C_{35}) showed restricted sporulation on few leaves (class 4) without any resistance reaction at 21 dpi. Surprisingly, the majority of these leaves showed delayed resistance response at 22-28 dpi. Delayed response appeared as necrotic lesions with slight chlorosis to stop further sporulation (looked like 3b reaction) compared to control which showed fused sporulation covering almost entire leaf. The delayed necrotic reaction created well defined boundary between healthy and infected area of transformed leaves. Similar to our observation, a systematic phenotypic analysis of apple root resistance to a fungal pathogen *Pythium ultimum* infection (a member in the ARD (Apple replant disease) pathogen complex) was performed and delayed resistance until 28 dpi was observed (Zhu and Saltzgiver, 2020). Although *Rvi12_Cd5* is expressed constitutively (Padmarasu *et al*., 2018), it might be possible that a delayed induction of *Rvi12_Cd5* expression happened due to inducible gene regulation nature of native promoter and presence of high number of WRKY binding motifs in promoter sequence (Fig. 3). WRKY binding motifs have been found in inducible promoters, such as for example, the barley Germin-Like GER promoter, that is highly inducible after infection by biotrophic and necrotrophic pathogens (Himmelbach *et al*., 2010). In addition, *Rvi12_Cd5* showed homology to receptor like kinases in *Xa21,* rice blast resistance gene (Padmarasu *et al*., 2018), which also contain WRKY biding sites for the positive regulation of blast resistance (Inoue *et al*., 2013). Anyhow, molecular analysis needs to be performed to understand defense signaling network of *Rvi12_Cd5* advanced and delayed activation.

After phenotypic analysis, assigned resistance classes (0-3b) and susceptibility symptom (class 4) were verified at microscopic level (Fig. 9-14). Overall, microscopic evaluation was well synchronized with macroscopic assigned resistance and susceptible phenotypes. In case of clean transformed leaves, presence of blocked conidial germination, indicated that assigned class 0 was not due to escape of infection but to an active reaction of the plant. In a particular case of microscopic analysis of transformed leaves assigned to class 2 phenotype 2 showed homology to hypersensitive response (class 1; HR) of *Rvi15* (*Vr2*) leaves as described by Galli *et al,* (2010a) due to similar number of browned cells (20-40) around conidia. However, the class 2 phenotype 2 showed macroscopic

homology to stellate chlorosis (SC) due to wrinkle effect and irregular chlorotic pattern (Fig 11; C). Previously, Padmarasu *et al* (2014) observed resistance phenotypes in mapping population of 'HB2 x Gala' for *Rvi12* locus and predicted that *Rvi12* resistance was responsible for 0-3b symptoms and an additional resistance locus was responsible for SC symptom, thus, scab resistance in 'HB2' was not considered monogenic and could be controlled by at least two unlinked genes. Surprisingly, 0-3b including SC like (class 2 phenotype 2) resistance reactions were induced by *Rvi12_Cd5*, suggesting that 'HB2' scab resistance might be monogenic. In contrast to Padmarasu *et al* (2014), Hemmat *et al* (2003) and Erdin *et al* (2006) did not mention presence of two resistance genes in 'HB2' in mapping studies of *Rvi12* (*Vb*). In the mapping studies of *Rvi12* (*Vb*), Hemmat *et al* (2003) observed phenotype like SC (chlorotic wrinkled with or without some sporulation), which is similar to SC phenotype observed by Padmarasu *et al* (2014), but Erdin *et al* (2006) did not mention SC phenotype except class 0-3b, which indicates that the variation in resistance reactions was most likely due to the use of mixed and different inoculum of *V. inaequalis*. In this study, broad resistance spectrum (0-3b and SC) was observed by *Rvi12_Cd5* against mixed inoculum of *V. inaequalis*, however, mono conidial (race/strain specific) inoculums are still required to understand which isolate of *V. inaequalis* triggered specific resistance response by *Rvi12_Cd5*, thus, can lead to better scab screening. The information from VINQUEST project data, has pointed out the most promising apple scab resistance genes such as *Rvi5*, *Rvi11*, *Rvi12*, *Rvi14*, and *Rvi15* for development of cultivars with durable resistance (Patocchi *et al*. 2009, 2021). If we consider *Rvi12_Cd5* alone as active *Rvi12*, it might add value for the durable scab resistance. Alternatively, if we agree with the findings of Padmarasu *et al* (2014) that two resistance loci are expected for 'HB2' resistance, then, only one of the two possible resistance genes has been transformed in present study. In that case, the consequences of not using the other gene responsible for only SC phenotype might not be valuable for durable resistance breeding because according to Padmarasu *et al* (2014), it is possible that this gene has already been overcome in the Bologna (Italy) during mapping study of Hemmat *et al*, (2003). Also in this case, *Rvi12_Cd5* as active *Rvi12* might add value alone to durable scab resistance without the second proposed gene of 'HB2'.

Regarding evaluation of T-DNA integration sites on chromosome, 40 transformed lines were analyzed by NGS method described in experimental procedure. By comparing Illumina sequence results (genomic DNA sequences of transformed lines) with the reference apple genome GDDH13 v1.1 (Daccord *et al*., 2017), 24 lines showed 90 to 100 % homology to 15 different chromosomes (Tab. 5). Some lines showed T-DNA integrations on different chromosomes, some showed integration on same chromosomes with different positions similar to T-DNA integration data found by Pompili *et al*, 2020. While some lines also showed integration on same chromosomes with almost same positions which happened probably due to experimental mistakes (e.g. lines belonging to the same

callus) or to the so called 'unaided natural homologous recombination', which was reviewed recently (Dong and Ronald, 2021), and explains that homology between T-DNA sequence and host plant DNA can induce natural targeted integration of T-DNA at the homologous part of host genome at low frequency (Bundock and Hooykaas, 1996). The homologous recombination concept has been proved in tobacco plants (Paszkowski *et al*., 1988; Risseeuw *et al*., 1995) and *Arabidopsis* plants (Halfter *et al*., 1992). In addition, micro-homologous DNA sequences between T-DNA borders (LB, RB) and host plant DNA can induce natural targeted insertions (Gheysen *et al*., 1991; Matsumoto *et al*., 1990). *In-silico* gene annotation analysis of chromosomes positions, used for T-DNA integrations, showed endogenous DNA was disrupted at integration sites except in case of two lines. Even so, validation of actual physical map of T-DNA integrations in transformed lines by PCR and sequencing is still required to make final conclusion.

We used transformation binary vector (Fig. 1) carrying heat inducible site specific recombination FLP/FRT system (Herzog *et al*., 2012; Würdig *et al*., 2013) to remove exogenous DNA (*nptII* marker gene) for the development of cisgenic lines. Eight transformed lines were treated with heat shock for the exogenous DNA removal. These eight lines were selected for marker gene removal before scab resistance evaluations due to time constraints, that's why we couldn't include all highly resistant lines except C_8 and C_{10}. However, other highly resistant lines such as C_6 and C_{12} are also chosen now in on-going analysis for marker gene removal. The *nptII* (the selection marker gene inserted in T-DNA cassette) copy number was analyzed by real-time PCR. The results showed 100% removal of *nptII* in heat treated sub-clones of 5 independent lines (Tab. 6, Fig. 16). The 0.0 CN of *nptII* directly correlates with the removal of the *nptII* marker gene and indicates that the site specific FLP/FRT recombination system was successful to remove exogenous DNA. However, another validation of *nptII* marker gene removal is still needed by PCR and sequencing (Dalla Costa *et al*., 2016). Theoretically, heat inducible site specific recombination FLP/FRT system used in this study can leave 34 to 96 bps of exogenous DNA (Fig. 1; RB, FRT, LB) after removal of marker gene. Trimming of left border, right border and FRT site (LB, RB and FRT) has been reported by Kost *et al*, (2015) and Würdig *et al*, (2015). These trimming results indicates that occasional border region truncations occur when the T-DNA is translocated during the *A. tumefaciens* mediated transformation into the plant genome (Bundock and Hooykaas, 1996). In the present study, Illumina sequence (Fig. 15; genomic sequence of transformed lines used for T-DNA integration sites analysis) was blasted against vector plasmid (Fig. 1) to see preliminary results of trimming of left T-DNA border and one FRT site next to left border. Trimming of FRT site next to LB was observed in 11 lines and trimming of LB was observed in 26 lines (Tab. 5). Other than exogenous DNA integrated in T-DNA cassette, presence of vector backbone needs to be checked for fine quality of cisgenic lines. In previous works, integration

of vector backbone has been reported. During the development of *Rvi6* cisgenic plants, for example, 80 % and 23 % integrated vector bone was reported by Vanblaere *et al*, (2011) and Würdig *et al*, (2015) respectively. Based on *nptII* marker gene removal, we achieved 5 cisgenic lines which can fit in cisgenic status defined by Schouten *et al*, (2006b, 2006a). Out of these 5 cisgenic lines, C_8 and C_{10} showed high scab resistant before marker gene removal (Scab test is required to be performed after marker gene removal), carrying 1 copy of *Rvi12_Cd5* gene on intergenic region of chromosome 4 and 16 respectively. However, scab resistance validation after heat treatment, validation of marker gene removal, T-DNA integration, trimming of LB, FRT, RB and presence or absence of vector backbone should be performed. The validation of all these aspects necessary for cisgenic lines mentioned before, is central part of on-going analysis.

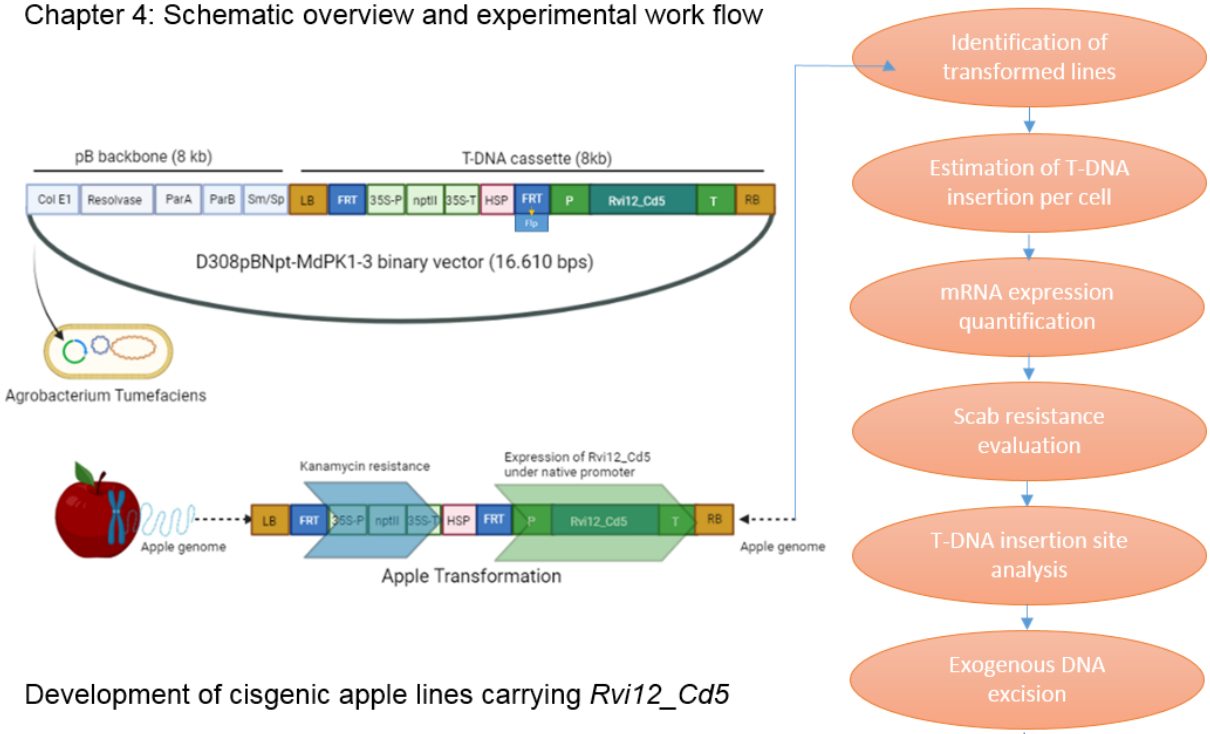
In European Union, cisgenesis underlies the GMO regulations except if cisgenic plant contains only 20 bps of exogenous/foreign DNA (Lusser *et al.*, 2011; Lusser and Davies, 2013). However, to achieve this level of exogenous DNA removal by heat inducible site specific recombination FLP/FRT system (Dalla Costa *et al*., 2020, 2016; Herzog *et al*., 2012; Kost *et al*., 2015; Luo *et al*., 2007; Pompili *et al*., 2020; Würdig *et al*., 2015) used in this study or by R/Rs system (58 bps will remain) (Krens *et al*., 2015; Righetti *et al*., 2014; Schaart *et al*., 2004; Vanblaere *et al*., 2011), seems impossible. To solve this issue, either European legislation laws needs to more flexible since cisgenic lines are quite similar to traditionally bred crops ((GMO), 2012) or there is a strong need to develop novel vectors which leave only 20 bps in final product or utilization of alternative methods in future.

4.6 Conclusion

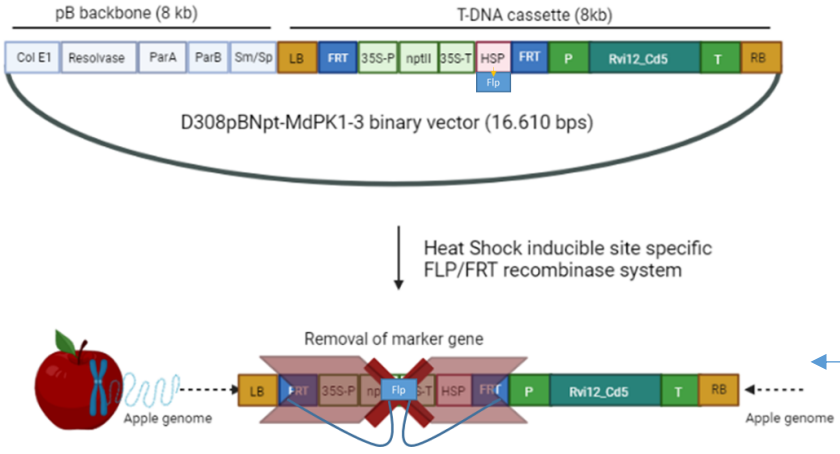
This study was able to give only a predictive knowledge of important cis-regulatory elements of *Rvi12_Cd5* native promoter. Based on *in-silico* analysis, native promoter used in this study, seems to be efficient for transcription initiation and quantitative expression of *Rvi12_Cd5*. Six major plant defense related transcription factors binding sites were predicted in promoter sequence, indicating its possible role in defense related gene signaling pathways against *V. inaequalis*. Thus, the native promoter, coding region and native terminator of *Rvi12_Cd5* were able to produce high, partial or low resistance against mixed inoculum of *V.inaequalis* in tranformed lines regardless of low mRNA expression level. Our designed vector machinery carrying site specific FLP/FRT recombination cassette to leave minimum number of exogenous DNA was efficient to produce cisgenic lines with 100% removal of marker gene. Further validation of T-DNA insertion site and exogenous DNA removal by PCR and sequencing may lead to true cisgenic lines. Which can be a next step forward to obtain in field cisgenic apple lines carrying new scab gene *Rvi12_Cd5* or for gene pyramidation with other

resistance genes such as *Rvi6* whose resistance stability has been compromised by pathogen in some cases.

Chapter 4: Schematic overview and experimental work flow



Development of cisgenic apple lines carrying *Rvi12_Cd5*



Promoter Sequence (2618 bps)

TTTCTTTTGCTTTCCGTGACGGCTTTTGGTGATGTCTACTTTCATCTACTCTATTTGTAATGGCTT
TGTGTCAACCAAATGATGCAAAATGCAAGCAAACATGCCCAAAACGACTCCGTTTTGCCTTCTCTT
CCAACGGAGGGATTGCCGAATGTGAAATAATCGCGACCGCAGAGGATACAAACCCCTTAAACAACG
GCTAAGCACCTTTCCATTTATTTTTTTCATTTATTTTTCAATTTTTGTCATTGACTGATTCGAAAT
TTTTTACAGCTCTTTAAGAGTAGGAACGCCATCGGTATGAATATGATCACTGAAACAAACATAGAA
AGTCTCCTCCGCTAGTCCAATCCAGCTGCTTCTGCAGAAAGCCAAAGATTTCAGCTGCAAATTCGA
AATCGCGATGTTCGCCAAGCGGCTTTTGCAGAAAGCCATAAACAACTCTCAGGTAAAATCGTATTC
CCAAGCATTCGATCGCCGCCATTTTTTTCTGCTTTTTTGGAATTTTTCAGTTTAGCTTCGTAATTT
GCGTTTTTTTTTAATTAAAATTCCATTTCTTTTTTGTTGGGGTGGATTAAATTCCGATGCTTTATC
TTGTTAGTATGCTGGAAAACTGGATATTGCATCAATGGATTATTTCTTATGATTTGTTTATTCATT
TCTGTGTATTTTTATGCTCGAATACTTGGGTTGTATAAATTTGGAGCCTCTTGGTATCGACATTTG
GACTGATACTTGCCCTTTTTTTTTAATTAGGATTTTGTATTGGAAATTGTTGGTTTCAGCATAATTT
GTTCACAGCATAATTGTTGGTTTCAGCTCAAATTTCCTTGTGAGCCCTGTCTATCCCATTTTTTTT
TTCTCGCAAAGCTTCATCTAATAGTGCTCTGGCTATGAAGAATCTATGGAGAATTGGTGTTTTAGT
AAGTCATTGACTAGATTTATTCATAACAACAACGATTATCAATAATCGCTTGGAAGTTTGTGAACT
ATTTAGCCCAAATGGGGAAAAGAAAAAAAAATCAGTAAGATCAAAAAAGAAAAACAAAGCTCATCC
AATGAAGACATACACAGAAAGGATGAAAGCCAAAAGCGAACGCCAAAGAAAAGTCATTTTCTCAAA
CCCATTTACATGCATTTCCTCACTTTCCCATTCCAATTTTCTGTAATTATTTCCAAGAACAACTAA
TTCTTTTCAAACTCCAATCAAGAAAAATAAAAAATTGTATGTAAATTTAAGTTGGGTTTGGACGGG
TTATAACTAAAAAAATACTCAATTGTCCGAATTTTGAAGTGGAATAAGGCATAGACAAATTAGGAT
TGACCCAATCCAAGTTTCACTCCTAATTGAGATACAGATGCCAACATGATGCCATAACATATCTAA
TATATTAATTATGTTATACTAACAAAATAAGTGAGAAAAAAAAAAAAAAAAAAAAAAGACTTGACT
TGACCTGTAAAGCTCTCTCTCCGTTGCTTCTCTCAATAGATCCCCACCAAAATACGGTTGCTTCTT
CTCCTTAGCTGTTCTATTGTTCAAATCGAAACCCAGTTACCCAGCAGCCTTGCTGTTGTAGACGAC
GTAGCCAACACAGTTGCAGACGCCGGTCCACGGTGAGAGTCTCTCCCACCATAGTCTGATTATCCT
TGAAAACGTATCCCGACAGTAGAATTCGCGTATCGAAAGCTTATCGACGACGTATCCAGAGGTCGA
CTGGTCAACGGATACGTTCAGATACGTATCCGATGCGTATTGGGCCGTATCGGGTGTGCTTGTCGT
ATCCCAAACCGTATCTGATACGGGATACGCCATAAAACCGGCGTGTCCGTGCTTCGTGGTGCGGAC
AAAGTCGTCTGACTCTTCCGTTCCTTATAAGCTGTGGACTAAGATTTCTTAATTTCTGTTGACTAA
TTATTAAGTTGGTCTATCGTAATTTCTCAAGTGCTTTTTCTTACATGTGATAAGATTTCTTAATTT
CTGTAGACTAACTACTAATTTGGTCCATCTTAATTTCTGTGGCCATCCAATGTAAGTGTTTTTTCT
TAGATGTGATAGGATTTCTTCTCTTTCGTGCTTTTGTGCAAATTAAACTTTGGTTTTCAGGTTTTC
CATTGTCTTTGAACAATGATGGAGCATTCACGTACTATTCGTAATCTGATTTTGTTGGAATGTGAT
TGATTACAAACATACATAGACATCCCTCTTATAGAGATTTTACAAAGACCAACACAAAACATAGGT
GTTCAAAAGTCTTTGCACATACACACTTTTACACACACCTTTAAACTTTTGTATACACACACATAG
CTTTCGGCCATTACAGAAACATTGACAGCTATCAAAGTAAAAGACAAGTATAGCTGTTGAAAAACA
AGGCAGCTATAGACAAAACACGCCAACTATTCCACCAATTTCGGCCACTTTATTTTGATTTGAACT
TCCAATACTTCCAACAGATTTCTTAATTTCTGTTGACTTACTACTAAGTTGGTCTATCTTAATTTC
TAGGGACATCCAATCTCGAGTGTTTTTTCTTACATGTGATAGGATTTCTTCTCTTTTGTGCTTTTG
TGCAAATTAAACTTTGATTTTTTTGGTTTTCCATTTGTCTTTGAACA

4.7 Bibliography

(GMO), E. P. on G. M. O. (2012). Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis. *EFSA Journal*, *10*(2), 2561.

Akhond, M. A. Y., & Machray, G. C. (2009). Biotech crops: technologies, achievements and prospects. *Euphytica*, *166*(1), 47–59.

Aldwinckle, H. S., & Malnoy, M. (2009). *Plant regeneration and transformation in the Rosaceae*.

Aman Beshir, J., & Kebede, M. (2021). *In silico* analysis of promoter regions and regulatory elements (motifs and CpG islands) of the genes encoding for alcohol production in Saccharomyces cerevisiaea S288C and Schizosaccharomyces pombe 972h. *Journal of Genetic Engineering and Biotechnology*, *19*(1), 1–14.

Andersen, E. J., Ali, S., Byamukama, E., Yen, Y., & Nepal, M. P. (2018). Disease resistance mechanisms in plants. *Genes*, *9*(7), 339.

Bakker, J. J. (1999). *19e Rassenlijst voor kleinfruit*. NFO.

Barbieri, M., Belfanti, E., Tartarini, S., Vinatzer, B. A., Sansavini, S., Silfverberg-Dilworth, E., Gianfranceschi, L., Hermann, D., Patocchi, A., & Gessler, C. (2003a). Progress of map-based cloning of the *Vf*-resistance gene and functional verification: Preliminary results from expression studies in transformed apple. *HortScience*, *38*(3), 329–331.

Barbieri, M., Belfanti, E., Tartarini, S., Vinatzer, B. A., Sansavini, S., Silfverberg-Dilworth, E., Gianfranceschi, L., Hermann, D., Patocchi, A., & Gessler, C. (2003b). Progress of Map-based Cloning of the *Vf*-resistance Gene and Functional Verification: Preliminary Results from Expression Studies in Transformed Apple. In *329 Hortscience* (Vol. 38, Issue 3).

Beckerman, J. L., Sundin, G. W., & Rosenberger, D. A. (2015). Do some IPM concepts contribute to the development of fungicide resistance? Lessons learned from the apple scab pathosystem in the United States. *Pest Management Science*, *71*(3), 331–342.

Belfanti, E., Barbieri, M., Tartarini, S., Vinatzer, B., Gennari, F., Paris, R., Sansavini, S., Silfverberg-Dilworth, E., Patocchi, A., Hermann, D., Gianfranceschi, L., & Gessler, C. (2004). Gala apple transformed with the putative scab resistance gene *HcrVf2*. *Acta Horticulturae*, *663*, 453–456. https://doi.org/10.17660/ActaHortic.2004.663.78

Belfanti, Enrico, Silfverberg-Dilworth, E., Tartarini, S., Patocchi, A., Barbieri, M., Zhu, J., Vinatzer, B. A., Gianfranceschi, L., Gessler, C., & Sansavini, S. (2004b). The *HcrVf2* gene from a wild

apple confers scab resistance to a transgenic cultivated variety (Vol. 101, Issue 3). www.pnas.orgcgidoi10.1073pnas.0304808101

Bengtsson, M., Lindhard, H., & Grauslund, J. (2000). Occurrence of races of *Venturia inaequalis* in an apple scab race screening orchard in Denmark. *IOBC Wprs Bulletin*, *23*(12), 225–230.

Bonaterra, A., Badosa, E., Cabrefiga, J., Francés, J., & Montesinos, E. (2012). Prospects and limitations of microbial pesticides for control of bacterial and fungal pomefruit tree diseases. *Trees*, *26*(1), 215–226.

Boone, D. M., Kline, D. M., & Keitt, G. W. (1957). *Venturia inaequalis* (Cke.) Wint. XIII. Pathogenicity of induced biochemical mutants. *American Journal of Botany*, 791–796.

Borejsza-Wysocka, E., Norelli, J. L., Aldwinckle, H. S., & Malnoy, M. (2010). Stable expression and phenotypic impact of attacin E transgene in orchard grown apple trees over a 12 year period. *BMC Biotechnology*, *10*(1), 1–9.

Borneman, A. R., Gianoulis, T. A., Zhang, Z. D., Yu, H., Rozowsky, J., Seringhaus, M. R., Wang, L. Y., Gerstein, M., & Snyder, M. (2007). Divergence of transcription factor binding sites across related yeast species. *Science*, *317*(5839), 815–819.

Bowen, J. K., Mesarich, C. H., Bus, V. G., Beresford, R. M., Plummer, K. M., & Templeton, M. D. (2011). *Venturia inaequalis*: the causal agent of apple scab. *Molecular Plant Pathology*, *12*(2), 105-122.

Breathnach, R., & Chambon, P. (1981). Organization and expression of eucaryotic split genes coding for proteins. *Annual Review of Biochemistry*, *50*(1), 349–383.

Bundock, P., & Hooykaas, P. J. J. (1996). Integration of *Agrobacterium tumefaciens* T-DNA in the Saccharomyces cerevisiae genome by illegitimate recombination. *Proceedings of the National Academy of Sciences*, *93*(26), 15272–15275.

Burley, S. K., & Roeder, R. G. (1996). Biochemistry and structural biology of transcription factor IID (TFIID). *Annual Review of Biochemistry*, *65*(1), 769–799.

Bus, V. G. M., Rikkerink, E. H. A., Caffier, V., Durel, C.-E., & Plummer, K. M. (2011). Revision of the Nomenclature of the Differential Host-Pathogen Interactions of *Venturia inaequalis* and Malus . *Annual Review of Phytopathology*, *49*(1), 391–413. https://doi.org/10.1146/annurev-phyto-072910-095339

Chetty, V. J., Ceballos, N., Garcia, D., Narváez-Vásquez, J., Lopez, W., & Orozco-Cárdenas, M. L.

(2012). Evaluation of four *Agrobacterium tumefaciens* strains for the genetic transformation of tomato (Solanum lycopersicum L.) cultivar Micro-Tom. *Plant Cell Reports 2012 32:2*, *32*(2), 239–247. https://doi.org/10.1007/S00299-012-1358-1

Chevalier, M., Lespinasse, Y., & Renaudin, S. (1991). A microscopic study of the different classes of symptoms coded by the *Vf* gene in apple for resistance to scab (*Venturia inaequalis*). *Plant Pathol*, *40*. https://doi.org/10.1111/j.1365-3059.1991.tb02374.x

Chow, C.-N., Lee, T.-Y., Hung, Y.-C., Li, G.-Z., Tseng, K.-C., Liu, Y.-H., Kuo, P.-L., Zheng, H.-Q., & Chang, W.-C. (2019). PlantPAN3. 0: a new and updated resource for reconstructing transcriptional regulatory networks from ChIP-seq experiments in plants. *Nucleic Acids Research*, *47*(D1), D1155–D1163.

Chow, C.-N., Zheng, H.-Q., Wu, N.-Y., Chien, C.-H., Huang, H.-D., Lee, T.-Y., Chiang-Hsieh, Y.-F., Hou, P.-F., Yang, T.-Y., & Chang, W.-C. (2016). PlantPAN 2.0: an update of plant promoter analysis navigator for reconstructing transcriptional regulatory networks in plants. *Nucleic Acids Research*, *44*(D1), D1154–D1160.

Crosby, J. A., Janick, J., Pecknold, P. C., Korban, S. S., O'Connon, P. A., Ries, S. M., Goffreda, J., & Voordeckers, A. (1992). Breeding apples for scab resistance: 1945-1990. *Fruit Var J*, *46*.

Cusin, R., Revers, L. F., & Maraschin, F. D. S. (2017). New biotechnological tools to accelerate scab-resistance trait transfer to apple. *Genet Mol Biol*, *40*. https://doi.org/10.1590/1678-4685-gmb-2016-0043

Czarnecka, E., Key, J. L., & Gurley, W. B. (1989). Regulatory domains of the Gmhsp17. 5-E heat shock promoter of soybean. *Molecular and Cellular Biology*, *9*(8), 3457–3463.

Daccord, N., Celton, J. M., Linsmith, G., Becker, C., Choisne, N., Schijlen, E., Geest, H., Bianco, L., Micheletti, D., Velasco, R., Pierro, E. A., Gouzy, J., Rees, D. J. G., Guérif, P., Muranty, H., Durel, C. E., Laurens, F., Lespinasse, Y., Gaillard, S., … Bucher, E. (2017). High-quality de novo assembly of the apple genome and methylome dynamics of early fruit development. *Nat Genet*, *49*. https://doi.org/10.1038/ng.3886

Dalla Costa, L., Bozzoli, M., Pompili, V., Piazza, S., Broggini, G. A. L., Patocchi, A., & Malnoy, M. (2019). Development of a Taqman real-time PCR method to quantify nptII in apple lines obtained with 'established'or 'new breeding'techniques of genetic modification. *European Food Research and Technology*, *245*(3), 643–652.

Dalla Costa, L., Piazza, S., Campa, M., Flachowsky, H., Hanke, M.-V., & Malnoy, M. (2016).

Efficient heat-shock removal of the selectable marker gene in genetically modified grapevine. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *124*(3), 471–481.

Dalla Costa, L., Piazza, S., Pompili, V., Salvagnin, U., Cestaro, A., Moffa, L., Vittani, L., Moser, C., & Malnoy, M. (2020). Strategies to produce T-DNA free CRISPRed fruit trees via *Agrobacterium tumefaciens* stable gene transfer. *Scientific Reports*, *10*(1), 1–14.

De Marchi, E., Cavaliere, A., Bacenetti, J., Milani, F., Pigliafreddo, S., & Banterle, A. (2019). Can consumer food choices contribute to reduce environmental impact? The case of cisgenic apples. *Science of the Total Environment*, *681*, 155–162.

Denancé, N., Sánchez-Vallet, A., Goffner, D., & Molina, A. (2013). Disease resistance or growth: the role of plant hormones in balancing immune responses and fitness costs. *Frontiers in Plant Science*, *4*, 155.

Dong, O. X., & Ronald, P. C. (2021). Targeted DNA insertion in plants. *Proceedings of the National Academy of Sciences*, *118*(22), e2004834117. https://doi.org/10.1073/pnas.2004834117

Erdin, N., Tartarini, S., Broggini, G. A., Gennari, F., Sansavini, S., Gessler, C., & Patocchi, A. (2006). Mapping of the apple scab-resistance gene *Vb*. *Genome*, *49*(10), 1238-1245.

EFSA, G. M. O. (2011). Panel (EFSA Panel on Genetically Modified Organisms), 2011. Scientific Opinion on Guidance on the Risk Assessment of Genetically Modified Microorganisms and Their Products Intended for Food and Feed Use. *EFSA Journal*, *9*(6), 2193.

Fenning, T. M., & Gershenzon, J. (2002). Where will the wood come from? Plantation forests and the role of biotechnology. *TRENDS in Biotechnology*, *20*(7), 291–296.

Flachowsky, H., Le Roux, P. M., Peil, A., Patocchi, A., Richter, K., & Hanke, M. V. (2011). Application of a high-speed breeding technology to apple (*Malus*×*domestica*) based on transgenic early flowering plants and marker-assisted selection. *New Phytologist*, *192*(2), 364–377. https://doi.org/10.1111/j.1469-8137.2011.03813.x

Flor, H. H. (1971). Current status of the gene for gene concept. *Annu Rev Phytopathol*, *9*. https://doi.org/10.1146/annurev.py.09.090171.001423

Galli, P., Broggini, G. A. L., Gessler, C., & Patocchi, A. (2010). Phenotypic characterization of the *Rvi15* (*Vr2*) apple scab resistance. *JPP*, *92*.

Gardiner, S. E., Bus, V. G. M., Rusholme, R. L., Chagné, D., & Rikkerink, E. H. A. (2007). Apple. In *Fruits and nuts* (pp. 1–62). Springer.

Gaskell, G., Stares, S., Allansdottir, A., Allum, N., Castro, P., Esmer, Y., Fischler, C., Jackson, J., Kronberger, N., & Hampel, J. (2010). *Europeans and Biotechnology in 2010. Winds of change?*

Gessler, C. (2011). Cisgenic disease resistant apples: a product with benefits for the environment, producer and consumer. *Outlooks on Pest Management*, *22*(5), 216–219.

Gessler, C., & Patocchi, A. (2007). Recombinant DNA technology in apple. *Advances in Biochemical Engineering/Biotechnology*, *107*, 113–132. https://doi.org/10.1007/10_2007_053

Gessler, C., & Pertot, I. (2012). *Vf* scab resistance of *Malus*. In *Trees - Structure and Function*. https://doi.org/10.1007/s00468-011-0618-y

Gessler, C., Vanblaere, T., Szankowski, I., & Broggini, G. (2009). Cisgenic approach to disease resistance in Apple. *Phytopathology*, *99*(6), S42–S42.

Gheysen, G., Villarroel, R., & Van Montagu, M. (1991). Illegitimate recombination in plants: a model for T-DNA integration. *Genes & Development*, *5*(2), 287–297.

Hemmat, M., Brown, S. K., Aldwinckle, H. S., Weeden, N. F., & Mehlenbacher, S. A. (2003). Identification and mapping of markers for resistance to apple scab from 'Antonovka' and 'Hansen's baccata# 2'. *Acta horticulturae*.

Halfter, U., Morris, P.-C., & Willmitzer, L. (1992). Gene targeting in Arabidopsis thaliana. *Molecular and General Genetics MGG*, *231*(2), 186–193.

Heng, Y., Yoon, S., & House, L. (2021). Explore Consumers' Willingness to Purchase Biotechnology Produced Fruit: An International Study. *Sustainability*, *13*(22), 12882.

Hernandez-Garcia, C. M., & Finer, J. J. (2014). Identification and validation of promoters and cis-acting regulatory elements. *Plant Science*, *217*, 109–119.

Herzog, K., Flachowsky, H., Deising, H. B., & Hanke, M.-V. (2012). Heat-shock-mediated elimination of the *nptII* marker gene in transgenic apple (*Malus*× *domestica* Borkh.). *Gene*, *498*(1), 41–49.

Himmelbach, A., Liu, L., Zierold, U., Altschmied, L., Maucher, H., Beier, F., Müller, D., Hensel, G., Heise, A., & Schützendübel, A. (2010). Promoters of the barley *germin-like GER4* gene cluster enable strong transgene expression in response to pathogen attack. *The Plant Cell*, *22*(3), 937–952.

Hood, E. E., Gelvin, S. B., Melchers, L. S., & Hoekema, A. (1993). New *Agrobacterium* helper

plasmids for gene transfer to plants. *Transgenic Research 1993 2:4*, *2*(4), 208–218. https://doi.org/10.1007/BF01977351

Houdebine, L.-M. (2007). Transgénique ou cisgénique: quel niveau de risque? *Cahiers Agricultures*, *16*(1), 63–64.

Inoue, H., Hayashi, N., Matsushita, A., Xinqiong, L., Nakayama, A., Sugano, S., Jiang, C.-J., & Takatsuji, H. (2013). Blast resistance of CC-NB-LRR protein *Pb1* is mediated by WRKY45 through protein–protein interaction. *Proceedings of the National Academy of Sciences*, *110*(23), 9577–9582.

Jacobsen, E., & Nataraja, K. N. (2008). Cisgenics-Facilitating the second green revolution in India by improved traditional plant breeding. *Current Science*, *94*(1), 1365–1366.

Jacobsen, E., & Schouten, H. J. (2008). Cisgenesis, a new tool for traditional plant breeding, should be exempted from the regulation on genetically modified organisms in a step by step approach. *Potato Res*, *51*. https://doi.org/10.1007/s11540-008-9097-y

Jha, G., Thakur, K., & Thakur, P. (2009). The *Venturia* apple pathosystem: Pathogenicity mechanisms and plant defense responses. *Journal of Biomedicine and Biotechnology*, *2009*. https://doi.org/10.1155/2009/680160

Joshi, S. G., Soriano, J. M., Kortstee, A., Schaart, J. G., Krens, F. A., Jacobsen, E., & Schouten, H. J. (2009). Development of cisgenic apples with durable resistance to apple scab. *Acta Horticulturae*. https://doi.org/10.17660/ActaHortic.2009.839.53

Joshi, S G, Schaart, J. G., Groenwold, R., Jacobsen, E., Schouten, H. J., & Krens, F. A. (2011). Functional analysis and expression profiling of *HcrVf1* and *HcrVf2* for development of scab resistant cisgenic and intragenic apples. *Plant Mol Biol*, *75*. https://doi.org/10.1007/s11103-011-9749-1

Joshi, S. G. (2010). Towards durabale resistance to apple scab using cisgenes. Wageningen University and Research.

Krens, F. A., Schaart, J. G., Van der Burgh, A. M., Tinnenbroek-Capel, I. E., Groenwold, R., Kodde, L. P., ... & Schouten, H. J. (2015). Cisgenic apple trees; development, characterization, and performance. *Frontiers in plant science*, *6*, 286.

Kong, Y., Zhou, G., Wang, G., & Wang, Y. (2000). Factors affecting transformation of Agrobacterium tumefaciens and their application on cereals. *Ying Yong Sheng tai xue bao= The*

Journal of Applied Ecology, *11*(5), 791-794.

Kost, T. D., Gessler, C., Jänsch, M., Flachowsky, H., Patocchi, A., & Broggini, G. A. L. (2015a). Development of the first cisgenic apple with increased resistance to fire blight. *PLoS One*, *10*(12), e0143980.

Kost, T. D., Gessler, C., Jänsch, M., Flachowsky, H., Patocchi, A., & Broggini, G. A. L. (2015b). Development of the first cisgenic apple with increased resistance to fire blight. *PLoS ONE*, *10*(12). https://doi.org/10.1371/journal.pone.0143980

Krens, F. A., Schaart, J. G., van der Burgh, A. M., TinnenbroekCapel, I. E. M., Groenwold, R., Kodde, L. P., Broggini, G. A. L., Gessler, C., & Schouten, H. J. (2015). Cisgenic apple trees; development, characterization, and performance. *Frontiers in Plant Science*. https://doi.org/10.3389/fpls.2015.00286

Kumar, S., Volz, R. K., Alspach, P. A., & Bus, V. G. M. (2010). Development of a recurrent apple breeding programme in New Zealand: a synthesis of results, and a proposed revised breeding strategy. *Euphytica*, *173*(2), 207–222.

Lammerts van Bueren, E. T., Verhoog, H., Tiemens-Hulscher, M., Struik, P. C., & Haring, M. A. (2007). Organic agriculture requires process rather than product evaluation of novel breeding techniques. *NJAS: Wageningen Journal of Life Sciences*, *54*(4), 401–412.

Laurens, F., Aranzana, M. J., Arus, P., Bassi, D., Bink, M., Bonany, J., Caprera, A., Corelli-Grappadelli, L., Costes, E., Durel, C. E., Mauroux, J. B., Muranty, H., Nazzicari, N., Pascal, T., Patocchi, A., Peil, A., Quilot-Turion, B., Rossini, L., Stella, A., … Van De Weg, E. (2018). An integrated approach for increasing breeding efficiency in apple and peach in Europe. *Horticulture Research*, *5*(1). https://doi.org/10.1038/s41438-018-0016-3

Lee, T. I., & Young, R. A. (2000). Transcription of eukaryotic protein-coding genes. *Annual Review of Genetics*, *34*(1), 77–137.

Lefrancq, B., & Lateur, M. (2009). Monitoring and occurrence of new races of *Venturia inaequalis* on apple in Belgium. *Communications in Agricultural and Applied Biological Sciences*, *74*(3), 623–631.

Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., Rouzé, P., & Rombauts, S. (2002). PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences. *Nucleic Acids Research*, *30*(1), 325–327.

Li, S., Cong, Y., Liu, Y., Wang, T., Shuai, Q., Chen, N., Gai, J., & Li, Y. (2017). Optimization of *Agrobacterium*-mediated transformation in soybean. *Frontiers in Plant Science*, *8*, 246.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods*, *25*(4), 402–408.

Luo, K., Duan, H., Zhao, D., Zheng, X., Deng, W., Chen, Y., Stewart Jr, C. N., McAvoy, R., Jiang, X., & Wu, Y. (2007). 'GM-gene-deletor': fused loxP-FRT recognition sequences dramatically improve the efficiency of FLP or CRE recombinase on transgene excision from pollen and seed of tobacco plants. *Plant Biotechnology Journal*, *5*(2), 263–374.

Lusk, J. L., & Sullivan, P. (2002). Consumer acceptance of genetically modified foods. *Food Technology*, *56*(10), 32–37.

Lusser, M, & Davies, H. V. (2013). Comparative regulatory approaches for groups of new plant breeding techniques. *New Biotechnol*, *30*. https://doi.org/10.1016/j.nbt.2013.02.004

Lusser, Maria, Parisi, C., Plan, D., & Rodríguez-Cerezo, E. (2011). New plant breeding techniques: state-of-the-art and prospects for commercial development. Publications office of the European Union Luxembourg.

MacHardy. (1996). Apple scab: biology, epidemiology, and management. *American Phytopathological Society (APS Press).*

MacHardy, W. E., Gadoury, D. M., & Gessler, C. (2001). Parasitic and biological fitness of *Venturia inaequalis*: relationship to disease management strategies. *Plant Disease*, *85*(10), 1036–1051.

Malnoy, M., Xu, M., Borejsza-Wysocka, E., Korban, S. S., & Aldwinckle, H. S. (2008). Two receptor-like genes, *Vfa1* and *Vfa2*, confer resistance to the fungal pathogen *Venturia inaequalis* inciting apple scab disease. *Mol Plant-Microbe Interact*, *21*. https://doi.org/10.1094/MPMI-21-4-0448

Manktelow, D. W. L., Beresford, R. M., Batchelor, T. A., & Walker, J. T. S. (1996). Use patterns and economics of fungicides for disease control in New Zealand apples. *Acta Horticulturae*, *422*, 187–192. https://doi.org/10.17660/ACTAHORTIC.1996.422.31

Mantovani, R. (1999). The molecular biology of the CCAAT-binding factor NF-Y. *Gene*, *239*(1), 15–27.

Marette, S., Disdier, A.-C., & Beghin, J. C. (2021). A comparison of EU and US consumers' willingness to pay for gene-edited food: Evidence from apples. *Appetite*, *159*, 105064.

Matsumoto, S., Ito, Y., Hosoi, T., Takahashi, Y., & Machida, Y. (1990). Integration of *Agrobacterium* T-DNA into a tobacco chromosome: possible involvement of DNA homology between T-DNA and plant DNA. *Molecular and General Genetics MGG*, *224*(3), 309–316.

Molina, C., & Grotewold, E. (2005). Genome wide analysis of Arabidopsis core promoters. *BMC Genomics*, *6*(1), 1–12.

Okonechnikov, K., Golosova, O., Fursov, M., & Team, U. (2012). Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics*, *28*(8), 1166–1167.

Padmarasu, S., Sargent, D. J., Jaensch, M., Kellerhals, M., Tartarini, S., Velasco, R., Troggio, M., & Patocchi, A. (2014). Fine-mapping of the apple scab resistance locus *Rvi12* (*Vb*) derived from 'Hansen's baccata #2.' *Molecular Breeding*. https://doi.org/10.1007/s11032-014-0167-3

Padmarasu, S., Sargent, D. J., Patocchi, A., Troggio, M., Baldi, P., Linsmith, G., Poles, L., Jänsch, M., Kellerhals, M., Tartarini, S., & Velasco, R. (2018). Identification of a leucine-rich repeat receptor-like serine/threonine-protein kinase as a candidate gene for *Rvi12* (*Vb*)-based apple scab resistance. *Molecular Breeding*, *38*(6). https://doi.org/10.1007/S11032-018-0825-Y

Papp, D., Gao, L., Thapa, R., Olmstead, D., & Khan, A. (2020). Field apple scab susceptibility of a diverse *Malus* germplasm collection identifies potential sources of resistance for apple breeding. *CABI Agriculture and Bioscience 2020 1:1*, *1*(1), 1–14. https://doi.org/10.1186/S43170-020-00017-4

Papp, D., Singh, J., Gadoury, D., & Khan, A. (2020). New North American isolates of *Venturia inaequalis* can overcome apple scab resistance of malus floribunda 821. *Plant Disease*, *104*(3), 649–655. https://doi.org/10.1094/PDIS-10-19-2082-RE

Parisi, L., Fouillet, V., Schouten, H. J., Groenwold, R., Laurens, F., Didelot, F., Evans, K., Fischer, C., Gennari, F., Kemp, H., Lateur, M., Patocchi, A., Thissen, J., & Tsipouridis, C. (2004). Variability of the pathogenicity of *Venturia inaequalis* in Europe. *Acta Horticulturae*, *663*, 107–114. https://doi.org/10.17660/ACTAHORTIC.2004.663.13

Paszkowski, J., Baur, M., Bogucki, A., & Potrykus, I. (1988). Gene targeting in plants. *The EMBO Journal*, *7*(13), 4021–4026.

Perini, P., Pasquali, G., Margis-Pinheiro, M., de Oliviera, P. R. D., & Revers, L. F. (2014). Reference genes for transcriptional analysis of flowering and fruit ripening stages in apple (*Malus*× *domestica* Borkh.). *Molecular Breeding*, *34*(3), 829–842.

Pompili, V., Dalla Costa, L., Piazza, S., Pindo, M., & Malnoy, M. (2020). Reduced fire blight susceptibility in apple cultivars using a high-efficiency CRISPR/Cas9-FLP/FRT-based gene editing system. *Plant Biotechnology Journal*, *18*(3), 845–858. https://doi.org/10.1111/pbi.13253

Righetti, L., Djennane, S., Berthelot, P., Cournol, R., Wilmot, N., Loridon, K., Vergne, E., & Chevreau, E. (2014). Elimination of the *nptII* marker gene in transgenic apple and pear with a chemically inducible R/Rs recombinase. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *117*(3), 335–348.

Risseeuw, E., Offringa, R., Franke-van Dijk, M. E. I., & Hooykaas, P. J. J. (1995). Targeted recombination in plants using *Agrobacterium* coincides with additional rearrangements at the target locus. *The Plant Journal*, *7*(1), 109–119.

Robertson, K. D. (2002). DNA methylation and chromatin–unraveling the tangled web. *Oncogene*, *21*(35), 5361–5379.

Schaart, J. G., Krens, F. A., Pelgrom, K. T. B., Mendes, O., & Rouwendal, G. J. A. (2004). Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene. *Plant Biotechnology Journal*, *2*(3), 233–240.

Schaart, J. G., Tinnenbroek-Capel, I. E. M., & Krens, F. A. (2011). Isolation and characterization of strong gene regulatory sequences from apple, *Malus× domestica*. *Tree Genetics & Genomes*, *7*(1), 135–142.

Schaart, J. G., van de Wiel, C. C. M., Lotz, L. A. P., & Smulders, M. J. M. (2016). Opportunities for products of new plant breeding techniques. *Trends in Plant Science*, *21*(5), 438–449.

Schouten, H J, Brinkhuis, J., Burgh, A. M., Schaart, J. G., Groenwold, R., Broggini, G. A. L., & Gessler, C. (2014). Cloning and functional characterization of the *Rvi15* (*Vr2*) gene for apple scab resistance. *Tree Genet Genomes*, *10*. https://doi.org/10.1007/s11295-013-0678-9

Schouten, H J, Soriano, J. M., Joshi, S. G., Kortstee, A. J., Krens, F. A., Schaart, J. G., van der Linden, K., Allan, A. C., Hellens, R. P., & Espley, R. V. (2007). Cisgenesis is a promising approach for fast, acceptable and safe breeding of pip fruit. *XII EUCARPIA Symposium on Fruit Breeding and Genetics 814*, 199–204.

Schouten, Henk J, Krens, F. A., & Jacobsen, E. (2006a). Do cisgenic plants warrant less stringent oversight? *Nature Biotechnology*, *24*(7), 753.

Schouten, Henk J, Krens, F. A., & Jacobsen, E. (2006b). Cisgenic plants are similar to traditionally bred plants: international regulations for genetically modified organisms should be altered to exempt cisgenesis. *EMBO Reports*, *7*(8), 750–753. https://doi.org/10.1038/sj.embor.7400769

Schubert, D., & Williams, D. (2006). 'Cisgenic'as a product designation. *Nature Biotechnology*, *24*(11), 1327–1329.

Setlow, J. K. (2012). *Genetic engineering: principles and methods* (Vol. 13). Springer Science & Business Media.

Silfverberg-Dilworth, E., Besse, S., Paris, R., Belfanti, E., Tartarini, S., Sansavini, S., Patocchi, A., & Gessler, C. (2005a). Identification of functional apple scab resistance gene promoters. *Theoretical and Applied Genetics*. https://doi.org/10.1007/s00122-005-1940-9

Silfverberg-Dilworth, E., Besse, S., Paris, R., Belfanti, E., Tartarini, S., Sansavini, S., Patocchi, A., & Gessler, C. (2005b). Identification of functional apple scab resistance gene promoters. *Theoretical and Applied Genetics*, *110*(6), 1119–1126. https://doi.org/10.1007/s00122-005-1940-9

Smale, S. T., & Baltimore, D. (1989). The "initiator" as a transcription control element. *Cell*, *57*(1), 103–113.

Smith, A. (2000). *Oxford dictionary of biochemistry and molecular biology: Revised Edition*. Oxford University Press.

Szankowski, I, Briviba, K., Fleschhut, J., Schönherr, J., Jacobsen, H. J., & Kiesecker, H. (2003). Transformation of apple (*Malus domestica* Borkh.) with the stilbene synthase gene from grapevine (*Vitis vinifera* L.) and a *PGIP* gene from kiwi (Actinidia deliciosa). *Plant Cell Reports*, *22*(2), 141–149.

Szankowski, I, Waidmann, S., Degenhardt, J., Patocchi, A., Paris, R., Silfverberg-Dilworth, E., Broggini, G., & Gessler, C. (2009). Highly scab-resistant transgenic apple lines achieved by introgression of *HcrVf2* controlled by different native promoter lengths. *Tree Genet Genomes*, *5*. https://doi.org/10.1007/s11295-008-0191-8

Tripathi, G. (2010). *Cellular and Biochemical Science*. IK International Pvt Ltd.

Tsuda, K., & Somssich, I. E. (2015). Transcriptional networks in plant immunity. *New Phytologist*, *206*(3), 932–947.

Vaillancourt, L. J., & Hartman, J. R. (2000). Apple scab. *The Plant Health Instructor*.

Vanblaere, T., Flachowsky, H., Gessler, C., & Broggini, G. A. L. (2014). Molecular characterization of cisgenic lines of apple 'Gala'carrying the *Rvi6* scab resistance gene. *Plant Biotechnology Journal*, *12*(1), 2–9.

Vanblaere, T., Szankowski, I., Schaart, J., Schouten, H., Flachowsky, H., Broggini, G. A. L., & Gessler, C. (2011). The development of a cisgenic apple plant. *Journal of Biotechnology*. https://doi.org/10.1016/j.jbiotec.2011.05.013

Vinatzer, B. A., Patocchi, A., Gianfranceschi, L., Tartarini, S., Zhang, H. B., Gessler, C., & Sansavini, S. (2001). Apple contains receptor-like genes homologous to the *Cladosporium fulvum* resistance gene family of tomato with a cluster of genes cosegregating with *Vf* apple scab resistance. *Molecular Plant-Microbe Interactions*, *14*(4), 508-515.

Williams, E. B., & Kuc, J. (1969). Resistance in *Malus* to *Venturia inaequalis*. *Annual Review of Phytopathology*, *7*(1), 223–246.

Williams, E. B., & Shay, J. R. (1957). The relationship of genes for pathogenicity and certain other characters in *Venturia inaequalis* (Cke.) Wint. *Genetics*, *42*(6), 704.

Würdig, J., Flachowsky, H., & Hanke, M.-V. (2013). Studies on heat shock induction and transgene expression in order to optimize the Flp/FRT recombinase system in apple (*Malus*× *domestica* Borkh.). *Plant Cell, Tissue and Organ Culture (PCTOC)*, *115*(3), 457–467.

Würdig, J., Flachowsky, H., Saß, A., Peil, A., & Hanke, M.-V. (2015). Improving resistance of different apple cultivars using the *Rvi6* scab resistance gene in a cisgenic approach based on the Flp/FRT recombinase system. *Molecular Breeding*, *35*(3), 1–18.

Xu, M. L., & Korban, S. S. (2002). AFLP derived SCARs facilitate construction of a 11 Mb sequence ready map of a region that spans the *Vf* locus in the apple genome. *Plant Mol Biol*, *50*. https://doi.org/10.1023/A:1019912419709

Yamaguchi-Shinozaki, K., & Shinozaki, K. (1994). A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *The Plant Cell*, *6*(2), 251–264.

Zhu, Y., & Saltzgiver, M. (2020). A systematic analysis of apple root resistance traits to *Pythium ultimum* infection and the underpinned molecular regulations of defense activation. *Horticulture Research*, *7*.

Zou, C., Sun, K., Mackaluso, J. D., Seddon, A. E., Jin, R., Thomashow, M. F., & Shiu, S.-H. (2011).

Cis-regulatory code of stress-responsive transcription in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences*, *108*(36), 14992–14997.

Chapter 5

General discussion and conclusion

Apple scab is a worldwide endemic and devastating disease of apple (*Malus* × *domestica*). In commercial orchards, scab is currently controlled by using up to 15-30 fungicides per year (Manktelow *et al*., 1996; Vaillancourt and Hartman, 2000). Excessive use of chemical fungicides, on the other hand, can pollute the environment and endanger human health (Gessler, 2011; Linhart *et al*., 2019). With the overwhelming goal of reducing the use of fungicides, disease-resistant cultivars are a top priority for most breeding programs (Gessler and Pertot, 2012).

Traditional breeding strategies to develop scab resistant cultivars, have several drawbacks such as undesirable characters due to linkage drag, requirement of multiple generations of pseudo-testcrossing, low efficiency rate due to long vegetative phase (Fenning and Gershenzon, 2002; Gardiner *et al*., 2007; Gessler and Pertot, 2012). An important example is *Rvi6*-resistant cultivars, as it took several decades for traditional breeding to deliver them with good fruit quality to the market (Bakker, 1999). Unfortunately, most scab resistant apple cultivars carrying the resistance gene *Rvi6* (*Vf*), have become susceptible to this disease due to the emergence of the pathogen avirulence gene *avrRvi6* (Lefrancq and Lateur, 2009; Papp *et al*., 2020c). Moreover, other than *Rvi6* (Belfanti *et al*., 2004), only one gene *Rvi15* has been cloned and functionally characterized so far (Schouten *et al*., 2014). To take a step forward and increase the number of cloned and functionally characterized apple scab resistance genes, the recently identified apple scab resistance *Rvi12_Cd5* gene was cloned and functionally characterized (Padmarasu *et al*., 2018). Advanced biotechnology provides plant breeders a rapid improvement of a cultivar with scab resistance by performing transgenesis or cisgenesis with the identified candidate genes (Aldwinckle and Malnoy, 2009; Cusin *et al*., 2017; Flachowsky *et al*., 2009; S. G. Joshi *et al*., 2009). Using transgenic approach, within one year, apple scab resistance genes such as *Rvi6* (*HcrVf2*) and *Rvi15* (*Vr2*) have been transformed to susceptible 'Gala' cultivar plants via *Agrobacterium tumefaciens* and resulting transgenic genotypes showing to be resistant to *V. inaequalis* (Belfanti *et al*., 2004; Schouten *et al*., 2014). Similarly, apple scab resistant cisgenic *Rvi6* (*HcrVf2*) 'Gala' cultivar has also been produced in short period of time (Vanblaere *et al*., 2011).

The candidate *Rvi12_Cd5* gene was cloned (binary vector D301P9OU10-35S-PK123) and used for *A. tumefaciens* mediated transformation of susceptible apple cultivar 'Gala Galaxy'. *Rvi12_Cd5* was overexpressed by the control of the cauliflower mosaic virus 35S promoter. After 7 months, 60 transformed 'Gala' lines were produced, indicating the good efficiency of the used transformation method. Overall, *A. tumefaciens* transformation method proved to be helpful for the transfer of *Rvi12_Cd5* into susceptible 'Gala' in a short period of time, similar to other studies conducted for transferring *Rvi6* (*HcrVf2*) and *Rvi15* (*Vr2*) as mentioned above. Consequently, these lines were further checked for determining the gene integration to contain constitutive transcript expression into

the plant. The 35S promoter was able to overexpress *Rvi12_Cd5* in all transgenic lines compared to 'HB2'. With characteristics like 1-2 T-DNA insertions after *in-vitro* root production and acclimatization, 7 promising transgenic lines were selected for further analysis.

The GM 'Gala' plants were used for the functional characterization of *Rvi12_Cd5* against apple scab. Three independent artificial scab infections with a mixed inoculum of *V. inaequalis* were performed plants for analysis of resistance phenotype development. Thorough phenotypic analysis of resistance reactions in transgenic 'Gala' lines at 21 dpi (days post inoculation), resistance reactions ranging from class 0 to 3b were observed, whereas untransformed 'Gala' showed susceptibility as class 4 (Chevalier *et al*., 1991). The overall resistance response of each line was defined on the basis of median values (chapter 3; Tab. 4), similarly to Belfanti *et al*, (2004). Transgenic 'Gala' lines T_{10}, T_{18}, T_{20}, T_{30}, and T_{34} showed class 0 (no symptom) resistance reaction while T_{18} and T_{20} also showed partial resistance as class 3a (necrotic/chlorotic lesion with light sporulation). Apart from assigning the class 0 and 3a to lines based on majority of leaves response (Median value) or symptom severity analysis, it was observed that few transformed leaves also showed class 2 phenotype (chlorotic lesion with small necrosis in case T_{30} and T_{35}), class 3b (necrotic/chlorotic lesion with sparse sporulation in case of T_{20}) and class 3b to 4 (restricted sporulation in case of T_{10}, T_{25} and T_{29}). Overall T_{30} and T_{34} showed full resistance as class 0/2 throughout the infection trails and exhibited highest mRNA expression level. While other transgenic lines showed certain degree of variation in resistant responses as mentioned above and no clear correlation could be observed between mRNA expression and resistance level or between T-DNA insertions and mRNA expression to explain the variation. Similarly, no correlation has also been observed in studies of Belfanti *et al*, (2004) and Joshi *et al*, (2011). So range of resistance reactions from class 0–3b probably happened due to the use of mixed inoculum (unknown strains of *V. inae*qualis), as we don't know which race is causing a specific reaction; similar range of resistance reactions by using of mixed inoculum has been observed in the mapping populations for *Rvi12* (*Vb*) locus as reported by Hemmat *et al*, (2003), Erdin *et al*, (2006b) and Padmarasu *et al*, (2018). Similarly, the diverse responses were also observed in *Rvi6*, *Rvi11*, *Rvi19* and *Rvi20* (Belfanti *et al*., 2004; Clark *et al*., 2014; Gygax *et al*., 2004; Malnoy *et al*., 2008), which range from 0 to 3b. Noteworthy, a few leaves of T_{10}, T_{25} and T_{29} showed class 4 with restricted sporulations which were macroscopically distinctive from the untransformed leaves (control) showing abundant sporulation. These transformed lines showed high constitutive mRNA expression compared to 'HB2' before infection. However no correlation was observed between mRNA expression and scab resistance among transgenic lines so it might happened due to the chimerism (regeneration from transformed and untransformed cells) (Butaye *et al*., 2005; Flachowsky *et al*., 2008) or mutation happened possibly in T-DNA insert or promoter region, or, alternatively, in the coding region by environmental

factors or transformation steps which affected translation or functionality of the *Rvi12_Cd5* protein (Jada *et al*., 2014; Pang *et al*., 1996; Qu *et al*., 2021). No transgenic line carrying *Rvi12_Cd5* gene showed class1 (hypersensitive response) in this study which is in accordance with all phenotypic studies performed for mapping of Rvi12 (*Vb*) locus (Erdin *et al*., 2006b; Hemmat *et al*., 2003; Padmarasu *et al*., 2018).

After phenotypic assessment of transgenic lines, all the resistant reactions and susceptibility symptoms were validated by microscopic observations (Chapter 3; Fig 9-14). The transformed symptomless leaves linked to class 0, showed no fungal growth beyond appresorium and penetration pegs at microscopic level. Since majority of transgenic lines showed symptomless leaves after inoculation, microscopy helped us to see that the healthy leaves observed, were due to the resistance response of plants, not due to inoculum escape. Necrotic/chlorotic lesions with no, slight or sparse sporulation in transgenic lines were well characterized by browning of epidermal and mesophyll cells around different stages of fungal development, well correlating with the infection severity. In contrast, untransformed leaves of susceptible 'Gala' (control) showed distinctive dense homogenous network of mycelium. Considering the combination of consistent indications, such as high level of mRNA expression in transgenic lines compared to 'HB2', ability of transgenic lines to produce range of resistance reactions against *V. inaequalis* compared to control and validation of resistant responses by detailed cytology, it can be said that the *Rvi12_Cd5* gene derived from 'HB2' is the functional *Rvi12* gene. According to Padmarasu *et al*, (2014), scab resistance in 'HB2' was not considered monogenic and could be controlled by at least two unlinked genes, thus, *Rvi12_Cd5* as active *Rvi12* is at least one of the two proposed genes in 'HB2'.

Broad-scale development of transgenic apple cultivars have been hampered in Europe due to indicated or perceived risks connected with genetic modification (GM) by a large population of consumers (Gaskell *et al*., 2010). The concept of "Cisgenesis" was created to answer some of the most common objections, with the purpose of reducing public fear and biosafety concerns to the greatest extent possible (Schouten *et al*., 2006a, 2006b). Cisgenesis is the addition of natural genes from crossable donor plants, including the native promoter, terminator, exons, and introns (Schouten *et al*., 2006a, 2006b). Traditional breeding leaves co-transferred DNA sequences along gene of interest in the new cultivar, while cisgenesis maintains the original cultivar characteristics (Borejsza-Wysocka *et al*., 2010; Joshi *et al*., 2009; Krens *et al*., 2015). Moreover, cisgenesis also eliminates the introduction of undesirable alleles, whereas traditional breeding removes these alleles gradually over generations (Jacobsen and Schouten, 2008; Schaart *et al*., 2016). Such cisgenic cultivars are viewed more acceptable by consumers (De Marchi *et al*., 2019; Gaskell *et al*., 2010; Heng *et al*., 2021; Lusk and

Sullivan, 2002; Marette *et al*., 2021). The European Food Safety Authority (EFSA Panel on Genetically Modified Organisms, 2012) concluded that the risks of cisgenic plants are the same as those of conventionally bred ones. Also in comparison to a transgenic product, cisgenic scab resistant apple lines may create fewer issues, such as allergenicity induced by the product of the inserted foreign gene (Vanblaere *et al*., 2014). Up to now, cisgenic cultivars produced for apple scab resistance carry only one gene *Rvi6* (*HcrVf2*) *(*Krens *et al., 2015;* Vanblaere *et al., 2011;* Würdig *et al*., 2015), which emphasizes the need for further development of cisgenic apple cultivars, carrying new apple scab resistance genes.

Keeping in view, all the problems associated with commercialization of transgenic apple cultivars at public level and positive aspects related to cisgenesis, after the functional characterization of the *Rvi12_Cd5* by transgenic approach, which is a prerequisite for producing cisgenic apple cultivar (Gessler and Pertot, 2012), a cisgenic apple cultivar carrying *Rvi12_Cd5* was developed. The development of cisgenic plants carrying *Rvi12_Cd5,* require the gene expression controlled by its natural promoter, thus, it was important to understand different factors involved in functional efficiency of promoter. Before starting *in-silico* analysis to find important promoter motifs involved in *Rvi12_Cd5* gene transcription and defense response, two lengths of promoter were defined, a short promoter (SP: already known sequence of 900 bps upstream to *Rvi12_Cd5* TSS; Padmarasu *et al*., 2018) and a long promoter (LP: new promoter sequence extended from 900 bps to 2.6 kb sequence via sanger sequencing at FEM Sequencing Platform Facility). The analysis predicted TATA box at -233 bps and CAAT box at -40 bps upstream which is theoretically indicating that both SP and LP, contains core promoter region and can induce quantitative gene expression. Other predicted defense related motifs were related to major six plant defense related TFs, like WRKY, AP2/ERF, bHLH, bZIP, MYB, and NAC (Andersen *et al*., 2018). Similar TFs were found in *in-silico* analysis of 'HB2' genome (Chen *et al*., 2019). The majority of defense related TFs were positioned on the core and proximal region of promoter compared to distal region, although, these results cannot ensure that core promoter or proximal promoter might give qualitative expression. Also, many enhancers exist in proximal and distal regions which can contribute to quality expression (Hernandez-Garcia and Finer, 2014). Out of major six defense related TFs, the highest number of matches were found for WRKY TF family which performs important role in plant immunity (Jiang *et al*., 2017; Jimmy and Babu, 2015; Pandey and Somssich, 2009). In addition, *Rvi12_Cd5* also showed homology to well-characterized receptor like kinases in *Xa21,* rice blast resistance gene (Padmarasu *et al*., 2018), which also contain WRKY binding sites for the positive regulation of blast resistance (Inoue *et al*., 2013). These predicted motifs may or may not be functional and practical demonstration of the contribution of these motifs to promoter activity is essential to confirm their role. Overall, *in-silico* analysis helped us to give an idea that the SP 900

bps used in *Rvi12_Cd5* expression construct, seems functional and may produce quantitative expression.

For the development of cisgenic plants, *Rvi12_Cd5* controlled by its own promoter (900 bps) was successfully transferred (binary vector D308pBNpt-MdPK1-3) to 45 'Gala' lines, indicating that the *A. tumefaciens* mediated transformation method used is efficient. Similar method has been used to transfer *Rvi6* (*HcrVf2*) controlled by its native promoter for production of cisgenic apple lines (Krens *et al*., 2015; Vanblaere *et al*., 2011; Wurdig *et al*., 2015). Consequently, 15 *in-vitro* transformed 'Gala' lines carrying 1 to 2 copy of the T-DNA per cell were examined for mRNA expression before scab inoculation test. Compared to the expression of mRNA of *Rvi12_Cd5* under the control of 35S promoter, the expression of mRNA of *Rvi12_Cd5* under the control of its own promoter was significantly lower than 'HB2' (positive control) except one-line C_6 (0.9-fold higher). The reason for low transcript expression could be the use of RNA material extracted from *in-vitro* transformed leaves rather than greenhouse acclimatized. This assumption is supported by the results of Malnoy *et al*, (2008) as transformed lines carrying *Rvi6* (*HcrVf2*) under control of its native promoter showed low level of mRNA expression before scab infection and significantly higher expression after scab infection. In contrast, Szankowski *et al*, (2009) observed no difference of *Rvi6* (*HcrVf2*) mRNA expression in *in-vitro* and greenhouse plants. However, it can be still interesting to check mRNA expression of greenhouse grown transformed plants before and after scab infection. Another important reason could be involvement of native promoter motifs and its length as they both conjunctively play important role in gene expression as discussed above. The *Rvi12_Cd5* SP 900 bps seemed to contain core and proximal promoter regions as indicated by *in-silico* motif analysis, which should activate transcription and provide quantitative expression theoretically (Gudynaite-Savitch *et al*., 2009; Juven-Gershon and Kadonaga, 2010). So, the possibility for low mRNA expression could be exclusion of distal promoter as fully functional promoter depends directly on the positional and combinatorial understanding of the motifs present in both the proximal and distal regions (Molina and Grotewold, 2005). Also, the transcription factors (TFs) which binds to these motifs can result in expression of the adjacent gene to be either constitutive, induced by external factors, tissue-specific (Hartmann *et al*., 2005; Maniatis *et al*., 1987), which may explain that possibly, the mRNA level risen up after inoculation due to combinational nature of promoter (constitutive and inducive) and presence of WRKY motifs in its sequence as mentioned earlier. Regarding native promoter length and mRNA expression, there are several studies conducted on *Rvi6* (*HcrVf2*), for example, Silfverberg-Dilworth *et al*. (2005) observed reduced mRNA expression from sequences longer than 288 bps, Malnoy *et al*, (2008) observed low mRNA expression using 2kb promoter, then, Szankowski *et al*, (2009) which observed high

mRNA expression by using 288 bps and 779 bps promoter. Instead Joshi *et al*, (2011) observed average mRNA independent of lengths of 288 bps and 2 kb. Würdig *et al*, (2015) observed also average mRNA expression from 1315 bps and 536 bps and Vanblaere *et al.* (2011) observed very low *Rvi6* mRNA expression from 220 bps length. Considering all these studies on *Rvi6* (*HcrVf2*), *Rvi12_ Cd5* 900 bps short promoter resulted an appropriate choice for resistance induction, despite the low mRNA expression. Despite the low mRNA expression. Similarly, significant resistance was observed with quite low level of *Rvi6* mRNA expression controlled by native promoter, in the study of Vanblaere *et al*, (2014).

A range of visible resistance reactions (class 0, 2, 3a, 3b; Chevalier *et al*., 1991) were observed in transformed lines compared to control. Based on media values and symptom severity analysis, seven lines C_3, C_5, C_6, C_7, C_8, C_{10} and C_{12} were highly resistant, assigned as class 0/2 while other seven lines showed partial resistance, assigned as 3a/3b. During macroscopic analysis, class 2 phenotype was also difficult to decide when resistance reactions were either chlorotic only (phenotype 1) or chlorotic lesion with necrotic center without sporulation at all (phenotype 2). That why, class 2 was assigned to two phenotypes. Interestingly, the class 2 phenotype 2 was slightly confusing with stellate chlorosis phenotype (SC) due to irregular shape of lesion. It might be possible that *Rvi12_Cd5* is responsible for resistance reaction from class 0 to 3b but also SC (class 2 phenotype 2) which is contradictory to phenotypic observation obtained from 'HB2 x Gala' mapping population by Padmarasu *et al*, (2014) who stated that *Rvi12* is responsible for resistance reactions ranging from class 0 to 3b and another unknown different gene is responsible for stellate chlorosis in 'HB2'. Regarding resistance reaction variability among transformed lines, all lines showed comparable mRNA levels among each other, but no correlation could be found between resistance and mRNA level (except one-line C_6), neither between T-DNA insertions and mRNA levels to explain range of resistance reaction variation among lines. So, most probably it could happened due to integration site of T-DNA on chromosome as chromatin-based regulation can also effect the transcription of gene as degree of regulation depends on where the transgene is integrated, an effect called as positional effect due to its location on genome (Hernandez-Garcia and Finer, 2014; Kooter *et al*., 1999). In addition, due to usage of mixed inoculum, it is not clear which race of *V. inaequalis* elucidate the resistance response, indicating the certain degree of resistance reaction variation among lines. In both scab experiments, 'Gala' produced heavy sporulation, assigned as class 4 (complete susceptibility). However, few transformed leaves of lines C_9, C_{11}, C_{14}, C_{15}, C_{26} and C_{35} also showed class 4 at 21 dpi, nevertheless, majority of these leaves showed delayed necrosis with slight chlorosis (3b) on infection sites at 22-28 dpi to restrict sporulation whereas control showed fused dense sporulation covering entire leaf surface. Now, this observation points towards possibility of combinational nature (constitutive or

inducible) of *Rvi12_Cd5* gene or its promoter. Here, it can be interesting to compare mRNA level before and after inoculation. Other plants have shown a delayed resistance response, such as transgenic *'Nicotiana benthamiana'* plants expressing the nuclear inclusion b (*NIb*) gene constitutively, were initially susceptible to Plum Pox Potyvirus infection but then they recovered (Guo and García, 1997). Delay in *RNAi-PtDDM1* (RNA inference-*Populus trichocarpa*-Daecreased DNA Methylation) gene expression has also been observed in other transgenic plants after a dormancy cycle and growth outdoors (Zhu *et al*., 2013). After macroscopic evaluation of resistant reactions (0-3b) and susceptibility symptoms (4), all symptoms were verified by microscopy. The leaves of transformed lines linked with class 0/2 resistance reactions, showed blocked growth of *V. inaequalis* conidia after formation of appresorium, germination tube, penetration pegs and subcuticular hyphae with browning of epidermal cells (class 2) or without browning of cells (class 0) at microscopic level. While leaves of transformed lines linked with 3a/3b showed necrosis of many epidermal and mesophyll cells around different stages of *V. inaequalis* germination such as advanced subcuticular hyphae (class 3a) to complete subcuticular stroma formation (class 3b). Susceptible 'Gala' (control) linked with class 4, showed active, dense and tight network of mycelium at microscopic level. Overall, it can be concluded that *Rvi12_Cd5* controlled by its natural promoter is able to induce high or partial resistance against mixed inoculum of *V. inaequalis*.

T-DNA integration sites were also checked in 40 transformed lines by NGS method described by Della Costa *et al*, (2020). Out of 40, 24 lines showed preliminary results of T-DNA integration on 16 different chromosomes based on their 90 to 100 % homology to reference genome (apple genome assembly GDDH13 v1.1). Some lines showed insertions on different chromosomes, some showed insertion on same chromosomes with different positions similar to the data of T-DNA integration found by Pompili *et al*, 2020. While some lines also showed integration on same chromosome with almost same positions. *A. tumefaciens* T-DNA normally integrates T-DNA into random positions in the plant genome (Chilton and Que, 2003). This happened probably either due to experimental mistake that lines belongs to same callus or due to the phenomena called natural homologous recombination (Dong and Ronald, 2021), which is based on homology between T-DNA and host genome DNA, thus, can produce unaided integration of T-DNA at the homologous site with low frequency (Bundock and Hooykaas, 1996; Kucherlapati *et al*., 1984; Risseeuw *et al*., 1995; Smithies *et al*., 1985) in higher plants (Hanin *et al*., 2001; Kempin *et al*., 1997; Terada *et al*., 2002). In addition, *in-silico* gene annotation analysis of chromosomes positions used by integrated T-DNA, revealed that no apple endogenous gene has been disrupted by T-DNA integration except at two chromosome positions. However, PCR and sequencing need to be performed to validate the actual physical map of T-DNA insertions sites.

After molecular characterization, and apple scab resistance evaluation of transformed lines carrying *Rvi12_Cd5* controlled by its native promoter (SP; 900 bps), the removal of exogenous DNA (*nptII* marker gene) from integrated T-DNA cassette was performed for production of cisgenic plants (Schouten *et al*., 2006b, 2006a). Out of several methods defined for exogenous DNA removal for (Schaart *et al*., 2011, 2004), the heat inducible site specific recombination FLP/FRT system (Herzog *et al*., 2012; Würdig *et al*., 2013) was chosen for this study because its leaves less exogenous DNA (34 bps of one FRT site) in final product than R/Rs system. The heat shock was induced in eight selected transformed lines and consequently, CN of *nptII* (marker gene) was checked. The results showed 100% removal in heat treated sub-clones of 5 independent lines. Correlating the *nptII* CN reduction with the removal of the marker gene itself indicates that the site specific FLP/FRT recombination system was successful to remove exogenous DNA. However, another validation of marker gene removal in cisgenic lines is still needed by PCR and sequencing (Dalla Costa *et al*., 2016; Pompili *et al*., 2020). Due to time constraints, *Rvi12_Cd5* cisgenic lines were only tested for scab resistance before exogenous DNA removal. So, it is also important to test scab resistance in *Rvi12_Cd5* cisgenic lines after heat shock treatment. However, in another study, no change in apple scab resistance was observed in *Rvi6* (*HcrVf2*) cisgenic apple cultivars after excision of the recombinase cassette (Würdig *et al*., 2015), which suggests *Rvi12_Cd5* cisgenic lines would likely to be scab resistant even after heat shock. In addition, durability of scab resistance and mRNA expression level in cisgenic lines carrying *Rvi12_Cd5* should be evaluated over period of time, similar to study of Krens *et al*, (2015) and Vanblaere *et al*, (2014) respectively.

Molecular characterization of cisgenic plants is required by the legal regulation of GM plants in Europe ((EC) Regulation No. 1829/2003; EFSA 2011), in this regard, 2 promising lines C_8 and C_{10} already fulfill that requirement in some aspects such as mRNA expression level (low mRNA expression compared to 'HB2'), T-DNA CN and insertion sites data (carry 1 copy of *Rvi12_Cd5* gene on intergenic region of chromosome 4 and 16 respectively without disruption of endogenous gene). Theoretically, these lines might contain minimum 34 bps (FRT) to maximum 96 bps (LB and RB) of exogenous DNA (Dalla Costa *et al*., 2020, 2016; Herzog *et al*., 2012; Kost *et al*., 2015; Luo *et al*., 2007; Pompili *et al*., 2020; Würdig *et al*., 2015) and may be vector backbone, however only PCR and sequencing can confirm extent of exogenous DNA left in final product. Due to possibility of minimum 34 bps of exogenous DNA left after heat shock treatment, *Rvi12_Cd5* cisgenic lines would be considered as GM according to European Union regulation, as it states that cisgenesis is similar to GM, except if only 20 bps left of exogenous DNA of final product (Lusser *et al.*, 2011; Lusser and Davies, 2013; (GMO), 2012). T-DNA (RB and LB) borders contain non-coding sequences so it seems

unconvincing that they might cause any phenotypic effect on plants, moreover, T-DNA border sequences are found homologous to many DNA sequences in plant genome (Conner *et al*., 2007). Similarly, one of the two FRT sites, its middle and back part also showed homology to apple genome in blast results (Würdig *et al*., 2013). However, the role of homology of T-DNA border or FRT short sequences to apple genome is not clear yet for flexibility of 20 bps rule (Lusser *et al*., 2011). To produce cisgenic plants satisfying 20 bps rule, designing of construct with minimum exogenous DNA is required. In past alternative methods has been studied such as transformation without a selection marker using a PCR-based selection strategy (Malnoy *et al*., 2010) or use of endogenous marker such as anthocyanin based visible selection marker (Kortstee *et al*., 2011). Besides these limitation factors of technique used in this study, cisgenic lines carrying *Rvi12_Cd5* may fit in the status of cigenesis defined by Schouten *et al* (2006b, 2006a).

On-going analysis is based on the validation of preliminary results obtained for cisgenic lines, particularly, C_8 and C_{10} which showed high resistance (class 0, 2) to *V. inaequalis* (scab test was performed before heat shock treatment). The on-going analysis centralized around accuracy of physical map of T-DNA integration sites and exogenous DNA removal from T-DNA (marker gene, T-DNA borders, vector backbone and FRT site) by PCR and sequencing. Furthermore, scab test evaluation after heat shock treatment is also going to be performed. Other reasons or hypothesis explained during discussion can provide starting point for further research such as: mRNA expression analysis of greenhouse plants before and after infection of *V. inaequalis* in order to understand nature of *Rvi12_Cd5* and its native promoter, heat shock treatment for marker gene removal in other resistant plants produced in present study and subsequent scab test, molecular characterization and sequencing, resistance durability check in cisgenic plants over period of time, phenotyping of transgenic or cisgenic lines after mono conidial inoculum, practical analysis to understand the functional role of defense related motifs and transcription factors to understand downstream signaling pathways involved in *Rvi12_Cd5* based defense action.

In conclusion, use of transgenic approach to overexpress the candidate gene *Rvi12_Cd5* controlled strong constitutive promoter into susceptible 'Gala Galaxy' and functional characterization of transgenic lines against mixed inoculum of *V. inaequalis*, supported that *Rvi12_Cd5* seems to be the active *Rvi12* resistance gene. Moreover, *in-silico* analysis provided predictive insights into functional efficiency of *Rvi12_Cd5* native promoter and presence of six major defense related motifs and corresponding transcription factors. The native promoter, coding region and native terminator of *Rvi12_Cd5* were also able to produce resistance against mixed inoculum of *V. inaequalis.* The site specific FLP/FRT recombination cassette was efficient to produce cisgenic lines with 100 % removal

of marker gene. Considering the capacity of *Rvi12_Cd5* to produce high scab resistance and production of *Rvi12_Cd5* cisgenic lines, it can be a next step forward to use *Rvi12_Cd5* in fields instead of *Rvi6* whose resistance stability has been compromised by pathogen and provide an opportunity to create durable resistance in susceptible cultivars by combining *Rvi12_Cd5* with other scab resistance genes like *Rvi16* and *Rvi15* in one background which can allow studying the mechanisms and downstream pathways of these three apple scab resistance genes in extensive detail.

5.0 Bibliography

(GMO), E. P. on G. M. O. (2012). Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis. *EFSA Journal*, *10*(2), 2561.

Aldwinckle, H. S., & Malnoy, M. (2009). *Plant regeneration and transformation in the Rosaceae*.

Andersen, E. J., Ali, S., Byamukama, E., Yen, Y., & Nepal, M. P. (2018). Disease resistance mechanisms in plants. *Genes*, *9*(7), 339.

Bakker, J. J. (1999). *19e Rassenlijst voor kleinfruit*. NFO.

Belfanti, E., Silfverberg-Dilworth, E., Tartarini, S., Patocchi, A., Barbieri, M., Zhu, J., ... & Sansavini, S. (2004). The *HcrVf2* gene from a wild apple confers scab resistance to a transgenic cultivated variety. *Proceedings of the National Academy of Sciences*, *101*(3), 886-890.

Borejsza-Wysocka, E., Norelli, J. L., Aldwinckle, H. S., & Malnoy, M. (2010). Stable expression and phenotypic impact of attacin E transgene in orchard grown apple trees over a 12 year period. *BMC Biotechnology*, *10*(1), 1–9.

Bundock, P., & Hooykaas, P. J. J. (1996). Integration of *Agrobacterium tumefaciens* T-DNA in the *Saccharomyces cerevisiae* genome by illegitimate recombination. *Proceedings of the National Academy of Sciences*, *93*(26), 15272–15275.

Butaye, K. M. J., Cammue, B., Delauré, S. L., & De Bolle, M. F. C. (2005). Approaches to minimize variation of transgene expression in plants. *Molecular Breeding*, *16*(1), 79–91.

Chen, X., Li, S., Zhang, D., Han, M., Jin, X., Zhao, C., ... & An, N. (2019). Sequencing of a wild apple (Malus baccata) genome unravels the differences between cultivated and wild apple species regarding disease resistance and cold tolerance. *G3: Genes, Genomes, Genetics*, *9*(7), 2051-2060.

Chevalier, M., Lespinasse, Y., & Renaudin, S. (1991). A microscopic study of the different classes of symptoms coded by the *Vf* gene in apple for resistance to scab (*Venturia inaequalis*). *Plant Pathol*, *40*. https://doi.org/10.1111/j.1365-3059.1991.tb02374.x

Chilton, M.-D. M., & Que, Q. (2003). Targeted integration of T-DNA into the tobacco genome at double-stranded breaks: new insights on the mechanism of T-DNA integration. *Plant Physiology*, *133*(3), 956–965.

Clark, M. D., Bus, V. G. M., Luby, J. J., & Bradeen, J. M. (2014). Characterization of the defence

response to *Venturia inaequalis* in ‘Honeycrisp’apple, its ancestors, and progeny. *European Journal of Plant Pathology*, *140*(1), 69–81.

Conner, A. J., Barrell, P. J., Baldwin, S. J., Lokerse, A. S., Cooper, P. A., Erasmuson, A. K., Nap, J.-P., & Jacobs, J. M. E. (2007). Intragenic vectors for gene transfer without foreign DNA. *Euphytica*, *154*(3), 341–353.

Cusin, R., Revers, L. F., & Maraschin, F. D. S. (2017). New biotechnological tools to accelerate scab-resistance trait transfer to apple. *Genetics and Molecular Biology*, *40*, 305-311.

Dalla Costa, L., Piazza, S., Campa, M., Flachowsky, H., Hanke, M.-V., & Malnoy, M. (2016). Efficient heat-shock removal of the selectable marker gene in genetically modified grapevine. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *124*(3), 471–481.

Dalla Costa, L., Piazza, S., Pompili, V., Salvagnin, U., Cestaro, A., Moffa, L., Vittani, L., Moser, C., & Malnoy, M. (2020). Strategies to produce T-DNA free CRISPRed fruit trees via *Agrobacterium tumefaciens* stable gene transfer. *Scientific Reports*, *10*(1), 1–14.

De Marchi, E., Cavaliere, A., Bacenetti, J., Milani, F., Pigliafreddo, S., & Banterle, A. (2019). Can consumer food choices contribute to reduce environmental impact? The case of cisgenic apples. *Science of the Total Environment*, *681*, 155–162.

Dong, O. X., & Ronald, P. C. (2021). Targeted DNA insertion in plants. *Proceedings of the National Academy of Sciences*, *118*(22), e2004834117. https://doi.org/10.1073/pnas.2004834117

Erdin, N., Tartarini, S., Broggini, G. A. L., Gennari, F., Sansavini, S., Gessler, C., & Patocchi, A. (2006). Mapping of the apple scab-resistance gene *Vb*. *Genome*, *49*(10), 1238–1245. https://doi.org/10.1139/G06-096

Fenning, T. M., & Gershenzon, J. (2002). Where will the wood come from? Plantation forests and the role of biotechnology. *TRENDS in Biotechnology*, *20*(7), 291–296.

Flachowsky, H, Hanke, M., Peil, A., Strauss, S. H., & Fladung, M. (2009). A review on transgenic approaches to accelerate breeding of woody plants. *Plant Breeding*, *128*(3), 217–226.

Flachowsky, Henryk, Riedel, M., Reim, S., & Hanke, M.-V. (2008). Evaluation of the uniformity and stability of T-DNA integration and gene expression in transgenic apple plants. *Electronic Journal of Biotechnology*, *11*(1), 26–40.

Gardiner, S. E., Bus, V. G. M., Rusholme, R. L., Chagné, D., & Rikkerink, E. H. A. (2007). Apple. In *Fruits and nuts* (pp. 1–62). Springer.

Gaskell, G., Stares, S., Allansdottir, A., Allum, N., Castro, P., Esmer, Y., Fischler, C., Jackson, J., Kronberger, N., & Hampel, J. (2010). *Europeans and Biotechnology in 2010. Winds of change?*

Gessler, C. (2011). Cisgenic disease resistant apples: a product with benefits for the environment, producer and consumer. *Outlooks on Pest Management*, *22*(5), 216–219.

Gessler, C., & Pertot, I. (2012). *Vf* scab resistance of *Malus*. In *Trees - Structure and Function*. https://doi.org/10.1007/s00468-011-0618-y

Gudynaite-Savitch, L., Johnson, D. A., & Miki, B. L. A. (2009). Strategies to mitigate transgene–promoter interactions. *Plant Biotechnology Journal*, *7*(5), 472–485.

Guo, H. S., & García, J. A. (1997). Delayed resistance to plum pox potyvirus mediated by a mutated RNA replicase gene: involvement of a gene-silencing mechanism. *Molecular Plant-Microbe Interactions*, *10*(2), 160–170.

Gygax, M., Gianfranceschi, L., Liebhard, R., Kellerhals, M., Gessler, C., & Patocchi, A. (2004). Molecular markers linked to the apple scab resistance gene *Vbj* derived from *Malus baccata* jackii. *Theoretical and Applied Genetics*. https://doi.org/10.1007/s00122-004-1803-9

Hanin, M., Volrath, S., Bogucki, A., Briker, M., Ward, E., & Paszkowski, J. (2001). Gene targeting in Arabidopsis. *The Plant Journal*, *28*(6), 671–677.

Hartmann, U., Sagasser, M., Mehrtens, F., Stracke, R., & Weisshaar, B. (2005). Differential combinatorial interactions of cis-acting elements recognized by R2R3-MYB, BZIP, and BHLH factors control light-responsive and tissue-specific activation of phenylpropanoid biosynthesis genes. *Plant Molecular Biology*, *57*(2), 155–171.

Hemmat, M., Brown, S. K., Aldwinckle, H. S., & Weeden, N. F. (2003). Identification and mapping of markers for resistance to apple scab from "Antonovka" and "Hansen's baccata #2." *Acta Hortic*, *622*. https://doi.org/10.17660/ActaHortic.2003.622.13

Heng, Y., Yoon, S., & House, L. (2021). Explore Consumers' Willingness to Purchase Biotechnology Produced Fruit: An International Study. *Sustainability*, *13*(22), 12882.

Hernandez-Garcia, C. M., & Finer, J. J. (2014). Identification and validation of promoters and cis-acting regulatory elements. *Plant Science*, *217*, 109–119.

Herzog, K., Flachowsky, H., Deising, H. B., & Hanke, M.-V. (2012). Heat-shock-mediated elimination of the *nptII* marker gene in transgenic apple (Malus× domestica Borkh.). *Gene*, *498*(1), 41–49.

Inoue, H., Hayashi, N., Matsushita, A., Xinqiong, L., Nakayama, A., Sugano, S., Jiang, C.-J., & Takatsuji, H. (2013). Blast resistance of CC-NB-LRR protein *Pb1* is mediated by WRKY45 through protein–protein interaction. *Proceedings of the National Academy of Sciences*, *110*(23), 9577–9582.

Jacobsen, E., & Schouten, H. J. (2008). Cisgenesis, a new tool for traditional plant breeding, should be exempted from the regulation on genetically modified organisms in a step by step approach. *Potato Res*, *51*. https://doi.org/10.1007/s11540-008-9097-y

Jada, B., Soitamo, A. J., Siddiqui, S. A., Murukesan, G., Aro, E.-M., Salakoski, T., & Lehto, K. (2014). Multiple different defense mechanisms are activated in the young transgenic tobacco plants which express the full length genome of the Tobacco mosaic virus, and are resistant against this virus. *PloS One*, *9*(9), e107778.

Jiang, J., Ma, S., Ye, N., Jiang, M., Cao, J., & Zhang, J. (2017). WRKY transcription factors in plant responses to stresses. *Journal of Integrative Plant Biology*, *59*(2), 86–101.

Jimmy, J. L., & Babu, S. (2015). Role of OsWRKY transcription factors in rice disease resistance. *Tropical Plant Pathology*, *40*(6), 355–361.

Joshi, S. G., Schaart, J. G., Groenwold, R., Jacobsen, E., Schouten, H. J., & Krens, F. A. (2011). Functional analysis and expression profiling of *HcrVf1* and *HcrVf2* for development of scab resistant cisgenic and intragenic apples. *Plant Mol Biol*, *75*. https://doi.org/10.1007/s11103-011-9749-1

Joshi, S. G., Soriano, J. M., Kortstee, A., Schaart, J. G., Krens, F. A., Jacobsen, E., & Schouten, H. J. (2009). Development of cisgenic apples with durable resistance to apple scab. *Acta Horticulturae*. https://doi.org/10.17660/ActaHortic.2009.839.53

Juven-Gershon, T., & Kadonaga, J. T. (2010). Regulation of gene expression via the core promoter and the basal transcriptional machinery. *Developmental Biology*, *339*(2), 225–229.

Kempin, S. A., Liljegren, S. J., Block, L. M., Rounsley, S. D., Yanofsky, M. F., & Lam, E. (1997). Targeted disruption in Arabidopsis. *Nature*, *389*(6653), 802–803.

Kooter, J. M., Matzke, M. A., & Meyer, P. (1999). Listening to the silent genes: transgene silencing, gene regulation and pathogen control. *Trends in Plant Science*, *4*(9), 340–347.

Kortstee, A. J., Khan, S. A., Helderman, C., Trindade, L. M., Wu, Y., Visser, R. G. F., Brendolise, C., Allan, A., Schouten, H. J., & Jacobsen, E. (2011). Anthocyanin production as a potential

visual selection marker during plant transformation. *Transgenic Research*, *20*(6), 1253–1264.

Kost, T. D., Gessler, C., Jänsch, M., Flachowsky, H., Patocchi, A., & Broggini, G. A. (2015). Development of the first cisgenic apple with increased resistance to fire blight. *PLoS One*, *10*(12), e0143980.

Krens, F. A., Schaart, J. G., van der Burgh, A. M., TinnenbroekCapel, I. E. M., Groenwold, R., Kodde, L. P., Broggini, G. A. L., Gessler, C., & Schouten, H. J. (2015). Cisgenic apple trees; development, characterization, and performance. *Frontiers in Plant Science*. https://doi.org/10.3389/fpls.2015.00286

Kucherlapati, R. S., Eves, E. M., Song, K.-Y., Morse, B. S., & Smithies, O. (1984). Homologous recombination between plasmids in mammalian cells can be enhanced by treatment of input DNA. *Proceedings of the National Academy of Sciences*, *81*(10), 3153–3157.

Lefrancq, B., & Lateur, M. (2009). Monitoring and occurrence of new races of *Venturia inaequalis* on apple in Belgium. *Communications in Agricultural and Applied Biological Sciences*, *74*(3), 623–631.

Linhart, C., Niedrist, G. H., Nagler, M., Nagrani, R., Temml, V., Bardelli, T., Wilhalm, T., Riedl, A., Zaller, J. G., & Clausing, P. (2019). Pesticide contamination and associated risk factors at public playgrounds near intensively managed apple and wine orchards. *Environmental Sciences Europe*, *31*(1), 28.

Luo, K., Duan, H., Zhao, D., Zheng, X., Deng, W., Chen, Y., Stewart Jr, C. N., McAvoy, R., Jiang, X., & Wu, Y. (2007). 'GM-gene-deletor': fused loxP-FRT recognition sequences dramatically improve the efficiency of FLP or CRE recombinase on transgene excision from pollen and seed of tobacco plants. *Plant Biotechnology Journal*, *5*(2), 263–374.

Lusk, J. L., & Sullivan, P. (2002). Consumer acceptance of genetically modified foods. *Food Technology*, *56*(10), 32–37.

Lusser, M, & Davies, H. V. (2013). Comparative regulatory approaches for groups of new plant breeding techniques. *New Biotechnol*, *30*. https://doi.org/10.1016/j.nbt.2013.02.004

Lusser, Maria, Parisi, C., Plan, D., & Rodríguez-Cerezo, E. (2011). New plant breeding techniques: state-of-the-art and prospects for commercial development. Publications office of the European Union Luxembourg.

Malnoy, M, Xu, M., Borejsza-Wysocka, E., Korban, S. S., & Aldwinckle, H. S. (2008). Two receptor-

like genes, *Vfa1* and *Vfa2*, confer resistance to the fungal pathogen *Venturia inaequalis* inciting apple scab disease. *Mol Plant-Microbe Interact*, *21*. https://doi.org/10.1094/MPMI-21-4-0448

Malnoy, Mickael, Boresjza-Wysocka, E. E., Norelli, J. L., Flaishman, M. A., Gidoni, D., & Aldwinckle, H. S. (2010). Genetic transformation of apple (*Malus x domestica*) without use of a selectable marker gene. *Tree Genetics & Genomes*, *6*(3), 423–433.

Maniatis, T., Goodbourn, S., & Fischer, J. A. (1987). Regulation of inducible and tissue-specific gene expression. *Science*, *236*(4806), 1237–1245.

Manktelow, D. W. L., Beresford, R. M., Batchelor, T. A., & Walker, J. T. S. (1996). Use patterns and economics of fungicides for disease control in New Zealand apples. *Acta Horticulturae*, *422*, 187–192. https://doi.org/10.17660/ACTAHORTIC.1996.422.31

Marette, S., Disdier, A.-C., & Beghin, J. C. (2021). A comparison of EU and US consumers' willingness to pay for gene-edited food: Evidence from apples. *Appetite*, *159*, 105064.

Molina, C., & Grotewold, E. (2005). Genome wide analysis of Arabidopsis core promoters. *BMC Genomics*, *6*(1), 1–12.

Padmarasu, S., Sargent, D. J., Jaensch, M., Kellerhals, M., Tartarini, S., Velasco, R., Troggio, M., & Patocchi, A. (2014). Fine-mapping of the apple scab resistance locus *Rvi12* (*Vb*) derived from 'Hansen's baccata #2.' *Molecular Breeding*. https://doi.org/10.1007/s11032-014-0167-3

Padmarasu, S., Sargent, D. J., Patocchi, A., Troggio, M., Baldi, P., Linsmith, G., Poles, L., Jänsch, M., Kellerhals, M., Tartarini, S., & Velasco, R. (2018). Identification of a leucine-rich repeat receptor-like serine/threonine-protein kinase as a candidate gene for *Rvi12* (*Vb*)-based apple scab resistance. *Molecular Breeding*, *38*(6). https://doi.org/10.1007/S11032-018-0825-Y

Pandey, S. P., & Somssich, I. E. (2009). The role of WRKY transcription factors in plant immunity. *Plant Physiology*, *150*(4), 1648–1655.

Pang, S., Ja, F., Carney, K., Stout, J., Tricoli, D. M., Quemada, H. D., & Gonsalves, D. (1996). Post-transcriptional transgene silencing and consequent tospovirus resistance in transgenic lettuce are affected by transgene dosage and plant development. *The Plant Journal*, *9*(6), 899–909.

Papp, D., Singh, J., Gadoury, D., & Khan, A. (2020). New North American isolates of *Venturia inaequalis* can overcome apple scab resistance of *Malus floribunda 821*. *Plant Disease*, *104*(3), 649-655.

Pompili, V., Dalla Costa, L., Piazza, S., Pindo, M., & Malnoy, M. (2020). Reduced fire blight

susceptibility in apple cultivars using a high-efficiency CRISPR/Cas9-FLP/FRT-based gene editing system. *Plant Biotechnology Journal*, *18*(3), 845–858. https://doi.org/10.1111/pbi.13253

Qu, J., Dry, I., Liu, L., Guo, Z., & Yin, L. (2021). Transcriptional profiling reveals multiple defense responses in downy mildew-resistant transgenic grapevine expressing a TIR-NBS-LRR gene located at the *MrRUN1/MrRPV1* locus. *Horticulture Research*, *8*(1), 1–12.

Risseeuw, E., Offringa, R., Franke-van Dijk, M. E. I., & Hooykaas, P. J. J. (1995). Targeted recombination in plants using *Agrobacterium* coincides with additional rearrangements at the target locus. *The Plant Journal*, *7*(1), 109–119.

Schaart, J. G., Krens, F. A., Pelgrom, K. T. B., Mendes, O., & Rouwendal, G. J. A. (2004). Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene. *Plant Biotechnology Journal*, *2*(3), 233–240.

Schaart, J. G., Tinnenbroek-Capel, I. E. M., & Krens, F. A. (2011). Isolation and characterization of strong gene regulatory sequences from apple, *Malus× domestica*. *Tree Genetics & Genomes*, *7*(1), 135–142.

Schaart, J. G., van de Wiel, C. C. M., Lotz, L. A. P., & Smulders, M. J. M. (2016). Opportunities for products of new plant breeding techniques. *Trends in Plant Science*, *21*(5), 438–449.

Schouten, H J, Brinkhuis, J., Burgh, A. M., Schaart, J. G., Groenwold, R., Broggini, G. A. L., & Gessler, C. (2014). Cloning and functional characterization of the *Rvi15* (*Vr2*) gene for apple scab resistance. *Tree Genet Genomes*, *10*. https://doi.org/10.1007/s11295-013-0678-9

Schouten, Henk J, Krens, F. A., & Jacobsen, E. (2006a). Do cisgenic plants warrant less stringent oversight? *Nature Biotechnology*, *24*(7), 753.

Schouten, Henk J, Krens, F. A., & Jacobsen, E. (2006b). Cisgenic plants are similar to traditionally bred plants: international regulations for genetically modified organisms should be altered to exempt cisgenesis. *EMBO Reports*, *7*(8), 750–753. https://doi.org/10.1038/sj.embor.7400769

Smithies, O., Gregg, R. G., Boggs, S. S., Koralewski, M. A., & Kucherlapati, R. S. (1985). Insertion of DNA sequences into the human chromosomal *β-globin* locus by homologous recombination. *Nature*, *317*(6034), 230–234.

Szankowski, I, Waidmann, S., Degenhardt, J., Patocchi, A., Paris, R., Silfverberg-Dilworth, E., Broggini, G., & Gessler, C. (2009). Highly scab-resistant transgenic apple lines achieved by

introgression of *HcrVf2* controlled by different native promoter lengths. *Tree Genet Genomes*, *5*. https://doi.org/10.1007/s11295-008-0191-8

Terada, R., Urawa, H., Inagaki, Y., Tsugane, K., & Iida, S. (2002). Efficient gene targeting by homologous recombination in rice. *Nature Biotechnology*, *20*(10), 1030–1034.

Vaillancourt, L. J., & Hartman, J. R. (2000). Apple scab. *The Plant Health Instructor*.

Vanblaere, T., Flachowsky, H., Gessler, C., & Broggini, G. A. L. (2014). Molecular characterization of cisgenic lines of apple 'Gala'carrying the *Rvi6* scab resistance gene. *Plant Biotechnology Journal*, *12*(1), 2–9.

Vanblaere, T., Szankowski, I., Schaart, J., Schouten, H., Flachowsky, H., Broggini, G. A., & Gessler, C. (2011). The development of a cisgenic apple plant. *Journal of biotechnology*, *154*(4), 304-311.

Würdig, J., Flachowsky, H., & Hanke, M.-V. (2013). Studies on heat shock induction and transgene expression in order to optimize the Flp/FRT recombinase system in apple (*Malus*× *domestica* Borkh.). *Plant Cell, Tissue and Organ Culture (PCTOC)*, *115*(3), 457–467.

Würdig, J., Flachowsky, H., Saß, A., Peil, A., & Hanke, M.-V. (2015). Improving resistance of different apple cultivars using the *Rvi6* scab resistance gene in a cisgenic approach based on the Flp/FRT recombinase system. *Molecular Breeding*, *35*(3), 1–18.

Zhu, R., Shevchenko, O., Ma, C., Maury, S., Freitag, M., & Strauss, S. H. (2013). Poplars with a *PtDDM1*-RNAi transgene have reduced DNA methylation and show aberrant post-dormancy morphology. *Planta*, *237*(6), 1483–1493.