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GENETICS OF RESISTANCE TO SCAB CAUSED BY *VENTURIA INAEQUALIS* IN 'PRÉSIDENT ROULIN' AND 'GENEVA' APPLE CULTIVARS

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Héloïse Bastiaanse (2015). Genetics of resistance to scab caused by *Venturia inaequalis* in 'Président Roulin' and 'Geneva' apple cultivars. (PhD Dissertation in English). Gembloux, Belgium, Gembloux Agro-Bio Tech, University of Liège.

Abstract

Apple scab caused by *Venturia inaequalis* is the major constraint to apple production worldwide. Today, an intensive use of pesticide is required to protect commercial orchards planted with cultivars (cvs) highly susceptible to scab. This intensive use of pesticides costs, and has a detrimental impact on the consumer health and the environment, but could be largely reduced by the introduction of resistant or partially resistant scab cvs in the orchard. This thesis aims to provide new insights into the mechanisms of resistance of two cvs presenting contrasting resistance phenotypes and durability: the durable partial resistance in 'Président Roulin' and the non durable resistance in 'Geneva', harboring a complex of complete and partial effects R genes. Preliminary, phytopathological tests were used in both cvs to differentiate the resistance loci. Resistance in both cultivars were shown to be race-specific and governed by at least five resistance loci. Expression signature of the partially resistant 'Président Roulin' challenged by *V. inaequalis* revealed common but also different molecular pathways as compared to the susceptible 'Gala' and a completely resistance *Rvi6* (*Vf*) 'Gala'-transformed line. Thirteen candidate defense genes (CDGs) were identified and their expression characterized over a time-course experiment during pathogen infection. Co-localizations of CDGs with apple genomic regions known to carry resistance factors were found (QTLs, major scab genes, or analogues of resistance genes). Finally, contribution to their functional assessment was made by investigating correlations between their expression and the level of resistance in a progeny derived from the cross between 'Gala' and 'Président Roulin'. Regulation of nine CDGs accounted for 46% of the phenotypic variance. In 'Geneva', five resistance loci (with dominant and more complex recessive control) were fine mapped in a region of 5 centimorgan (cM) located at the distal end of chromosome 4. This region corresponded to a 2 megabase pairs (Mbp) region containing nine CDGs encoding for nucleotide binding site-leucine rich repeat (NBS-LRR) family proteins on the 'Golden Delicious' apple reference genome. Overall this thesis contributed to better understand the genetic determinism of the resistance of apple against scab and its durability. Results were also at the basis of development of molecular marker tools to speed up the selection in a genome-informed breeding program.

Héloïse Bastiaanse (2015). Déterminisme génétique de la résistance des variétés 'Président Roulin' et 'Geneva' à la tavelure du pommier cause par *Venturia inaequalis* (Thèse de doctorat en anglais). Gembloux, Belgium, Gembloux Agro-Bio Tech, University of Liège.

Résumé

La tavelure, causée par *Venturia inaequalis*, est la principale maladie présente dans les vergers commerciaux de pommiers. Aujourd'hui la plupart de ces vergers sont plantés de variétés extrêmement sensibles à la maladie. La lutte contre la tavelure requiert ainsi l'usage intensif de fongicides, ce qui a un impact tant sur le coût de production que sur l'environnement et la santé des consommateurs. Une méthode alternative à un tel usage de pesticides réside dans la plantation de variétés résistantes ou partiellement résistantes à la tavelure. Dans ce contexte, cette thèse a pour objectif d'étudier le déterminisme génétique de deux variétés de pommiers aux formes contrastées de résistance : 'Président Roulin', ancienne variété belge présentant une résistance partielle et durable à la tavelure, et 'Geneva', variété à chair rouge présentant un complexe de plusieurs gènes majeurs et mineurs de résistance, non durables en vergers. Premièrement, afin de différencier les loci de résistance au sein des deux variétés, des tests phytopathologiques ont été réalisés par l'inoculation de différentes races de *V. inaequalis*. Les deux formes de résistance ont démontré être spécifiques aux races de tavelure et être sous le contrôle d'au moins cinq loci de résistance. Une analyse de données transcriptomiques semble démontrer que l'expression de la résistance partielle de 'Président Roulin' empreinte des voies métaboliques communes mais aussi différentes de celles observées dans le cas d'une réaction compatible (variété sensible 'Gala') ou incompatible (ligne 'Gala' transformée par le gène majeur de résistance *Rvi6* (*Vf*)). Treize gènes candidats de défense (GCD) ont été sélectionnés, et leur expression respective a été caractérisée après différentes périodes de temps au cours de l'infection par *V. inaequalis*. Certains GCD semblent être localisés dans des régions génomiques du pommier porteuses de facteurs de résistance déjà connus (gènes majeurs, loci de caractères quantitatifs et analogues de gènes de résistance). Enfin, la fonction de ces GCD dans les mécanismes de résistance partielle à la tavelure de 'Président Roulin' a été évaluée par l'estimation des corrélations existant entre la régulation de ces gènes et l'expression de la résistance dans une population en ségrégation dérivée du croisement entre 'Gala' et 'Président Roulin' : 46% de la proportion de la variance phénotypique a pu être expliquée par la régulation de neuf GCD. Chez 'Geneva', cinq loci de résistance (avec un contrôle dominant, ainsi qu'un contrôle récessif plus complexe) ont pu être finement cartographiés dans une région de 5 centimorgans (cM) située à l'extrémité du chromosome 4 du pommier. Cette région correspond à 2 mégabases dans le génome de référence de 'Golden Delicious' et contient neuf GCD encodant des protéines de résistance de la famille 'nucleotide binding site-leucine rich repeat' (NBS-LRR). En conclusion, cette thèse contribue à une meilleure compréhension à la fois du déterminisme génétique et des mécanismes biologiques de résistance à la tavelure du pommier. Cette thèse a de plus contribué au développement d'outils de marqueurs moléculaires visant à accélérer le processus de sélection dans des programmes modernes d'amélioration du pommier.

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My PhD has been an epic journey, across a wide diversity of offices, labs, orchards and greenhouses across Belgium, New Zealand, and even the USA from where I am writing the present lines. This provided me the opportunity to meet a great number of people to whom I would like to express my sincerest gratitude. Without your help, your support, your knowledge and advice, I would not have achieved this work.

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let's be honest, some quality belgian draft beers over these weekends! I am definitely missing all these very special moments with you!

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- David, I have to confess that I was really scared of moving to your lab, in the 'lovely' city of Palmerston North. According to my colleagues of the sunny side of Hawke's Bay, the city of 'Palmy' was as 'lovely' as a cold, stormy day could be, with some extra gusts of wind that were strong enough to blow you off of your bicycle if you even dare crossing the Manawatu river bridge.

Really ‘lovely’ they said, with a big ironic smile, sunglasses on, and a distinct smell of sunscreen floating around them! I did encounter some rainy days and strong gusts, but more importantly I found an awesome supervisor and a great team at PFR Palmerston North. Thank you for your training and your broad expertise in plant genetics and in the molecular mapping of phenotypic traits. It has been a really steep learning curve for me during these 6 months spent with the team. Thank you also for your contagious energy, as well as your great sense of humor. To know in how many research projects you were involved, I have always wondered how you managed so well to stay relaxed and available for all your students, and their daily troubleshooting and fair amount of questions about molecular mapping! Thank you very much for this! With the PhD student team, we were not too scared of having the Sauron eyes glued on the window separating your office space from ours. We had in fact the promise of some nice scooter rides along the corridors as well as interpretive ‘harlem shake’ dances in the lab! I also remember your creativity on the Belgian king’s picture in the office, replacing the flowers by a bouquet of mussels and frites. Yes, I am Belgian, don’t be jealous! ;o)

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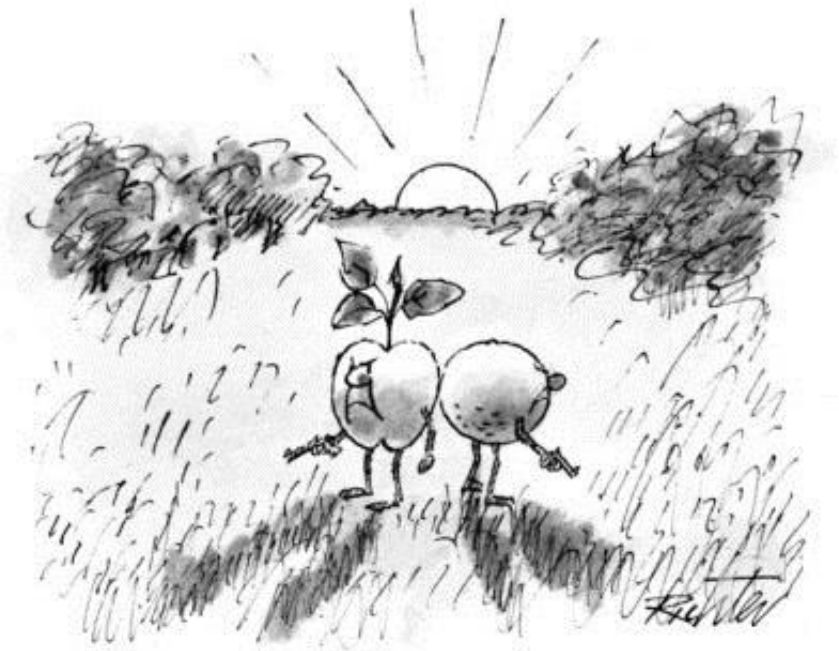

Plant defense against pathogens

Contents

List of abbreviations

Avr	Avirulence
ASPV	Apple stem pitting virus
Bp	Base pair
CC	Coiled coil
CDG	Candidate defense gene
cDNA-AFLP	cDNA amplified fragment length polymorphism
Chl	Chlorosis
Cv	Cultivar
eQTL	Expression quantitative trait loci
EST	Expressed sequence tag
ETI	Effector triggered immunity
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBS	Genotyping by sequencing
'GD'	'Golden Delicious'
gDNA	genomic DNA
GfG	Gene for gene
GMO	Genetically modified organism
GO	Gene ontology
GWA	Genome wide association
GWS	Genome wide selection
HR	Hypersensitive response
LD	Linkage desequilibrum
LG	Linkage group
LK	LRR-kinase
LRR	Leucine-rich repeat
MAB	Marker-assisted breeding
MAS	Marker-assisted selection
N	Necrosis
NBS	Nucleotide-binding site
PAMP	Pathogen-associated molecular pattern
PME	Pectin methylesterase
PR	Pathogenesis related
PTI	PAMP-triggered immunity
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
QTL	Quantitative trait loci
qPCR	real-time quantitative polymerase chain reaction
R	ResistantRGA Resistance gene analogue
R-gene	Resistance gene
RH	Relative humidity
RLP	Receptor-like protein
S	Susceptible
SC	Stellate chlorosis
SN	Stellate necrosis
SNP	Single nucleotide polymorphism
SSR	Single sequence repeat
TDF	Transcript derivied fragment
TIR	Toll interleukin1 receptor

General introduction

Context

Apple scab caused by the fungus *Venturia inaequalis* is the major constraint to apple production worldwide, causing severe economic losses. Currently, multiple applications of fungicides are required for effective control in commercial orchards mainly planted with highly susceptible cultivars. Depending on the year and the region, as many as 18 to 29 fungicide treatments may be necessary in one season to control the disease (Van Melckebeke, 1990; Way et al., 1991). This intensive use of fungicides raises ecological problems and human health concerns, in addition to the economic costs. The most effective alternative to chemical control is the use of apple cultivars resistant to scab. For more than 50 years, the major scab resistance genes (R gene), *Rvi6* (*Vf*) from *Malus floribunda* 821, has provided effective resistance against apple scab and allowed a reduction of 75% in the number of fungicide treatments (Parisi et al., 1995). However, this intensive use of a single R-gene in almost all commercial cultivars put a high selection of pressure on the pathogen and resulted in the emergence of *V. inaequalis* strains able to overcome *Rvi6*, rendering these cultivars highly susceptible to the disease (Parisi et al., 1993). Additional races of *V. inaequalis* (monoconidial isolates able to overcome the resistance in a host) have been demonstrated for other major scab R genes (Bus et al., 2011), showing the urgent need to characterize further R genes that can be used in a strategy to introduce durable resistance into apple breeding programs.

One method of delaying the breakdown of scab resistance is to pyramid in one cultivar multiple R genes showing complete and/or partial effects (Laurens et al., 2004; Joshi and Nayak, 2010). When complete resistance conditions strong resistance symptoms (such as the hypersensitive response, HR), partial resistance allows a reduction of the fungal development and sporulation as compared to a susceptible cultivar. Partial resistance has been mapped as quantitative trait loci (QTLs) in the apple genome (Gessler et al., 2006; Silverberg-Dilworth et al., 2006; Calenge et al., 2004; Durel et al., 2003, 2004; Liebhard et al., 2003; Bus et al., 2011). However, its molecular determinants remain largely unknown.

Aim of the thesis

The aim of this thesis is to provide new insights into the mechanisms of plant-pathogen interactions between various isolates of *V. inaequalis* and two apple cultivars showing contrasting nature of resistance genes and durability. The first apple cultivar, ‘Président Roulin’, is an old Belgian cultivar showing partial and durable resistance in the orchard. Its genetic determinism is largely unknown. The second apple cultivar ‘Geneva’, a scab-resistant open-pollinated selection of *Malus pumila* (Jefferson, 1970), is believed to carry multiple R-genes with major and minor effects (Bus et al., 2011), but despite its gene pyramidization, its resistance has been shown to be non durable in the field (Bengtsson et al. 2000; Beckerman et al. 2009). To have new insights into the apple-*V. inaequalis* interactions involved in these two cultivars, two approaches will be employed: a transcriptomics approach for the partial resistance in ‘Président Roulin’, and a genetics approach for the complex resistance in ‘Geneva’. Along with its intrinsinct interest for the understanding of the apple - *V. inaequalis* interactions, this investigation will provide new opportunities for the development of molecular marker tools to be used in genome-informed breeding programs as well as a better understanding of how these resistance genes could be employed to provide durable apple scab resistance.

Outline

This manuscript is structured as follows. First, a literature review on the genetics of host-pathogen relationships between *V. inaequalis* and *Malus* sp. is provided in Chapter 1. Then, the following chapters investigate the genetic determinism of the scab resistance in 'Président Roulin' and 'Geneva' apple cultivars, from the two different perspectives: the transcriptomics approach for the partial resistance of 'Président Roulin' (Chapter 2 and 3) and the genetics approach for the complex resistance in 'Geneva' (Chapter 4). Finally, Chapter 5 compils the main findings of this thesis and their future prospects in apple breeding.

Framework

This thesis was initiated in the framework of the POMINNO project: "Recherche de méthodes rapides de sélection de variétés innovantes de pommes de qualités différenciées et adaptées à une agriculture durable" supported by the Moerman funds (CRA-w) and jointly conducted by the Life Sciences Department (Breeding and Biodiversity Unit, Bioengineering unit) of the Walloon Agricultural Research Centre (CRA-w, Gembloux, Belgium) and the Phytopathology Unit of Gembloux Agro-Bio Tech, University of Liège (GxABT - ULg, Gembloux, Belgium; previously Faculté universitaire des Sciences Agronomiques de Gembloux). Investigations on the partially resistant cultivar 'Président Roulin' were made in the framework of this project.

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Chapter 1.

Genetics of host-pathogen relationships between *Venturia inaequalis* and *Malus* spp.: a review

Outline

Apple scab caused by the pathogen *Venturia inaequalis*, which has a long-term coevolution with its *Malus* host, is one of the most damaging diseases in orchards. Today, in a context of growing concerns about safe and healthy food produced in sustainable agricultural systems, durable resistance is a major goal of apple breeding programs worldwide. Achieving this objective will be facilitated by a comprehensive understanding of the host-pathogen relationships involving gene-for-gene (GfG) relationships between resistance genes in the host and avirulence genes in the pathogen. Here we present a review of the recent literature about the genetics of the apple - *V. inaequalis* interaction and the prospects in breeding for durable scab resistance, with an emphasis on recent developments and the new opportunities enabled by the revolution in DNA sequencing technologies.

Introduction

Apple (*Malus* x *domestica* Borkh.) is the second largest fruit crop produced in the world (after banana, FAOSTAT, 2012) and is also one of the most emblematic and widespread fruit crops in temperate regions. Even with the existence of more than 10,000 documented cultivars of apple (Janick et al., 1996; Jackson et al., 2011), commercial apple production is dominated by a limited number of cultivars, all highly susceptible to apple scab caused by the ascomycete fungus *Venturia inaequalis*. When it is not strictly controlled by large amounts of fungicides (from 18 to 29 treatments per season in some high disease regions, VanMelckebeke, 1990; Way et al., 1991), the fungus may defoliate trees, which can reduce tree growth and yield, and blemish fruit to a point where it is unmarketable. Such a large number of treatments raises numerous ecological and consumer health concerns, in addition to direct financial costs to growers. With the aim to find an alternative to the intensive use of pesticides, breeding for scab resistance became a high priority in most apple breeding programs throughout the world (Laurens, 1996). Growing resistant varieties has been shown to reduce the number of fungicidal treatments by 75% compared with susceptible cultivars (Parisi et al. 1995). Moreover, the recent availability of genomic techniques has brought more insight into the interaction of *V. inaequalis* and apple. In this review, a summary of the recent literature about the genetics of the *V. inaequalis*-apple interaction is presentend, as well as the resistance mechanisms of apple against scab. The prospects of breeding for scab resistance are also discussed, along with recent developments and new opportunities enabled by the revolution in DNA sequencing technologies.

The host plant

Malus (family *Rosaceae*, tribe *Pyreae*) is a complex genus consisting of a broad range of at least 24 primary species, distributed in Europe, Central and Eastern Asia, and three in North America (Way et al., 1990; Janick, 2005). Most of the wild apples are small (<3cm diameter) and bitter with the exception of the Kazak *M. sieversii*, which includes accessions containing all characters of modern apples in term of size, quality and colors (**Figure 1**). The first records of a form of domestic apples appeared in the Near East around 4,000 years ago (Zohary and Hopf, 2000) that also corresponds to the use of grafting techniques. The cultivated apple *M.* x *domestica* that was then introduced into Europe and North Africa by the Greeks and the Roman is thought to be the result of various interspecific hybridizations between wild *Malus* species over 2000 years of travel and trades along the Silk Road stretching from Rome in Italy through Samarkand in Uzbekistan to Luoyang in China. The ‘x’ in the species binomial denotes these various hybridizations.

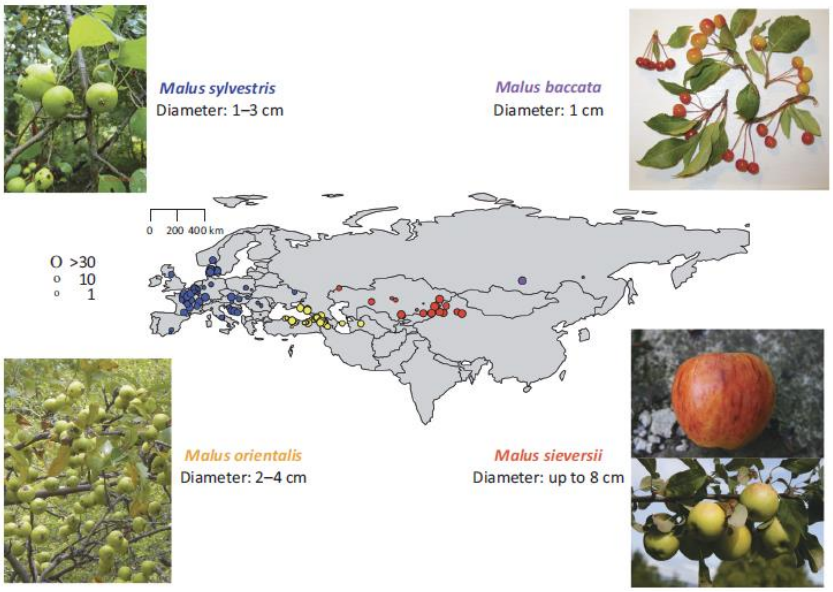


Figure 1: Distribution and key morphological features of wild apples: *Malus sylvestris* (blue), *Malus orientalis* (yellow), *Malus sieversii* (red) and *Malus baccata* (purple). Disk areas are proportional to the number of accessions. Pictures of the fruit of the different wild apples are provided, as well as their respective diameters (adapted from Cornille et al., 2014).

Recently, *M. sieversii* has been proved to be the main ancestral progenitor, with significant contributions of other wild species present along the Silk route, mainly *M. sylvestris* (European crabapple), a species native to Europe, but also to a lesser extent *M. baccata* native to Siberia and *M. orientalis* originating from eastern Asia (Harris *et al*., 2002; Coart et al., 2006; Cornille et al., 2012). This history was revealed by recent population studies using different types of molecular markers for evolutionary inferences (**Figure 2**). Interestingly, a high level of genetic diversity, similar to those found in wild apples, has been been maintained in the domesticated apple (Cornille et al., 2012; 2014). This could be due to the self-incompatible nature of *Malus* species and the traditional breeding strategy mainly based on the selection of the best phenotypes grown from open-pollinated seeds (‘chance seedlings’). Also, the isolation of farms and differences in taste preferences across regions probably favored the incorporation of diverse genetic material from multiple wild sources. Then, the spread of the grafting technique, together with the lengthy juvenile phase (5–10 years) and the long lifespan of apple, may have contributed to the maintenance of this high level of genetic diversity (Cornille et al., 2012; 2014). Our knowledge of the domestication history of apple has important implications for breeding programs. It highlights the need to invest efforts into the conservation of wild apple species and old apple cultivars as they are original genetic resources that could further improve the breeding value of domesticated apple germplasm, such as disease resistance (Lateur et al., 1996; Laurens et al., 2004; Coart et al., 2006).

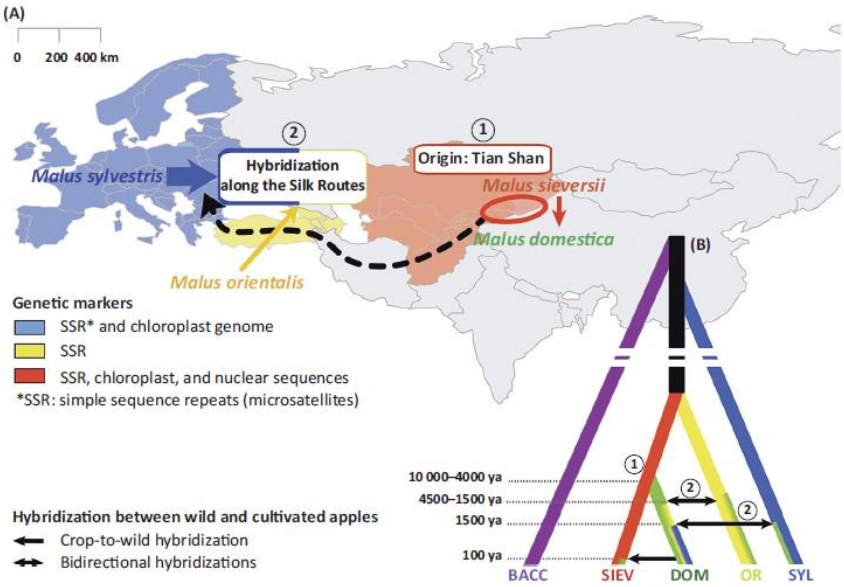


Figure 2: Evolutionary history of the cultivated apple (A) and genealogical relationships between wild and cultivated apples (B). (1) Origin in the Tian Shan Mountains from *Malus sieversii*, followed by (2) dispersal from Asia to Europe along the Silk Route, facilitating hybridization and introgression from the Caucasian and European crabapples. Arrow thickness is proportional to the genetic contribution of various wild species to the genetic makeup of *Malus domestica*. Approximate dates of the domestication and hybridization events between wild and cultivated species are detailed in the legend. Abbreviations: BACC, *Malus baccata*; DOM, *Malus domestica*; OR, *Malus orientalis*; SIEV, *Malus sieversii*; SYL, *Malus sylvestris*; ya, years ago (adapted from Cornille et al. 2014).

Whereas the haploid (x) chromosome numbers of most *Rosaceae* are 7, 8 or 9, *Pyreae*, (including the genus *Malus*) have a distinctive x = 17. This was demonstrated in the first extensive genetic map by Maliepaard et al. (1998) and was later confirmed in the recent publication of the whole genome sequencing of the domesticated apple (Velasco et al., 2010). A number of models have been proposed to explain the uniquely high number of chromosomes in *Pyreae* (Figure 3). Results from genome sequencing project support the hypothesis of a recent genome-wide duplication event from a 9-chromosome ancestor followed by a simple and parsimonious pattern of chromosome breakage and fusion leading to the current x = 17 *Pyrae* karyotype. The estimated genome size of the domesticated apple is 742.3 Mb with 57,386 genes (excluding transposable element–related genes, alleles, short predictions and low-level functional annotate genes). This putative gene content in apple is the highest reported so far, as compared to *Arabidopsis thaliana* (27,228 genes) or to other sequenced fruit species as papaya (28,027), or grape (33,514). The apple genome also has a relatively high number of repeated sequences (Velasco et al., 2010).

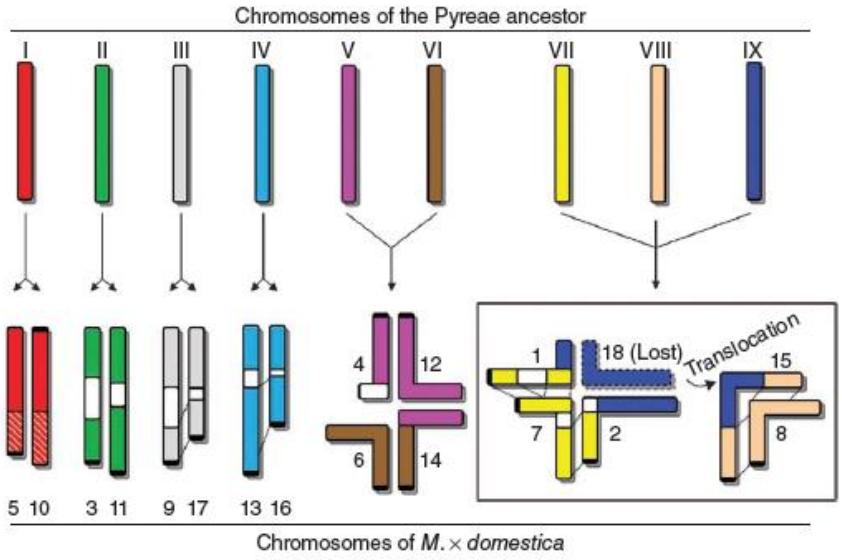


Figure 3 : A model explaining the evolution from a 9-chromosome ancestor to the 17-chromosome karyotype of extant *Pyreae*, including the genus *Malus*. A genome-wide duplication followed by a parsimony model of chromosome rearrangements is postulated. Shared colors indicate homology between extant chromosomes. White fragments of chromosomes indicate lack of a duplicated counterpart. The white-hatched portions of chromosomes 5 and 10 indicate partial homology (adapted from Velasco *et al*., 2010).

The pathogen

V. inaequalis (Cke.) belongs to the Ascomycetes class and is a hemibiotroph that infects members of the genus *Malus* as well as different hosts belonging to the subfamily *Maloideae*, such as *Crataegus*, *Sorbus*, *Pyracantha* and *Eriobotrya* (Raabe and Gardner, 1972; McHardy, 1996). However, since the isolates originating from the other species than *Malus* do not infect apple, but are characterized as *V. inaequalis* on the basis of morphological criteria, suggests that there is a need to further subdivide the species into either *formae speciales* or distinct species with separate host specificities (Le Cam et al., 2002; Bowen et al., 2011).

The oldest available report of scab is in the year 1819 by a Swedish scientist, Fries, however, it is likely that apple scab is much more ancient (McHardy, 1996). Genetic studies on populations of *V. inaequalis* suggest that the fungus originates from Central Asia, which also is the center of origin of wild *Malus* species (Gladieux et al., 2008, 2010). Consistent with this observation, the greater genetic diversity amongst populations of *V. inaequalis* has been found on *M. sieversii*, the major progenitor of the domesticated apple (Gladieux et al., 2010), as compared with the European populations found on *Malus* x *domestica*. It is likely that the pathogen tracked its hosts by following the dispersal of domesticated apple that accompanied Europeans as they migrated to new territories (Gladieux et al., 2008, 2010; Bowen et al., 2011). In the United States, Japan and Australia, *V. inaequalis* introductions have been relatively recent and occurred much later than the introduction of apple itself (McHardy, 1996). The genome size of *V. inaequalis* is estimated to be around 100 Mb (Broggini et al., 2007), while most of the other ascomycetes genomes are mostly in the range of 40 Mb. The whole genome sequence assembly of *V. inaequalis* is currently under process, but phytopathologists dispose of two recently published genetic maps of *V. inaequalis* constituted of 11, and 15 linkage groups spanning 1106 and 972

cM, respectively (Xu et al., 2009; Broggini et al., 2011). These maps do not span the entire *V. inaequalis* genome as it is composed of many more linkage groups than would be expected for a fungus with a base chromosome number of x = 7, as revealed during histological studies (Day et al., 1956). However, these give new insight into the genetics of *V. inaequalis* and provide valuable tools for the study of scab-apple interactions.

The life cycle of the fungus consists of an obligate sexual reproduction phase on infected apple leaf litter during the winter resulting in pseudothecia, followed by several cycles of asexual reproduction during the apple growing season (McHardy, 1996). The fungus is self-sterile, and hence opposite mating types are required for mating to succeed (Keitt and Palmiter, 1937). Briefly (**Figure 4**), epidemics begin in spring, when suitable temperatures and moisture promote the release of ascospores (sexual spore) from mature pseudothecia. These spores rise into the air, land on the surface of the leaf cuticle, where they germinate by producing a germ tube that differentiates into an appressorium held fast to the leaf surface by means of a mucilaginous sheath. Then, minute infection hyphae are formed, at or near the center of the pore in the appressorium, which directly penetrate the host surface and differentiate into subcuticular stroma. Under disease-conducive environmental conditions, conidiophores are initiated within 10 to 14 days. Conidiophores enlarge, pierce or split the cuticular layer of the leave to produce and release conidia. These asexual spores continue the cycle of secondary infection throughout the summer by germinating and infecting other areas of the host tree. At the beginning of the winter, with the onset of leaf fall, the mycelium penetrates a bit deeper into leaf tissue, forms pseudothecial initials and enter a period of dormancy. In the early spring, *V. inaequalis* switches from the vegetative to a reproductive phase, wherein the mycelia of two different mating types undergo sexual reproduction (mating). A new generation of ascospores is produced, constituting the primary inoculum (MacHardy, 1996; Vaillancourt and Hartman, 2000).

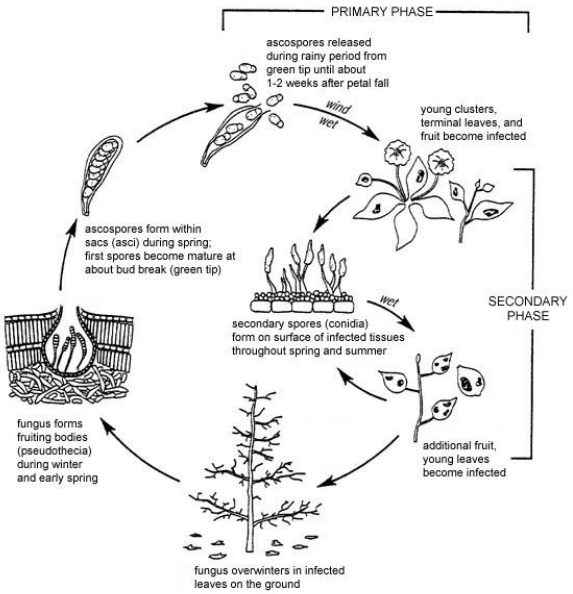


Figure 4 : The life cycle of *Venturia inaequalis* consists of a single obligate sexual generation and several asexual generations per year. During winter, when the fungus is saprophytic on foliar litter, ascospores are produced by sexual reproduction between strains of compatible mating types. In spring, ascospores cause primary lesions on young apple leaves (primary phase), which is followed by several asexual generations producing conidia that spread the disease primarily through rain splashing on leaves and fruits (secondary phase). Adapted from Vaillancourt and Hartman (2000).

Venturia-Malus interaction

The *V. inaequalis-Malus* interaction was one of the first examples for which gene-for-gene (GfG) relationships were suggested (Boone and Keitt, 1957; William and Shay, 1957). This assumption was based on the differential interaction observed using single-spore isolates of *V. inaequalis* in segregating populations of both *Malus* and *V. inaequalis* (Bonne, 1971, Williams and Shay, 1957, Williams and Kuc, 1969). In this model, the resistance relationship is hypothesized to be the result of a specific recognition event, either direct or indirect, between a host resistance gene (R-gene) product and a corresponding avirulence (*Avr*) gene product in the pathogen (Jones and Dangl, 2006). The classical receptor-ligand model (Bonas and Lahaye, 2002) proposes a direct recognition interaction between an elicitor protein of the *Avr* gene and a receptor protein in the host (**Figure 5a**). The recognition event by the resistance protein triggers a signal cascade leading to a hypersensitive response (HR) in the plant, which can include rapid localized cell death, formation of reactive oxygen species, accumulation of phytoalexins, cell wall fortification, and/or formation of pathogenesis-related proteins (Jha et al., 2010; Bowen et al., 2011; Daniëls, 2013). Conversely, in the absence of the R gene, no recognition takes place and a cascade of pathogenicity genes in the pathogen is activated, for example, leading to the assembly of infection and feeding structures, secretion of enzymes degrading the cuticle and cell walls, and production of host-selective toxins (Jha et al., 2010; Bowen et al., 2011). Each single-spore isolate of the pathogen able to overcome completely the resistance in the host was defined as a "race". A mutation of the *Avr* locus in the pathogen would be at the origin of the non-recognition by the host, hence leading to complete susceptibility (Hulbert et al., 1997; Ellis et al., 1999; Bus et al., 2011). Several other models for indirect interaction have been proposed (Bonas and Lahaye, 2002), such as the co-receptor model in which the *Avr* protein binds first to a high-affinity binding site of the co-receptor, which interacts with the R protein to elicit the defense response (**Figure 5b**); and guard protein model (**Figure 5c**) where the resistance protein safeguards a matching pathogenicity target (Bonas and Lahaye, 2002).

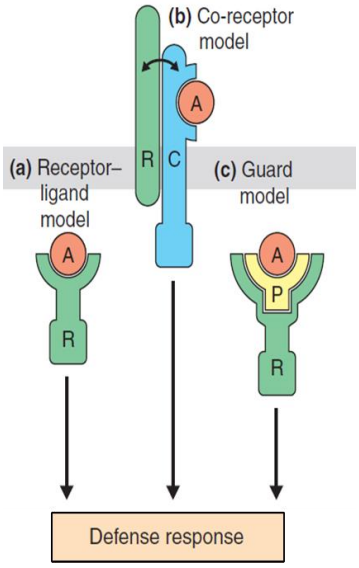


Figure 5 : Biochemical models for the gene-for-gene relationship. These models are not mutually exclusive. A = avirulence protein; R = resistance gene protein; C = co-receptor; P = pathogenicity target. Adapted from Bonas and Lahaye (2002).

Figure 6 illustrates differential interactions observed in *M. sieversii* accessions after inoculation with various races of *V. inaequalis* that permitted the identification and mapping of the *Rvi8* (*Vh8*) scab resistance locus (Bus et al., 2005b). Based on the hypothesis that differential interactions result from GfG relationships between the host and the pathogen, a GfG relationship model was proposed: isolate EU-B05 and 1639 are incompatible (-) with hosts carrying two different R-genes conditioning stellate necrosis (SN) and chlorosis reactions, respectively, while NZ188B.2 is compatible (+) with hosts carrying either gene.

	EU-B05	1639	NZ188B.2
SN gene	-	+	+
Chl gene	+	-	+

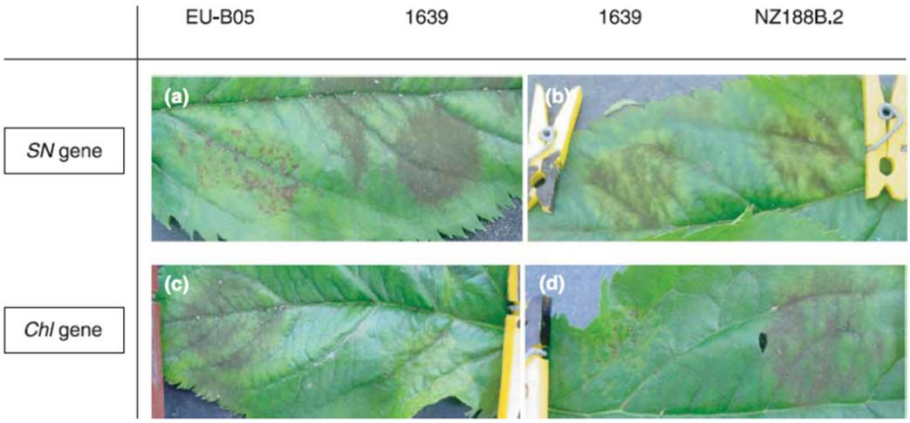


Figure 6: At the top, (left) droplet inoculation technique involving the use of small inoculation chambers allowing the observation of resistance reactions of two different isolates on the same leaf, and (right) the model of gene-for-gene relationships hypothesized from the differential interactions observed on the leaves using this method (a-d). Inoculation with four single-spore isolates of *Venturia inaequalis* on *Malus sieversii* accessions carrying a stellate necrosis R-gene (SN) corresponding to the *Rvi8* (*Vh8*) locus or a chlorosis (Chl) R-gene showed isolates EU-B05 and 1639 to be incompatible (-) with hosts carrying the SN and Chl gene, respectively, while NZ188B.2 was compatible (+) with hosts carrying either gene. Adapted from Bus et al. (2005b).

Different classification systems were developed to characterize the multiplicity of symptoms observed on scab infected leaves, ranging from complete resistance to susceptibility. These help plant breeders to group the progeny of controlled crosses in identifiable classes of resistance reactions (Hough, 1944, Chevalier *et al*., 1991). The most informative classification systems provide quantitative and qualitative descriptions of the host response, e.g. the qualitative scale of Chevalier et al. (1991) assigning the foliar symptoms into five classes coupled with the quantitative scale of Lateur and Populer (1994) for scab incidence or sporulation severity. With these Chevalier classes, the rate of response generally determines the type and intensity of the resistance reaction. Class 1 corresponds to the fast hypersensitive response (HR), which results in the complete inhibition of pathogen development through a local programmed cell death and the apparition of pinpoint-pit reactions on the epidermal cells from two to six days after infection.

The class 2 of reaction appears later during the infection (between 7 and 10 days), and is characterized by chlorotic lesions (Chl) with irregular edges would centre can be slightly necrotic. When the density of symptoms is high, the leaves can take on a crinkled aspect. To the original publication of Chevalier et al. (1991), we can add in the class 2 of reaction the distinctive stellate necrotic (SN) and stellate chlorotic (SC) reactions. In this type of host defense reaction, the delay in the onset of resistance allows some stroma development, where strands of subcuticular mycelium radiate out from the initial point of infection, resulting in growth in the shape of a star (Bus et al., 2005b). Within such reactions, conidiophores can sometime differentiate, but are never able to sporulate. In contrast, slower resistance reaction are expressed as necrosis (N) and/or chlorosis (Chl) and involve sporulation to different degrees from low (class 3A) to high (class 3B). In these classes, a mix of aborted and sporulating conidiophores are found. Finally, class 4 symptom includes the abundantly sporulating lesions with no host reaction.

All these resistance reaction types are represented by the plethora of major resistance genes that have been mapped to date. **Figure 7** illustrates various classes of reaction symptoms on apple leaves observed macroscopically by Gessler et al. (2006), or microscopically under brightfield or autofluorescence (Bastiaanse, unpublished). Microscopic observations suggest that HR and SN resistance reactions involve many more cells than those in direct contact with the appressorium and or mycelium of the fungus. This suggests that extensive cell-to-cell signaling of the resistance response takes place in an attempt to contain the infection.

Venturia-Malus co-evolution

While the secondary infections with asexual spores (conidia) cause severe damage on apple because of the multiplicity of cycles during the season, sexual reproduction of *V. inaequalis* is of great importance for the management of apple scab resistance in the orchard. It offers the possibility to the pathogen to rapidly adapt to its perennial host, maintained for years in orchards, and to its changing environment through the accumulation of virulence genes in new pathotypes (Lê Van et al., 2012). This adaptive potential of the pathogen, coupled with low host species diversity in commercial orchards, favours a high selection pressure towards virulence in the pathogen. As a consequence, constant efforts by apple breeders are required to avoid, or at least delay, the breakdown of host resistance by the pathogen, which is unevitable if not managed even for a gene, such as the major scab resistance gene *Rvi6* (*Vf*) (Parisi et al., 1993) that seemed durable on its own. For breeding apple cultivars with durable resistance to *V. inaequalis*, it is essential to understand how the pathogen virulence can adapt to its host and the extent to which evolutionary forces the pathogen to shift its virulence. Apple scab is now well established worldwide and has been shown to display within-population diversity from country to country (Tenzer and Gessler, 1999; Gladieux et al., 2008), region to region (Tenzer and Gessler, 1997; Guérin et al., 2007), or even from tree to tree inside the same orchard planted with various cultivars (Guérin and Le Cam, 2004; Barbara et al., 2008).

Although more information on within-population variability is needed to better understand the forces acting on the host-pathogen co-evolution, it is now clear that the best germplasm for resistance breeding is generally found at the host's centre of origin and diversity, where host and pathogen have co-evolved for the longest period of time, as stated by the theory of Nikolai Vavilov (Zhukovsky, 1961). In apple, the lowest diversity in both the cultivated hosts *M.* x *domestica* and the associated *V. inaequalis* population was found in regions away from the centre

of diversity. As a result, wild *Malus* species of East Asia, e.g. *M. floribunda*, *M. micromalus*, *M. doumeri* and *M. hupehensis*, remain a substantial resource for apple breeding programs worldwide (Bowen et al., 2011).

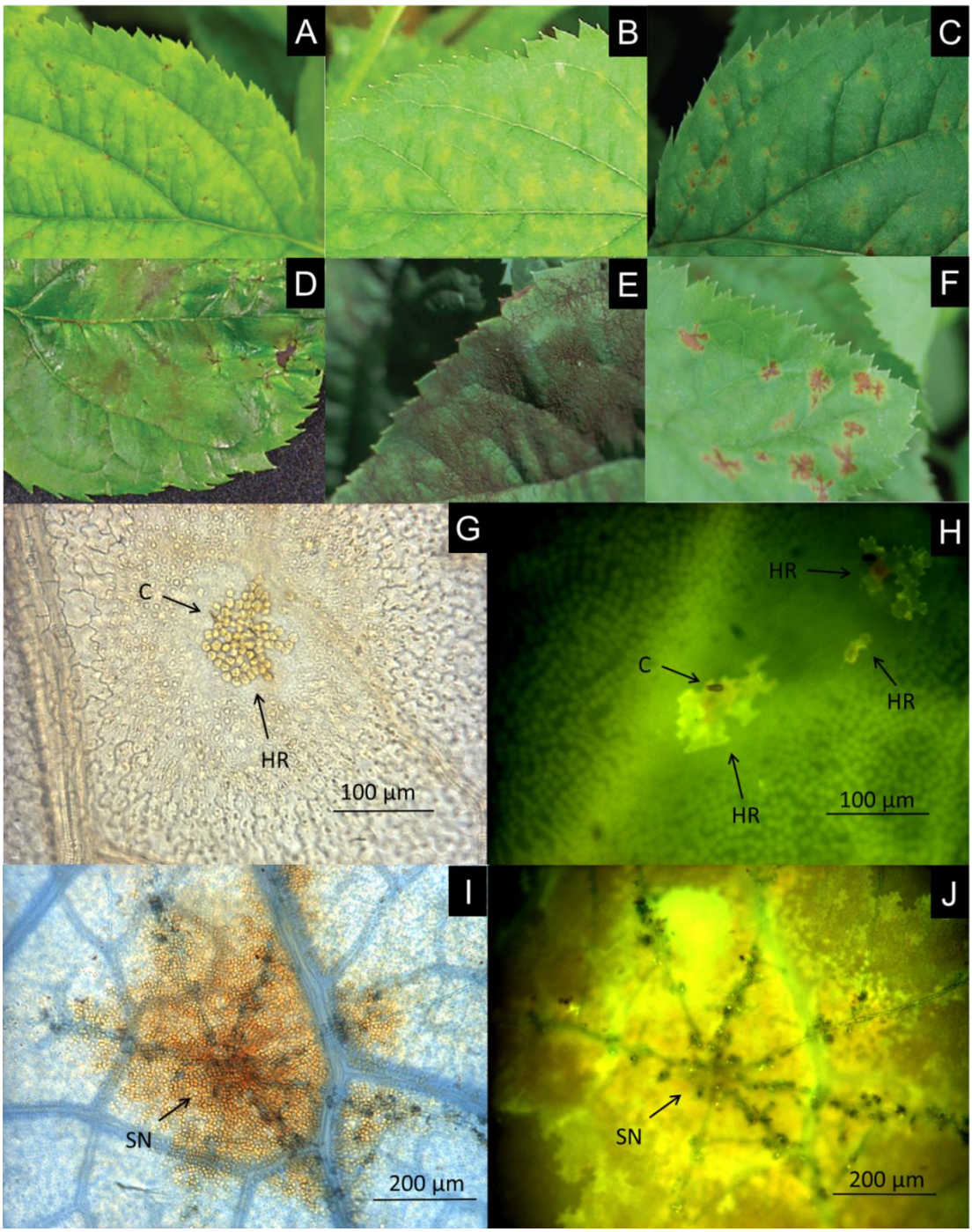


Figure 7: Macroscopic (A to F, adapted from Gessler et al., 2006) and microscopic scab reactions on apple leaves: (A) class 1 with HR; (B) class 2 with chlorotic lesions; (C) and (D) class 3A and 3B with chlorotic and necrotic with slight sporulation and clearly sporulating lesions; (E) class 4 complete susceptibility; (F) stellate necrotic reaction; (G) and (H) germinated conidia and collapsed epidermal cells of an hypersensitive response observed under brightfield and autofluorescence microscopy; (I) and (J) stellate necrosis reaction conditioned by the *Rvi8(Vh8)* scab resistance gene observed under birghtfield and autofluorescence microscopy. Under autofluorescence microscopy, resistance reactions showed multiple fluorescent cells to be involved. *HR* hypersensitive reaction, *C* conidia, *SN* stellate necrosis.

Concept of complete and partial resistance and resistance spectrum

As in many plant pathogen interactions, two types of disease resistance, complete and partial resistance, have been recognized to govern the interactions between *Malus* and *V. inaequalis*. Many different definitions have been used to describe these resistances, including horizontal vs vertical, complete vs incomplete, major-gene vs minor-gene and narrow-spectrum vs broad-spectrum, these definitions having different meanings and interpretations (Van der Plank, 1963; Parlevliet and Zadoks, 1977). In this review, complete and partial resistances are defined phenotypically. Complete resistance usually confers high levels of resistance, and fully inhibits pathogen reproduction. In contrast, partial resistance confers a reduction rather than a lack of disease. In such cultivars, pathogen reproduction is limited and significantly reduced as compared to susceptible one.

In host (*vs* nonhost) resistance, complete resistance is often conferred by single gene of major effect and is transmitted to the progeny in a simple Mendelian fashion (ratio 1:1). Partial resistance is controlled by multiple loci of partial effects, referred as quantitative trait loci (QTLs). These complex resistance phenotypes tend to be measured quantitatively. The size of the lesions, the amount of sporulation, latency period, and number of symptoms resulting from the infection may differ depending on the cultivar and the environment. Partial resistance usually does not fit simple Mendelian ratios. When partial resistant cultivars are crossed with highly susceptible ones, this resistance can be traced back in just a very small proportion of the resulting progenies. As compared to major R-genes that can be easily manipulated, partial resistance has brought little attention in breeding programs, and mechanisms of defense underlying such resistance remain largely unknown.

Another concept, informative for the type of resistance encountered in apple cultivars, is the concept of spectrum of the resistance (Bus et al., 2011). This concept refers to the proportion of the pathogen against which the resistance is effective. In this respect, a narrow spectrum resistance would be effective against only a few isolates of the pathogen population, when a broad spectrum resistance would be effective against most, if no all the isolates of the pathogen population. In any genetics study of apple scab resistance, it is recommended to describe independently the spectrum of the resistance gene (narrow/ broad spectrum) from its effect (complete-partial).

Major scab R-genes in the host

A plethora of very narrow spectrum resistance genes, with GfG relationships are present in many apple cultivars (Shay and Williams, 1956; Boone and Keitt, 1957; Bagga and Boone, 1968a,b; Boone, 1971; Sierotzki et al., 1994; Koch et al., 2000; Barbara et al., 2008), but these genes are of poor interest for apple breeding since they confer insufficient field resistance. To date 18 major scab R-genes have been described and mapped in apple (Shay and Hough, 1952; Shay et al., 1953; Bagga and Boone, 1968b; William and Kuc, 1969, Durel *et al*., 2000; Patocchi *et al*., 2004; Tartarini *et al*., 2004; Bus *et al*., 2004; Bus *et al*., 2005a, 2005b, Soriano et al., 2014). Recently, a new gene nomenclature system based on international standard used for *Arabidiopsis* has been proposed for the *Malus-V. inaequalis* interaction (Bus et al., 2011). In this nomenclature system, each major resistance gene is designed as *Rvik* (R refers to resistance gene, *vi* to *Venturia inaequalis*, and *k* to the differential host), and the corresponding *Avr* gene of the pathogen is

named *AvrRvik*. This nomenclature is complemented by a set of apple differential hosts each carrying only one *Rvik* gene, and single-spore reference isolates of the pathogen having altered or lost only the complementary allele, identified as *avrRvik* at the *AvrRvik* locus. These unique *Rvik-AvrRvik* interactions will aid breeders in the preservation of known R genes and the identification of new ones.

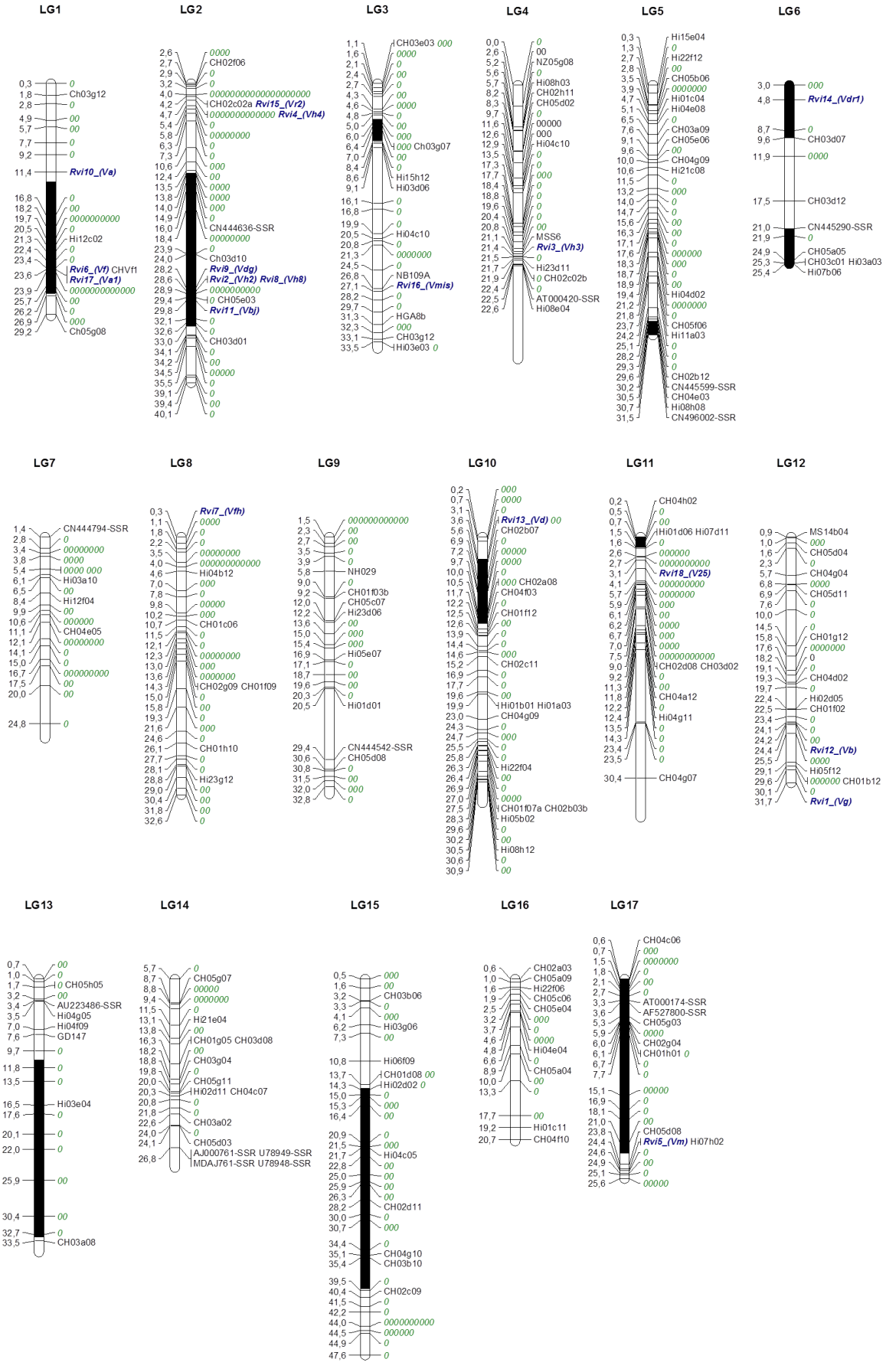
Table 1: Nomenclature of the gene-for-gene relationships between *V. inaequalis* and *Malus.* The races are defined by the avirulence genes they are lacking, hence resulting in susceptibility on the complementary host. Adapted from Bus et al. (2011), with addition of the *Rvi18* (*V25*) recently described by Soriano et al. (2014).

		Malus				***Venturia inaequalis***			
Differential host			**Resistance locus**			**Avirulence locus**			
Nb	**Accession**	**Pheno-type**	**Historical**	**LG [a]**	**New**	**New**	**Old**	**LG[e]**	**Race**
h(0)	Royal Gala	S							(0)
h(1)	Golden delicious	N	*Vg*	12	*Rvi1*	*AvrRvi1*			(1)
h(2)	TSR34T15	SN	*Vh2*	2	*Rvi2*	*AvrRvi2*	p-9	A6	(2)
h(3)	Geneva [b]	SN	*Vh3*	4	*Rvi3*	*AvrRvi3* [d]	p-10		(3)
h(4)	TSR33T239	HR	*Vh4=Vx=Vr1*	2	*Rvi4*	*AvrRvi4* [d]		4	(4)
h(5)	9-AR2T196	HR	*Vm*	17	*Rvi5*	*AvrRvi5*			(5)
h(6)	Priscilla	Chl	*Vf*	1	*Rvi6*	*AvrRvi6*		2	(6)
h(7)	LPG3-29 [f]	HR	*Vfh*	8	*Rvi7*	*AvrRvi7*			(7)
h(8)	B45	SN	*Vh8*	2	*Rvi8*	*AvrRvi8*		A4	(8)
h(9)	K2	SN	*Vdg*	2	*Rvi9*	*AvrRvi9*	p-8	A6	(9)
h(10)	A723-6 [b]	HR	*Va*	1[c]	*Rvi10*	*AvrRvi10* [d]			(10)
h(11)	A722-7	SN/Chl	*Vbj*	2	*Rvi11*	*AvrRvi11* [d]			(11)
h(12)	Hansen's baccata#2[b]	Chl	*Vb*	12	*Rvi12*	*AvrRvi12* [d]			(12)
h(13)	Durello di Forli	SN	*Vd*	10	*Rvi13*	*AvrRvi13* [d]			(13)
h(14)	Dülmener Rosenapfel [b]	Chl	*Vdr1*	6	*Rvi14*	*AvrRvi14* [d]			(14)
h(15)	GMAL 2473	HR	*Vr2*	2	*Rvi15*	*AvrRvi15* [d]			(15)
h(16)	MIS op 93.051 G07-098 [b]	HR	*Vmis*	3	*Rvi16*	*AvrRvi16* [d]			(16)
h(17)	Antonovka APF22 [b]	Chl	*Va1*	1	*Rvi17*	*AvrRvi17* [d]			(17)
h(18)	1980-015-025[b]	HR	*V25*	11	*Rvi18*	*AvrRvi18*			(18)

[a] LG = linkage group of apple.
[b] Temporary differential host until the host has been confirmed as being monogenic, or a monogenic progeny from this polygenic host has been selected.
[c] Provisional placement based on the assumption that the resistance in sources PI 172623 and PI 172633 are identical.
[d] Gene-for-gene relationship not confirmed to date.
[e] from Broggini et al. (2011). A6 and A4 refer to minor LG in *V. inaequalis*.
[f] Differential host proposed by Caffier et al., 2015.

The 18 major scab R-genes and their corresponding *Avr* genes are presented in **Table 1**, many of which originated from small-fruited Asian *Malus* species. Finally, for 7 additional R-genes, the status of differential hosts still has to be confirmed since some uncertainty remains on the presence of a unique R-gene on this host. For this reason, a *Rvik* number as assigned and a temporary host designated until the host has been confirmed as being monogenic, or a monogenic progeny from this polygenic host has been selected (Bus et al., 2011).

Major scab R-genes were mapped on 10 out of the 17 LG of apple. To date, only two of them have been cloned. This gave new insight into the molecular mechanisms or the apple defense system against scab. While the *Rvi6* resistance locus revealed the presence of a cluster of four resistance gene paralogs (called *HcrVf* genes), similar to the tomato *Cladiosporium fulvum* R-gene family of tomato encoding leucine-rich repeats receptor-like proteins (LRR-RLP) (Vinatzer et al., 2001), the *Rvi15* (*Vr2*) was reported to contain three TIR-NBS-LRR genes (Galli et al., 2010). The function of all these genes was analysed and only two of them, *HcrVf2* (Belfanti et al, 2004; Joshi et al., 2011) and *Vr2-C* (Schouten et al., 2014) for *Rvi6* and *Rvi15*, respectively, were proven to be functional against *V. inaequalis*. Literature is not in agreement on *Vf1a* (syn. *HcrVf1*) function (Joshi et al., 2011; Malnoy et al., 2008). Paralogs of such family proteins have been found in the apple genome and could underlie the resistance of the resistance loci: about 22 *HcrVf* paralogs were found on LG1 and LG6 in cultivars 'Florina' (Broggini et al., 2009), and 992 resistance genes analogues (RGAs) belonging to the NBS-LRR family proteins and 320 LRR-kinase (LK) genes have also been annotated on the whole genome sequence assembly of 'Golden Delicious'. This is much more than what has been found in other plant genomes, such as in *Arabidiopsis* (178 NBS-LRR and 216 LK genes) (Troggio et al., 2012). A high fraction (80%) of the NBS-LRR occurred in clusters, indicating frequent tandem duplication and ectopic translocation events (Perazzolli et al., 2014). The genomic distribution of RGAs is not uniform, with apple chromosomes 2, 7 8, 11 and 15 containing almost twice as many resistance genes than the others (Troggio et al., 2012; Perazzolli et al., 2014). Organization of major genes, QTLs for apple scab resistance and clusters of RGAs on the physical map of the reference cultivars 'Golden Delicious' is presented in **Figure 8**. This map has been created by anchoring on 'Golden Delicious' the flanking molecular markers of major R-genes and QTLs for apple scab resistance (Gessler et al., 2006; Silverberg-Dilworth et al., 2006; Calenge et al., 2004; Durel et al., 2003, 2004; Liebhard et al., 2003; Bus et al., 2011; Soriano et al., 2014). Positions of individual and clusters of RGAs were retrieved from (Perazzoli et al., 2014).


LG1
LG2
LG3
LG4
LG5
LG6
LG7
LG8
LG9
LG10
LG11
LG12
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LG14
LG15
LG16
LG17

Figure 8: Genome organization (in Mbp) of apple scab resistance genes (R-genes), quantitative trait loci (QTLs), and resistance gene analogues (RGAs) on the physical map of the 'Golden Delicious' reference genome (Velasco et al., 2010). QTLs and major R-genes published in the literature were anchored on the map by blasting their flanking molecular markers (Gessler et al., 2006; Silverberg-Dilworth et al., 2006; Calenge et al., 2004; Durel et al., 2003, 2004; Liebhard et al., 2003; Bus et al., 2011). Major R-genes are represented in blue, confidence intervals of QTLs are represented by the back bars. Dots indicate the position of individual and clusters of RGAs retrieved from Perazzolli et al., (2014). Number of dots is representative of the number of RGAs inside a cluster.

Avr genes in *V. inaequalis*

Recent efforts have been made to fully characterize the interactions of reference *V. inaequalis* isolates with the apple scab differential hosts, and hence their *Avr* genes (Caffier et al., 2015). Knowing the race status of these isolates regarding the full set of major apple scab resistance genes constitutes a precious tool for breeder for the correct genetics characterization of their progenies. Description of these status is presented in **Table 2**. Resistance reactions were showed to form a continuum from weak resistance to complete susceptibility as shown by Chevalier et al. (1991).

Table 2: Description of the race status of 23 Venturia inaequalis isolates inoculated on 14 apple differential hosts using two inoculation techniques (spray inoculation or droplet inoculation). Adapted from Caffier et al., 2015.

Isolate	Race[a]	Host not tested[b]
2410 = US4 [c]	(-[d])	h(3)
104	(1)	h(9)
301	(1)	h(3), h(9)
EU-B05	(1)	h(7)
405	(1,4)	h(9)
1634	(1,4)	h(9)
1638	(1,4)	h(9)
302	(1,6)	h(3), h(9)
2199	(1,6)	h(9)
2409 = 1774-1	(1,7)	h(4)
EU-B04	(1,10)	
EU-NL19	(1,10)	h(7)
413	(1,2,10)	h(3), h(9)
1680	(1,6,7)	h(8), h(9)
EU-D42[a]	(1,6,10)	
147	(5,7)	h(9)
1639VB	(1,2,8,9)	
2408 = NZ188B	(1,7,8)	
EU-NL24	(1,3,6,7)	h(2), h(4)
1066e	(6,7,8,10)	
1127	(6,7,8,10)	h(9), h(13)
EU-NL05	(6,7,8,10,13)	
174[c]	(1,7,8,10,13)[e] or (1,5,7,8,10,13)[f]	

[a] Description of the virulence pattern following the nomenclature described in Bus et al. (2011)
[b] Differential hosts h(11), h(16), h(17) were not tested in the study; h(3), h(4), h(7), h(8), h(9) and h(13) were missing when indicated
[c] Isolates 2410 and 174 were avirulent on the susceptible host h(0) with the droplet technique
[d] Isolate 2410 was avirulent on the 14 hosts carrying major resistance genes
[e] Race status following spraying technique for isolate 174
[f] Race status following droplet inoculation technique for isolate 174

Furthermore, on a quest to gain more information about the genomic organization and genetic control of *Avr* genes and their primary function in *V. inaequalis*, Broggini et al. (2011) investigated the inheritance of *Avr* traits on resistant *Malus* accessions with a progeny of a cross between race (1, 10) isolate EU-B04 and race (1,2,8,9) isolate 1639 (**Figure 9**). Most *Avr* loci were inherited independently as it was demonstrated earlier (Bénaouf and Parisi, 2000; Sierotzki

and Gessler, 1998a, b; Williams and Shay, 1957). Independent segregation of *Avr* loci provides the pathogen with a large potential to develop new pathotypes during the sexual reproduction phase (Boone, 1971). Nevertheless, existence of clusters of *Avr* genes in the pathogen was also demonstrated. These Avr genes also corresponded to R-genes in the host derived from unrelated sources, suggesting that the *Avr* proteins are related targets of the different resistance genes (William and Shay, 1957; Broggini et al., 2011).

Most of the *Avr* genes identified in this study involved narrow spectrum resistance genes, of poor interest for apple breeding, but also gave new insights into the location *Avr* loci corresponding to some broad spectrum R-genes: *AvrRvi6* mapped on LG2, *AvrRvi8* on A4 (minor LG) and, finally, *AvrRvi2* and *AvrRvi9* were linked on A6. Finally, the interaction, direct or indirect, of multiple resistance gene products with one *Avr* gene product has been proposed (Jones and Dangl, 2006; Broggini et al. 2011) to explain segregation distortion observed among the *Avr* genes phenotypes. This scenario would have important consequences for apple breeding. If one virulent strain was able to overcome multiple R-gene, pyramiding of these corresponding R-genes will not be effective in the breeding for durable resistance.

None of the *Avr* identified in *V. inaequalis* has been cloned successfully to date (Broggini et al., 2007), but studies on the primary function of such effectors have been initiated (Win et al., 2003; Kucheryava et al., 2008; Bowen et al., 2009). For instance, in order to identify the product of the *AvrRvi5* gene of *V. inaequalis*, a set of proteins from an *in vitro* fungal culture was isolated that induced a necrosis similar to an HR reaction following injection in an *Rvi5*-resistant cultivar, but not in a susceptible one (Win et al., 2003). However, the gene encoding for this protein could not be identified.

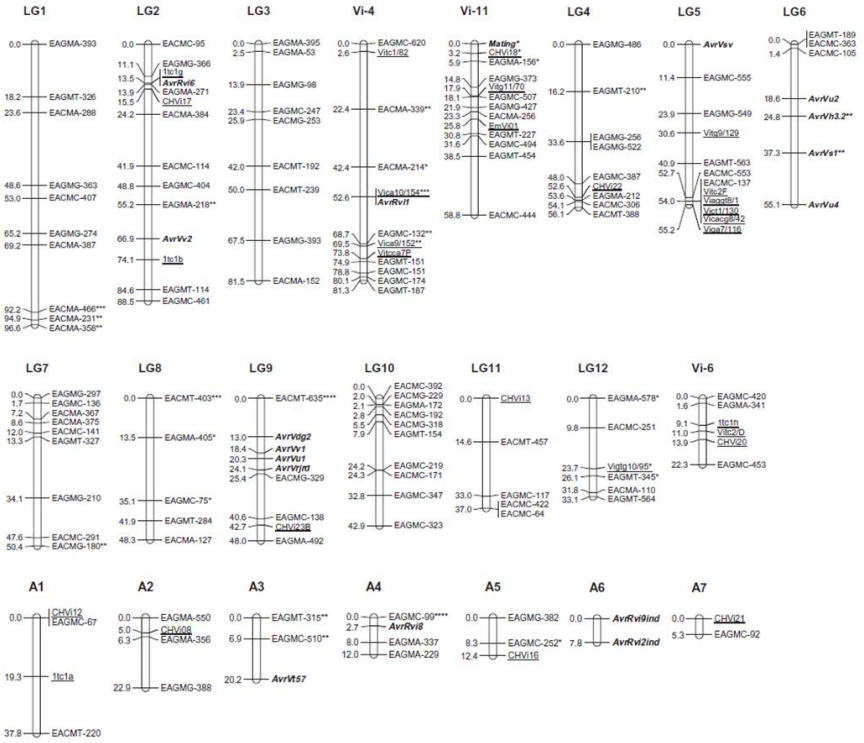

Figure 9: The 15 major and seven minor linkage groups of 83 progeny derived from a cross of *Venturia inaequalis* isolates EU-B04 and 1639. Simple sequence repeat (SSR) markers are underlined, avirulence loci based on the phenotype of the progeny are shown in bold and italics. Linkage groups were ordered by size. Segregation distortion is indicated by means of significance level p of the chi-square test: *= 0.05, **=0.01, ***= 0.005, **** = 0.001, *****= 0.0005. Figure adapted from Broggini et al. (2011).

Quantitative trait loci for apple scab resistance

In order to diversify the resistance sources used in breeding programs (complete vs. partial), efforts have been renewed to identify partial apple scab resistance among apple cultivars. Hence, some apple breeding programs worldwide, such as the apple breeding program of CRA-w (Gembloux, Belgium), mainly base their progenies on the use of apple cultivars carrying partial apple scab resistance, to be crossed with modern commercial cultivars or with cultivars carrying major scab resistance genes. At CRA-w, old Belgian cultivars are collected from public collections, farms or private gardens and screened for their moderate susceptibility to scab, powdery mildew and canker as well as for their pomological characters (Lateur and Populer, 1994; Lateur et al., 1996).

A European initiative, the DARE project (Durable Apple Resistance in Europe) funded by the European Union (Durel et al., 1999, 2003; Liebhard et al., 2003; Calenge et al., 2004), followed by the later study of Soufflet-Freslon et al. (2008) made significant improvement for the use of partial resistance in breeding programs by mapping QTLs for scab resistance on the apple genome. Genetic studies were carried out on six mapping populations against field inoculum and several races of *V. inaequalis* to identify QTLs providing broad-spectrum resistance. The polygenic resistance sources used in these experiments were 'Discovery,' TN10-8 (a selection from Schmidt's 'Antonovka' PI 172632), 'Durello di Forlì' and 'Dülmener Rosenapfel'. They were crossed with the susceptible cultivar 'Gala', the moderately susceptible cultivar 'Fiesta' or with the cultivar 'Prima' carrying the *Rvi6* and *Rvi1* major resistance genes. These studies highlighted five major genomic regions involved in broad-spectrum scab resistance: they were located on LG1, LG2, LG6, LG11, and LG17. In addition, other QTLs with rather strong specificities towards the tested isolates were identified on LG3, LG5, LG10, LG13 and LG15 in five different progenies. Effects of individual QTLs on the phenotypes, measured as the proportion of phenotypic variance explained by the genotype (R^2), ranged from 4% to 68%. Interestingly, the highest R^2 values were attributed to LG1 and LG17. While LG1 carries major R-genes, LG17 is not known to carry such genes for scab resistance.

Genetic organization of major R-genes, QTLs and RGAs in apple

The availability of the whole genome sequence assembly of 'Golden Delicious', and the annotation of numerous RGAs belonging to the NBS-LRR family resistance as well as defense-related genes (Velasco et al., 2010), provided an opportunity to test here their co-localization with major R-genes and QTLs for apple scab resistance. This analysis undergone in the frame of this thesis demonstrates (**Table 3**) that RGAs more frequently co-localized with regions shown to be involved in scab disease resistance, either major genes or QTLs compared with a random set of cDNA from the uninfected apple leaves cDNA library AELA of Newcomb et al. (2006). While RGAs belonging to the NBS-LRR family proteins have been recognized to be at the origin of most of the GfG relationships observed in complete plant resistance to pathogens (McHale et al., 2006), including the scab R-gene *Rvi15* (*Vr2*) (Schouten et al., 2014), the genes governing partial plant resistance remain largely unknown, with a very few minor genes for partial resistance having been cloned (Poland et al., 2009; Fukuoka et al., 2009; Krattinger et al., 2009; St Clair, 2010), with none in apple.

Table 3 : Frequencies of co-localization of candidate genes (resistance gene analogues and defense-related genes) with major R-genes and QTLs for apple scab resistance

	Total sequences tested	Mapped scab R gene [a] (%)	QTL [a]
RGA[b]	902	5,3 % ***	22,1% *
Defense gene [b]	112	0 %	13%
Random cDNA [a]	3795	0,8%	18,5%

[a] QTLs and scab R-genes identified in the literature (Gessler et al., 2006; Soufflet-Freslon, 2008; Bus et al., 2011) were mapped *in silico* on the 'Golden delicious' whole genome sequence assembly (Velasco et al., 2010) by blasting the flanking molecular markers. Frequencies of co-localization of RGAs and defense genes with R-genes and QTLs were compared with frequencies observed with an uninfected apple library AELA (Newcomb et al., 2006) using $\chi 2$ test. P values are indicated as follows: ***= P <0.001 and *= 0.01 < P <0.05.

[b] Positions of RGAs and defense genes were retrieved from Velasco et al. (2010).

Although not all R-genes have been demonstrated to reside in clusters, tight linkage associations of many RGAs have been found (Perazzolli et al., 2014) (**Figure 8**). Clustering of R-genes suggests that a common genetic mechanism has been involved in their evolution, e.g. unequal crossing-over and gene conversion (Sudapak et al., 1993). It is unlikely that each member of an evolving gene family will remain functional. Without positive or negative selection acting to retain function of gene copies within a gene family, the accumulation of deleterious mutations would quickly result in the silencing of redundant genes (Kanazin et al., 1996). However, within an environment in which a population is continually challenged by a broad range of pathogen isolates, we can assume that a rich repertoire of functionally similar genes responsive to unique signals develops. Alternatively, there is increasing evidence of pairs of R-genes, which physically associate to cooperatively trigger immunity against a range of pathogens (Cesari et al., 2014; Griebel et al., 2014). Action of complementary R-gene pairs has been found to function in the recognition of bacterial, viral, oomycete and fungal pathogens in both monocotyledonous and dicotyledonous plants, suggesting this is a common and widespread mechanism in plant resistance. Interestingly, all these R-protein pairs identified in the literature are encoded by two tightly linked resistance loci, most of which are transcribed in opposite directions with a relatively short intergenic region. This conserved genomic organization could be important for co-regulation of these R-genes or to prevent recombination events leading to separation or inappropriate pairing of R-genes, which could cause a loss of resistance or spontaneous necrosis (Cesari et al., 2014). This might also form the basis of the resistance of apple against *V. inaequalis*.

Is partial resistance different from complete resistance

Qualitative resistance conferred by major R-genes is often described as mono- or oligogenic and vertical (race-specific). In contrast, quantitative resistance is considered polygenic and horizontal (non race-specific, uniform) (VanderPlank, 1963; 1968). Nevertheless, there is increasing evidence that such generalization is misleading. First, the term "polygenic" conjures an inappropriate impression of many minor genes, each of them having an approximately equal effect on the phenotype. QTL mapping results indicate that quantitative resistance is often controlled by one or two QTLs with large effects, in association with a few minor QTLs. For instance, the total phenotypic variation explained by all QTLs for resistance in the cross 'Discovery'x 'TN10-8' to one isolate ranged from 13 to 84% (Calenge et al., 2004). Second, race

non-specificity of quantitative resistance seems to be an exception rather than a rule, since differential interactions between isolates and QTLs have been demonstrated for most of partial resistant hosts tested with various isolates of *V. inaequalis* (Calenge et al., 2004; Durel et al., 2004).

For these reasons, after VanderPlank's definitions of horizontal and vertical resistance (Van der Plank, 1963, 1968), it has been suggested that the same genes could govern the quantitative and qualitative plant resistance against pathogen. One hypothesis would be that 'defeated' R-genes, whose effectiveness has been partially overcome by pathogen mutation to virulence would trigger weak defense response and lead to partially resistant phenotypes. This phenomenon has been demonstrated for the major R-genes *Rvi4* (*Vh4*) (Bus et al., 2011) and *Rvi6* (*Vf*) (Durel et al., 2000) in the *V. inaequalis* - *M.* x *domestica* interaction, as well in other plant-pathogen interactions (Nass et al., 1981; Brodny et al., 1986; Li et al., 1999). In apple, this hypothesis is also supported by the observation of co-localization of resistance QTLs with major scab R-genes (Durel et al., 2003; Bus et al., 2004; Soufflet-Freslon et al., 2008), as well as with RGAs belonging to the NBS-LRR family as demonstrated in this review (**Table 3)**. Another hypothesis suggests that partial resistance would be governed by genes with other functions than the classic NBS-LRR resistance genes, as demonstrated by the cloning of partial resistance loci, some of them encoding for proteins of unknown functions (Poland et al., 2009; Fukuoka et al., 2009; Krattinger et al., 2009; St Clair, 2010). However, to my knowledge, none of the apple scab partial resistance loci have been cloned .

Durability of resistance in *Malus*

Introduction of new apple cultivars showing durable resistance is the most important aim of apple breeding programs worldwide. This objective is particularly difficult to achieve with apple scab, since *V. inaequalis* is a pathogen that is well-known for overcoming resistance genes introgressed into cultivars from *M.* x *domestica* germplasm and from wild genetic resources of *Malus* (virulent isolates have been identified for 13 out of 18 GfG relationships defined to date) (Bus et al., 2011; Soriano et al., 2014). This is mainly due to the high evolutionary potential of *V. inaequalis*, characterized by a mixed reproduction system and a large effective population size (meaning that a large number of individuals in the population contributes to the offspring to the next generation; Kiliman et al., 2008) that would facilitate the pathogen to adapt to specific R-genes of the host by both increasing risks of avirulence losses and spreading of new virulences. Moreover, population genetic studies have shown that apple resistance genes might induce specialisation in *V. inaequalis* populations, thus favouring host-related adaptations (Guérin et al., 2007; Gladieux et al. 2011). For these reasons, phytopathologists (McDonald and Linde, 2002a) classified *V. inaequalis* among the pathogens with the highest risks or R-genes breakdown (expected risk of 8-9 on a scale of 11).

Shift in pathogen virulence that was associated with economic consequences, was the emergence of *V. inaequalis* strains able to overcome the major R-gene *Rvi6* (*Vf*) from *M. floribunda* clone 821. This gene has been widely introgressed into modern apple cultivars (Crosby et al., 1992; Gessler and Pertot, 2012) planted in monocultures over 10-15 years. This exerted a high selection pressure on *V. inaequalis* with the potential to initiate major epidemics. The first breakdown was reported in Germany in the 1980s (Parisi et al., 1993), and spread to several European countries (Roberts, 1994; Lespinasse et al., 2000; Parisi et al., 2000; 2006; Lateur et al.,

2001). It has been suggested that a single mutational event in the *Avr* locus of *V. inaequalis* in a limited geographic area is at the origin of the breakdown of resistance (Guerin and Le Cam, 2004; Guerin et al., 2007). Then, rather than from a continuous propagation of an epidemic front, a limited number of virulent strains were disseminated to the other European countries in incidental long distance dispersal events, with transportation of infected leaves by wind or human activities (e.g. exchange of agricultural machines or technical visits of orchards) (Guerin et al., 2007). The low diversity observed among race (6) isolates and the absence of gene flow between these and avirulent populations of *V. inaequalis* (Guerin et al., 2007; Leroy et al., 2013) also suggested that a change of fitness occurred in these isolates. It would result in a reduced capacity to compete on non-*Rvi6* cultivars and reproduce (and hence exchange genes) with avirulent strains (Guerin et al., 2007).

Strategies have been proposed to extend the duration of resistance in apple cultivars. The first approach depends upon the pyramiding of several (at least two, if not three or more) major R-genes in one cultivar to prevent the acquisition of virulences in sequential order by the races (Mundt, 1990; Bus et al., 2011). This approach requires the careful characterization of the resistance spectrum of the genes in question, such that the 'gene pyramid' is effective against the target pathogen population. Finally, it has also been suggested that gene pyramiding will likely be durable if the resistance genes impose a high penalty to the pathogen for adaptation (concept of 'fitness cost' of virulence, e.g. if virulent pathogens perform less well) (Leach et al., 2001; Montarry et al., 2010). The second approach is based on the used of quantitative resistance which is frequently assumed to be more durable than qualitative resistance (Parlevliet, 2002), presumably due to the smaller effects of the resistance, therefore resulting in a lower selection pressure on the pathogen. Moreover since partial resistance is believed to be conferred by multiple genes of partial effects, pathogen isolates that overcome one of the genes would gain only a marginal advantage.

For apple scab, durability of partial resistance of local European cultivars has been experimentally demonstrated over up to 30 years in various local initiatives aiming to collect and evaluate pome fruit genetic resources for disease resistance (Lateur and Populer, 1994; Lateur et al., 1996; Laurens et al., 2004; Kellerhals et al., 2012). However in the apple-*V. inaequalis* pathosystem, as it is the case in other pathosystems, recent studies suggested that directional selection of isolates (e.g. with an increased rate of infection, a shortened latent period or an increased size of lesions) may also occur over generation in pathogen populations that infect cultivars with quantitative resistance (Gandon and Michalakis, 2000; McDonald and Linde, 2002a; 2002b, Lê Van et al., 2013). In the case of apple, such selection seemed to occur only when using broad spectrum QTLs (Lê Van et al., 2013), highlighting the importance of characterizing the resistance spectra of cultivars in apple breeding programs. This selection would slowly lead to an erosion of resistance, as demonstrated by Caffier et al., (2014) in an experimental approach involving progeny segregating for two QTLs (F11 and F17) planted in different locations (Caffier et al., 2014). They demonstrated that the incidence of apple scab increased up to two-fold over an 8-year period. In one location, a breakdown of resistance was even recorded on some genotypes carrying the two QTLs. Nevertheless, authors assumed that the erosion observed may have been exacerbated by the experimental conditions (e.g., climatic conditions highly favorable to scab development in the place where the breakdown was observed, the presence of apple trees from the same genetic background would have favored an unbalanced selection of isolates).

Proof of the concept of durability for both a pyramided complex of major R-genes and/or QTLs in a particular apple cultivar can be showed until a particular race of the pathogen finds his way to overcome the resistance in the host. However, breeders can take advantage of the knowledge on genetics of apple scab resistance to design the optimal breeding strategy. Knowledge about the effect of the genes (complete/partial), race specificity (broad/narrow spectrum), stability of resistance in different environments, mode of action (same/distinct and complementary modes of action), linkage to undesirable traits, pleiotropic effects, etc. provides the breeder with criteria that are instrumental to making decisions as to which set of genes/QTLs to select in a particular cultivar to a resistance in the orchard stable over years (Lindhout, 2002).

Downstream reactions

Efforts have been made to characterize the downstream responses triggered by major scab R-genes in apple. The induced defense mechanisms are difficult to study, since they are part of a complex combined action of different factors. Transcripts profiling were mainly investigated on apple genotypes carrying the *Rvi6* (*Vf*) gene, either present 'naturally' in the commercial variety 'Florina' or introgressed solely in susceptible line 'Gala' (Komjanc et al., 1999; Gau et al. 2004; Holzapfel et al., 2012; Paris et al., 2009, 2012). The various apple cDNA stress collection comprised a broad repertoire of genes that were modulated in each step of the defense response of the *Malus–V. inaequalis* interaction, starting from pathogen recognition to signal transduction and gene expression re-programming after pathogen challenge. Nevertheless, to date little is known about the downstream defense response to infection by apple scab involving quantitative disease resistance.

Defense reactions associated with *Rvi6* resistance gene involved putative LRR receptor-like protein kinases (LRPKm) acting in signal transduction after pathogen recognition (Cova et al., 2010) and several defense-related proteins, such as b-1,3-glucanase, pathogenesis-related (PR) proteins (PR-1, PR-2, PR-3, PR5, PR-8, ribonuclease-like PR-10), cysteine protease inhibitor, chitinase and endochitinase, ferrochelatase, lipoxygenase and ADP-ribosylation factor (Gau et al., 2004; Degenhardt et al., 2005; Paris et al., 2009). Another pathogen-induced gene expression experiments showed that defense of the *Rvi6* cultivar 'Rewena' was based on different mechanisms than the response of the susceptible 'Golden Delicious' to *V. inaequalis* infection: whereas lignification seems to play an essential role in scab defense of 'Rewena', a thaumatin-like as well as a flavonoid gene are assumed to be involved in the mostly insufficient defensive response of 'Golden Delicious' (Holzapfel et al., 2012). In *HcrVf2*-transformed 'Gala', increased expression of catalase, ascorbate peroxidase, polyphenol oxidase, glutathione S-transferase and reductase were found after infection (Paris et al., 2009). These enzymes are probably involved in the onset of an oxidative burst leading to a programmed cell death and HR response, and in the detoxification of ROS that could damage the plant if not controlled. Methallothionein may also play a role in plant defense against *V. inaequalis* as it is present in large amounts in the apoplast of the *Rvi6* cultivar 'Remo' (Degenhardt et al., 2005).

These studies also suggested the importance of constitutive expression of defense-related genes over a pathogen-induced action of the same gene for the onset of the resistance reaction (Gau et al., 2004; Degenhardt et al., 2005). Nevertheless, since the accumulation of defense proteins demands energy of the plant, it can be considered to be a disadvantage in absence of the pathogen. When induced upon pathogen infection, the kinetics of expression of defense-related

genes might be at the origin of the variability of symptoms observed on resistant cultivars: a rapid onset of defense-related genes would culminate in hypersensitivity such as with *Rvi5* (*Vm*). A somewhat slower reaction would lead to a more or less rapid blocking of the pathogen without an HR, but rather a stellar necrosis such as with *Rvi2* (*Vh2*) and *Rvi8* (*Vh8*). Finally, chlorotic genes such as *Rvi6* (*Vf*) condition an even more delayed reaction that allows the fungus to develop running stroma, in some cases with limited sporulation.

At the protein level, Daniëls (2013) highlighted changes in the leaf proteome of the *Rvi6* (*Vf*) resistant cultivar 'Topaz' in response to *V. inaequalis*. The up-regulated stress response related proteins at 5 days post inoculation were a pathogenesis-related protein (Mal d 1), a ROS detoxification enzyme (thioredoxin), a protein involved in defense signaling mediated by R proteins (EDS1), a protein that participates in the synthesis of phenolic compounds with antifungal action (chalcone synthase), proteins that play a role in protein folding (HSP 60, HSP 70 and TCP domain class transcription factor), proteins involved in cell wall metabolism (linoleate 13S-lipoxygenase and alpha-1,4-glucan-protein synthase), and proteins that participate in nucleotide biosynthesis (nucleoside diphosphate kinase 1 and 'bifunctional purine biosynthesis protein'). The down-regulated proteins identified were mainly involved in central anabolic processes, i.e. in photosynthesis carbon fixation and in assimilary metabolism. It has been suggested that such parts of the plant metabolism were switched off during pathogen attack to allow the onset of plant defense, which is energy demanding. Downstream defense reactions triggered by partial apple scab resistance remain largely unknown since no transcriptomic/proteomic studies have been carried out on apple cultivars carrying QTLs for scab resistance.

Transcript modulation of *V. inaequalis* has also been investigatied on a *de novo* assembly of the transcriptome expressed during its growth on laboratory medium and that expressed during its biotrophic stage of infection on apple (Thakur et al., 2013). This provided a better understanding of the biology of the apple scab pathogen and identified candidate genes/functions required for its pathogenesis. Several cell wall degrading enzymes were identified as well as a few host-translocated putative RxLR effectors, orthologs of several known candidate effectors and known pathogenicity determinants of various pathogenic fungi. Finally, a large number of transcripts encoding membrane transporters were identified and the comparative analysis unraveled that the number of transporters encoded by *Venturia* is significantly higher than that encoded by several other important plant fungal pathogens.

Apple scab resistance from genome to breeding

Apple tree breeding is a slow process because of its long juvenility period, while large size of mature plants, inbreeding depression, reproductive self-incompatibility, and complex phenotypes related to grafted trees add to the challenge (Brown and Maloney, 2003). When combined with classical plant breeding selection methods, the use of molecular markers for marker-assisted breeding (MAB) has the potential to alleviate this process by increasing the percentage of desired genotypes and associated phenotypes early on in the breeding pipeline, and assisting breeders in combining desirable traits from different parents into breeding progenies. In **Figure 10**, it has been proposed that marker assisted selection (MAS) could reduce by at least 4 years the length of each breeding cycle in apple. However, I would like to suggest the importance of phenotypic evaluations of the progenies in complement to the use of molecular markers for

MAB. Hence, the use of genomic selection tools might not be adopted as a general tool for reducing the length of the breeding cycle, however, it could be applied to reduce extension growth and to focus phenotypic efforts on the elite genotypes where space is at a premium. Above all, marker usage must be cost-effective in order to replace any preceding and well-established phenotypic screening procedure (Collard and Mackill, 2008; Hospital, 2009). Genotyping costs for MAS have been investigated for beans (Yu et al., 2000), wheat (Kuchel et al., 2005; Van Sanford et al., 2001), barley (Van Sanford et al., 2001), maize (Dreher et al., 2003), rice (Collard and Mackill, 2008) and potato (Slater et al., 2013), but not for apple.

For apple scab resistance, linkage maps have been developed around various major resistance loci, as well as the identification of QTLs for partial resistance (Gardiner et al., 2007, Gessler et al., 2006). In apple, genetic markers have been utilized to varying degrees for at least 18 years to increase the efficiency of apple scab resistance breeding programs. Applications include screening of germplasm with markers for *Rvi5* (*Vm*) (Cheng et al., 1998; Mattison and Nybom, 2005), selection of parents with desired combinations of different resistance genes (Bus et al., 2002), and early selection for gene pyramids for resistance that cannot be selected when phenotyping large breeding population (Bus et al., 2002). Among the limitations of such techniques is the low density of genetic maps leading to recombinants between the molecular markers and the phenotype, hence in reduced efficiency of MAB in breeding programs (Bus et al., 2009). Using multi-allelic SSR markers with specific alleles closely linked to each of the genes will improve the efficiency of MAB, but the goal of 100% efficacy for targeted MAS will only be achieved by using gene-specific functional markers for gene pyramiding (Bus et al. 2009; Kumar et al., 2012). In QTL studies, another main limitation are the small genetic effects of individual QTLs and their poor transferability from one genetic background to another: it restricts their application to only a few families and therefore their potential usefulness of the favorable QTL alleles for MAB (Kenis et al.; 2008; Troggio et al., 2012). A possible approach to increase the transferability of QTL in various progenies is the use of the pedigree genotyping allowing the identification and exploitation of most alleles present in an ongoing breeding program. This is achieved by including breeding material itself in QTL detection, thus covering multiple generations and linking many crosses through their common ancestors in the pedigree (van de Weg et al., 2003).

However, over the past 20 years, genome resources for apple have been greatly developed. It culminated with the sequencing of the diploid 'Golden Delicious' genome (Velasco et al., 2010), a widely grown cultivar (Noiton and Alspach 1996). This was followed by the sequencing of 27 apple cultivars that are the founders in global apple breeding programs in the frame of the RosBREED consortium (Iezzoni et al., 2010). These efforts allowed the identification of millions of single nucleotide polymorphism markers (SNPs) spanning the whole apple genome in and around coding regions (Chagné et al., 2008, 2012; Khan et al., 2012) and permitted the development of the first apple Infinium SNP chip, comprising nearly 8,000 markers (Chagné et al., 2012). Depending on the germplam collections tested, only a subset of these markers might be informative for genetics analysis of particular traits of interest (Keulemans, personal communication). Another straightforward and cost-effective technique that combines marker discovery and genotyping, the genotyping-by-sequencing method (GBS) (Elshire et al., 2011), is based on next-generation sequencing technologies and has been efficiently applied on heterozygous apple cultivars (Gardner et al., 2014). These techniques, together with the availability of the whole genome apple sequence, are likely to lead to new apple scab resistance gene discovery, improved efficiency of MAB by increasing the density of genetic maps, and the

identification of positional candidate genes between two flanking molecular markers from apple genetic maps. But more importantly, these techniques, and particularly GBS due to its cost efficiency, represent an ultimate MAB tool for genomic selection in large scale apple breeding programs, based on statistical approaches such as the genome wide association (GWA) mapping and the genome wide selection (GWS).

One of the key goals of GWA mapping is to overcome the disadvantage of low transferability of QTL analysis by identifying large-effect marker-trait associations that can be deployed through MAB in subsequent generations of cultivar development populations. Moreover, contrary to individual QTLs with relatively small genetics effect, GWA is a more powerful tool since it tends to select for most (if not all) QTLs that affect the trait. To date, the only GWA that was applied on apple (Kumar et al., 2013) aimed to detect linkage disequilibrium (LD) between genotyped markers from the 8K Illumina Infinium SNPs array of Chagné et al. (2012) and QTL alleles for fruit quality traits in a family-based design (e.g. firmness, acidity, susceptibility to physiological disorders). The study demonstrated that while the majority of the SNPs individually explained only a small proportion of trait variation, fitting 2,500 markers simultaneously captured nearly all of the genetic variance (i.e. heritability) for most of the traits. Moreover, since the markers provided by the chip encompassed SNPs in the coding region of predicted gene models, the GWA provided some potential candidate genes underlying the quantitative traits under study (Kumar et al., 2013). These findings gave promising results for its application to apple scab resistance and suggested that genome-based methods could potentially increase the efficiency of the traditional phenotypic selection in apple. First established in cattle breeding programs, the GWS method involves estimation of the effects of chromosome segments (e.g. SNP alleles) from genotypic and phenotypic data in a 'training' population, followed by prediction of phenotypes of individuals in a 'selection' population based on their genomic estimated breeding values alone (Kumar et al., 2012). Successfully applied for apple disease resistance characters (Bus et al., 2009, Volz et al., 2009), GWS is an alternative to phenotypic selection to speed up the introgression of value traits into commercial cultivars by dramatically reducing the length of the breeding cycle and the costs involved in phenotyping. Now that the availability of SNPs and the high-throughput genotyping technologies are no longer a barrier, the subsequent challenge is overcoming the 'phenotyping bottleneck' that limits the ability of apple breeders to capitalize on sequencing and genotyping information. The phenotyping of large populations, albeit still very costly at this stage, will enable a better understanding of integrated plant performance, and lead to the achievement of fast targeted breeding (Troggio et al., 2012).

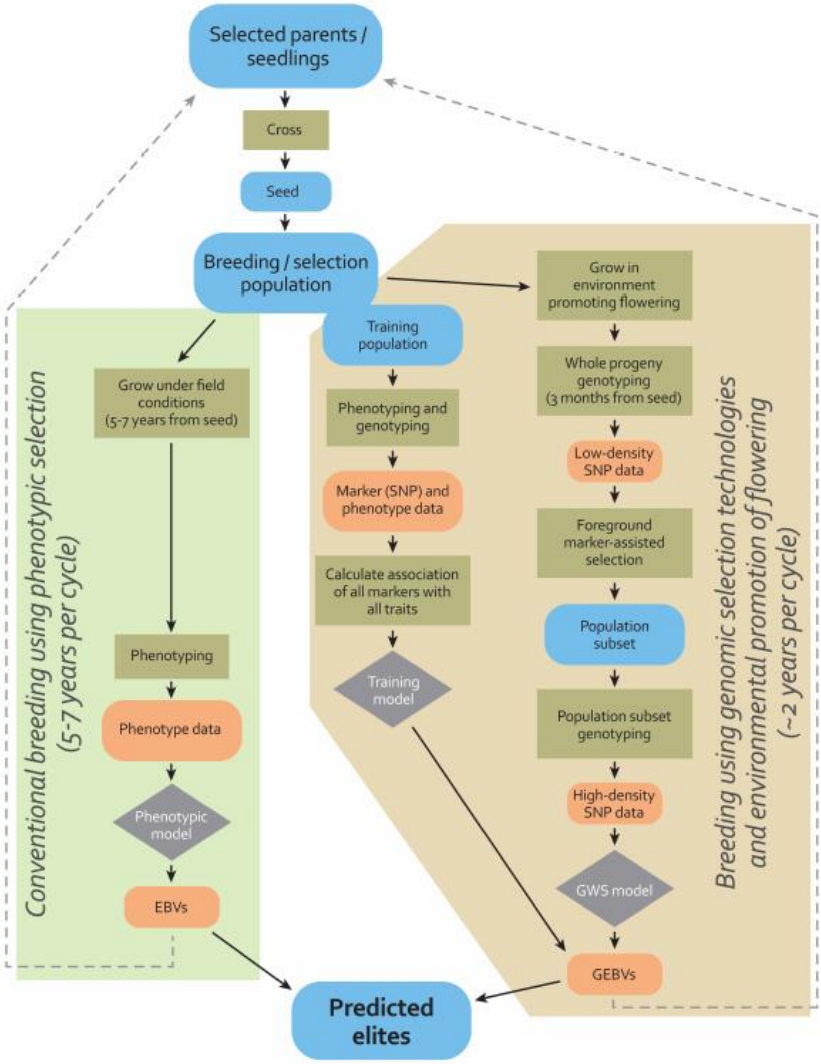

Figure 10: Comparison of apple breeding parameters between standard breeding using phenotypic selection, and genomics-assisted breeding where progeny are raised in conditions that promote flowering, and foreground marker assisted selection (MAS) is applied for major gene 'must-have' traits, followed by genome wide selection (GWS) for traits controlled by multiple loci. In standard breeding programs (pathway at left), elites are selected on the basis of estimated breeding values (EBVs) for traits calculated from phenotypic data recorded after a minimum of 5 years from seed. This is also the earliest that elites can be advanced as parents to the next cycle of breeding and material can be further evaluated for potential as commercial cultivars. Breeding programs using genomic technologies for selection of elites (pathways at right), coupled with promotion of early flowering, can advance selected progeny to phenotypic evaluation for cultivar development as early as 2 years from seed. In effect, the length of each breeding cycle is reduced by at least 4 years. Two cycles of genotyping are employed. First, MAS is used to identify plants possessing simple traits critical to the success of a cultivar, such as pest and disease resistance. Then, only this subset of the original breeding population is genotyped, employing a dense marker set (SNP array or genotyping-by-sequencing) to enable GWS for fruit traits under more complex genetic control, thus minimizing the cost of genotyping. GEBVs are calculated from genotypic data exclusively. The application of GWS relies on the use of a training population for development of the model for association of genetic markers with phenotypic traits and this population must be both genotyped and phenotyped. It should be genetically closely related to the selection population, and in practice the model is cyclically redeveloped following phenotyping of each generation of progeny. Adapted from van Nocker and Gardiner (2014).

As cloning and characterization of resistance genes proceeds, it will become progressively easier to manipulate genetic resistance by transferring clusters of resistance genes within and across species. This resistance gene pyramiding would be achieved more quickly through a single transformation step rather than through a long series of hybridizations and backcrosses in classical breeding (a form of gene therapy). Engineering resistance to apple scab has been the focus of several laboratories. Transformation of susceptible apple lines was made with genes present at the *Rvi6* and the *Rvi15* loci (Belfanti et al. 2004; Malnoy et al., 2008; Joshi et al., 2011 and Schouten at al., 2014). Bolar et al. (2001) inserted endochitinase and exochitinase genes from the biocontrol fungus *Trichoderma atroviride* and increased resistance to scab in transgenic 'Marshall McIntosh' apple. Finally, Faize et al. (2004) showed that expression of wheat puroindoline-b reduced scab susceptibility in transgenic apple 'Galaxy'. However, while genetic engineering technologies offer great potential, with for instance the potential insertion of clusters of R-genes in one go in apple cultivars, the initial enthusiasm was dampened after public acceptance of GMO products was affected by the perceived risk inherent in a new technology. Acceptance also was diminished because GMOs present a number of uncharacterized risks to agroecosystems (hybridization with weedy species, effects on non-target organisms, horizontal gene transfer) that require further investigation. In that regard, cisgenesis represents a step toward a new generation of GM crops. The lack of selectable genes (e.g. antibiotic or herbicide resistance) in the final product and the fact that the inserted gene(s) derive from organisms sexually compatible with the target crop should rise less environmental concerns and increase consumer's acceptance. Such cisgenic apple plants have been developed with the *HcrVf2* (*Rvi6*) scab resistance gene (Vanblaere et al., 2011). Recently, the US Department of Agriculture (USDA) approved the authorization of commercialisation of two cisgenic apple varieties (Artic ® Granny and Arctic ® Golden), genetically engineered to resist browning (USDA, 2015). This shows the potential development of such technologies. Nevertheless, the same precautions regarding the durability of the resistance genes should be taken while engineering apple cultivars for resistance to V. inaequalis, as it has to be done by traditional breeding methods.

Conclusion and future challenges

In a context of growing concern of consumers and society about safe and healthy food produced in sustainable agricultural systems, but also because of the emergence of strains of pathogen resistant to fungicides, durable scab resistance has been a major, but elusive goal of apple breeding program worldwide to date. Genetic studies on both the pathogen, *V. inaequalis*, and the apple host have considerably increased our understanding of the genetic basis of the host-pathogen relationships. Molecular marker mapping work and large-scale sequencing revealed the detailed organization of resistance gene clusters and some genetic mechanisms involved in resistance specificities. Global functional analyses have shone light onto complex regulatory networks and the diversity of proteins involved in resistance and susceptibility. However, to date, despite the great potential in breeding for partial resistance, most efforts have been made in the characterization of major R-genes, and genes underlying the durable quantitative resistance of apple to scab remains poorly understood.

Today, the availability of high throughput genotyping techniques at low cost will allow the application of novel genomic approaches that will have a significant impact on efforts to ameliorate plant disease resistance by increasing access to, and evaluation of genepools available for apple improvement. These approaches will involve the detailed characterization of the many

genes that confer resistance, as well as technologies for the precise selection and deployment of partial and complete resistance genes. Finally, genomic studies on *V. inaequalis* will provide an understanding of the molecular basis of specificity and the opportunity to select targets for more durable resistance. Considering the long history of coevolution of *Malus* and its pathogen *V. inaequalis*, it seems unlikely that we will be able to eliminate apple scab simply by increasing our knowledge of the genetics of the host-pathogen relationships and by the deployment of multiple resistance genes in cultivars. Of course, *Malus* species already have evolved clusters of linked resistance genes over evolutionary time scales, while *V. inaequalis* is still with us. But the current state of the art in science and also our increased experience in efficient selection systems offer a great potential to stay a few steps ahead of the pathogen.

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Chapter 2.

Gene expression profiling by cDNA-AFLP reveals potential candidate genes for partial resistance of 'Président Roulin' against *Venturia inaequalis*

Outline

'Président Roulin' is an old Belgian apple cultivar presenting partial quantitative resistance to *V. inaequalis*. In this chapter, the transcript signature of 'Président Roulin' was analysed and compared to the signature of the susceptible cultivar 'Gala' after pathogen challenge. Then, in order to gain insight into the biological mechanisms involved in the partial resistance, a gene ontology (GO) enrichement analysis was performed. GO terms from 'Président Roulin' were compared to various public cDNA libraries: two independent uninfected apple leaf libraries, and one library extracted from a *Rvi6* (*Hcrvf2*)-transformed 'Gala' line (complete resistance) challenged by *V. inaequalis*. cDNA were *in silico* mapped on the 'Golden Delicious' apple reference genome and candidate defense genes for partial resistance were identified.

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Gene expression profiling by cDNA-AFLP reveals potential candidate genes for partial resistance of 'Président Roulin' against *Venturia inaequalis*

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Abstract

Background: Scab, caused by the fungus *Venturia inaequalis*, is one of the most important diseases of cultivated apple. While a few scab resistance genes (R genes) governing qualitative resistance have been isolated and characterized, the biological roles of genes governing quantitative resistance, supposed to be more durable, are still unknown. This study aims to investigate the molecular mechanisms involved in the partial resistance of the old Belgian apple cultivar 'Président Roulin' against *V. inaequalis*.

Results: A global gene expression analysis was conducted in 'Président Roulin' (partially resistant) and in 'Gala' (susceptible) challenged by *V. inaequalis* by using the cDNA-AFLP method (cDNA-Amplified Fragment Length Polymorphism). Transcriptome analysis revealed significant modulation (up- or down-regulation) of 281 out of approximately 20,500 transcript derived fragments (TDFs) in 'Président Roulin' 48 hours after inoculation. Sequence annotation revealed similarities to several genes encoding for proteins belonging to the NBS-LRR and LRR-RLK classes of plant R genes and to other defense-related proteins. Differentially expressed genes were sorted into functional categories according to their gene ontology annotation and this expression signature was compared to published apple cDNA libraries by Gene Enrichment Analysis. The first comparison was made with two cDNA libraries from *Malus* x *domestica* uninfected leaves, and revealed in both libraries a signature of enhanced expression in 'Président Roulin' of genes involved in response to stress and photosynthesis. In the second comparison, the pathogen-responsive TDFs from the partially resistant cultivar were compared to the cDNA library from inoculated leaves of *Rvi6* (*HcrVf2*)-transformed 'Gala' lines (complete disease resistance) and revealed both common physiological events, and notably differences in the regulation of defense response, the regulation of hydrolase activity, and response to DNA damage. TDFs were *in silico* mapped on the 'Golden Delicious' apple reference genome and significant co-localizations with major scab R genes, but not with quantitative trait loci (QTLs) for scab resistance nor resistance gene analogues (RGAs) were found.

Conclusions: This study highlights possible candidate genes that may play a role in the partial scab resistance mechanisms of 'Président Roulin' and increase our understanding of the molecular mechanisms involved in the partial resistance against apple scab.

Keywords

cDNA-AFLP, Partial resistance, Apple scab, *Venturia inaequalis.*

Background

Apple scab caused by the hemi-biotrophic ascomycete *Venturia inaequalis* (Cke.) Wint. is one of the most serious diseases of apple (*Malus* x *domestica*, Borkh.) worldwide, causing huge economic losses. Scab infection leads to deformation in the shape and size of fruits, premature leaf and fruit drop, and enhances susceptibility of the tree to chilling and freezing injuries (Jha et al., 2009). Currently, multiple applications of fungicides are required for effective control in commercial orchards planted with susceptible cultivars. Depending on the year and region, as many as 18 to 29 fungicide treatments may be necessary in one season to control the disease. For apple orchards in France, pesticide treatments costs account for about 10% of the fixed production expenses, representing a substantial cost per kg of apple (0.031 €) (Sauphanor B, Dirwimmer, 2009). This intensive use of fungicides in orchards raises ecological problems and human health concerns in addition to the economic cost.

An effective alternative to chemical control is the use of scab-resistant apple cultivars. Phenotypically, the effect of resistance genes against *V. inaequalis* has been showed to cover a continuum from complete immunity to near-susceptibility depending on genetic background, pathogen and environment (Bus et al., 2011). Despite the great deal of gray area between the extremes (Poland et al., 2009) hampering the classification of some genotypes, apple scab resistance is often qualified either as complete, when the pathogen growth is fully inhibited (complete or qualitative resistance), or partial, when the resistance allows limited but significantly reduced pathogen growth as compared to a susceptible genotype (partial or quantitative resistance). Based upon the extent of pathogen growth and nature of symptoms, Chevalier et al. (1991) classified the macroscopic foliar symptoms into four classes; classes from 0 to 3 are categorized as resistance responses while class 4 is considered to be a susceptible response. It is usually referred that complete resistance is determined by major resistance gene (R gene) while incomplete resistance involved multiple genes or loci of partial effect (QTLs, Quantitative Trait Loci).

R genes, typically providing high levels of resistance, are relatively easy to manipulate. For these reasons, they were extensively used in both basic research and applied breeding programs. To date, at least 17 major scab resistance genes have been identified and mapped across nine linkage groups of the apple genome (Bus et al., 20011). For more than 50 years, one of these R genes, the *Rvi6* (*Vf*) gene from *Malus floribunda* 821, has provided effective resistance against apple scab by allowing a reduction of 75% in the number of fungicide treatments (Parisi et al., 1995). Nevertheless, the use of single R gene-mediated resistance for crop protection is hampered by a lack of durability, particularly with pathogens having high evolutionary potential, as with *V. inaequalis* (Lê Van , 2012). This ephemeral nature of R gene-mediated resistance is highlighted by the recent emergence of some races of *V. inaequalis* that are virulent on cultivars carrying the widely-deployed *Rvi6* (*Vf*) gene (Parisi et al., 1993). In contrast to major genes, the performance of partially resistant cultivars in the orchard is a function of the gene effects and spectra, which is thought to be more durable than single R gene-mediated resistance (Lateur, 1998). This durability could be explained by the smaller effects of partial resistance that result in a lower selection pressure on the pathogen and/or its presumed broader spectrum. Also, because partial resistance is controlled by multiple genes, pathogen isolates that overcome one of the genes would gain only a marginal advantage (Poland, 2009). In apple, partial resistance has been mapped as QTLs to 10

out of the 17 linkage groups of the genome (Durel et al., 2003; Liebhard et al., 2003; Calenge et al., 2004; Soufflet-Freslon et al., 2008; Gessler et al., 2008).

Extensive efforts have been made to clone and characterize major scab R genes, and the downstream responses that they trigger have become better understood. R genes typically encode proteins that recognize pathogen effectors or modifications of plant proteins that are targets of those effectors (Poland et al., 2009). In this respect, the *Rvi6* (*Vf*) resistance locus revealed the presence of a cluster of four resistance gene paralogs (called *HcrVf* genes), similar to the tomato *Cf* resistance gene, encoding leucine-rich repeats receptor-like proteins (LRR-RLP) (Vinatzer et al., 2001) and the *Rvi15* (*Vr2*) was reported to contain three TIR-NBS-LRR genes (Galli et al., 2010). The function of all these genes was analyzed and only two of them, *HcrVf2* (Belfanti et al., 2004; Joshi et al., 2011) and *Vr2-C* (Schouten et al., 2014) for *Rvi6* and *Rvi15*, respectively, were proven to be functional against *V. inaequalis*. Literature is not in agreement on *Vf1a* (syn. *HcrVf1*) function (Joshi et al., 2011; Malnoy et al., 2011). Recognition of *V. inaequalis* by these proteins triggers downstream defense reactions involving putative LRR receptor-like protein kinases (Komjanc et al., 1999; Cova et al., 2010) and several defense-related proteins, such as b-1,3-glucanase, ribonuclease-like PR-10, cysteine protease inhibitor, endochitinase, ferrochelatase, and ADP-ribosylation factor (Gau et al., 2004; Degenhardt et al., 2005). Methallothionein may also play a role in plant defense against *V. inaequalis* as it is present in large amounts in the apoplast of the *Rvi6* cultivar 'Remo'. Finally, recent publications explored the network of defense response triggered in *Rvi6* (*HcrVf2*)-transformed apple plants using wide genome expression techniques, such as the PCR-based suppression subtractive hybridization and the dHPLC for cDNA-Amplified Fragment Length Polymorphism (cDNA-AFLP) transcript profiling (Paris et al., 2009; Paris et al., 2012). These studies gave new insight into the understanding of the plant pathogen-interaction that results in complete scab resistance. Nevertheless, the function of genes underlying the QTLs for partial apple scab resistance remains unknown. They are believed not to be based on pathogen recognition systems, as it is the case with most major R genes, but the possibility cannot be excluded (Vanderplank, 1984).

The partially resistant apple cultivar 'Président Roulin' is an old Belgian cultivar that is used in apple breeding programs of the Walloon Agricultural Research Center (CRA-W) to broaden genetic apple scab resistance, and therefore reduce the risk of resistance breakdown. Under heavy scab infection, this cultivar shows typical resistance symptoms of chlorosis and necrosis with only slight sporulation (Chevalier class 3a). Its resistance against *V. inaequalis* has been durable for over 25 years in scab evaluation in different orchards of the CRA-W in Belgium without any fungicide treatment (Lateur, 2000). The partial resistance has been shown to be polygenic, but highly heritable (Lateur, 1998).

In this study we investigated the defense response of 'Président Roulin' by identifying genes differentially expressed between this cultivar and the susceptible 'Gala' cultivar after pathogen challenge. For this purpose, cDNA-AFLP technology was chosen as it allowed a survey of transcriptional changes with no prior assumptions about which genes might be involved in the plant response (Bachem et al., 1998). This technique constitutes a robust solution for differential display, detecting changes in gene expression between samples, and it has been successfully applied in several quantitative expression studies in apple (Qubbaj et al., 2004; Massart et al., 2006; Yao et al., 2007; Aldaghi et al., 2012; Paris et al., 2012). The genes identified in this study were annotated and sorted into Gene Ontology (GO) categories. Comparisons were then made with various *Malus* x *domestica* libraries: two EST libraries from uninfected young expanding

leaves (Newcomb et al., 2006; Gasic et al., 2009) and a cDNA library from an apple susceptible line transformed with the major scab R gene *Rvi6* (*HcrVf2*) (Paris et al., 2009). Common as well as different defense pathways were revealed and are discussed. Finally, we checked for co-localizations of our differentially expressed genes with resistance gene analogues (RGAs), QTLs and mapped R genes for apple scab resistance published in the literature.

Material and methods

Plant material and inoculation with Venturia inaequalis

Plants of the partially resistant Belgian cultivar 'Président Roulin' and the susceptible cultivar 'Gala' were grafted on M9 rootstocks and grown in a greenhouse at 20°C under 16 hours of illumination by daylight-incandescent lights. In the frame of the DARE European project, 'Président Roulin' has been shown to be resistant to a large range of inocula, including local mix inocula and monoconidal *V. inaequalis* strains belonging to the race 1, 6, 7 (Laurens et al., 2004) and 2, 8, 9 (data not showed).

In this study we used six monoconidial strains of race 1 *V. inaequalis* originating from the INRA collection at Angers, France (EU-B04, EU-B16, EU-D49, EU-F05, EU-F11 and EU-I09) to prepare the inoculum. Each strain, first grown in Petri dishes for 10 days on malt extract agar and covered by a cellophane membrane, was multiplied separately on young seedlings raised from open-pollinated 'Golden Delicious'. Infected leaves were dried and stored at −20°C for not more than 1 year before use. Conidia were harvested by shaking the leaves in sterile water. A conidial suspension was prepared by mixing conidia of the 6 strains at a final concentration of 2.5 x 10^5 conidia per milliliter. The conidial suspension was sprayed onto young leaves of actively growing 'Gala' and 'Président Roulin' plants in quantities sufficient to form small droplets on the leaf surface before run off. The inoculated plants were incubated at 20°C under maximum relative humidity (RH) for two days and were then transferred to the greenhouse (20°C, 60-80% RH). Control plants were inoculated with sterile water. Conidia vitality was verified after 24 h by determining the fraction of germinated conidia (~70% for all the isolates). Levels of scab infection were scored for each plant 21 days after inoculation. To reduce the level of biological variation among samples, two plants per treatment were used. For each plant, the three youngest leaves were collected at 48 hpi, immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction.

Microscopic investigation of fungal development

To identify the optimal timing of sampling for the subsequent cDNA-AFLP experiment, progress of pathogen development was followed on 'Président Roulin' and 'Gala' using light microscopy. Plants were spray-inoculated and incubated as described above. At 1, 4, 6, 16, 20, 24, 32, 48, 54 and 120 hpi, the youngest expanding leaf of each cultivar was sampled (one leaf/sampling time/cultivar), cleared overnight in 99% methanol and stained with periodic acid-basic fuchsine according to the method by Preece (1959). Samples were thoroughly rinsed with water and mounted onto glass slides. Pathogen development stages were examined at the different time points by observing at least 200 conidia per sampling time for each of the cultivars.

In silico cDNA-AFLP simulations

A total of 450 full-length apple cDNAs sequences from the study by Newcomb et al. (Newcomb et al., 2006) were analyzed with the AFLPinSilico program (Rombauts et al., 2003). The combinations of restriction enzymes used here were *Sau3*A, *Taq*I, *Dde*I, *EcoR*I, *EcoR*II, and *Apo*I as first sites in combination with the restriction enzyme *Mse*I as second site, and *vice-versa*. The following parameters were estimated: percentage of cleaved cDNA, percentage of cDNA visualized on a gel, number of cleavage sites per cDNA, average distance between the last recognition site and the polyadenylation site, and the mean size of the restriction fragments.

RNA extraction and cDNA-AFLP

Total RNA was isolated from 100 mg of leaf material collected at 48 hpi (two plants per treatment), using the extraction method described by Gasic et al. (2004). After DNase I treatment (Fermentas Inc), purification of mRNA was performed starting from 250 µg total RNA using the Qiagen Oligotex mRNA kit (Qiagen Inc.). RNA purity and concentration was measured with the Nanodrop technology (Thermo Scientific Inc.). Double stranded cDNA was finally obtained starting from 500 ng mRNA following the instructions of the Superscript Double Stranded cDNA Synthesis kit (Invitrogen Inc.).

cDNA-AFLP analysis was performed with the AFLP Core Reagent kit (Invitrogen Inc.) as recommended by the manufacturer. The double-stranded cDNA was digested with *EcoR*I and *Mse*I and ligated to the corresponding *EcoR*I and *Mse*I adapters. The pre-amplification step was carried out using 20 cycles of amplification (94°C for 30 s; 56°C for 1 min; 72°C for 1 min) starting from a 5 µl aliquot of a 1:2 dilution of the ligation reaction and 75 ng of primer corresponding to the *Mse*I and *EcoR*I adapter sequence without any extension, in 50 µl total volume. After 10-fold dilution of the PCR fragments, specific amplifications were carried out with a total of 141 *EcoR*I and *Mse*I primer combinations containing two (123 primer combinations with EcoRI + 2/MseI + 2), or three additional selective bases at the 3' end (18 primer combinations with EcoRI + 2/MseI + 3, EcoRI + 3/MseI + 3 or EcoRI + 3/MseI + 1). The *Eco* primers were labeled with [γ33P] dATP. Amplification products were separated by electrophoresis at 60 W on a vertical denaturing polyacrylamide gel (5%) containing 7 M urea for 3 hours 30 minutes. Gels were transferred onto Whatman 3MM paper before drying. Repeatability of the technique was checked trough an experimental replication of the selective amplification step (for a few selective primer pairs) starting from the same pre-amplified cDNA samples.

Bands corresponding to the TDFs were visualized on the polyacrylamide gel by autoradiography using X-ray films. Band intensities were digitized using the PhosphorImager tool (Biorad) and were quantified using QuantityOne software (Biorad). For each primer pair combination tested, only cDNA-AFLP profiles with the same global band intensity among genotypes and treatments were compared. This is presumed to reflect the fact that equivalent amounts of amplified cDNA were compared. We considered all the TDFs whose expression ratio between inoculated and non-inoculated control treatments was above the threshold of two (fold-change >2) as significantly up-regulated, and all those whose ratio was below the inverse threshold (fold-change <1/2) as significantly down-regulated.

Bands of interest belonging to the cultivar 'Président Roulin' were cut from the polyacrylamide gels, eluted in 50 µl of water, re-amplified with the selective primers and cloned

into Pjet 1.2 vector (Fermentas). Two transformed colonies per TDF with an insert of the expected size were subsequently sequenced (Macrogen Inc.). cDNA sequences were manually checked for overall quality, and vector and primer contaminations were trimmed.

Bioinformatics analysis

In order to limit redundancy and to produce a longer consensus sequence, the sequences were clustered using Egassembler (Masoudi-Nejad et al., 2006). We then compared the contigs and singletons identified with the whole apple genome sequence assembly v1.0 (Velasco et al., 2010) by BLAST sequence similarity search. This second step allowed us to identify the TDFs that originated from the same original contig (and thus from the same gene) of the apple genome assembly. The functional annotation of this non-redundant database was performed using the automatic Blast2GO bioinformatics tool (Conesa et al., 2005). Basically, input sequences were queried by BLAST-X against the GenBank non-redundant sequences and ESTs database at the National Center for Biotechnology Information (NCBI) database, taking similarities with an E-value < 10^{-3} as significant matches. Then, the program extracts the GO terms associated to each of the obtained hits and returns an evaluated GO annotation for the query sequences (E-value < $1e^{-6}$). These E-values were the default values proposed by Blast2GO.

In order to detect GO annotations whose abundance was significantly different between different sets of annotated genes, a gene function enrichment analysis was performed using Blast2GO (Conesa et al., 2005). This software employs Fisher's exact test to estimate the significance of associations between two categorical variables using a single test p-value. A set of GO terms that are under- or over-represented at a specified significance value ($P < 0.05$) were obtained as a result of performing the enrichment analysis. For this analysis, two subsets of our cDNA-AFLP library were compared: the TDFs being up- and down-regulated in 'Président Roulin' after pathogen infection (group II + and II -). We also compared our cDNA-AFLP library (group I and group II) from infected leaves of 'Président Roulin' to: (1) two EST libraries from uninfected young expanding leaves of *Malus* x *domestica* 'Royal Gala' (AELA) (Newcomb et al., 2006) and 'Wijcik' (Mdstw) (Gasic et al., 2009), and (2) a cDNA library (Paris et al., 2009) of differentially expressed transcripts from *Rvi6* (*HcrVf2*)-resistant transgenic 'Gala' lines challenged by *V. inaequalis* (obtained by suppression subtractive hybridization between *Rvi6* (*HcrVf2*)-transformed 'Gala' lines and susceptible 'Gala' plants). The last three libraries were assembled in contigs and singletons using the EGassembler tool (Masoudi-Nejad et al., 2006) before performing the analysis. Sequences were annotated with the bioinformatics tool Blast2GO using the same parameters as those applied for the annotation of the cDNA-AFLP fragments.

Quantitative real-time reverse transcription PCR (qRT-PCR)

qRT-PCR was carried out on the same RNA samples used for the cDNA-AFLP analysis and on RNA derived from one independent biological replication of the experiment. Specific TDF primers were designed with the software Primer3 (Rozen et al., 2000) and qRT-PCR was performed using Biorad CFX96 and Maxima SYBR Green qPCR master mix (Fermentas Inc.), following the instruction of the manufacturer. A list of the specific primer pairs used for each TDF and product lengths is given in **Additional Table 3**. PCR conditions were the same for all primer pairs: initial denaturation at 95°C for 10' followed by 40 cycles of denaturation at 95°C for 15", annealing at 60°C for 30" and extension at 72°C for 30". Fluorescence data were collected at

the end of the annealing step. Following cycling, samples were denatured at 95°C for 10". The melting curve was analyzed to differentiate between the desired amplicons and any primer dimers or DNA contaminants (in the range 65°–95°C, with a temperature increment of 0.5°C for 5"). Each reaction was run in duplicate. The LinRegPCR software was used to confirm that individual PCR efficiencies were about 2 for each primer pair [102]. The relative expression ratio of the target genes between scab-inoculated and water-treated plants was evaluated using the ΔΔCt method described by Applied Biosystems (Relative expression ratio = $2^{\Delta\Delta Ct}$), with the glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) as the internal reference (primers sequence F-5'CAAGGTCATCCATGACAACTTTG3', R-5' GTCCACCACCCTGTTGCTGTAG3'). In fact, as it was the case in other qRT-PCR studies conducted on apple (Aldaghi et al., 2012; Gadiou and Kundu, 2012), the *GAPDH* gene appeared to be the best housekeeping gene in our experimental conditions. Contrary to the elongation factor gene (*EF*, primers sequence F-5'TACTGGAACATCACAGGCTGAC3', R-5'TGGACCTCTCAATCATGTTGTC3'), expression of the *GAPDH* was stable in scab-inoculated and water-treated leaf samples (**Additional Figure 1**). Individual relative expression values were then subjected to the ANOVA procedure, using Minitab 16 software, at a statistical significance level of $P < 0.01$.

In Silico mapping and co-localization of the TDFs with RGAs, QTL or apple scab major R genes

TDFs sequences were searched by BLAST-N within the whole genome sequence assembly v1.0 of apple (Velasco et al., 2010) on the Genome Database of Rosaceae (GDR) (http://www.rosaceae.org/) taking into account the best BLAST result (E-value < 0.001). Clusters of apple resistance genes analogues (RGAs) (Perazzolli et al., 2014), QTLs and major scab resistance genes already identified in apple (Gessler et al., 2006; Bus et al., 2011; Soufflet-Freslon et al., 2008) were also anchored *in silico* to the apple genome sequence. Molecular markers flanking the major scab R genes and QTLs were obtained from the HIDRAS (High-quality Disease Resistant Apples for Sustainable Agriculture) website (http://www.hidras.unimi.it/HiDRAS-SSRdb/pages/index.php) and searched in the apple genome sequence by BLAST-N. Only SSR markers having an E-value ≤3e-3, a ratio of matched bases to marker sequence equal to 100% and a position on the expected chromosome on the 'Golden Delicious genome sequence assembly (according to their genetic position) were anchored to the physical map. Clusters of RGAs and their physical position on the 'Golden Delicious' genome assembly were retrieved from the publication of Perazzolli et al., (2014). Only TDFs mapping inside a QTL confidence interval or mapping within 250 kb from any cluster of RGAs or major scab R gene were considered to co-localize in the genomic regions involved in resistance. This distance has been used in previous publication as the average distance separating genes inside a cluster (Perazzolli et al., 2014). In order to determine if TDFs co-localized only by chance, frequencies of co-localization were compared to frequencies observed for ESTs from an uninfected apple library (Newcomb et al., 2006) using a $\chi 2$ test employing the statistical software Minitab 16.

Results

Fungal development across post inoculation time points

Microscopic observations revealed no significant difference between 'Président Roulin' (partially resistant) and 'Gala' (susceptible) for pathogen development at the early stages of infection. This comprises conidia germination, formation of appressoria and cuticle penetration (**Figure 11**). On both cultivars, conidia germination began within 4 hours post inoculation (hpi), and at 16 hpi most of the conidia had produced germ tubes. At this time, the rudimentary germ tubes had bulged at the tip to form characteristic appressoria adhering to the cuticle. At the end of the 24 hour period, the process was further advanced. Shortly after the formation of the appressoria, infection hyphae appeared and penetrated the host. At 48 hpi, 80% of the appressoria were formed and invasion of the host plant started with the expansion of the primary stroma. With the staining method used in this study, the subcuticular growth of the fungus was difficult to observe because it was poorly stained. Nevertheless, we can assume that, when the stroma was visible, at 120 hpi, subcuticular development on the susceptible host, 'Gala', had significantly exceeded that of the partially resistant 'Président Roulin' (data not shown). This difference in the extent of colonization of the fungus between the two cultivars remained throughout the whole infection cycle of the fungus. Between 7 and 12 days after inoculation, apple scab symptoms became macroscopically visible on 'Président Roulin' and 'Gala'. After 21 days, 90% of the leaf surface of 'Gala' (susceptible) was covered by sporulating apple scab lesions (class 4) (Chevalier et al., 1991). However, typical chlorotic and necrotic lesions with slight sporulation were observed on leaves of 'Président Roulin' (partially resistant), covering less than 15% of the leaf surface. These symptoms were considered as resistance responses and were classified in the class 3b, as described by Chevalier et al. (1991).

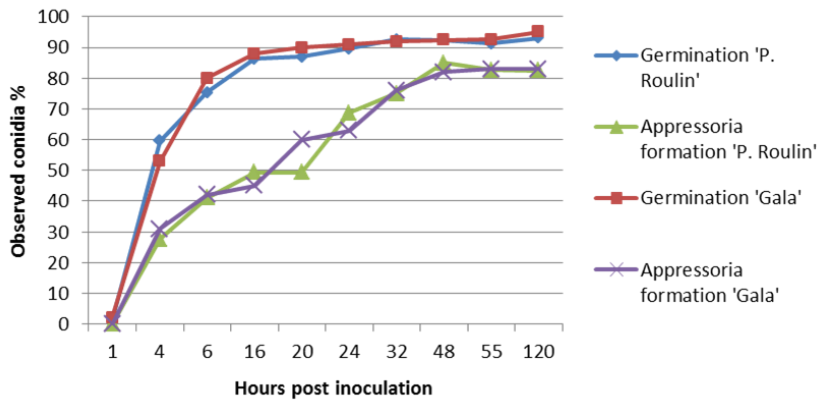

Figure 11 : Kinetics of *V. inaequalis* conidial development on 'Président Roulin' and 'Gala' leaves. Germination of conidia and formation of appressoria were observed under light microscopy over time post inoculation. Fungal tissues were stained on whole leaves with periodic acid-basic fuchsin according to the method of Preece (1959).

cDNA-AFLP fingerprints: optimization and identification of differentially expressed transcripts

We used the AFLPinSilico application to choose the optimal restriction enzyme combination for the cDNA-AFLP experiments. The results of the different parameters for each enzyme pair combination are shown in **Table 4**. Enzymes with 4- or 5-base recognition sites

yielded the highest number of Transcript Derived Fragments (TDFs), although these were generally relatively short and highly redundant, with up to 5 cleavage sites per cDNA. In opposite, the *Eco*RI/*Mse*I recognized cleavage sites on less than half (34%) of the apple full-length cDNA tested, but this combination of restriction enzymes generated more informative TDFs than all the other enzyme pairs tested. In fact, *Eco*RI provided the longest TDF (mean size of 234 bp) with the lowest redundancy (0.5 restriction sites per cDNA) and derived at least partially from coding regions (683 bp from the poly(A) + tail). This enzyme was therefore the most appropriate and it was chosen in combination with *Mse*I for cDNA-AFLP analysis.

Table 4: Suitability of restriction enzymes for use in cDNA-AFLP analysis in apple

Enzyme[a]	Restriction site	TDF visualized[b]	N° cleavage sites	Position (bp)[c]	Length (bp) [d]
SAU3A	GATC	69%	5.5	723	133
TAQI	TCGA	67%	4.5	680	147
DDEI	CTNAG	70%	4.5	650	156
ECORII	CCWGG	66%	2.0	605	222
APOI	RAATTY	59%	2.4	676	167
ECORI	GAATTC	34%	0.5	683	234

[a] 450 Full Length cDNA from *Malus* x *domestica* were analyzed *in silico* for patterns of cleavage by different restriction enzymes in combination with *Mse*I
[b] Percentage of cDNAs that yielded a TDF of a size that could be resolved on a 5% polyacrylamide gel (between 50 and 1000 bp) after cleavage with the particular enzyme in combination with *Mse*I
[c] Average distance between the last recognition site and the polyadenylation site
[d] Mean size of restriction fragments, expressed in base pairs

The cDNA-AFLP analysis using 141 primer combinations in 'Président Roulin' and 'Gala' resulted in 30 to 100 TDFs per primer combination, depending on the number of additional bases used for the selective amplification step, and a total of about 10,250 TDFs in each cultivar (representing a total of 20,500 for both cultivars). TDFs ranged in size from 30 to 800 bp. **Figure 12** shows a typical cDNA-AFLP profile of the two apple genotypes challenged by *V. inaequalis* or water. Considering that 123 primer pairs with two additional selective nucleotides (*Eco*RI + 2/*Mse*I + 2) were tested out of the 256 possible primer pair combinations, and taking into account that about 40% of the apple cDNA could potentially be visualized with the restriction enzyme *Eco*RI and *Mse*I, we estimated that we analyzed a representative sample of approximatively 19% of the apple genes expressed in the tissues.

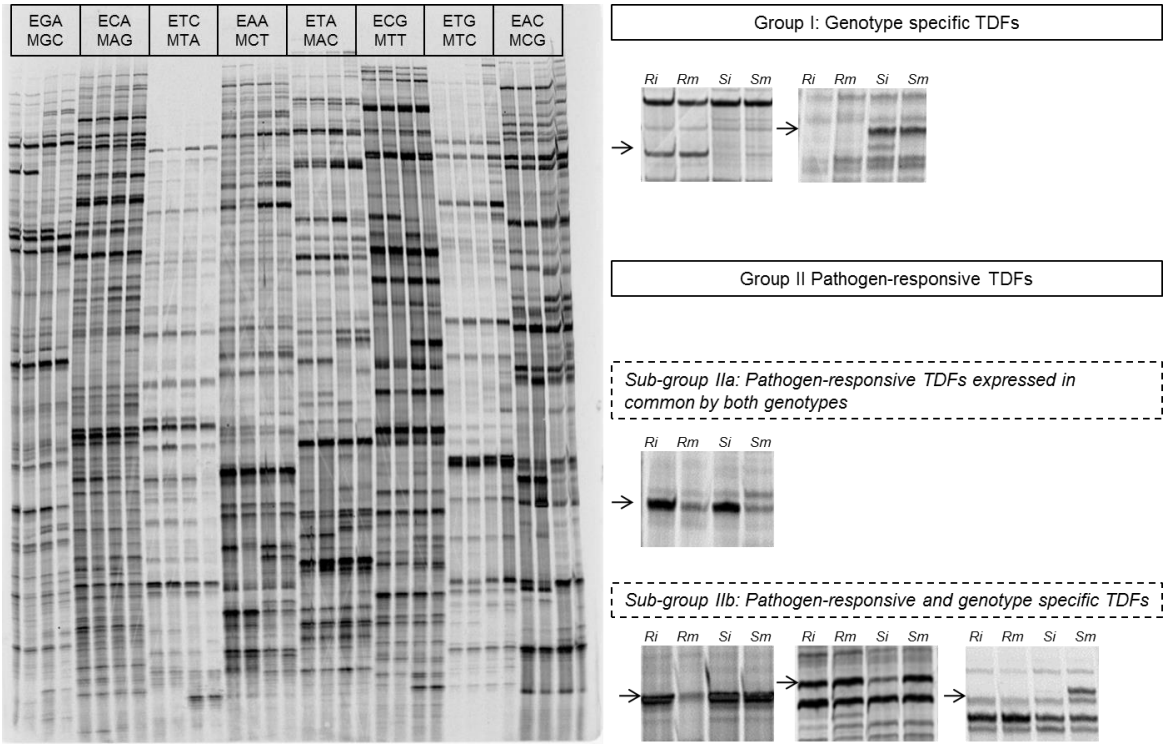

Figure 12: Expression patterns of apple genes displayed by cDNA-AFLP fingerprints. The cDNA-AFLP compares transcriptional profiles from 'Président Roulin' (partially resistant) and 'Gala' (susceptible) mock-inoculated or challenged by *V. inaequalis* at 48 hpi. The 32 samples are arranged in 8 groups according to the different specific primers tested during the selective amplification step of the AFLP procedure. E and M refer to the *Eco*RI and *Mse*I primers, followed by the selective nucleotides used. Within each of the 8 groups samples are ordered as follows: 'Président Roulin' infected (Ri) and mock-inoculated (Rm), and 'Gala' infected (Si) and mock-inoculated (Sm). Differentially expressed TDFs were classified into 2 categories: genotype-specific TDFs (group I) and pathogen-responsive TDFs (group II), further divided into two sub-groups; pathogen-responsive TDFs expressed in common by both genotypes (sub-group IIa) and pathogen-responsive and genotype specific TDFs (sub-group IIb). Illustrations are given.

At the stringent threshold used, only 4.6% of all the 20,500 generated fragments exhibited significant differences in intensity among the different genotypes or treatments. According to their banding patterns, the differentially expressed TDFs were classified into two groups: (I) genotype-specific TDF, for all banding patterns differing between the genotypes but not affected by fungal infection and (II) pathogen-responsive TDFs representing all the TDFs showing differential expression during infection. Group II was further divided into two subgroups: (IIa) pathogen-responsive TDFs, representing the TDFs with differential expression upon pathogen challenge in both genotypes and (IIb) pathogen-responsive and genotype-specific TDFs, representing the TDFs showing differential expression induced by the pathogen in one of the two cultivars.

The first group (group I, genotype-specific) accounted for about 1.7% of all generated fragments (232 TDFs present only in 'Président Roulin' and 115 in 'Gala'). In contrast, 281 (230 up-and 51 down-regulated) and 311 TDFs (241 up- and 70-down regulated), respectively, were identified in 'Président Roulin' and 'Gala' as significantly differentially expressed after fungal infection (**Figure 13**). These pathogen-responsive TDFs (group II) accounted for about 2.9% of all the 20,500 TDFs analyzed for both cultivars. Among them, 125 (111 up-regulated and 14 down-regulated) overlapped between the two genotypes (subgroup IIa). The remaining 156 and 186 TDFs, for 'Président Roulin' and 'Gala', respectively, displayed differential expression after

fungal attack that depended on the genotype. They were classified into subgroup IIb (pathogen-responsive and genotype-specific) and represented about 1.7% of the fragments. From our point of view, these bands differentially expressed in ‘Président Roulin’ only, are the most interesting as they could be involved in specific plant defense reaction against *V. inaequalis*. In fact, genes that showed only a genotype effect may reflect the effect of the genetic background, whereas genes exhibiting only a treatment effect may represent a general plant response to pathogen challenge that does not determine the final different phenotype.

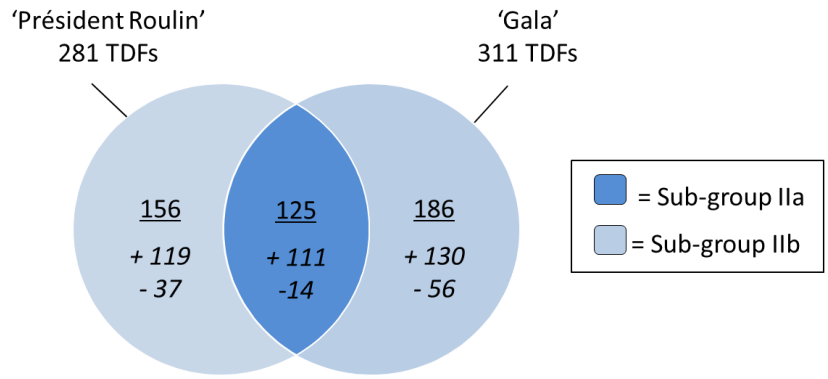

Figure 13: Venn diagram showing number of pathogen-responsive TDFs in ‘Président Roulin’ (partially resistant) and/or ‘Gala’ (susceptible). Group II of TDFs was classified into two sub-groups: pathogen-responsive TDFs expressed in common by both genotypes (sub-group IIa) and pathogen-responsive and genotype specific TDFs (sub-group IIb). ‘+’ and ‘-’ represent up- and down-regulation, respectively.

We then considered the amplitude and direction of expression changes for all pathogen-responsive TDFs (group II). We plotted the log10 transformed expression ratios of ‘Président Roulin’ against ‘Gala’ and distinguished the TDFs that were differentially expressed in only one of the two lines (blue squares for ‘Président Roulin’ and red squares for ‘Gala’, **Figure 14**) from those being differentially expressed by both cultivars (green triangle, intersect of the two circles in the Venn diagram of **Figure 13**). This graph showed the overall similarity and specificity of gene expression in the TDFs differentially expressed in common by ‘Président Roulin’ and ‘Gala’: most of these TDFs were differentially regulated in the same direction (up or down-regulation) by both cultivars. Fold-changes from 2 to 70 were observed, with the majority of the TDFs showing a difference in expression less than 7-fold.

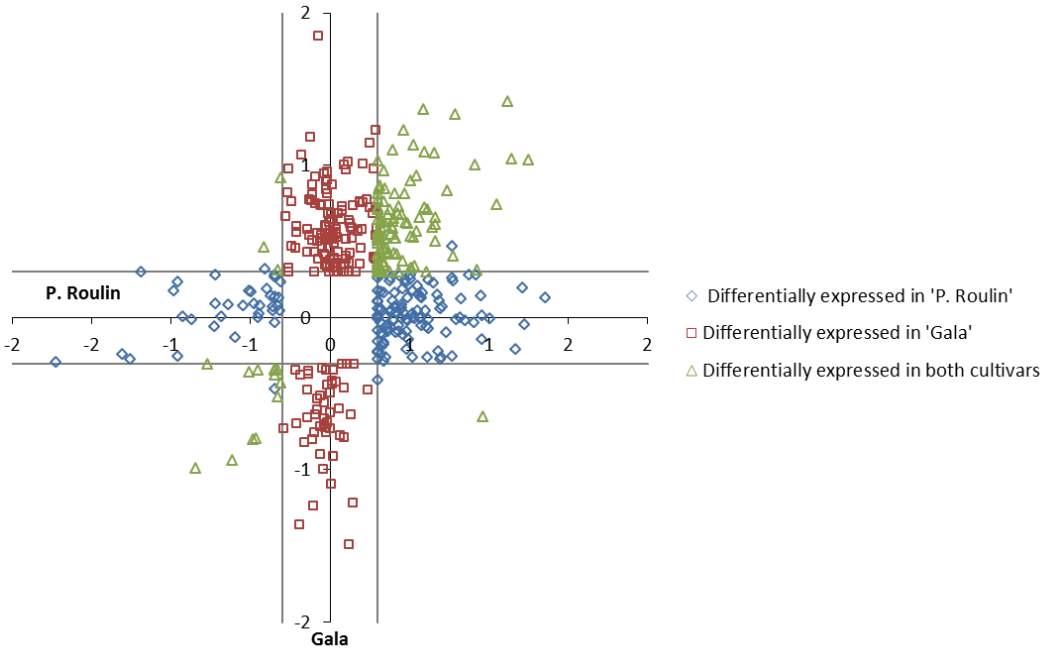

Figure 14 : **Scatter plot of log10-gene expression fold changes of pathogen-responsive TDFs from 'Président Roulin' and 'Gala'.** Fold changes are relative to mock inoculation. Colour-coded plots represent TDFs differentially regulated in one of the cultivars (cultivars-specific) or in both cultivars (common). Log ratios >0 or <0 indicate up- or down-regulation, respectively, dashed lines set at 0.3 and −0.3 (+ − $\log_{10}2$) correspond to the threshold of a two-fold change in gene expression that was used as the cut-off value for biological significance.

Sequences annotation

Two-hundred and fifty nine TDFs out of the 281 pathogen-responsive TDFs from the resistant cultivar and part of the genotype-specific TDFs (131 TDFs) were excised from the gels, re-amplified and cloned (390 TDFs in total). No bands belonging to the susceptible cultivar 'Gala' were excised and a priority was given to TDFs of sufficient length (upper part of the gel), to facilitate their functional characterization. Two clones were sequenced for each re-amplified TDF and for 38% of them (146 TDFs) a different sequence between the two clones was found, leading to a total of 536 sequences. To limit redundancy, sequences were clustered using the software Egassembler (http://egassembler.hgc.jp**)** (Masoudi-Nejad et al., 2006) and then compared to the whole apple genome sequence assembly in order to identify the unigenes (Velasco et al., 2010). This returned 497 unique sequences (29 contigs and 468 singletons) from 53 bp to 803 bp that were annotated and submitted to the NCBI database with accession numbers assigned (**Additional Table 1**).

Among the 497 sequences, 69% (344 TDFs) were similar to known expressed sequences in public databases (319 could be annotated, 25 were similar to encoded proteins with unclear function), while 153 sequences had no matches in the NCBI database. With the exception of 15 sequences similar to genes belonging to the apple stem pitting virus (ASPV), all TDFs were similar to sequences from model plants, with *Vitis vinifera*, *Ricinus communis* and *Populus trichocarpa* being the three most represented species. As expected, most TDFs (80%) were found to be similar (BLAST E-value <1e-4) to *Malus* x *domestica* sequences derived from the whole genome sequence of apple (Velasco et al., 2010). The ASPV is a widespread pathogen of pome fruit usually transmitted by the rootstock and frequently latent in the host (Mathioudakis et al., 2010).

As the cDNA encoding for ASPV coat proteins was found in both inoculated and controlled plants and as no symptoms of ASPV were observed in apple trees used for the experiments we believe that the virus was latent and did not interfere with gene expression data.

Putative functions of the apple TDFs were then classified according to the GO vocabulary. **Figure 15** shows the percentages of apple genes assigned to the biological processes. About 27% of the annotated sequences have metabolic roles (particularly in protein and carbohydrate metabolism) and 24% are involved in cellular processes. Other relevant groups, accounting for 12, 10 and 7% of the TDFs, respectively, include single-organism process, response to stimulus (particularly response to biotic stress, oxidative stress, response to wounding and defense response, listed in **Table 5)** and biological regulation. In addition to TDFs related to plant defense response according to the GO classification, we also found TDFs showing similarities with genes encoding for proteins reported to have a potential role in the general defense response pathway through careful analysis of the scientific literature. These TDFs are also potential candidate genes for partial resistance of 'Président Roulin' against *V. inaequalis*. These TDFs encoded proteins involved in the recognition of the pathogen, signal transduction, oxidation reduction process, photosynthesis, transport, response to environmental stress, metabolism, transcription, and cell wall organization (**Table 6**). Expression of TDFs of interest was further investigated by qRT-PCR and putative functions of some of these proteins in the partial resistance against *V. inaequalis* are discussed.

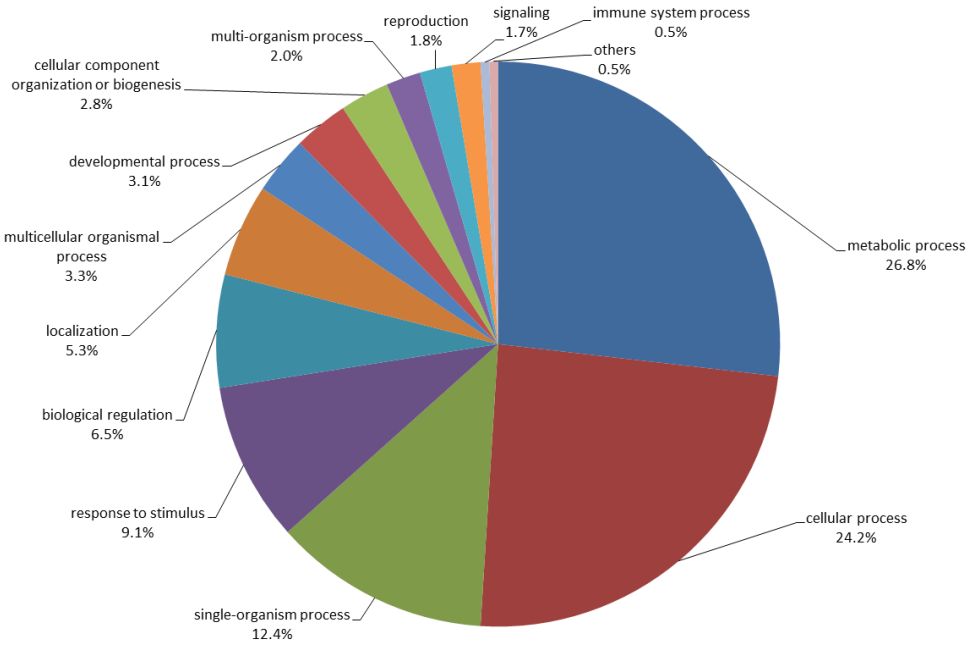

Figure 15: Distribution of differentially expressed TDFs within the GO categories of biological processes. GO annotations were made according to the International Gene Ontology Consortium using the automatic bioinformatics software Blast2GO.

Table 5: TDFs associated with plant defense response, response to oxidative stress and response to wounding

TDF	Expression pattern[a]		Annotation	GO annotations[b]
1AU61'	IIb	+	e3 sumo-protein ligase siz1	P:induced systemic resistance; P:negative regulation of systemic acquired resistance
37DU41	IIb	+	cysteine proteinase inhibitor	P:defense response
43CU118	IIb	-	tmv resistance protein	P:defense response; P:innate immune response
43DU149	IIb	+	peroxidase	P:response to oxidative stress
43DU149'	IIb	+	nucleotide binding site leucine-rich repeat disease resistance protein	P:defense response
44AU9	IIb	+	LRR receptor kinase-like protein	P:defense response
51HU129'	IIb	+	tocopherol cyclase	P:regulation of defense response
56AU33'	IIb	+	nbs-lrr resistance protein	P:defense response
14GU213	IIa	+	TMV resistance protein N-like	P:innate immune response; P:defense response
33FU130'	IIa	-	Avr9/Cf-9 rapidly elicited protein	P:response to wounding
34EU#2	IIa	+	12-oxophytodienoate reductase	P:response to wounding
34CU81'	IIa	+	nadp-dependent oxidoreductase	P:response to oxidative stress
34FU145'	IIa	+	disease resistance protein	P:defense response; P:innate immune response
41CU29'	IIa	+	protein bonzai 3-like	P:positive regulation of cellular defense response
42AU1	IIa	+	nad-dependent epimerase dehydratase	P:defense response to bacterium
46CU57'	IIa	+	cc-nbs-lrr resistance protein	P:defense response
47CU77'	IIa	+	ferredoxin-nadp + reductase	P:defense response to bacterium
54DU58	IIa	+	progesterone 5-beta-reductase	P:response to wounding
55BU33	IIa	+	multidrug resistance protein abc transporter family	P:response to wounding
34DU#2	I		adp-ribosylation factor gtpase-activating protein agd2-like	P:systemic acquired resistance
43BU45	I		proteasome subunit beta type-6	P:regulation of plant-type hypersensitive response
43CU113'	I		type ii peroxiredoxin	P:response to oxidative stress
43HU225	I		cell wall-associated hydrolase	P:response to oxidative stress
44CU85	I		acetylornithine aminotransferase	P:defense response to bacterium
44HU193'	I		disease resistance protein at3g14460-like	P:defense response
45CU49	I		formamidopyrimidine-dna glycosylase	P:response to oxidative stress
52BU9	I		nad-dependent epimerase dehydratase	P:defense response to bacterium

[a] TDF Expression pattern according to the cDNA-AFLP (induced + or repressed -) at 48 hpi by *V. inaequalis*: genotype-specific TDFs (I), pathogen-responsive TDFs expressed in common by both genotypes (IIa) and pathogen-responsive and genotype specific TDFs (IIb).

[b] GO annotations were made using the automatic bioinformatics software Blast2GO.

Table 6: Gene expression analysis of selected TDFs by qRT-PCR in 'Président Roulin' (resistant) and 'Gala' (susceptible)

TDF	Annotation (blastx)	Exp.[a]	Fold induction/repression			
			cDNA-AFLP samples		Biological repetition	
			Resistant.	Susceptible	Resistant	Susceptible
	Defense response					
43DU149'	cc-nbs-lrr resistance protein	IIb +	+7.9 ± 2.6*[b]	+1.9 ± 0.0	+2.8 ± 1.1	+1.4 ± 0.3
56AU33'	cc-nbs-lrr resistance protein	IIb +	+2.6 ± 0.1*	+1.5 ± 0.1	+2.9 ± 0.5	+1.3 ± 0.5
42BUHcrVf	HcrVf paralog	IIb +	+1.0 ± 0.1	−1.9 ± 0.2*	−1.2 ± 0.1	−1.2 ± 0.2
43CU118	TMV resistance protein	IIb -	+1.6 ± 0.1	+1.5 ± 0.2	-	-
44 AU9	LRR receptor kinase-like protein	IIb +	+ 5.1 ± 1.7*	+1.4 ± 0.4	+2.2 ± 0.4*	+1.7 ± 0.0*
44GU169	2-cys peroxiredoxin	IIb +	+10.3 ± 0.1*	+2.2 ± 0.1*	+1.4 ± 0.5	+1.2 ± 0.0
54CU21	Phi class glutathione transferase	IIb +	+3.5 ± 0.0*	−1.2 ± 0.5	+1.1 ± 0.1	−1.5 ± 0.1
	Signal transduction					
2EU181	Putative MAP kinase	IIb +	+2.2 ± 0.0*	+1.4 ± 0.2	+2.1 ± 0.3*	−1.3 ± 0.0
39AU13	MAP kinase phosphatase	IIb +	+1.4 ± 0.0	+1.1 ± 0.2	−1.0 ± 0.1	−1.6 ± 0.0*
	Transporter					
46EU122	ABC transporter	IIb -	−2.4 ± 0.1*	+1.3 ± 0.0	−1.5 ± 0.1	+2.4 ± 0.0*
	Oxidation reduction process					
51DU17	Cytochrome P450	IIb +	+2.0 ± 0.3*	+1.1 ± 0.3	+1.0 ± 0.2	+1.1 ± 0.0
53DU34	Cytochrome P450	IIb -	−4.8 ± 0.0*	+1.5 ± 0.2	−1.9 ± 0.0*	−2.6 ± 0.1*
	Photosyntesis					
56 AU5'	Uroporphyrinogen decarboxylase	IIb +	+6.5 ± 1.3*	+3.9 ± 1.1	−1.0 ± 0.0	−1.4 ± 0.2
	Response to environmental stress					
43DU149	Peroxidase 12	IIb +	+3.4 ± 0.2*	+1.2 ± 0.1	+4.0 ± 0.2*	+1.9 ± 0.2*
51HU129'	Tocopherol cyclase	IIb +	+8.1 ± 0.0*	+2.0 ± 0.1*	+6.9 ± 0.0*	+1.5 ± 0.0*
	Metabolism					
Consensus 44EU122/ 44EU118	Cysteine protease	IIa -	- 12.7 ± 4.5*	- 4.1 ± 1.2*	−68.8 ± 0.0*	−3.2 ± 0.3
37DU41	Cysteine protease inhibitor	IIb +	+ 2.4 ± 0.1*	+1.2 ± 0.1	+2.9 ± 0.0*	+1.4 ± 0.1
1AU61'	Sumo ligase	IIb +	+1.5 ± 0.3	+1.6 ± 0.1	-	-
56AU29	Chitinase	IIb +	+2.3 ± 0.1*	+1.4 ± 0.1	+2.7 ± 0.2*	+1.2 ± 0.1*
44GU182	Lysosomal Pro-X carboxypeptidase	IIb -	- 28.4 ± 4.9*	+ 1.4 ± 0.1	−22.0 ± 0.0*	−1.9 ± 0.1*
	Transcription factor					
53HU89	Zinc finger homeodomain protein1	IIb +	+10.3 ± 0.2*	+1.7 ± 0.1	+3.3 ± 0.1*	+1.8 ± 0.1*
	Cell wall organization					
44GU173	Pectin methylesterase inhibitor	IIb +	+3.3 ± 0.8*	+1.1 ± 0.1	+3.6 ± 0.9*	+1.2 ± 0.2*
	Unknown functions					
55FU102	No homology	IIa +	+3.0 ± 0.1*	+1.4 ± 0.5	+3.8 ± 0.2*	+1.3 ± 0.0
55HU125'	No homology	IIb +	+4.8 ± 0.0*	- 1.5 ± 0.1	+1.7 ± 0.0	+1.5 ± 0.2

[a] Expression pattern according to the cDNA-AFLP. Group IIa represents pathogen-responsive TDFs expressed in common by both genotypes and group IIb pathogen-responsive and genotype specific TDFs. "+" = induced and "–" = repressed TDF.

[b] Means and SD of fold induction (+) or repression (−) calculated by the ΔΔCt method applied using qRT-PCR. Significant fold changes were judged considering the following criteria: statistical significance of individual ΔCt values at P <0.01 (*) and biological significance at fold change ≥2.

Gene enrichment analysis

The gene enrichment analysis revealed that genes accounting for the different functional GO categories were similarly represented (P > 0.05) in two subsets of our cDNA-AFLP: the TDFs being up- or down-regulated in 'Président Roulin' after pathogen attack (group II + and II -). Nevertheless, when compared to two different collections of ESTs (Newcomb et al., 2006; Gasic et al., 2009) from uninfected expanding apple leaves (AELA and Mdstw), some biological pathways appeared to be significantly over-represented (P < 0.05) in the 'Président Roulin' cDNA-AFLP library (**Figure 16**). In both comparisons, it was notably the case for genes involved in photosynthesis and in response to stress. Additionally, other GO categories were significantly over-represented in 'Président Roulin' but depended on the library being compared: e.g. oxidation reduction process, regulation of gene expression in the AELA library, and homeostatic process in the Mdstw library. All these genes could be involved in the general defense response pathway of 'Président Roulin' against *V. inaequalis*. No biological pathways appeared to be significantly under-represented in our collection.

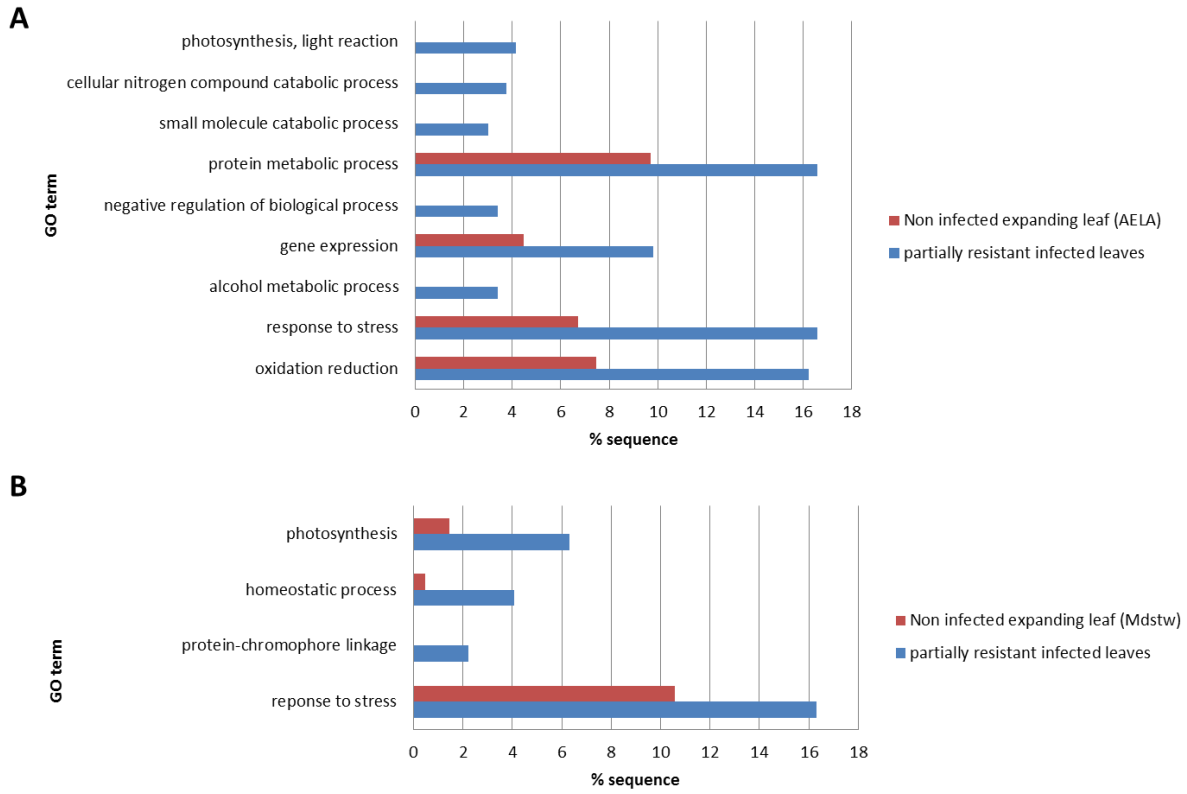

Figure 16: Over-representation of GO categories in 'Président Roulin' cDNA-AFLP library compared to non-infected EST apple libraries. The comparison has been made by gene enrichment analysis for Biological Process GO categories between our cDNA-AFLP library from scab-infected leaves of 'Président Roulin' (partially resistant) and two EST libraries from uninfected actively growing shoot of: **(A)** cultivar 'Royal Gala' in the library AELA (Newcomb et al., 2006) and **(B)** cultivar 'Wijcik' in the library Mdstw (Gasic et al., 2005). Gene enrichment analysis was conducted with the software Blast2Go using Fisher's Exact Test at a p-value <0.05. No GO categories were shown to be under-represented.

In order to assess if mechanisms leading to partial resistance against *V. inaequalis* might involve biological pathways different to a complete resistance response, we compared by gene enrichment analysis our 'Président Roulin' TDFs dataset to the SSH library from *Rvi6 (HcrVf2)*-transformed 'Gala' line challenged by *V. inaequalis* (Paris et al., 2009). Some biological processes were over-represented in our partial resistance compared to the complete resistance mediated by *Rvi6* (*HcrVf2*) gene (**Figure 17**, **Additional Table 2**). This was the case for genes involved in the plant response to DNA damage stimulus, particularly DNA repair, and those involved in the regulation of hydrolase activity. A significant over-representation of differentially regulated genes classified as 'regulation of defense response' was also found in 'Président Roulin'. Again, no under-representation of any biological pathways was found.

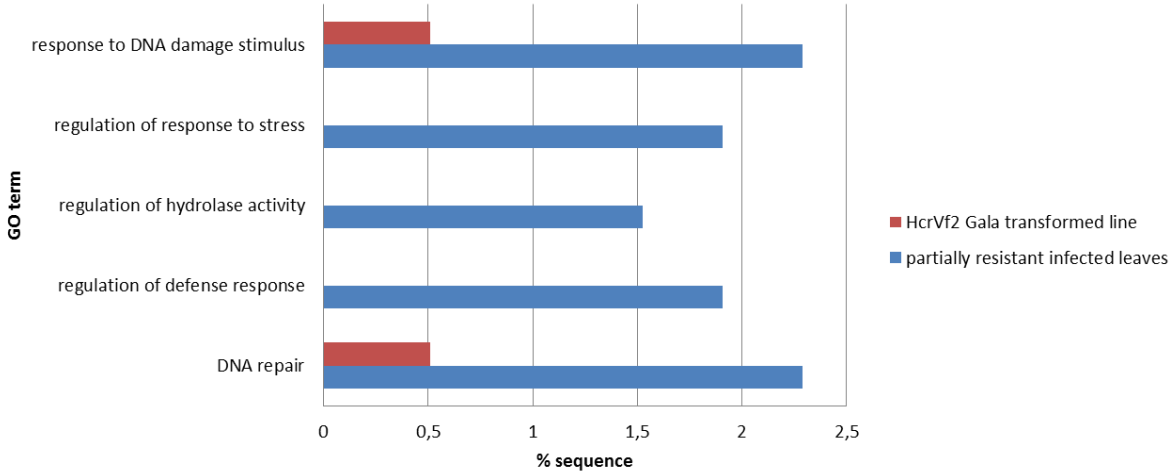

Figure 17: Over-representation of GO categories in 'Président Roulin'cDNA-AFLP library compared to *Rvi6* (*HcrVf2*)-'Gala' transformed library. The comparison has been made by gene enrichment analysis for the Biological Process GO category between our cDNA-AFLP library (scab-infected leaves of 'Président Roulin', partially resistant) compared with a cDNA library from completely resistant *Rvi6* (*HcrVf2*)-transformed 'Gala' lines challenged with *V. inaequalis* (Paris et al., 2009). Gene enrichment analysis was conducted with the software Blast2Go using Fisher's Exact Test at a p-value <0.05.

Quantitative real-time reverse transcriptase PCR

To validate the reliability of our cDNA-AFLP analysis, qRT-PCR was performed on 24 pathogen-responsive TDFs representative of almost all functional categories identified, with a preference for defense-related genes and genes possibly involved in pathogenesis. **Table 6** presents separately the qRT-PCR results carried out on the same RNA samples used for the cDNA-AFLP analysis and on a biological replication of the experiment. Results were expressed as fold-change of 'Président Roulin' (partially resistant) and 'Gala' (susceptible) after pathogen attack, in respect to mock-inoculated leaves. In general, expression data provided by qRT-PCR were in good agreement with profiles detected by cDNA-AFLP. When performed on RNA samples used for cDNA-AFLP, qRT-PCR confirmed the pattern of gene expression of 20 TDFs (83%). Expression of 22 of these RNA samples was then further verified by qRT-PCR on a biological replicate of the experiments, and the pattern of expression of 13 of these samples was confirmed (59%). TDFs that were not in accordance with the cDNA-AFLP showed no significant changes in expression in 'Président Roulin' (i.e. 42BUHcrVf, 43CU118, 39 AU13). For three TDFs, strong changes in gene expression (more than five-fold) were detected in infected leaves of

'Président Roulin' in both experiments (51HU129', 44GU182 and 44EU122). Two different TDFs (43DU149 and 43DU149') cloned from the same band and showing a significant increase in intensity after pathogen challenge were both confirmed to be up-regulated after pathogen attack. In most cases, no significant changes of expression were detected in infected leaves of the susceptible 'Gala' cultivar.

Co-localization of the TDFs with RGA, QTL or apple scab major R genes

Approximatively, 40% of the TDFs anchored *in silico* on one of the 17 chromosomes of apple were localized in the proximity (within 250 kb) of RGAs clusters, QTLs or major R genes for apple scab resistance (**Table 7**). Nevertheless, this frequency was not significantly greater than those calculated for random ESTs derived from an uninfected apple leaves library (Newcomb et al., 2006). So co-localization could have occurred purely by chance. However, considering separately the three classes of TDFs (group I, IIa and IIb), we found that group I and IIb of TDFs mapped at a greater frequency in the vicinity of major R genes than EST from AELA library (5% instead of 1%).

Table 7 : Frequency of co-localization of TDFs from 'Président Roulin' with RGAs, QTLs and major apple scab R genes

Mapped	Number [b]	Cluster RGA[c] (%)	QTL[d](%)	Major R gene[d] (%)	QTL/cluster RGA/major gene (%)
TDFs[a]	387	22	21	4 **	40
TDFs group I	130	24	17	5***	38
TDFs group IIa	110	22	24	4	38
TDFs group IIb	147	22	22	5**	44
Uninfected apple library AELA	501	20	21	1	38

[a] Transcript-derived fragments (TDFs) at 48 hours after inoculation by *V. inaequalis*: genotype-specific TDFs (group I), pathogen-responsive TDFs expressed in common by both genotypes (group IIa) and pathogen-responsive and genotype specific TDFs (group IIb). Frequencies of co-localization of TDFs were compared to frequencies observed with an uninfected apple library AELA (Newcomb et al., 2006) using a χ^2 test. P values are indicated as follows: *** = P <0.001, ** =0.001 < P <0.01, and * =0.01 < P <0.05.

[b] Number of cDNA mapping onto the apple chromosome with a threshold E-value < 0.001 by BLAST analysis against homologous apple genomic sequences with known chromosomal locations (Velasco et al., 2010)]

[c] clusters of resistance gene analogues (RGAs) (Perazzolli et al., 2004).

[d] Quantitative trait loci (QTLs) and major R genes for apple scab resistance (Gessler et al., 2006; Soufflet-Freslon et al., 2008; Bus et al., 2011).

Discussion

Plant disease resistance and susceptibility are governed by the combined host and pathogen genotypes, and depend on a complex exchange of signals and responses occurring under given environmental conditions. During the long process of host-pathogen co-evolution, plants have developed various elaborate mechanisms to ward off pathogen attack (Rausher, 2001). In addition to constitutive defense, it has been postulated that a key difference between resistant and susceptible plants is the timely recognition of the invading pathogen, and the rapid and effective activation of host defense mechanisms. Such induced resistance mechanisms have been demonstrated at transcriptional level in numerous studies on plant-pathogen interactions involving either complete (Durrant et al., 2000; Gabriëls et al., 2006; Wang et al., 2010; Shi et al., 2011; Paris et al., 2012) or partial disease resistance (Alignan et al., 2006; Li et al., 2012). In both types of resistance, pathogen attack was accompanied by activation of host plant response genes and accumulation of corresponding gene products. Based on these findings, our cDNA-AFLP study attempted to elucidate the molecular mechanisms underlying partial resistance against apple scab by the identification of differentially expressed transcripts between 'Président Roulin' (partially resistant) and 'Gala' (susceptible) after pathogen attack.

Effective identification of differentially expressed transcripts after scab infection requires the determination of the stage of pathogen development at which it is first affected by the host's defense mechanisms. This is important particularly in investigations built upon the hypothesis that resistance involves the induction of defense pathways. To identify the appropriate time-point for the extraction of RNA, we compared the kinetic of pathogen development on leaves of 'Président Roulin' (partially resistant) and 'Gala' (susceptible). Light microscopic assessment identified 48 hpi as the critical time-point when the pathogen is first affected by the host's responses during formation of appressoria and stroma. In fact, whereas the early stages of pathogen growth occurred at the same rate in both cultivars, significant differences were observed in later stages, when development of subcuticular stroma subsequent to cuticle penetration was reduced by greater extent in the partially resistant cultivar compared to the susceptible one (**Figure 11**). These differences are concordant with the literature with various susceptible and resistant apple cultivars (Valsangiacomo and Gessler, 1988; Silfverberg-Dilworth, 2006) and led to a reduced disease severity and sporulation on 'Président Roulin' 21 days after infection. This also suggests that no particular pre-formed chemical or structural defense barriers are present at the leaf surface of 'Président Roulin'. The choice of 48 hpi as the key time for our gene expression study is further motivated by the fact that in various plant-pathogen interactions the accumulation of defense-related transcripts in the host is often concomitant to the formation of appressoria and the development of the intracellular hyphae (Clark et al., 1993; Ruiz-Lozano et al., 1999; Lo et al., 1999; Kleemann et al., 2012). When this cytological event occurs, the molecular dialog between the pathogen and the host presumably begins. The host detects the pathogen and initiates defense-related transcriptional responses (Chisholm et al., 2006).

As for any transcriptome study, a genome-wide screen for differentially expressed genes based on cDNA-AFLP requires that as many transcripts as possible are analyzed in a unique way, and that the data obtained are informative enough to allow characterization of the transcripts. Considering criteria such as the genomic region of the restriction sites (coding or non-coding region), the redundancy and the length of the TDFs being produced, we showed *in silico* that the restriction enzyme pair *Eco*RI/*Mse*I was the most appropriate for our cDNA-AFLP analysis. This

restriction enzyme combination was successfully used in similar experiments on apple (Yao et al., 2007; Dal Cin et al., 2009; Paris et al., 2009; Baldo et al., 2010; Paris et al., 2012). In our study, this enzyme combination yielded a very high informative content (69% of the TDFs could be effectively annotated) with limited redundancy (sequence assembly yielded only 29 contigs out of the 536 unclustered sequences).

The cDNA-AFLP method has the advantage that all treatments, time points and genotypes under investigation can be concurrently compared (Breyne and Zabeau 2001; Breyne et al., 2003). In our study, we estimated that we analyzed a representative sample of approximatively 19% of the genes expressed in the leaves of 'Président Roulin' and 'Gala' in response to *V. inaequalis* infection at 48 hpi. Our estimation corroborates with the data from the recent sequencing of the apple genome project since our 10,250 bands would represent about 18% of the 57,386 putative genes in apple (Velasco et al., 2010). We highlighted that the differences between partially resistant and susceptible responses to *V. inaequalis*, were more quantitative (different regulation of expression of the same genes in both cultivars) than qualitative (different genes being expressed in both cultivars), and that quantitative differences mainly involved up-regulation of genes (71% of up-regulations). As similarly demonstrated in the literature (Maleck et al., 2000; Katagiri et al., 2004), we can argue that, despite the very clear phenotypic differences observed between the partially resistant and susceptible responses, the timing and amplitude of gene induction could have a greater influence on the disease resistance status of the plant than the identity of genes activated. Under-representation of down-regulated TDFs has also been reported in apple - *V. inaequalis* interaction (Paris et al., 2009) and in other pathosystems (Wang et al., 1994; Eckey et al., 2004; Steiner et al., 2008) but it has been suggested that such under-representation could be mainly due to the two-step PCR of the cDNA-AFLP method (Eckey et al., 2004). The overall similarity of transcriptional signature between 'Président Roulin' and 'Gala' is further demonstrated by the observation that genes were regulated in the same direction in both cultivars: when a TDF was up-regulated in one cultivar, up-regulation was also observed in the other one (and inversely) (**Figure 14**). Finally, all 'Président Roulin' pathogen-responsive TDFs were also found to be expressed in the mock-control from high, to intermediate or low levels, supporting the idea that many genes involved in the defense reaction were already expressed in resistant genotype before pathogen challenge (Gilroy et al., 2007; Paris et al., 2012).

The potential presence of multiple sequences in one band cut from the gel is the major disadvantage of cDNA-AFLP fingerprinting. In our study, this mis-cloning has been observed for 38% of the excised bands and could be an underestimate of the multiplicity of sequences per band since only two clones per band were analyzed. Each restriction enzyme pair may in fact result in different fragments of the same size which are visualized as a single band on a cDNA-AFLP gel autoradiography. Therefore, confirmation of gene expression by an independent technique is required, as for hybridization-based assay like microarray. In this study, expression profiles observed by cDNA-AFLP were confirmed by qRT-PCR in 83% of the 24 TDFs under examination (on the same cDNA samples used for the AFLP, **Table 6**). Results were similar to those reported by Baldo et al. (2010) (79% of 28 ESTs) and Paris et al. (2012) (81% of 32 TDFs). The percentage of confirmation decreased when using an independent biological replication (59%). According to these results it can be assumed that the modulation of about 83% of the observed TDFs is confirmed; however extending data to at least a biological repetition is highly recommended to further confirm that gene modulation strictly depends on the biological system under study (i.e. the plant-pathogen interaction). This consideration is valid not only for cDNA-AFLP but for all transcriptional analysis as modulation of gene expression can be influenced by

other factors (environment, biotic stresses…). However, in our opinion, two or more sequences from one band can contribute to the change of band intensity observed on the autoradiography, as suggested by the fact that two different sequences cloned from the same band were both confirmed to be up-regulated after pathogen attack (43DU149 and 43DU149').

The GO analysis of differentially expressed TDFs revealed that they are represented by a high diversity of functional categories (**Figure 15**). This is not surprising since, with the emerging of genome-wide gene expression profiling technologies, it is now clear that plant response to pathogens is associated with massive changes in gene expression. For example, in an *Arabidopsis* microarray, more than 2000 genes (out of 8000 genes) involved in a broad range of biological responses were regulated in response to the bacterial pathogen *Pseudomonas syringae* (Tao et al., 2003). In our study, the annotation of 'Président Roulin' pathogen-responsive TDFs indicates that they may act in early events of plant defense response such as pathogen recognition (e.g. some TDFs encoded for putative nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins (McHale et al., 2006), or in signal transduction (e.g., mitogen-activated protein (MAP) kinase, Asai et al., 2002). Other TDFs identified may play a role in the later stages of the 'Président Roulin' defense response with the induction of genes aiming to stop or reduce the invasion of the host by pathogens. In plants, this often leads to the hypersensitive response, a form of localized programmed cell death orchestrated by the oxidative burst. TDFs involved in such reactions encoded for gluthatione-S-transferase (Marrs, 1996), peroxidase (Bindschedler et al., 2006), E3 ubiquitin protein ligase (Yang et al., 2006) and cysteine protease/cysteine protease inhibitor (Solomon et al., 1999).

In order to understand the global molecular pathways involved in partial resistance, gene enrichment analysis were conducted to statistically determine whether specific GO terms were enriched in different sets of cDNA. First we demonstrated that the same functional categories were involved in our up- and down-regulated set of TDFs (group II + and II -). These results differ from the findings of Paris et al. (2012) who found that TDFs similar to genes putatively involved in defense responses were generally up-regulated in R gene-mediated resistance (*Rvi6*/*HcrVf2*), while those putatively involved in general metabolism were down-regulated. In contrast, we demonstrated that genes involved in stress response (including disease/defense response) and in photosynthesis were preferentially regulated in the partially resistant cultivar 'Président Roulin' after pathogen attack, as compared to two published EST libraries from *Malus* x *domestica* uninfected young expanding leaves (Newcomb et al., 2006; Gasic et al., 2009) (**Figure 16**). When stress response genes could participate in the partial resistance of 'Président Roulin' against *V. inaequalis*, regulation of genes of the photosynthetic pathway might be the first step towards the appearance of chlorotic spots on infected leaves, which in the apple - *V. inaequalis* incompatible interaction appear about 8–12 days after inoculation (Paris et al., 2009). It is also well known that defense responses is energy intensive (Bolton, 2009) and requires transcriptional activation of genes (Rushton and Somssich, 1998). These could be the reason why we found in 'Président Roulin' an over-representation of genes involved in different catabolic/metabolic processes, that might 'fuel' the implementation of downstream defense response, and in the regulation of gene expression. Nevertheless, as these later GO categories appear to be significantly over-represented in only one of the two libraries being compared, their involvement in the resistance of 'Président Roulin' against *V. inaequalis* still has to be confirmed. Finally, our comparison between the 'Président Roulin' cDNA library with the completely resistant *Rvi6* (*HcrVf2*)-transformed 'Gala' lines (Paris et al., 2009) revealed both similar events (e.g. transport, photosynthesis functions), and differences in some biological process categories

(**Figure 17**). Among the differences (**Additional Table 2**) we noticed an over-representation in 'Président Roulin' of genes involved in (1) the regulation of defense response (e.g. E3 sumo ligase SIZ1 proteins (Lee et al., 2007) and tocopherol cyclase (Sattler et al., 2006; Maeda and DellaPenna, 2007); (2) proteins involved in the regulation of hydrolase activity, also recognized to be key enzymes in the regulation of programmed cell death in incompatible plant-pathogen interactions (e.g. cysteine protease inhibitor and a 13-fold repressed cysteine protease (Solomon et al., 1999; Avrova et al., 1999; Hao et al., 2006); (3) proteins involved in the response to DNA damage stimuli. This latter category is thought to be involved in the plant response to abiotic stress such as UV-B (Jansen et al., 1998) and osmotic stress (Xiong and Zhu, 2002), but to our knowledge, these proteins were not yet known to be involved in quantitative disease resistance.

Beside these slight differences between complete and partial resistance, large parts of the transcriptional signatures did not demonstrate enrichment for genes in particular functional categories. This finding is consistent with the hypothesis that partial resistance could be due in part to the same genes governing complete resistance. To illustrate this hypothesis, some classical R genes encoding for the NBS-LRR family protein were found to be up-regulated in the partially resistant cultivar 'Président Roulin' after pathogen attack. In that context, partial resistance could be due to defective R genes that recognize with low efficiency pathogens and trigger weak defense response (McHale et al., 2006). This may result either from mutation in the R genes themselves or at the corresponding avirulence locus of the pathogen. In fact, there are compelling lines of evidence that allelic variants of R genes account for quantitative disease resistance in plants (e.g. *Xa21* in rice-blast interaction, Andaya and Ronald, 2003). In the same way, when a pathogen strain overcomes an R gene due to a mutation at the avirulence locus, it has been proved that the "defeated" R gene still has a residual effect and can act as a QTL against virulent strains of the pathogen. This phenomenon has been observed for the major resistance genes *Rvi4* (*Vh4*) [3] and *Rvi6* (*Vf*) (Durel et al., 2000) in the *V. inaequalis* - apple interaction, and also in rice bacterial blight (Li et al., 1999), wheat stem rust (Brodny et al., 1986) and powdery mildew pathosystems (Nass et al., 1981). Likewise, in the same way as major resistance genes, several QTLs have been shown to be isolate-specific (Calenge et al., 2004; Talukder et al., 2004; Perchepied et al., 2005) and co-localization of QTLs and R genes has been noted in several species including apple (Wang et al., 1994; Gebhardt and Valkonen, 2001 ; Calenge et al., 2005; Xiao et al., 2007). Another evidence suggesting that genes controlling partial resistance in 'Président Roulin' could share structural and functional similarities with R genes resides in the fact that some subsets of our cDNA library mapped at greater frequency in apple genomic regions known to carry major scab R genes (Gessler et al., 2006; Bus et al., 2011) (as compared to random EST from uninfected apple library). The co-localization of ESTs with genomic regions carrying disease resistance factors (R genes, QTLs or RGAs) has been already reported in various genome-wide analyses studies [90-93]. No significant co-localization of our cDNA library with apple scab QTLs (Gessler et al., 2006; Soufflet-Freslon et al., 2008; Bus et al., 2011) nor apple RGAs (Perazzolli et al., 2014) has been found. Obviously, we could only check for co-localizations with R genes and QTLs that have been detected so far. Moreover, information on the genomic loci that can regulate the expression level of the TDF of interest is still lacking from this analysis. In fact, the measured mRNA levels can either be the product of regulation of the parent gene or of another gene, mapping somewhere else in the genome (cis- or trans- regulatory elements) (Gilad et al., 2008).

Conclusions

In conclusion, this study provides a wide transcriptional profile analysis for the comprehension of key events in partial resistance of ‘Président Roulin’ and highlights possible candidate resistance genes. We found altered gene expression in resistant and susceptible plants in response to *V. inaequalis* that involved many functional categories. Genes acting in pathogen recognition (NBS-LRR) as well functioning downstream of the initiated defense signaling pathways were identified. Biological processes related to stress response and photosynthesis were found to be over-represented in infected leaves of the partially resistant cultivar compared with two published libraries of uninfected apple leaves. In addition, through comparison between partial and complete resistance, the pathogen-responsive cDNA library revealed common physiological events, but differences in regulation of defense response, in the regulation of hydrolase activity, and in response to DNA damage stimuli. Finally, TDFs from ‘Président Roulin’ mapped more frequently in the vicinity of major R genes for apple scab resistance, suggesting that quantitative and complete resistance could be governed by the same types of genes. A functional analysis of the differentially expressed genes will allow more insights into their possible role in the quantitative resistance reaction of ‘Président Roulin’ against *V. inaequalis*. For example, an assessment of the differential expression of candidate resistance genes over different time points after infection could be investigated to find out how resistance is regulated by quantitative and/or kinetic enhancements. Also, analysis of candidate gene expression data in a segregating population could infer causal relationship between the differential expression of the genes and the resistance phenotype of the progeny. Finally, these candidate resistance genes might be at the basis of the development of molecular marker tools to be used in a genome-informed breeding program to speed-up the selection process of resistant plants.

AVAILIBILITY OF SUPPORTING DATA

The whole cDNA-AFLP library isolated from ‘Président Roulin’ has been deposited at DDBJ/EMBL/GenBank under the accession numbers JZ719314 to JZ719813. Other supporting data are included as Additional Table 1,Additional Table 2, Additional Table 3 and Additional Figure 1.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS’ CONTRIBUTIONS

HB, DM, YM, OP and ML conceptualized and designed the study. ML and DM coordinated the study. HB did the inoculation and the phenotyping of the plants with the advice of ML. OP helped in the setting up of the cDNA-AFLP protocol. HB carried out the cDNA-AFLP and qRT-PCR experiments under the supervision of YM and DM. HB annotated the sequences and analyzed the results. HB, DM, YM, RP, OP and ML participated in the results discussion. HB drafted the manuscript. DM, YM and RP reviewed the manuscript. All authors read and approved the final manuscript.

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Additional tables and figures

Additional Table 1 : Sequence annotation using the bioinformatics software Blast2GO. This Table provides a full list of differentially expressed TDFs isolated from ‘Président Roulin’, their expression pattern according to the cDNA-AFLP and their annotation using the bioinformatics software Blast2GO.

This table can be consulted at **http://www.biomedcentral.com/1471-2164/15/1043** (Bastiaanse et al., 2014)

Additional Table 2: Over-representation of GO categories in our cDNA-AFLP library compared to Rvi6 (HcrVf2)-'Gala' transformed library of Paris et al., 2009.

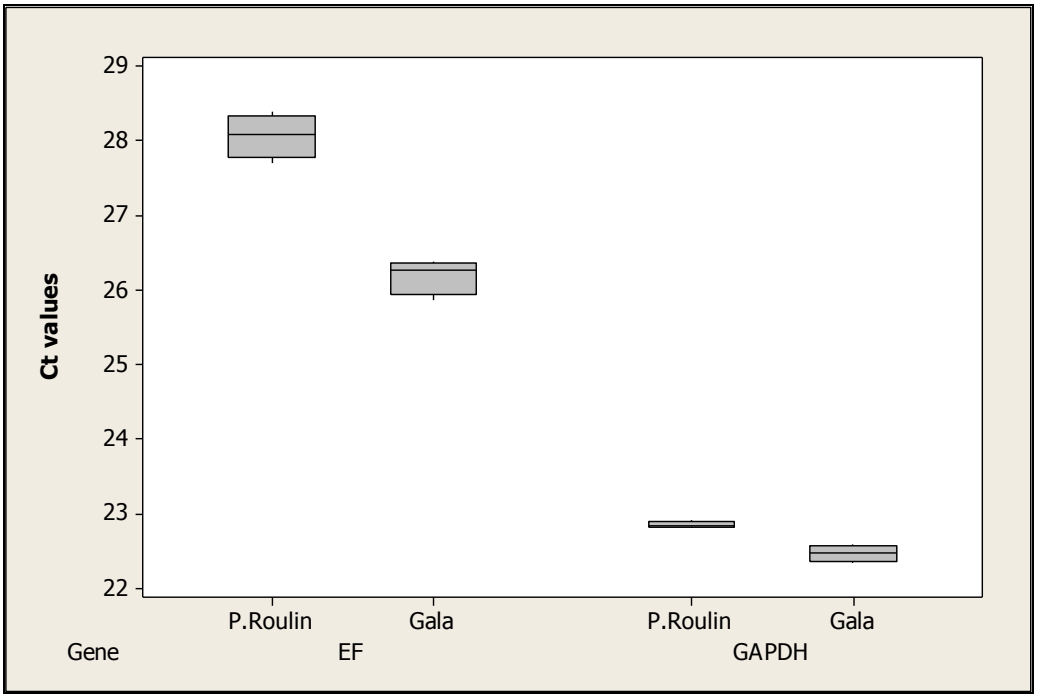
GO-ID	Term	P-Value [a]	#Test	#Ref.	#Annot. Test	#Annot. Ref.	Over/Under	Test Seq.	Test Seq. description
GO:0006281	DNA repair	0,049	6	2	256	391	over	33DU42'	structural maintenance of chromosomes 1
								Unigene7_34DU101 _38EU85	dna-3-methyladenine glycosylase i
								45CU49	formamidopyrimidine-dna glycosylase
								56BU69'	auxin-resistance protein 1
								33DU42	structural maintenance of chromosomes 1
								53CU21	dna-3-methyladenine glycosylase i
GO:0019220	regulation of phosphate metabolic process	0,040	5	1	257	392	over	37EU53	rab gdp-dissociation inhibitor
								49HU129	adp-ribosylation factor gtpase-activating protein agd8
								1AU85'	n-terminal domain containing expressed
								39FU141	d3-type cyclin
								34DU#2	adp-ribosylation factor gtpase-activating protein agd2-like
GO:0051336	regulation of hydrolase activity	0,025	4	0	258	393	over	37EU53	rab gdp-dissociation inhibitor
								49HU129	adp-ribosylation factor gtpase-activating protein agd8
								34DU#2	adp-ribosylation factor gtpase-activating protein agd2-like
								37DU41	cysteine proteinase inhibitor
GO:0031347	regulation of defense response	0,010	5	0	257	393	over	38FU97	structural constituent of ribosome
								1AU61'	e3 sumo-protein ligase siz1
								43BU45	proteasome subunit beta type-6
								41CU29'	protein bonzai 3-like
								51HU129'	tocopherol cyclase

[a] Gene enrichment analysis was conducted with the software Blast2Go using Fisher's Exact Test at a p-value <0.05

Additional Table 3 : List of primer pairs used for qRT-PCR validation of differential gene expression revealed by cDNA-AFLP.

TDF	Primer forward (5′–3′)	Primer reverse (5′–3′)	Size
43DU149'	TCCAAAGATGAGCCTAGTAGTGT	TCGTTAGAGACGGATACCCATA	155
56AU33'	CACCCAATTCTCCCATACTGA	ACCGAAGAGCGTGAAAAGTG	151
42BUHcrVf	AGTCTAGCTTCGTCGGCAAC	GTCTTCGAGTAAACGGTATCCTCCT	125
43CU118	CCTCCAGTTCATCGTTTCCA	GGACATATTGCCCAGTCACA	93
44AU9	AAGGGCCGACTTTCAAACTT	AAATCTGGCGGTGACAACTC	188
44GU169	AGTGTGTTTTCCCACCTTGC	TCTCAAAGCGATTCCCTGAT	147
54CU21	AACACGCAGTACCTGGTGAA	ACTGCAGGGCTGATCAACTT	154
2EU181	CCTTGACCGTCCAGTTCCTA	TCCCTCGTGGAAGTTTGTTT	186
39AU13	GAGGCACATCAGTCTCACGA	CTATGGCCTCCCAGTGAAGA	178
46EU122	GGAGAAGAGAAACCGTGCTG	CGAGCCTCTTGCCTGAATAG	190
51DU17	TGTTGAGCCTTCTGTTGCAC	TGTGGATGCGTTGCTTACAT	168
53DU34	GCTGGGTACAGACGGAATGT	TTGCCGGAACAGATACAACA	196
56AU5'	CAAAGGAAAGAGCTCAACCAA	AAGCCGGTAGAGGGAAACAT	160
43DU149	CCAGACCTTGTTCTTTGAGC	CGAGCAATCTGCACGAATTT	100
51HU129'	CTTTGGCATCAAGGTTCCAT	CATATTTGCCAATGGGGTTC	182
Consensus 44EU122/ 44EU118	GAAAATGCCCAACAACAACC	GCCCACTGAATCAACCTCAT	150
37DU41	GAAGCTGTACGAAGCGAAGG	ACAGATTGCCACCCAGGA	153
44DU105' (primer pair 1)[1]	GCTTATGGTGCTCCTGGCTA	CCTGAGGAGATTCGCAAGAC	-
44DU105' (primer pair 2)[1]	GGCTACGGGGGTACTTGTTT	GATTCGCAAGACGTTCAACA	-
2EU169 (primer pair 1)[1]	TGGAAGAATGCCAGCTACAA	TGTGGACAAATTTGGTGGTG	-
2EU169 (primer pair 2)[1]	CATGCAAAGCAGAAAAGCAA	AGTGCAACACTCCCATGACA	-
1AU61'	TAATCGCGGTCTCACAGAAC	CAGAGAAGGTGCCATGGATT	148
56AU29	GAGGTGGATGACCCCTATCA	CCTTGCCACAAACTGATTCC	155
53HU89	ACCAATTCTCCTCGGGCTAC	TCGACACGTCCTCTTCCTCT	150
44GU173	GATTACAAGACGGTGCAGGAG	CTATCCCGTCACCCAAGAAC	151
55FU102	GACACACATTCCCTACTGCAA	ACACTTTGGCCAGTTGATCC	172
55HU125'	CGGATAGTGAATGCGAATGA	GTTGCAGCCAAAAGAAGAGG	127
44GU182	AAGGTGGGAACATGGAATCA	TGAACCCAGAAACCTATCTGAAA	104

[1] None of these primer pairs led to any specific amplification of the TDFs



Additional Figure 1: Expression variation of candidate housekeeping genes in apple leaves challenged by *V. inaequalis* and mock-inoculated. Two housekeeping genes have been tested on 'Président Roulin' and 'Gala' leaves: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and elongation factor gene (*EF*). For each cultivar and each gene, boxes represent lower and upper quartiles of cycle thresholds range obtained in inoculated (2 qRT-PCR technical replications) and control mock-inoculated (2 qRT-PCR technical replications) plants, with medians indicated. Whiskers represent 10th and 90th percentiles. Graph was plotted using Minitab 16.0.

Chapter 3.

Candidate defense genes as predictors of partial resistance in 'Président Roulin' against apple scab caused by *Venturia inaequalis*

Outline

In this chapter, the effect (partial vs. complete) and spectrum of action (narrow- vs. broad-spectrum) of the resistance loci present in 'Président Roulin' were analysed using pathological tests in a F1 segregating population derived from the resistance cultivar. From the cDNA-AFLP transcript profiling, candidate defense genes (CDGs) were selected based on their specific expression in 'Président Roulin' after pathogen challenge as well as their known role in plant defense. Expression of the CDGs was investigated over a time course experiment after pathogen inoculation. Co-localization with known scab resistance factors (R genes, QTLs or RGAs) was also investigated by mapping *in silico* the cDNA sequences on the reference 'Golden Delicious' apple genome. Finally, a modest contribution of the functional assessment of the CDGs has been made by estimating the extent of correlation between their expression and the resistance levels of individuals composing a F1 segregating population derived from 'Président Roulin'.

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Candidate defense genes as predictors of partial resistance in 'Président Roulin' against apple scab caused by *Venturia inaequalis*

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Abstract

Scab, caused by the fungus *Venturia inaequalis*, is one of the most important diseases of apple. Although major scab resistance genes (R-gene) have been widely studied, little is known about the molecular mechanisms underlying partial resistance, thought to be more durable. We used a candidate gene approach to decipher the genetic determinism of the durable partial resistance in ‘Président Roulin’, an old Belgian apple cultivar. Pathological tests using monoconidial isolates of *V. inaequalis* on F1 ‘Gala’ x ‘Président Roulin’ progeny suggested that partial resistance was broad spectrum but resulted from the combination of several race-specific interactions and was governed by at least five R-genes. From an earlier transcript profiling study, we selected 13 pathogen-regulated genes in ‘Président Roulin’ with a known role in plant defense and characterized their expression over a time-course experiment. These candidate defense genes (CDGs) were regulated between 6 and 120 hours after inoculation. Most were significantly up or down regulated in incompatible interactions only, or were induced earlier compared with compatible interactions. Among them, eight were mapped *in silico* within chromosomal regions containing disease-resistance factors (R-gene analogues, major scab R-genes or quantitative trait loci). We also investigated the extent of the correlation between CDG expression data and phenotypic variation in the progeny. We estimated that the induction of nine out of 10 CDGs accounted for up to 46% of the phenotypic variance. Our study has improved the understanding of partial apple scab resistance and could be used in developing functional molecular markers for breeding new ‘spray-free’ cultivars with durable scab resistance.

Keywords:

Apple, partial resistance, *Venturia inaequalis*, gene expression, candidate resistance gene, segregating population.

Introduction

Apple scab, caused by *Venturia inaequalis*, is one of the most severe and widespread fungal diseases, affecting most of the apple growing areas in the temperate regions of Europe, Asia, the Americas and Australasia (Dunemann and Egerer 2010). Currently, apple scab is controlled by the intensive use of pesticides in the orchard. Due to its major impact on economic cost, environment and human health, apple scab has received a lot of attention by researchers and has become the most studied plant-pathogen interaction involving a woody species (Cova et al. 2010). The growing of resistant varieties has been proposed as an effective alternative to the use of pesticides, significantly reducing the number of fungicidal treatments needed to protect susceptible cultivars (Parisi et al. 2000).

Apple genetic resistance to scab is complex, involving loci for both complete and partial resistance to the pathogen, and a 'great deal of gray area' between these two extremes (Poland et al. 2009). Complete resistance confers high levels of protection, and fully inhibits pathogen reproduction. This resistance is governed by a single major resistance gene in the host and involves a gene-for-gene relationship (GfG) with the avirulence (*Avr*) gene of the pathogen (Flor, 1971). It is often non-durable since it facilitates a strong directional selection on pathogen isolates presenting higher virulence, which can lead to resistance breakdown (Gessler et al. 2006), as illustrated with the *Rvi6* (*Vf*) scab R-gene (Parisi et al. 1993). In contrast, partial resistance confers a reduction rather than a lack of disease, and the disease phenotypes tend to be measured quantitatively. It is generally controlled by multiple loci of partial effects, referred to as quantitative trait loci (QTLs). Although experimental evidence for durability remains scarce and despite the fact that partial resistance could also be subjected to erosion (Caffier et al., 2014), such resistance is frequently assumed to be more durable than complete resistance (Parvlevliet 2002; Kou and Wang 2010), presumably due to the smaller effects and the multiplicity of the partial resistance genes (Poland et al. 2009). Exploring partial resistance has therefore become an attractive alternative for controlling apple scab.

To date, at least 18 major resistance genes (R-genes) have been identified in *Malus* species and domesticated apples (Bus et al. 2011; Soriano et al. 2014). Most of them have been mapped to the apple genome and their GfG relationships with *Avr* genes in *V. inaequalis* defined (Bus et al. 2011; Caffier et al. 2015), but so far only two have been cloned. The *Rvi6* (*Vf*) resistance locus revealed the presence of a cluster of four resistance gene paralogs (called *HcrVf* genes), similar to the tomato *Cf* resistance gene, encoding leucine-rich repeat receptor-like proteins (LRR-RLP) (Vinatzer et al. 2001), and *Rvi15* (*Vr2*) has been reported to contain three toll interleukin1 receptor nucleotide binding site LRR (TIR-NBS-LRR) genes (Galli et al. 2010). The function of all these genes was analyzed and only two, *HcrVf2* (Belfanti et al. 2004; Joshi et al. 2011) and *Vr2-C* (Schouten et al. 2014) for *Rvi6* and *Rvi15*, respectively, were shown to be functional against *V. inaequalis*. There is disagreement in the literature on the *Vf1a* (syn. HcrVf1) function (Malnoy et al. 2008; Joshi et al. 2011).

QTLs for scab resistance have been identified and mapped to 10 out of the 17 linkage groups (LGs) of apple (Durel at al. 2003; Liebhard et al. 2003; Durel et al. 2004; Calenge et al. 2004; Soufflet-Freslon et al. 2008). Genetic studies suggested that proteins underlying partial plant resistance were involved in diverse mechanisms related to complete resistance (defeated R-genes theory), basal defense and unknown mechanisms since the function of some proteins underlying QTL has not been described yet (Ballini et al. 2008; Poland et al. 2009). Although geneticists are now equipped with the whole genome sequence assembly of *Malus* x *domestica* (Velasco et al. 2010), the nature of proteins governing partial scab resistance remains largely unknown. This is likely mainly due to the high number of candidate defense genes located inside the large confidence interval of the identified apple scab QTLs. An alternative strategy for deciphering the molecular basis of QTLs is to identify transcriptional responses of genes as key drivers of quantitative traits in experiments that combine both positional information and functional sequence tags such as cDNAs. In fact, the molecular basis underlying allelic variation at QTLs is similar to the identified variation for simple Mendelian loci, namely, alterations in gene expression or protein function (Paran and Zamir 2003). Expression gene profiling could be carried out between contrasting QTL genotypes (Wayne and McIntyre, 2002; Hazen et al. 2005; Baxter et al. 2005; Steiner et al. 2009) or between each individual of a segregating population (Damerval et al. 1994; Jansen and Nap 2001; Liu et al. 2011). In this latter case, gene expression data can be analyzed in combination with molecular marker data, making possible the use of QTL analysis for the identification of influential genes and gene products (expression QTL or eQTL).

'Président Roulin' is an old Belgian cultivar with durable resistance and is used in apple breeding programs at the Walloon Agricultural Research Center (CRA-W) to broaden genetic apple scab resistance and thus reduce the risk of resistance breakdown. Symptoms of resistance range from chlorosis to necrosis with only slight sporulation (Chevalier class 3a) (Chevalier et al. 1991). Its main components of resistance are reduced incidence and severity compared with the susceptible cultivars 'Jonagold' and 'Golden Delicious', as well as a delay in the appearance of the first symptoms (Villette 2000). In our breeding program, the resistance in 'Président Roulin' has also been shown to be polygenically inherited (unpublished data), but the number and identity of resistance loci remain unknown. Previously, using cDNA Amplified Length Polymorphism (cDNA-AFLP), we analyzed the transcript profiling of 'Président Roulin' (partially resistant) and 'Gala' (susceptible) at 48 hours post inoculation (hpi) (Bastiaanse et al. 2014). Among the 20,500 transcript-derived fragments (TDFs) generated during the analysis, we identified potential candidate defense genes (CDGs) that could form the basis of the partial resistance in 'Président Roulin'. These genes were shown to be significantly (up to twofold) induced or repressed 48 hours after pathogen challenge, compared with the susceptible cultivar 'Gala', and encoded for proteins known to act in disease resistance in other plant-pathogen interactions.

In this study, we first characterized the genetic determinism of the partial resistance of 'Président Roulin' by looking for differential interactions between various races of *V. inaequalis* and genotypes of F1 'Gala' x 'Président Roulin'. Then we proposed a candidate gene approach (Pflieger et al., 2001) to identify potential CDGs underlying the partial resistance of 'Président Roulin' consisting of three steps: the choice, screening, and functional assessment of CDGs.

Thirteen CDGs were chosen among the set of CDGs identified previously in 'Président Roulin' (Bastiaanse et al. 2014). Then, these CDGs were screened by investigating their dynamic of expression across a time-course experiment during scab infection and their co-localization with resistance gene analogues (RGAs), major R-genes or apple scab QTLs. Finally, contributions to the functional assessment of a subset of 10 CDGs were achieved by investigating the extent of correlation between their expression and the level of resistance/susceptibility in the segregating population.

Materials and methods

Evaluation of the partial scab resistance of 'Président Roulin' in unsprayed orchards

Over some 25 years (from 1984 to 2009), scab incidence on the leaves of the partially resistant 'Président Roulin' cultivar has been recorded at the Walloon Agricultural Research Centre in Gembloux, Belgium in various orchards under natural scab infection and in the absence of fungicide treatments. Scab incidence was scored using a 1-9 scale adapted from Lateur and Populer (1994), where 1 = no incidence and 9 = tree completely infected. Depending on the year, scab incidence was scored on between 1 and 17 trees. The highly susceptible 'Golden Delicious' cultivar was used as the control. Typical scab resistance reactions on 'Président Roulin' under natural infection in the field were described.

Plant material and inoculation with Venturia inaequalis

Inoculation was performed on the clonal parents 'Président Roulin' (partially resistant) and 'Gala' (susceptible) and their progeny, comprising 120 seedlings. The clonal 'Président Roulin' and 'Gala' trees were grafted on M9 rootstocks and grown in 3l plastic pots with a potting mix in the greenhouse at 20°C under 16 hours of illumination by daylight-incandescent lights. The seedlings were sown in trays with a seed-raising mix containing a general slow-release fertilizer. The trays were saturated with water, lined with plastic and stored in a cool store for a stratification period of 80 days, followed by germination in the glasshouse. The seedlings were then grown in individual plastic pots on their own roots for 2 years. After this, they were grafted in triplicate on M9 rootstocks and grown in 3 liters plastic pots with a potting mix in the glasshouse, as previously described.

In this study, various monoconidial isolates of *V. inaequalis* obtained from the Institut National de la Recherche Agronomique **(**INRA) collection at Angers, France and from the Plant Research International (PRI) collection at Wageningen, The Netherlands were used (**Additional Table 4**). For the gene expression analysis, young leaves of actively growing plants were sprayed with a suspension mix of conidia from six strains of *V. inaequalis* belonging to race 1: EU-B04, EU-B16, EU-D49, EU-F05, EU-F11 and EU-I09 (Caffier et al. 2015; Bus, personal communication). Isolates were grown separately and mixed together to constitute the inoculum calibrated at 2.5×10^5 conidia ml^{-1}. The leaves were sprayed in sufficient quantities to form small

droplets on the leaf surface before run off. The control plants were inoculated with sterile water. In a phytopathological test, we also inoculated monoconidial isolates belonging to races 1, 2, 3, 6, 7, 8, 9, 10 and 13 (1639, EU-NL24, EU-B04, 1066, EU-D42, EU-NL05, EU-B05, EU-D49) (Caffier et al. 2015; Bus, personal communication), separately, on 'Gala' x 'Président Roulin' progeny and their parents, using the droplet inoculation technique described by Bus et al. (2005). A 130 µl droplet containing the inoculum calibrated at 1×10^5 conidia ml^{-1} was deposited in the small inoculation chambers clipped onto the youngest fully expanded leaf of actively growing shoots. The inoculations were performed once on each triplicate of the 120 genotypes of the segregating population.

Isolate cultivation, storage and inoculum preparation were carried out as described by Bastiaanse et al. (2014). After inoculation, the plants were incubated for 2 days under optimal conditions for infection (at 20°C under maximum relative humidity, RH) and were then transferred to the greenhouse (20°C, 60-80% RH). The small chambers used in the droplet inoculation technique were removed after the 2-day incubation stage. Symptoms were recorded 21 days after inoculation. We scored sporulation severity on a scale of 1 to 5 (1 = no sporulation, 5 = heavy sporulation). These scores were used to compute maximum sporulation severity (MSPOR) over the genotype replications, taking account of the most susceptible plant. Where appropriate, a distinction was made in the resistance symptoms between hypersensitive reactions (HR), necrosis (N), chlorosis (Chl), stellate necrosis (SN) and stellate chlorosis (SC). The plants were classified as being resistant (R) or susceptible (S) according to the MSPOR index (R from 1-3 and S >3) and the presence of resistance reactions.

RNA extraction

Leaf tissues challenged by the inoculum mix of race 1 of *V. inaequalis* were harvested from the youngest leaves of 89 individuals randomly selected from the 120 'Gala' x 'Président Roulin' seedlings (one plant per leaf sampling) at 48 hpi and from mock-inoculated plants of each genotype. Leaf tissues were also harvested from the clonal 'Président Roulin' and 'Gala' cultivars (two plants per leaf sampling) at 6, 24, 48, 72, 96 and 120 hpi, as well as from control plants mock-inoculated with water at each of the corresponding time points. The leaves were quickly frozen in liquid nitrogen and stored at -80°C prior to total RNA extraction. Total RNA was isolated from 100 mg of ground leaf tissue, using the extraction method described by Gasic et al. (2004), and DNase I treatment was performed (Fermentas Inc). With total RNA extracted from the leaves of the clonal 'Président Roulin' and 'Gala' trees only, a further mRNA purification step was performed starting from 250 µg total RNA, using the Qiagen Oligotex mRNA kit (Qiagen Inc.). For all samples, RNA purity and concentration were measured using Nanodrop technology (Thermo Scientific Inc.) and double stranded cDNA was finally obtained starting from 500 ng mRNA, using the Superscript Double Stranded cDNA Synthesis kit (Invitrogen Inc.) and following the manufacturer's instructions.

Selection of partial resistance candidate genes and quantitative real-time reverse transcription PCR (qRT-PCR)

From our previous cDNA-AFLP study (Bastiaanse et al. 2014), we selected 13 TDFs as potential CDGs for partial resistance in 'Président Roulin' against *V. inaequalis*. They were specifically induced in 'Président Roulin' at 48 hpi and encoded for proteins reported in the literature to have a potential role in the general defense response pathway (**Table 8**). They were involved in the pathogen recognition, signal transduction, transcription, reactive oxygen species (ROS) production, protein modification, carbohydrate metabolism and cell wall organization. Among them, only two CDGs (44EU122/44EU118) were also differentially regulated in the susceptible 'Gala' cultivar. They were selected because they encoded for a cysteine protease that could balance the action of 37DU41, a cysteine protease inhibitor that is part of the TDF selection.

Primers specific to the TDFs were designed using Primer3 software (Rozen and Skaletsky, 2000). qRT-PCR was performed using the Biorad CFX96 and Maxima SYBR Green qPCR master mix (Fermentas Inc.) following the manufacturer's instructions. Amplification of the 13 TDFs was achieved using the mRNA extracted from the clonal trees at different time points after infection, while a subset of 10 TDFs was chosen for qRT-PCR using the mRNA from the segregating populations. This later study aimed to identify the extent of correlation between gene expression of the CDGs and resistance level in each individual of the population. The subset of 10 CDGs was selected based on the diversity of their biological function and on their mapped locations in regions known to contain disease resistance factors (RGAs, QTLs or major apple scab R-genes). Two CDGs were involved in pathogen recognition (43DU149' and 44AU9), two in gene transcription (53HU89) and signal transduction (2EU181), and five in plant defense reactions (44EU122, 44GU182, 43DU149, 37DU41 and 44GU173).

The PCR conditions were the same for all primer pairs: initial denaturation at 95°C for 10', followed by 40 cycles of denaturation at 95°C for 15", annealing at 60°C for 30" and extension at 72°C for 30". Fluorescence data were collected at the end of the annealing step. After cycling, the samples were denatured at 95°C for 10". The melting curve was then determined in order to differentiate between the desired amplicons and any primer dimers or DNA contaminants (in the 65-95°C range, with a temperature increment of 0.5°C for 5"). Each reaction was run in duplicate (technical replicate). LinRegPCR software was used to confirm that individual PCR efficiencies were about 2 for each primer pair (Ramakers et al. 2003). A list of the specific primer pairs used for each TDF is given in (**Additional Table 5**).

Table 8 : List of the candidate defensegenes (CDGs) and their role in plant defense responses according to the literature. The CDGs were differentially expressed in the partially resistant cv. 'Président Roulin' but not in the susceptible cv. 'Gala' after scab inoculation (except for the consensus sequence 44EU122/44EU118)

CDG	Genbank ID	Role in plant defense	Literature references	Plant-pathogen interaction under study
		Pathogen recognition		
43DU149'	JZ719417.1	cc-nbs-lrr resistance protein	McHale et al., 2006	numerous plant-pathogen interactions
56AU33'	JZ719506.1	cc-nbs-lrr resistance protein	McHale et al., 2006	numerous plant-pathogen interactions
44AU9	JZ719419.1	LRR receptor kinase-like protein	Komjanc et al., 1999; Song et al., 1995	apple-*V. inaequalis* rice *Xanthomonas oryzae*
		Signal transduction		
2EU181	JZ719320.1	Putative MAP kinase	Zhang and Klessig, 2001 Pedley and Martin, 2004 Eckey et al., 2004	*Arabidiopsis*-several pathogens tomato-*Pseudomonas syringae* barley-*Blumeria graminis*
		Transcription		
53HU89	JZ719483.1	Zinc finger homeodomain protein1	Korfhage et al., 1994 Park et al., 2007	parsley-various fungal and bacterial elicitors soybean-*Pseudomonas syringae*
		Reactive oxygen species production		
43DU149	JZ719416.1	Peroxidase 12, class III peroxidase	Almagro et al., 2009	numerous plant-pathogen interactions
51HU129'	JZ719472.1	Tocopherol cyclase	De Gara et al., 2003	numerous plant-pathogen interactions
		Protein modification		
Consensus 44EU122/ 44EU118	JZ719578.1 JZ719577.1	Cysteine protease	Krüger et al., 2002 Avrova et al., 1999 Hao et al., 2006 D'Silva et al., 1998 Solomon et al., 1999	tomato-*Cladosporium fulvum* potato-*Phytophthora infestans* tomato-*Pseudomonas syringae* cowpea-*Uromyces vignae* soybean-*Pseudomonas syringae*
37DU41	JZ719360.1	Cysteine protease inhibitor	Gutierrez et al., 1999 Solomon et al., 1999 Liu et al., 2008	tobacco-potyvirus soybean-*Pseudomonas syringae* rice-*Magnaporthe grisea*
44GU182	JZ719427.1	Lysosomal Pro-X carboxypeptidase	Moura et al., 2001	*Arabidiopsis-Pseudomonas syringae* *Arabidiopsis-Alternaria brassicicola* tomato-insects
		Defense response		
56AU29	JZ719503.1	Chitinase	Collinge, 1993 Métraux and Boller, 1986 Jongedijk et al., 1995 Vögeli-Lange et al., 1988 Krishnaveni et al., 1999 Sahai and Manocha, 1993.	numerous plant-fungi interactions cucumber-numerous pathogens tomato- *Fusarium oxysporum* tobacco-*TMV* sorghum- *Fusarium moniliforme* numerous plant-fungi interactions
		Cell wall organization		
44GU173	JZ719426.1	Pectin methylesterase inhibitor	Lionetti et al., 2007 An et al., 2008	*Arabidiopsis-Botrytis cinerea* pepper-*Xanthomonas campestris*

Data analysis

Severity of scab infection on the cultivars 'Président Roulin' and 'Golden Delicious' in the orchard over the 17 years of observation were subjected to the ANOVA procedure and a Pearson correlation coefficient between both cultivar disease scores over years was calculated.

In all the gene expression experiments, the relative expression ratio between scab-inoculated and water-treated plants was evaluated using the ΔΔCt method described by Applied Biosystems (fold change=$2^{\Delta\Delta Ct}$), with the glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) as the internal reference (primers sequence F-5'CAAGGTCATCCATGACAACTTTG3', R-5' GTCCACCACCCTGTTGCTGTAG3'). GAPDH has appeared to be stably expressed in other qRT-PCR studies conducted on apple (Aldaghi et al. 2012; Gadiou and Kundu 2012), as well as under our experimental conditions (Bastiaanse et al. 2014). Individual ΔCt values from each technical replicate of the qRT-PCR experiments were subjected to the ANOVA procedure at a statistically significant level of $P<0.05$. The biological level of significance for differential gene expression was set at the minimum level of twofold change between inoculated and non-inoculated plants. In the segregating population, the effect of the resistant vs susceptible status of the genotypes (R/S, considered as an explanatory factor) on the log-transformed relative expression of the individual CDGs (log $2^{\Delta\Delta Ct}$) was tested using a single factor ANOVA. The significance level was fixed at $P<0.05$, and the proportion of variation of log $2^{\Delta\Delta Ct}$ explained by the R/S factor (R^2) was computed. Then in order to estimate the contribution to the sporulation severity of several CDGs jointly considered, a multiple regression of MSPOR (as the explained variable) was performed on CDGs $\log 2^{\Delta\Delta Ct}$ values (multiple explanatory variables). In this regression analysis only the CDGs significantly associated with resistance by the ANOVA test were included. All the statistical analyses were performed using Minitab 16 software.

In silico mapping and co-localization with RGAs, QTLs or apple scab major R-genes

The CDG sequences were compared with the whole genome sequence assembly v1.0 of the 'Golden Delicious' apple cultivar (Velasco et al. 2010) using a BLAST-N sequence similarity search and taking account of the best blast result. QTLs and major scab R-genes already identified in apple (Bus et al. 2011; Gessler et al. 2006; Soufflet-Freslon et al. 2008) were also anchored on this physical map by blasting the sequences of the flanking SSR molecular markers (retrieved from http://www.hidras.unimi.it) on the apple genome sequence assembly. We added to the map only those markers with an E-value ≤ 3e-3, a ratio of matched bases to a marker sequence equal to 100% and a position on the expected chromosome of 'Golden Delicious' (according to their genetic position). Chromosomal positions of RGAs (individual or organized in clusters) were obtained from the work reported by Perazzolli et al. (2014). Only CDGs mapping inside a QTL confidence interval or mapping within 250 kb from any RGAs or a major scab R-gene were considered to co-localize with genomic regions involved in resistance.

Results

Scab incidence on 'Président Roulin' under natural infection in the field

Since the first evaluation in 1984, scab incidence in the field has always been low for 'Président Roulin', with a mean incidence ranging from 1 to 3.75, depending on the year under investigation **(Figure 18**). Compared with 'Président Roulin', the 'Golden Delicious' cultivar has always been significantly more susceptible (scab incidence mean ranging from 3 to 8.3) ($P<0.001$). When more scab lesions were observed on 'Président Roulin' leaves, a tendency of higher scab incidence levels was also observed on 'Golden Delicious' (Pearson correlation coefficient between both cultivar disease scores of 0.285, significant at $P<0.05$). Fluctuation in scab severity in both cultivars could reflect the variation in scab pressure over the years in the orchard. In the field (**Additional Figure 2**), typical scab resistance reactions in 'Président Roulin' corresponded with a mix of Chl and N reactions with no or limited sporulation (class 3a of Chevalier et al. 1991).

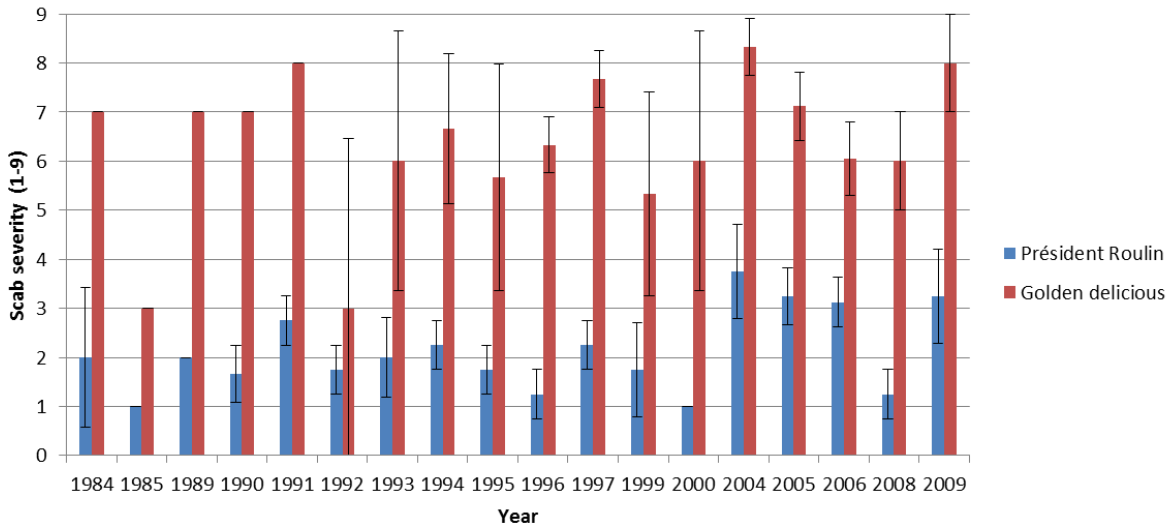

Figure 18 : Scab incidence recorded on 'Président Roulin' (partially resistant) and 'Golden Delicious' (susceptible) over some 25 years in different orchards in Gembloux, Belgium. Scab incidence was evaluated under natural infection and no fungicide treatments using the scale proposed by Lateur and Populer (1994) where 1 = no incidence and 9 = tree completely infected. Bars represent the scab incidence mean recorded on between 1 and 17 trees, depending on the year and the cultivar. Associated standard deviations of each histogram are represented.

Pathological tests and disease assessment in the glasshouse

Pathological tests on 'Gala' x 'Président Roulin' progeny and their parents revealed differential interactions between the various monoconidial isolates of *V. inaequalis* and the genotypes. Representative symptoms for each parent, as well as the segregation ratio (R:S) observed in the progeny for each inoculum, are presented in **Table 9**. 'Gala' was highly susceptible (MSPOR 5) to all isolates tested, without any resistance reaction. In contrast, 'Président Roulin' was resistant to all isolates, with strong resistance reactions (Chl, N or HR) and

sporulation levels ranging from 1 (no sporulation) to 3 (50% sporulation), depending on the isolate. **Additional Figure 3** illustrates reactions observed after inoculation with the various scab isolates. Different segregation ratios between resistant and susceptible seedlings (R:S ratio) were observed for each inoculum (**Table 9**): R:S = 1:1 for the isolates 1639, EU-B05, EU-D49 and the inoculum mix; R:S = 1:2 for the isolates EU-D42 and EU-NL05; and R:S = 1:3 for the isolates EU-NL24, EU-B04 and 1066 (P>0.05).

Table 9 : Phenotypic class and segregations for the 'Président Roulin' (partially resistant) and 'Gala' (susceptible) parents and their progeny after inoculation with various single-spore *V. inaequalis* isolates.

Isolate	Phenotypic class[a]		Segregation[b]		R:S ratio		
	'Président Roulin'	'Gala'	R	S	ratio	χ^2	P
1639	HR/N/1	4/5	57	63	1:1	0.3	0.584
EU-NL24	Ch/N/2	4/5	22	98	1:3	2.8	0.092
EU-B04	Ch/N/3	4/5	34	86	1:3	0.7	0.399
1066	Ch/N/3	4/5	34	86	1:3	0.7	0.399
EU-D42	Ch/N/2	4/5	42	78	1:2	6.4	0.699
EU-NL05	HR/Ch/2	4/5	46	74	1:2	6.5	0.245
EU-B05	HR/Ch/2	4/5	63	57	1:1	0.3	0.584
EU-D49	Ch/N/3	4/5	63	57	1:1	0.3	0.584
Inoculum mix [c]	Ch/N/2	4/5	63	57	1:1	0.3	0.584

[a] Parents and their progeny were grafted in triplicate, and symptoms were recorded 21 days after inoculation using the droplet inoculation technique (Bus et al., 2005). The phenotypic classes (Ch=chlorosis, N=necrosis, HR=hypersensitive reaction, 4=no resistance reaction) are followed by the maximum sporulation index (MSPOR, scale 1-5, with 1=no sporulation) recorded on the three replicates.
[b] R=resistant (presence of resistance reactions and MSPOR=1-3), S=susceptible (absence of resistance reactions and MSPOR=4-5)
[c] Inoculum mix is made up of the isolates EU-B04, EU-I09, EU-F11, EU-D49, EU-F05 and EU-B16

From our pathological tests (**Table 10**), we observed that 22 seedlings out of the 120 tested were resistant for all nine inoculums and 45 were fully susceptible. The remaining 53 seedlings showed differential interactions with *V. inaequalis*. The same resistance behavior was observed for some inocula: no differential interactions were observed between EU-B04 and 1066, or between EU-B05, EU-D49 and the inoculum mix. Also, some seedlings were incompatible with only one isolate among the set of isolates tested (the other isolates were all compatible with these particular seedlings). This was particularly so for 1639 and the isolates EU-B05/EU-D49/inoculum mix, which had the same resistance behavior. In contrast, more complex differential interactions were observed with the other isolates. For example, we noticed that isolate EU-NL24 showed resistance symptoms only on genotypes that were incompatible with all the isolates tested, and for eight genotypes the resistance reactions were recorded after inoculation of all isolates, except for EU-NL24.

Table 10 : Differential interactions of single-spore *V. inaequalis* isolates with genotypes of 'Gala' x 'Président Roulin' progeny. Phenotypes for each isolate were arranged along with a combination of phenotypes (R=Resistance, S=Susceptibility) and seedling numbers.

V. inaequalis isolates[a]						Nb seedlings
1639	**EU-NL24**	**EU-B04/ 1066**	**EU-D42**	**EU-NL05**	**EU-B05/EU-D49/ Mix[b]**	
R	R	R	R	R	R	22
R	S	R	R	R	R	8
S	S	S	S	S	R	9
R	S	S	S	S	R	8
R	S	R	S	R	R	4
S	S	S	R	R	R	9
R	S	S	R	R	R	3
R	S	S	S	S	S	12
S	S	S	S	S	S	45

[a] Progeny were grafted in triplicate, and phenotypes were assessed 21 days after inoculation using the droplet inoculation technique (Bus et al., 2005). Symptoms of resistance (chlorosis, necrosis, HR) and the severity of sporulation (maximum sporulation index, MSPOR, scale 1-5) were recorded. Plants were classified as resistant (R) or susceptible (S) as follows: R=presence of resistance reactions and MSPOR= 1-3; S=absence of resistance reactions and MSPOR=4-5

[b] Inoculum mix is made up of the isolates EU-B04, EU-I09, EU-F11, EU-D49, EU-F05 and EU-B16

Co-localization of CDGs with QTLs or apple scab major R-genes

Anchoring the existing apple genetic map to the whole genome sequence assembly of 'Golden Delicious' (Velasco et al. 2010) revealed a few discrepancies in the order of the microsatellites markers compared with the position on the genetic map produced by Gessler et al. (2006). Also, the relationship between genetic distance in centimorgans (cM) and the physical distance in mega base pairs (Mbp) was found to vary from 0.232 Mbp to 0.838 Mbp per cM, depending on the LG, with an average of 0.398 Mbp/cM.

All the CDGs could be anchored accurately on 11 out of the 17 apple LG (E-value<1e-28). Of the 13 CDGs tested, eight mapped *in silico* on the QTL interval for apple scab resistance and/or near major apple scab R-genes or RGAs (**Figure 19**).

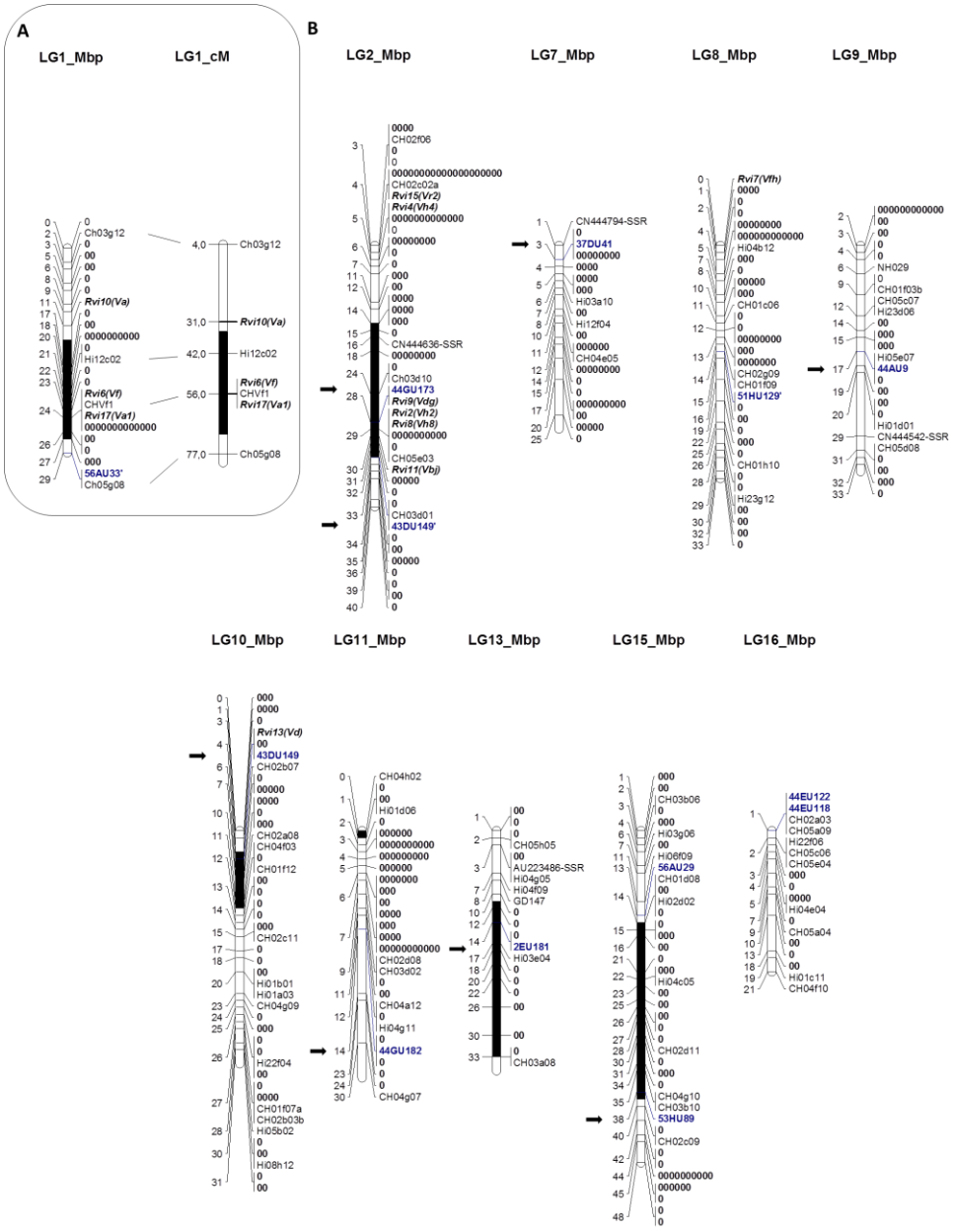

Figure 19 : *In silico* mapping of the CDGs for partial apple scab resistance identified by cDNA-AFLP and co-localization with RGAs, major R-genes and QTLs for apple scab resistance. (A) Example of comparison of the genetic map (cM) created by Gessler et al. (2006) with the physical map (Mbp) obtained by alignment of SSR molecular markers on the whole genome sequence assembly of apple (by BLAST sequence similarity search) (Velasco et al. 2010) (B) Physical map obtained for the other LGs. Black bars represent the interval of confidence of apple scab resistance QTLs as given by Gessler et al. (2006). Dots represent single and clusters of RGAs according to Perazzolli et al. (2014); number of dots reflects the number of RGAs inside the clusters. Italic bold indicates the published major scab resistance genes; bold indicates our disease CGRs for partial scab resistance. Arrows indicate disease CDGs showing co-localization (<250 kb) with RGAs, QTLs or major apple scab R-genes.

Investigation of the time-course expression during V. inaequalis- Malus interactions

The expression profiles of the 13 CDGs were analyzed by qRT-PCR over a time-course experiment during the *V. inaequalis-Malus* interaction (at 6, 24, 48, 72, 96 and 120 hpi). For 7 out of the 13 CDGs, a significant change in expression (more than twofold) was observed for 'Président Roulin' (partially resistant), but not for 'Gala' (susceptible) over the different time-points tested. When a significant change was observed for 'Gala', it happened later than in 'Président Roulin', except for 43DU149 where regulation happened earlier in the susceptible cultivar but in the opposite direction (repression instead of over-expression). Finally, except for one CDG, gene expression regulation in 'Président Roulin' occurred between 24 hpi and 120 hpi, the latest time point tested. 44GU182 only, a gene encoding a carboxypeptidase, was regulated at the early time point of 6 hpi. Expression profiles of the CDGs are presented in **Figure 20**, along with their annotation in plant defense response according to the literature (the other possible roles of these CDGs in the general plant metabolism are not presented here).

Expression studies of candidate resistance genes in a segregating F1 population

A study of the regulation of expression of a subset of 10 CDGs in a segregating population (out of the 13 CDGs initially selected) was made in order to identify the extent of correlation between gene expression and resistance level in each genotype. The significance of the effect of the R/S status on the regulation of gene expression, and the proportion of variance "explained" by the R/S levels conditioned by various *V. inaequalis* isolates are presented in **Table 11**. The results of EU-B04/1066 and EU-B05/EU-D49/inoculum mix were grouped because no differential interactions between those isolates were observed.

Non-significant effect (P-value>0.05) was found for only one CDG (37DU41) during the ANOVA analysis. This CDG showed homology with a cysteine protease inhibitor gene. For the other CDGs tested, significant effects were obtained for at least two inocula and the R2 varied between 4.6 and 21.3%, depending on the inoculum tested. The largest coefficient was found for the phenotypes of EU-B05/EU-D49/inoculum mix and the expression data of 43DU149' (CC-NBS-LRR resistance protein). When the regulation of expression of the whole set of TDFs was considered, the percentage of phenotypic variance of MSPOR accounted by the regulation of CDGs expression ranged from 16.1% (isolate EU-NL24) to 46.0% (EU-B05/EU-D49/inoculum mix).

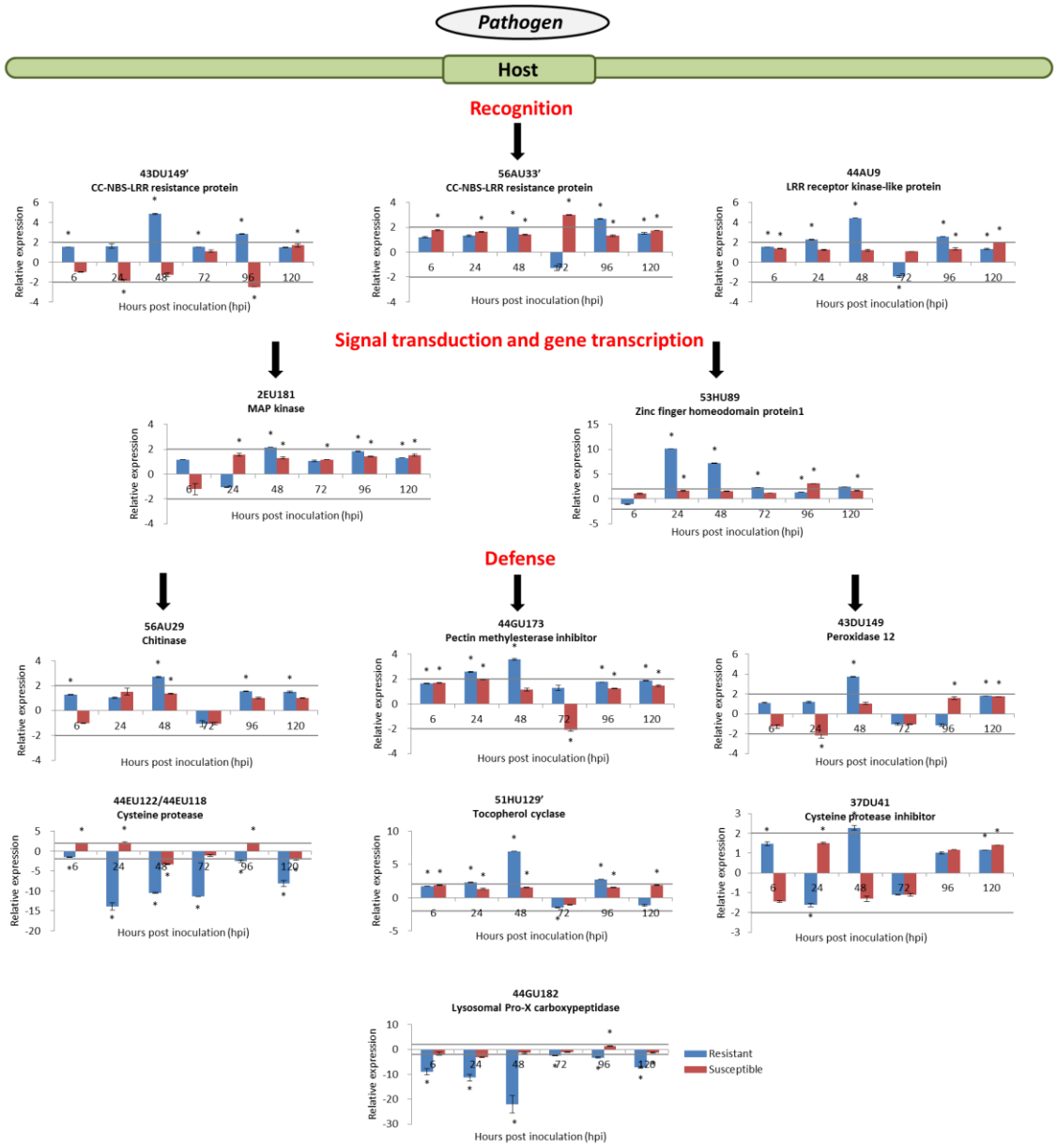

Figure 20 : Time course expression analysis of CDGs analyzed by qRT-PCR during *V. inaequalis-Malus* interaction. The possible involvement in the recognition, the transduction of the signal and the plant defense responses of specific genes was inferred from their involvement in other plant-pathogen interactions reported in the literature. The other possible roles of these CDGs in the general plant metabolism are not presented here. Blue histograms indicate response (induction for positive values and repression for negative values) in 'Président Roulin' (partially resistant), red histograms in 'Gala' (susceptible) at 6, 24, 48, 72, 96 or 120 hpi. The relative expression of CDGs (ΔΔCt) was expressed in 'fold-change' between infected and mock-inoculated plants at each time-point of the experiment, and was based on the expression of the housekeeping gene GAPDH. Individual ΔCt values from the qRT-PCR experiments were subjected to the ANOVA procedure, at a statistically significance level of $P<0.05$ (indicated by *). The bar indicates the biologically significant level of up to twofold induction or repression.

Table 11: Probabilities and correlations between the expression regulation of 10 candidate defense genes (CDGs) measured by qRT-PCR and phenotypes of 89 seedlings (resistant vs susceptible)[a] of the segregating population 'Gala' (susceptible) x 'Président Roulin'(partially resistant). For each CDG, correlations associated with the highest probabilities and R2 were indicated in italic.

Isolates	**1639**		**EU-NL24**		**EU-B04/1066**		**EU-D42**		**EU-NL05**		**EU-05/EU-D49/ Mix**	
CDGs	P	R^2 % [b]	P	R^2 %	P	R^2 %	P	R^2 %	P	R^2 %	P	R^2 %
43DU149'	0.000	15.6	NS	-	0.034	5.1	0.004	9.2	0.003	9.7	**0.000**	**21.3**
44AU9	0.038	5.0	NS	-	NS	-	0.037	5.0	0.036	5.1	**0.028**	**5.6**
53HU89	NS	-	0.029	5.5	NS	-	0.001	13.3	0.002	10.4	**0.000**	**15.2**
2EU181	NS	-	NS	-	0.044	4.7	NS	-	NS	-	**0.033**	**5.3**
44EU122/44EU118	0.044	4.7	0.044	4.7	0.015	6.7	0.008	8.0	0.010	7.6	**0.002**	**10.6**
44GU182	NS	-	0.037	5.1	0.046	4.6	0.002	11.0	**0.001**	**13.1**	0.006	8.8
43DU149	0.027	5.63	0.001	12.5	0.002	10.9	**0.000**	**14.7**	0.001	12.8	0.003	9.9
37DU41	NS	-	NS	-	NS	-	NS	-	NS	-	NS	-
44GU173	0.002	10.5	NS	-	0.019	6.3	0.029	5.5	0.007	8.2	**0.000**	**14.5**
% of phenotypic variation explained by the CDG [c]	**19.1%**		**16.1%**		**23.6%**		**35.9%**		**30.7%**		**46.0%**	

[a] Plants were considered as resistant when maximum sporulation severity (MSPOR)=1-3 and in the presence of resistance reactions; and susceptible when MSPOR=4-5 and in the absence of resistance reactions

[b] The significance of ANOVA (P) and the proportion of the phenotypic variance (R/S) "explained" (R^2) by the regulation of CDGs expression (log $2^{\Delta\Delta Ct}$) in each individual of the segregating population. Fold-changes between *V. inaequalis* inoculated and mock-inoculated controlled plants at 48 hpi were calculated by qRT-PCR over two technical repetitions using the 2ΔΔCt method, taking the glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) as internal reference.

Discussion

Scab resistance in 'Président Roulin' is partial and race specific

The resistance in 'Président Roulin' corresponded with partial resistance as defined by Parleviet and van Ommeren (1975). Under natural infection in the orchard (**Additional Figure 2**) or when inoculated with various *V. inaequalis* isolates (**Table 9**), the resistance in 'Président Roulin' was incomplete, allowing limited and significantly reduced pathogen growth and reproduction as compared with a susceptible genotype. Only with the 1639 isolate did 'Président Roulin' exhibit strong resistance reactions of HR with no sporulation. As these symptoms have never been seen before with a spray inoculation technique using this particular isolate (data not shown) or under natural infection in the field, we believe that they were conditioned by the particular conditions in the small inoculation chambers. As already demonstrated in the apple-*V. inaequalis* interactions (Caffier et al. 2015), the droplet method may overexpress the resistance cascade in an incompatible reaction due to a high concentration of effectors in a limited area of the leaf. Although it would never be possible to test this thoroughly, resistance of 'Président Roulin' also seems to be broad spectrum, since it provided a level of resistance against all the isolates artificially inoculated as well as the isolate population present in the orchard in Belgium over 25 years of experimentation.

Pathological tests conducted on the F1 segregating population revealed differential interactions between the isolates tested and the host genotypes (**Table 10**). In addition, under natural infection in the field, we observed a combination of resistance reactions with various degrees of sporulation on the leaf surfaces of 'Président Roulin' (**Additional Figure 3)**. These observations suggest that, along with possible basal defense response, some components of the partial resistance also show race specificity against *V. inaequalis*: the differential interactions would reflect the interactions between *Avr* genes in *V. inaequalis* and R-genes in the host. Race-specificity of QTLs has been widely demonstrated in the *V. inaequalis*-apple interaction (Calenge et al. 2004, Durel et al. 2003) as well as in other pathosystems (Caranta et al. 1997; Qi 1999; Dogimont et al. 2000; Chen et al. 2003; Talukder et al. 2004; González et al. 2012). One hypothesis would be that similar GfG relationships would govern complete and partial resistance. In the latter case (referred as minor gene-for-minor gene interaction), defective R-genes would recognize, with low efficiency, pathogens and would trigger a weaker (partial) defense response (Parlevliet and Zadoks 1977; McHale et al. 2006). An observation supporting this hypothesis is that 'defeated' R-genes have been shown to have a residual effect and act as a QTL against virulent strains of the pathogen. This phenomenon has been observed for the major resistance genes *Rvi4* (*Vh4*) (Bus et al. 2011) and *Rvi6* (*Vf*) (Durel et al. 2000) in the *V. inaequalis*-apple interaction and in other plant-pathogen interactions (Nass et al. 1981; Brodny et al. 1986; Li et al. 1999). So far as we know, however, evidence of a direct interaction between the product of a QTL for resistance and the matching *Avr* factor is still lacking. Genes controlling partial resistance to pathogens remain poorly documented and the molecular mechanisms underlying this kind of resistance are still unknown.

A hypothetical model of GfG relationships governing the partial resistance of ‘Président Roulin’

Based on the patterns of differential interactions observed in the segregating population, and the theory that for each R-gene in the host, there is an *Avr* gene in the pathogen (Flor et al. 1971), we suggested that the partial resistance of ‘Président Roulin’ would be governed by at least 5 race-specific minor genes. The presence of the first two loci was based on the observation that some seedlings were incompatible with only one isolate among the set of isolates tested (1639 and EU-B05/EU-D49/inoculum mix having the same resistance behavior), and overcome by the other one (**Table 10**). A third locus was hypothesized from the observation that isolate EU-NL24 showed incompatible interactions only with seedlings resistant to the full set of isolates. This same isolate, EU-NL24, could also sporulate on genotypes for which resistance symptoms were observed for all the other isolates tested, suggesting the presence of the resistance locus 4 in our GfG relationship model. Finally, additional differential interactions, notably plants being compatible with isolates 1639, NL24, B04/1066, and incompatible for all the other isolates tested, suggested the existence of a fifth resistance locus.

From these observations, we hypothesized a minor gene-for-minor gene relationships model in ‘Président Roulin’ (**Table 12**) with: loci 1 and 2 providing resistance to isolate 1639 and to the EU-B05/EU-D49/inoculum mix, respectively; locus 3 providing a broad spectrum resistance to the whole set of isolates tested, as would locus 4, except for EU-NL24. Finally, locus 5 would be incompatible with EU-D42, EU-NL05 and EU-B05/EU-D49/inoculum mix. These resistance loci do not seem to be tightly linked on the same chromosome since a relatively high number of seedlings (corresponding to the recombination frequency between R-genes) was observed for each pattern of differential interaction (from three to 12 plants). These loci are probably involved in the recognition of the pathogen by the host, rather than the defense-related genes acting downstream of the plant defense response. Also, we do not discard the hypothesis that other minor gene-for-minor gene interactions, not revealed by the *V. inaequalis* tested, could be involved in the partial resistance, or loci governing more basal resistance (non-race specific). This polygenic control seems to be durable, with a demonstrated resistance in ‘Président Roulin’ over 25 years under high disease pressure conditions (in absence of fungicides application). Pathogen isolates that overcome one of the genes would gain only a marginal advantage (Poland et al. 2009).

Table 12 : Model of gene-for-gene relationships between 'Président Roulin' and various *V. inaequalis* isolates (- indicates incompatible interaction, + compatible interaction) *

Resistance loci #	***Venturia inaequalis* isolates**					
	1639	**EU-NL24**	**EU-B04/ 1066**	**EU-D42**	**EU-NL05**	**EU-B05/ EU-D49/ MIX**
Locus 1	-	+	+	+	+	+
Locus 2	+	+	+	+	+	-
Locus 3	-	-	-	-	-	-
Locus 4	-	+	-	-	-	-
Locus 5	+	+	+	-	-	-

* The model was inferred from the differential interactions observed between the various single-spore *V. inaequalis* isolates and each genotype of a 'Gala' x 'Président Roulin' segregating population.

Segregation ratios suggest a complex genetic control of partial resistance

Interestingly, some *V. inaequalis* isolates showed a segregation that was not significantly different from R:S = 1:3 (EU-NL24, EU-B04, 1066) and 1:2 (EU-D42, EU-NL05). When the R:S = 1:1 segregation ratio indicates the inheritance of a dominant R-gene, segregation of resistance in less than half of the progeny suggests more complex genetic interactions, as already observed in the apple-*V. inaequalis* interaction (Durel et al. 2003; Bastiaanse et al. 2015). For instance, a ratio of R:S = 1:3 could involve a recessive genetic control of plant resistance (Büschges et al. 1997; Deslandes et al. 2002; Iyer and McCouch 2004; Diaz-Pendon et al. 2004; Iyer-Pascuzzi and McCouch 2007; Antony et al. 2010) which, in some cases, has been demonstrated to be broad spectrum and durable (van Schie and Takken, 2014). Another hypothesis would be the action of epistatic interactions among R-genes in 'Président Roulin' that we cannot explain with the data in this study. Further genetics studies, including the creation of new segregating populations involving backcrosses, DNA molecular marker work, or the cloning of the resistance loci, could help confirm the status of the genetic control of the complex resistance of 'Président Roulin' against *V. inaequalis*.

A candidate gene approach to dissect the partial resistance of 'Président Roulin'

In the current study, we proposed a candidate gene approach to better understand the molecular basis of the partial scab resistance of 'Président Roulin' consisting of three steps: the choice, screening, and functional validation of CDGs (Pflieger et al. 2001).

Choice of the CDGs

Thirteen CDGs were selected from a previous study (Bastiaanse et al. 2014) aiming to identify differentially expressed genes in 'Président Roulin', 48 hours after *V. inaequalis* inoculation, as compared to the susceptible cultivar 'Gala'. CDGs specifically induced or repressed in 'Président Roulin', and showing homology with plant defense genes were chosen (**Table 8**). In fact, numerous studies demonstrated that, beside basal host resistance, the key difference between resistant and susceptible hosts is the timely recognition of the invading

pathogen and the activation of plant defense genes which is accompanied by the accumulation of corresponding gene products (Durrant et al. 2000; Aligan et al. 2006; Gabriëls et al. 2006; Wang et al. 2010; Shi et al. 2011; Li et al. 2012; Paris et al. 2012).

The selected CDGs could act at different levels of the plant defense system, from the early recognition of the pathogen, which could be at the basis of the interpretation of our scab inoculation tests, to the activation of downstream plant defense responses. In fact, signaling and defense genes could also be invoked as good candidates for explaining the resistance of 'Président Roulin'. Among the set of selected CDGs, 43DU149'/56AU33' and 44AU9, encoded for R-proteins that are at the basis of the direct or indirect recognition of the pathogen (McHale et al. 2006). These R-proteins are part of membrane-associated protein complexes, including the NBS-LRR family proteins (to date, the widest class of R-genes cloned in plants) and LRR receptor kinase-like proteins. Then, external stimuli are usually transduced into an intracellular host defense response consisting of signalling proteins such as mitogen-activated protein kinase (MAPK) homologous with 2EU181 (Zhang and Klessig 2001), or serine carboxypeptidases similar to 44GU182. This later protein could also act in response to wounding (Moura et al. 2001; Liu et al. 2008). The subsequent stage of elicitation of defense-related proteins is the transcriptional activation of specific genes to redirect and alter the flow of metabolites required to sustain a pathogen attack. Transcription factors such as zinc finger homeodomain proteins (HD) (53HU89) might be a key element (Park et al. 2007). Finally, downstream events in plant defense response include the generation of highly toxic environments by massively producing reactive oxygen species (ROS) (involving peroxidase, 43DU149, and tocopherol cyclase, 51HU129') and the activation of HR, a form of local programmed cell death restricting pathogen invasion (involving both cysteine proteinase, 44EU122/44EU118, and cysteine proteinase inhibitors, 37DU41). Plants can also secrete hydrolytic enzymes that target pathogen cell walls (e.g. chitinases, 56AU29) and trigger local cell wall fortifications (e.g. pectin methylesterase, PME) and the pectin methylesterase inhibitor (PMEis, such as 44GU173). Since most of these CDGs (apart from the typical NBS-LRR resistance proteins), encode for proteins not strictly involved in plant disease resistance, but also act in other plant physiological traits, their real involvement in the partial resistance in 'Président Roulin' still has to be investigated.

Screening of the CDGs: co-localization with scab major R-genes, QTLs or analogues of resistance genes

The availability of the whole genome sequence assembly of apple (Velasco et al. 2010), together with the annotation of RGAs and the mapping of numerous scab R-genes and QTLs (Gessler et al. 2006; Bus et al. 2011), enabled us to investigate the co-localization of our CDGs with genomic regions known to control resistance. In an apple F1 segregating population, this approach has been previously used to identify genes whose steady-state transcript abundance was associated with the inheritance of resistance to powdery mildew disease and woolly apple aphid (Jensen et al. 2014). It has also been used as a criterion to select CDGs for partial resistance in various plant-pathogen interactions (Wang et al. 2001; Gonzalez et al. 2010; Schweizer and Stein 2011). Since expressed sequence tags (ESTs) generally have a biological meaning by representing

a particular gene (Matthews et al. 2001), compared with neutral markers used to map QTL, it may increase the knowledge about the genes underlying the agronomic traits.

Our CDGs were anchored to the existing 'Golden delicious' genetic map (Velasco et al. 2010). Eight out of the 13 pathogen-regulated CDGs co-localized with apple genomic regions known to act in scab resistance (QTLs, major scab R-loci, or RGAs) (**Figure 19**). We cannot discard the hypothesis that such co-localization occurred only by chance, regarding the high number of RGAs (868 RGAs) annotated in the reference apple genome (Perazzolli et al. 2014) but this could constitute a further indication of the potential role of our CDGs in the partial resistance of 'Président Roulin'. Obviously, in our analysis we could check only for co-localizations with R genes and QTLs that had been detected so far. In addition, information on the genomic loci that effectively regulate the expression level of the TDF of interest is still lacking from this analysis. The measured mRNA levels could be the product of regulation of the parent gene or another gene, mapping somewhere else in the genome (cis- or trans- regulatory elements) (Gilad et al. 2008). In our study, an e-QTL analysis of our segregating population would help to throw some light on these issues.

Screening of the CDGs: expression regulation in 'Président Roulin' challenged by V. inaequalis

Since timely recognition of the pathogen could determine the resistance status of a plant, we investigated expression regulation of our CDGs in a time-course experiment.

First, our time-course experiment confirmed the expression pattern obtained at 48 hpi in our previous cDNA-AFLP study (Bastiaanse et al. 2014). Results also showed an induction or a repression of the CDGs during partially incompatible interaction with *V. inaequalis*, or a delay in this regulation during compatible interaction (**Figure 20**). If these genes effectively act in the resistance of 'Président Roulin', this would suggest that, as important as the identity itself of the gene being activated, the quicker expression of some key genes could be crucial for the fate of the interaction between 'Président Roulin' and *V. inaequalis*. Sequences of the CDGs showed homologies to genes acting in the recognition of the pathogen, the transduction of the signal or the activation of defense-related proteins. Our previous study also demonstrated that most of the transcripts that were expressed in 'Président Roulin', were also expressed in 'Gala' (Bastiaanse et al. 2014). This preponderance of quantitative and/or kinetic trancriptomic differences between resistant and susceptible responses, over the qualitative one, corroborates findings in other plant-pathogen interactions (Maleck et al. 2000; Jwa et al. 2001; Tao et al. 2003; Eulgem 2005; Li et al. 2006).

Finally, our knowledge of the dynamic of leaf infection by *V. inaequalis* enabled us to compare the timing of CDG expression with particular events of pathogen development. First to be activated at 24 and 48 hpi were the genes that could act in the early events of pathogen perception, signal transduction and gene transcription. This might reflect physical and/or chemical stress experienced at the beginning of infection processes. At this phase of infection, appressoria

are formed and runner hyphae spread under the cuticle in direct contact with the epidermal cells (Nusbaum and Keitt 1938; Ortega et al. 1998). At 48 hpi, and up to 120 hpi, the latest time point tested, transcriptional responses of other defense-related CDGs occurred: PR proteins (peroxidase, chitinase), a pectin methylesterase inhibitor, a cysteine protease, a tocopherol cyclase, a cysteine protease inhibitor and a carboxypeptidase. This could lead to the appearance of the first resistance reactions in the host: collapsed epidermal cells under the subcuticular primary hyphae at 48 hpi and macroscopically visible necrosis, corresponding with large areas of collapsed epidermis cells, around 96 hpi (Nusbaum and Keitt 1938; Chevalier et al. 1991).

Based on their putative functions and their gene expression regulation during pathogen attack, we suggest in **Figure 20** a possible representation of the sequential activation of the CDGs based on their involvement during the plant-pathogen interaction: (1) genes involved in the recognition of the pathogen (43DU149', 56AU33' and 44AU9); (2) genes involved in the signal transduction and gene transcription (2EU181 and 53HU89); and (3) genes involved in plant defense responses (56AU29, 44GU173, 43DU149, 44EU122/44EU118, 51HU129', 37DU41 and 44GU182).

Functional assessment

In an attempt to functionally assess the involvement of our CDGs in the partial resistance of 'Président Roulin', we estimated the extent of correlations (in term of % of "explained variance") between expression regulation of a subset of CDGs and level of scab resistance of individuals composing a segregating population 'Président Roulin' x 'Gala'. The term "explained variance" could be misleading here since correlation between two variables does not necessarily imply causation (that one causes another), but could constitute the step forward for further evaluations of the CDGs (e.g. plant transformation with the CDGs). In our study, regulation of several CDGs (considered jointly) contributed from 16 to 46% of the sporulation severity (MSPOR) of the individuals (**Table 11**). This proportion of phenotypic variance "explained" by the regulation of our CDGs is modest, but is of the same magnitude as the ratio found for QTL markers controlling apple scab resistance in various progenies (Durel et al. 2003, Liebhard et al. 2003, Calenge et al. 2004, Soufflet-Freslon et al. 2008). This is probably due to the minor effect of loci governing partial resistance.

Apart from 44EU122/44EU118 (encoding for the same protein), the CDGs all mapped *in silico* in apple genomic regions known to contain disease resistance factors. Interestingly, the highest proportion (21.3%) of individual CDG expression variance explained by the R/S levels was the regulation of 43DU149', encoding a classic NBS-LRR resistance protein. Such larger effect could be due to its action upstream of plant defense reactions (recognition of the pathogen). Conversely, the effect of genes acting downstream could be diluted among the numerous defense-related genes participating in this stage of plant pathogen-interaction. In our study an important part of the phenotypic variation of MSPOR remains "unexplained" (54%). This might result from gene actions not covered by our study, either defense-related genes identified in 'Président Roulin' but not investigated here (Bastiaanse et al. 2014) or genes not previously reported to be

involved in disease resistance. Such genes with no similarity to any previously reported defense genes were shown to underlie some partial resistance QTL (Fukuoka et al. 2007; Zenbayashi-Sawata et al. 2007).

Conclusion and perspectives

In this study we identified potential CDGs for the polygenic (at least five R-loci) and durable partial resistance in 'Président Roulin'. These CDGs were shown to be specifically regulated in 'Président Roulin' or were induced earlier, compared with the susceptible 'Gala'. They encoded for proteins that could act at various stages of plant-pathogen interaction, from pathogen perception to the activation of downstream defense reactions. Nine CDGs accounted for 46% of the phenotypic variance of the disease severity, eight of them mapped within chromosomal regions containing disease resistance factors (RGAs, major scab R-genes or QTL). This is a modest indicator of the potential involvement of these CDGs in partial resistance against apple scab, regarding notably the small number of CDGs tested on a limited segregating population, and the absence of a genetic map for 'Président Roulin'. Future studies, such as plant transformation with the CDGs and eQTL analysis with larger populations, will determine how strategies might be developed to incorporate these genes into breeding programs. If functional markers for partial disease resistance could be developed based on these CDGs, as illustrated by various studies (Liu et al. 2004; Liu et al. 2011), these markers would have greater breeding value and alleviate the recombination problems associated with the use of neutral molecular markers in genome-informed breeding programs (Andersen and Luebberstedt 2003).

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DATA ARCHIVING STATEMENT

The cDNA sequences that formed the basis of our gene expression study were deposited at DDBJ/EMBL/GenBank in the library LIBEST_028504 under the following accession numbers: 43DU149'/JZ719417, 56AU33'/JZ719506, 44AU9/JZ719419, 2EU181/JZ719320, 53HU89/JZ719483, 43DU149/JZ719416, 51HU129'/JZ719472, 44EU122/ JZ719578, 44EU118/ JZ719577, 37DU41/ JZ719360, 44GU182/JZ719427, 56AU29/ JZ719503, 44GU173/JZ719426.

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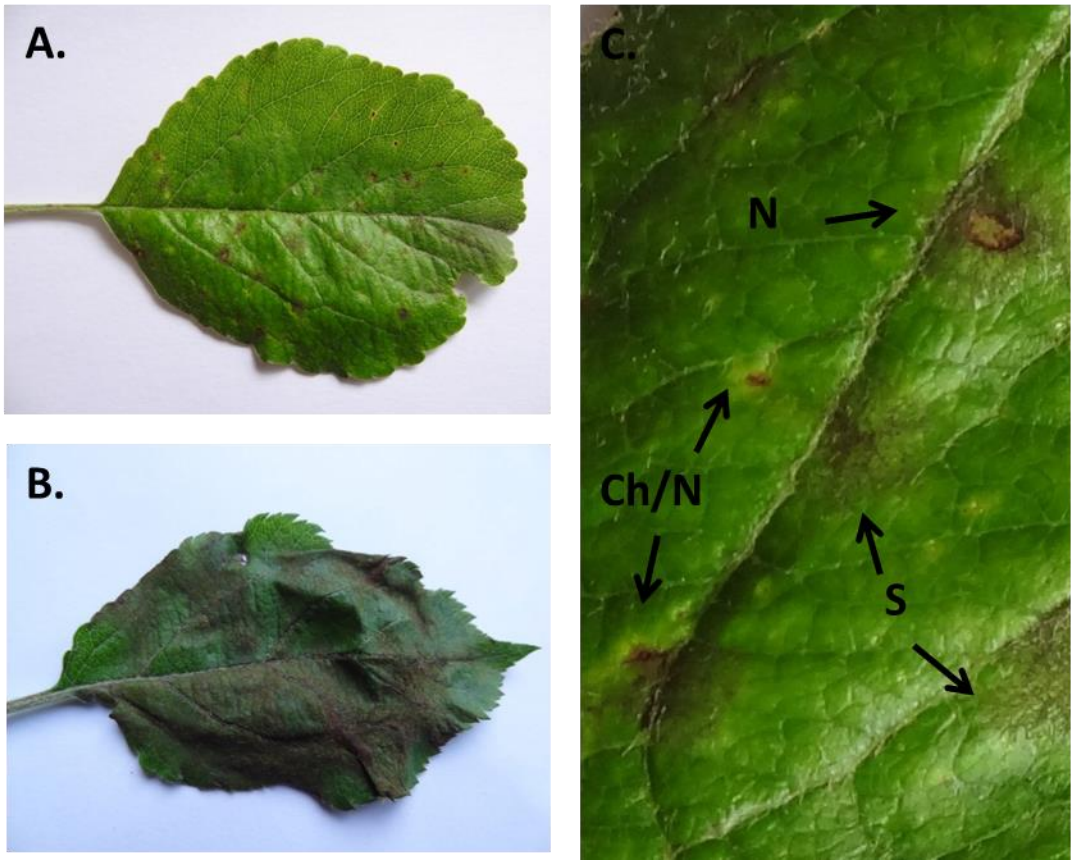
Additional tables and figures

Additional Table 4 : Origin of monoconidial isolates of *Venturia inaequalis* used in this study

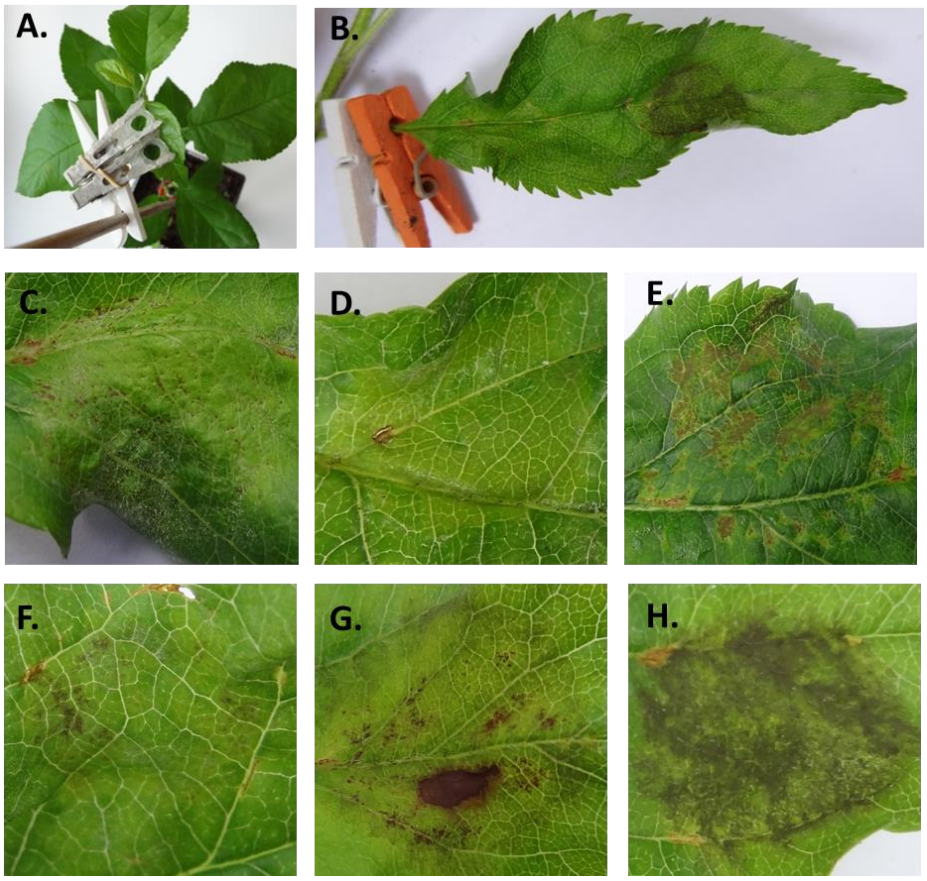
Race	Isolate	Origin	Collection
1, 10	EU-B04	Belgium	INRA, Angers
1	EU-B05	Belgium	PRI, Wageningen
1	EU-B16	Belgium	INRA, Angers
1	EU-D49	Germany	INRA, Angers
1	EU-F05	France	INRA, Angers
1	EU-F11	France	INRA, Angers
1	EU-I09	Italy	INRA, Angers
1,2,8,9	1639	France	PRI, Wageningen
1,6,10	EU-D42	Germany	PRI, Wageningen
6,7,8,10	1066	France	PRI, Wageningen
6,7,8,10,13	EU-NL05	The Netherlands	PRI, Wageningen
1,3,6,7	EU-NL24	The Netherlands	PRI, Wageningen

Additional Table 5 : List of the specific primer pairs used for qRT-PCR analysis

TDF	Primer forward (5′–3′)	Primer reverse (5′–3′)
43DU149'	TCCAAAGATGAGCCTAGTAGTGT	TCGTTAGAGACGGATACCCATA
56AU33'	CACCCAATTCTCCCATACTGA	ACCGAAGAGCGTGAAAAGTG
44AU9	AAGGGCCGACTTTCAAACTT	AAATCTGGCGGTGACAACTC
2EU181	CCTTGACCGTCCAGTTCCTA	TCCCTCGTGGAAGTTTGTTT
43DU149	CCAGACCTTGTTCTTTGAGC	CGAGCAATCTGCACGAATTT
51HU129'	CTTTGGCATCAAGGTTCCAT	CATATTTGCCAATGGGGTTC
Consensus 44EU122/ 44EU118	GAAAATGCCCAACAACAACC	GCCCACTGAATCAACCTCAT
37DU41	GAAGCTGTACGAAGCGAAGG	ACAGATTGCCACCCAGGA
44GU182	AAGGTGGGAACATGGAATCA	TGAACCCAGAAACCTATCTGAAA
56AU29	GAGGTGGATGACCCCTATCA	CCTTGCCACAAACTGATTCC
53HU89	ACCAATTCTCCTCGGGCTAC	TCGACACGTCCTCTTCCTCT
44GU173	GATTACAAGACGGTGCAGGAG	CTATCCCGTCACCCAAGAAC



Additional Figure 2 : (A.) Resistance reactions observed on 'Président Roulin' leaves under natural infection in the orchard compared with (B.) the heavy sporulation on a susceptible 'Gala' leaf. (C.) close-up of the different reactions observed on 'Président Roulin': Necrosis (N) with limited sporulation, Chlorosis-Necrosis symptoms (Ch/N) and No Reaction (NR) with limited sporulation.



Additional Figure 3 : Scab resistance reactions observed on a ‘Gala’ x ‘Président Roulin’ progeny 21 days after controlled inoculation with various monoconidial *V. inaequalis* isolates using the small chambers inoculation technique (Bus et al. 2005). (A.) Setting up the inoculation chambers on the leaf, a different isolate being inoculated in each well. (B.) Differential interactions obtained with the incompatible isolate 1639 (left, pin-point) and the compatible isolate EU-NL24 (right, heavy sporulation), alongside a close-up of different scab symptoms: (C) HR reactions (pin point), (D) chlorosis, (E) stellate necrosis, (F) chlorosis with limited sporulation, (G) chlorosis and necrosis with limited sporulation, (H) susceptibility.

Chapter 4.

Scab resistance in 'Geneva' apple is conditioned by a resistance gene cluster with complex genetic control

Outline

The red-fleshed cultivar 'Geneva' is an important source of scab resistance in breeding programs but its genetic control remains largely unknown. In this chapter, the dissection and fine mapping of a cluster of resistance loci with complex genetic control are described. Pathological tests with monoconidial isolates of *V. inaequalis* isolates were used to differentiate the resistance loci. Primers specific to candidate resistance genes found in the homologous region of the 'Golden Delicious' apple reference genome were designed and used to construct a detailed genetic map around the 'Geneva' resistance loci. The consequences of the complexity of the 'Geneva' scab resistance for breeding for durable scab resistance and the selection of a differential host for *V. inaequalis* pathotype monitoring are discussed.

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Scab resistance in ‘Geneva’ apple is conditioned by a resistance gene cluster with complex genetic control

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Abstract

Apple scab, caused by the fungal pathogen Venturia inaequalis, is one of the most severe diseases of apple worldwide. It is the most studied plant–pathogen interaction involving a woody species using modern genetic, genomic, proteomic and bioinformatics approaches in both species. Although ‘Geneva’ apple was recognized long ago as a potential source of resistance to scab, this resistance has not been characterized previously. Differential interactions between various monoconidial isolates of V. inaequalis and six segregating F1 and F2 populations indicate the presence of at least five loci governing the resistance in ‘Geneva’.The 17 chromosomes of apple were screened using genotyping-by-sequencing, as well as single marker mapping, to position loci controlling the *V. inaequalis* resistance on linkage group 4. Next, we fine mapped a 5-cM region containing five loci conferring both dominant and recessive scab resistance to the distal end of the linkage group.This region corresponds to 2.2 Mbp (from 20.3 to 22.5 Mbp) on the physical map of ‘Golden Delicious’ containing nine candidate nucleotide-binding site leucine-rich repeat (NBS-LRR) resistance genes. This study increases our understanding of the complex genetic basis of apple scab resistance conferred by‘Geneva’, as well as the gene-for-gene (GfG) relationships between the effector genes in the pathogen and resistance genes in the host.

Keywords: apple scab, differential host, fine genetic mapping, gene-for-gene relationships, molecular marker, resistance, *Venturia inaequalis*.

Introduction

Scab, caused by the fungus *Venturia inaequalis*, is one of the most serious diseases of apple (*Malus* × *domestica* Borkh.) worldwide. In-plant resistance to pathogens offers a promising alternative to disease control methods based on the use of pesticides, but often lacks durability as a result of the rapid evolution of pathogen populations (Caffier *et al*., 2014). Each year, *V. inaequalis* undergoes a sexual reproduction phase that is the basis for rapid changes in the virulence distributions in populations and raises a continuous threat to the effectiveness of existing scab-resistant varieties planted for 10–15 years in commercial orchards. Hence, there is an urgent need for strategies to develop varieties with durable resistance to scab. Pyramiding of multiple resistance (*R*) genes into one cultivar has been an effective route to avoid the selection of new pathogen races and to create more durable resistance in numerous plant–pathogen systems (Huang *et al*., 1997; Liu *et al*., 2000; Werner *et al*., 2005; Witcombe and Hash, 2000). However, this approach is time consuming for classical breeding in tree crops with a long juvenile period, such as apple. Moreover, plants combining two or more *R* genes are difficult to distinguish phenotypically from plants that have inherited a single resistance. The speed of selection of these plants with pyramided *R* genes can be greatly increased by the utilization of markerassisted selection (MAS) to identify the seedlings that carry multiple resistance alleles.

In the past, at least 17 major scab *R* genes and 13 quantitative trait loci (QTLs) have been identified and mapped across 11 apple linkage groups (LGs) (Bus *et al*., 2011; Calenge *et al*., 2004; Durel *et al*., 2003, 2004; Gessler *et al*., 2006; Liebhard *et al*., 2003; Soufflet-Freslon *et al*., 2008). The identity of the genes underlying the resistance loci remains largely unknown as only two major apple scab *R* genes have been cloned to date: *Rvi6* (*Vf*) encoding for a leucine-rich repeat receptor-like family protein (LRR-RLP) similar to the tomato *Cf R* gene (Vinatzer *et al*., 2001), and *Rvi15* (*Vr2*) encoding a nucleotide-binding site leucine-rich repeat (NBS LRR) family protein (Galli *et al*., 2010).

The process of resistance mapping and gene discovery has been made more efficient recently, as the draft genome sequence of the domesticated apple cultivar 'Golden Delicious' (GD) (Velasco *et al*., 2010) has given apple researchers access to millions of single nucleotide polymorphism (SNP) markers spanning the whole apple genome. Moreover, the annotation in GD of numerous *R* gene analogues (RGAs) encoding for NBS-LRR proteins (Perazzolli *et al*., 2014) could be the basis of a candidate gene approach to decipher the plant–pathogen interactions between apple and *V. inaequalis*. These genes show gene-forgene (GfG) relationships with effector genes in the pathogen and, if the corresponding effectors are not essential, are likely to be of a non-durable nature. In apple, SNP arrays have been developed for the construction of very dense genetic maps (Bianco *et al*., 2014; Chagné *et al*., 2012), and genotyping-by-sequencing (GBS) based on next-generation sequencing technologies (Elshire *et al*., 2011) has been used for association studies and genomicsassisted breeding (Deng *et al*., 2014; Gardner *et al*., 2014).

'Geneva' is a scab-resistant, open-pollinated selection of *Malus pumila* (Jefferson, 1970). Resistance symptoms on adult plant material were Class 2 non-sporulating, irregular chlorotic or necrotic reactions (Shay and Hough, 1952), with the same resistance reactions induced by seven isolates from the USA, the Netherlands and Germany. In contrast, monoconidial isolates from Canada induced symptoms ranging from hypersensitive reactions (HRs) to susceptibility, the latter by an isolate collected from 'Geneva' (Julien and Spangelo, 1957), which indicated differential interactions with multiple *R* genes. Williams and Shay (1957) discerned two distinct

resistance classes (Classes 2 and 3) associated with the *p-10* and *p-11* avirulence genes in *V. inaequalis* identified in the progeny of virulent isolate 651 collected from 'Geneva' in Nova Scotia in 1951 (Shay and Williams, 1956).With the 2 → 4 phenotype (Shay and Williams, 1956) assumed to be different from the Classes 2 and 3 resistance reactions, the 'Geneva' scab resistance was interpreted as being conditioned by three scab *R* genes (Bus *et al*., 2011). In this study, we use genetic maps constructed by GBS and traditional marker technologies to show that apple scab resistance of 'Geneva' is conditioned by at least five resistance loci, suggesting both dominant and recessive genetic control, which all mapped to the lower end of LG4. Primers specific to candidate NBS-LRR *R* genes found in the homologous region of the GD genome assembly were designed and used to construct a detailed genetic map around the 'Geneva' resistance loci. The consequences of the complexity of the 'Geneva' scab resistance for breeding for durable scab resistance and the selection of a differential host (3) (Bus *et al*., 2011) for *V. inaequalis* pathotype monitoring (www.vinquest.ch) are discussed.

Experimental procedure

Plant material and V. inaequalis isolates

The characterization of host–pathogen interactions was performed at Plant Research International in Wageningen, the Netherlands on F1 and F2 progenies derived from 'Geneva' using nine reference monoconidial isolates of *V. inaequalis* (Caffier *et al*., 2015), including isolate 1774-1, which was distributed as a race (3) isolate (Bus, 2006), but recently characterized as race (1,7) (Caffier *et al*., 2015) (**Table 13** and **Additional Table 6**, see Supporting Information).

Table 13 : F1 and F2 families and *Venturia inaequalis* isolate combinations used in this study to investigate the genetics of scab resistance of 'Geneva'.

Family	Parents		Gene-ration	Nb of prog eny	Year of pheno-typing	Isolates								
	Female	Male				1639	1774--1	1770-3	EU-B04	EU-B05	EU-D42	EU-NL19	EU-NL24	NZ188B
M	'Elstar'	'Geneva'	F1	125	2005		x	x				x		x
Q	'Geneva'	'Braeburn'	F1	91	2005	x	x		x	x			x	x
2011-050	'Golden Delicious'	M33	F2	118	2012	x	x	x			x	x		
2012-047	'Elstar'	Q35	F2	119	2013	x	x	x	x		x	x		
2012-037	Q49	'Gala'	F2	40	2013	(x)[a]	x	x	(x)					
2012-041	'Gala'	Q71	F2	119	2013	x	x	x	x			x		

[a] (x) means inoculum applied, but data not sufficient for genetic analysis.

Seeds of the F1 families 'Geneva' × BB and E × Geneva' were stratified at 0–1 °C for 8 weeks, and then germinated in the glasshouse.At the four to five-leaf stage, they were transplanted and raised under a 20 °C day/ 14 °C night temperature and 16 h light/8 h dark regime using daylightincandescent lights when required. Inoculations started at the 10-leaf stage. For the identification of the four resistant parents required to develop F2 populations, 'Geneva' and selected F1 progeny grafted onto 'M9' rootstock (clonal trees) were inoculated with 12 reference isolates, with each host–pathogen interaction evaluated in triplicate. These were crossed with susceptible parents GD, E and G, and the F2 seedlings were raised as described for the F1 progenies (**Table 13**).

Inoculation procedure and phenotyping

Inoculum was prepared from dried scab-infected leaves stored at −20 °C, as described by Bus *et al*. (2005). *Venturia inaequalis* inoculations were performed using 130 μL of inoculum at a concentration of 1 × 105 conidia/mL using the droplet inoculation technique (Bus *et al*., 2005), followed by symptom development in the glasshouse with the roof ventilation set at 20 °C and 80% relative humidity. Symptoms were recorded 21 days after inoculation. On clonal trees and on F1 progenies, phenotypic data were scored according to the classification of Chevalier *et al*. (1991), with additional descriptions of resistance reactions when observed, e.g. necrotic (N), stellate necrotic (SN) or stellate chlorotic (SC). On F2 progenies, sporulation severity was also scored using a quantitative scale, ranging from '1' (slight) to '5' (heavy). For genetic mapping, plants were classified as being resistant (R) in the presence of resistance reactions with low/moderate sporulation (levels 1–3) and as susceptible (S) in the absence of resistance reactions with moderate to high sporulation (level 4–5) or in the presence of chlorosis with very high sporulation (level 5). Finally, plants with an indeterminate phenotype were scored as 'I'. Uninoculated leaves from each individual of the progenies and their parents were harvested and immediately frozen in dry ice and lyophilized for storage at −80 °C until DNA extraction.

GBS

GBS was used to screen 58 of the 119 individuals from the F2 segregating population G × Q71. Genomic DNA (gDNA) was extracted from leaf material of both parents and each individual by milling with ball bearings for 1 min at 3.55 m/s in a cetyltrimethylammonium bromide (CTAB)-based buffer (**Additional Table 7**, see Supporting Information). The homogenate was incubated at 65 °C for 30 min, cooled and a chloroform extraction was performed. gDNA was precipitated with the addition of a two-thirds volume of isopropanol and centrifuged. The gDNA pellet was washed twice with 70% ethanol and resuspended in TE buffer [10 : 0.1, 10 mMTris-Cl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 8]. gDNA concentrations were determined by fluorometry (Qubit, Life Technologies, Carlsbad, CA, USA) and quality was checked by running 100–150 ng of each gDNA sample on a 1% w/v agarose gel. Fragment libraries were prepared from 1 μg of gDNA using a modified GBS protocol (Elshire *et al*., 2011). gDNA was digested with *Ape*KI type II restriction endonuclease (New England Biolabs, Ipswich, MA, USA).

Fragments were barcoded and common linker DNA oligonucleotides were annealed and ligated to the gDNA ends using T4 DNA ligase (Promega, Madison, WI, USA). The libraries were amplified with high-fidelity Taq DNA polymerase (Accuprime, Life Technologies) as follows: an initial denaturation step at 94 °C for 2 min, followed by 25 amplification cycles of [94 °C for 30 s, 65 °C for 30 s, 68 °C for 30 s], and then a final extension step at 68 °C for 5 min. An

aliquot from each library amplification was run on a 3% agarose gel to check for product. Amplified libraries were pooled and purified on a spin column (Qiagen, Venlo, Netherlands) prior to sequencing. Sequencing was performed by Macrogen Inc. (Seoul, South Korea) on the HiSeq2000 Illumina® platform. GBS data were analysed using the TASSEL-GBS pipeline (Glaubitz *et al*., 2014). The sequencing reads from the barcoded 58 genotypes and both parents were deconvoluted and aligned to the GD genome assembly v1.0 (http://www.rosaceae.org/). GBS-derived SNPs were called using TASSEL for the efficient processing of raw GBS sequence data into SNP genotypes, with SNP nomenclature indicating the position of the SNP on the apple LG and position in base pair (bp) in the assembly. SNPs heterozygous for the Q71 resistant parent and homozygous for the susceptible G parent were used for linkage map analysis.

SSR and SNP genotyping

The F1 and F2 families were genotyped using SSRs (Liebhard *et al*., 2002; Silfverberg-Dilworth *et al*., 2006; Yamamoto *et al*., 2002) and SNPs. DNA was extracted from the lyophilized leaves by Slipstream Automation Ltd. (Palmerston North, New Zealand) **(**http://www.slipstream-automation.co.nz**)** on a subset of the F1 families (94 and 91 individuals for the E × 'Geneva' and 'Geneva' × BB populations, respectively) and on all the F2 progenies. Both SNP and SSR markers were initially screened over DNA extracted from both parents and six individuals of each progeny to identify polymorphic markers, which were then screened over the entire populations. The primer sequences used to generate these are listed in **Additional Table 8** (see Supporting Information). SNP markers were genotyped using the high-resolution melting (HRM) technique (Liew *et al*., 2004) as described by Chagné *et al*. (2008). SNPs were scored using the LightCycler 480 Gene Scanning Software (Roche, Basel, Switzerland). PCR amplifications for SSR markers were performed in a final volume of 12.5 μL containing 5 ng of genomic DNA,1 × buffer, 1.5 mM MgCl2, 200 μM of each deoxynucleoside diphosphate (dNTP), 13 nM forward primer with the 5'-end labelled with the fluorescent FAM, HEX or NED, 200 nM reverse primer and 0.5 U of Platinum Taq DNA Polymerase (Life Technologies). All SSR amplifications were performed under touchdown PCR conditions as described by Bus *et al*. (2005). The amplified fragments were run on an ABI 3500 Genetic Analyzer (Applied Biosystems by Life Technologies) with an internal 50–500-bp size standard, and fragment sizing was performed with GeneMarker software v. 2.2.0 (SoftGenetics LLC, State College, PA, USA).

Candidate gene mining and design of primers

To delimit the physical interval in which the scab resistance loci are located, the sequences of the molecular markers flanking the resistance loci were searched in silico in the GD genome assembly v1.0 (Velasco et al., 2010) using BLASTN.This interval was mined for RGAs of the NBS-LRR family and the *Cladiosporium fulvum* R gene family, using the apple GenomeBrowser. Primers were designed using Primer3 (Rozen and Skaletsky, 2000) to amplify amplicons of 50 to 120 bp in size located in the promoter region of each candidate R gene. NBS-LRR candidate R genes identified in the homologous region of GD and their specific primer sequences used in this study are listed in Tables S3 and S4. PCR was conducted on both parents and the individuals of the F2 progenies G × Q71, GD × M33 and E × Q35, and SNPs were detected using the HRM technique as described above.

Genetic map construction

Genetic maps were constructed for the resistant parents for each family using JoinMap 3.0 and 4.1 with the double pseudo-testcross mapping strategy (Grattapaglia and Sederoff, 1994) and a LOD score of 6 for grouping. The phenotypic data obtained with the various monoconidial isolates of *V. inaequalis* were integrated into the JoinMap file, with the phenotypes scored as I and plants showing genotype–phenotype incongruence (GPI plants; Gygax *et al*., 2004) recorded as missing data.The final locus order was determined following extensive proofreading and by minimizing the number of double crossovers flanking single loci. Chisquared tests were used to identify any segregation distortions of parental alleles and segregation ratios of resistance phenotypes. For the GBS mapping analysis, the SNP data were divided into 17 files comprising SNPs informative for the resistant Q71 parent (ab × aa backcross-type segregation). Each file contained SNPs located on the GD primary assembly for a particular LG. A LOD score of 5 was used for grouping and genetic maps were drawn using MapChart 2.2. A consensus map using the phenotypic and genotypic data of the F2 populations derived from M33, Q35 and Q71 was constructed using JoinMap 3.0.

Results

Resistance phenotypes and segregation ratios

Although the F1 families (**Table 13**) showed very similar segregation ratios of resistant to susceptible (R : S) = 1 : 1 for isolates 1639, EU-B04 and EU-NL19, variable segregation ratios were observed for isolates 1770-3, 1774-1, NZ188B and EU-B05, with each time less than one-half of the seedling population being incompatible (R : S = 1 : 2, 1 : 3 and 1 : 7) (**Table 14**). The plants resistant to the latter isolates were always also resistant to the former isolates. A notable finding was that isolates 1774-1 and NZ188B showed segregation ratios in the R : S = 1 : 2 to 1 : 3 range in the cross with ‘Elstar’ (E), but in the 1 : 3 to 1 : 7 range with ‘Braeburn’ (BB). Most plants that were resistant to all isolates exhibited Class 2 resistance reactions, sometimes with some HR as the result of the high inoculum density, whereas greater proportions of Classes 3A, 3B and 4 were found in plants showing more complex interactions between isolates. Isolate EU-NL24 was compatible with all ‘Geneva’ × BB progeny (**Table 14**).

Table 14 : The segregation data for the various families derived from 'Geneva' 21 days after inoculation with conidia from monospore cultures of *Venturia inaequalis* isolates

	Family	1639	EU-NL19	EU-B04	EU-D42	1770-3	1774-1	NZ188B	EU-B05	EU-NL24
F1	**E x 'Geneva'**[a]									
	R	67	54	-	-	36	40	41	-	-
	I	2	9	-	-	8	4	9	-	-
	S	56	62	-	-	81	81	75	-	-
	Segregation ratios[b]	*1:1***	*1:1***	-	-	*1:2***	*1:2***	*1:2***	-	-
						*1:3***	*1:3***	*1:3***	-	-
F1	**'Geneva' x BB**									
	R	40	-	40	-	-	13	17	13	0
	I	4	-	4	-	-	14	5	5	0
	S	47	-	47	-	-	64	69	73	91
	Segregation ratios	*1:1****	-	*1:1****	-	-	*1:3****	*1:3****	*1:3***	-
			-		-	-	*1:7****	*1:7***	*1:7****	-
F2	**GxQ71**									
	R	46	62	63	-	14	39	-	-	-
	I	10	11	5	-	8	10	-	-	-
	S	63	46	51	-	97	70	-	-	-
	Segregation ratios	*1:1****	*1:1****	*1:1****	-	*1:3**	*1:3**	-	-	-
					-	*1:7****	*1:2****	-	-	-
F2	**GDxM33**									
	R	58	35	-	62	46	50	-	-	-
	I	9	18	-	8	5	4	-	-	-
	S	51	65	-	48	67	64	-	-	-
	Segregation ratios	*1:1****	*1:3***	-	*1:1****	*1:1***	*1:1****	-	-	-
			*1:2****	-				-	-	-
F2	**ExQ35**									
	R	15	71	57	79	15	18	-	-	-
	I	17	25	7	6	5	11	-	-	-
	S	90	26	58	37	102	93	-	-	-
	Segregation ratios	*1:3***	*3:1****	*1:1****	*3:1***	*1:3**	*1:3***	-	-	-
		*1:7****				*1:7****	*1:7****	-	-	-
F2	**Q49xG**									
	R	-	-	-	-	13	3	-	-	-
	I	-	-	-	-	3	0	-	-	-
	S	-	-	-	-	24	37	-	-	-
	Segregation ratios	-	-	-	-	*1:1***		-	-	-
						*1 :2****				
						*1 :3****				

[a] R = resistant, S = susceptible, I = indeterminate, 'I' seedlings were discarded from the segregation ratio calculations
[b] Significant Mendelian segregation ratios judges at the following levels for P (χ2) values: *** P>0.1; ** P>0.01; * P>0.001

From these F1 families, 10 seedlings were selected to represent the main patterns of differential interactions observed among isolates and subjected, with 'Geneva', to a replicated clonal evaluation. Most of the differential interactions were confirmed, with additional isolates providing complementary information (**Table 15**). Being adult clonal material, the plants showed a higher degree of resistance in that some plants showed no symptoms and/or Class 1 (HR) reactions, which were not observed on the young F1 seedlings. Other plants showed symptoms ranging from Classes 2 to 4, with some isolates inducing varied resistance reactions on the same leaf. Some isolates, such as NZ188B, 1774-1 and 1770-3, showed some inconsistencies among the replicates as both resistance and susceptibility symptoms were observed. Eleven of the 12 reference isolates were incompatible with 'Geneva', whereas EU-NL24 was compatible with this accession and all of its F1 derivatives. Isolate 1639 was the only one incompatible with 'Geneva' and all of its selected resistant progeny.

Genotypes M33 from the E × 'Geneva' progeny, and Q35, Q71 and Q49 from the 'Geneva' × BB progeny, were selected as the resistant parents for the F2 families and were crossed with susceptible parents GD, E or 'Gala' (G). (**Table 13**). Symptoms varied from Class 1 to Class 4 and segregation for resistance was found, even with 1774-1 and 1770-3 isolates showing (weak) compatibility with parents M33, Q35 and/or Q71 (**Table 14**). In these particular cases, less than one-half of the population was found to be resistant to these isolates (R : S = 1 : 2, 1 : 3 and 1 : 7). Conversely, when these isolates were incompatible with the resistant parent, the resistance often segregated following a ratio of R : S = 1 : 1 in the corresponding F2 progeny (e.g. 1774-1 in GD × M33 and 1770-3 in Q49 × G). Resistance to isolates 1639, EU-NL19, EU-B04 and EU-D42 was found in one-half of the various F2 families (R : S = 1 : 1) or less (R : S = 1 : 2, 1 : 3 and 1 : 7), with only isolates EU-D42 and EU-NL19 showing a higher proportion of seedlings resistant, with a tendency towards a R : S = 3 : 1 segregation in the E × Q35 family (**Table 14**).

Differential interactions

From 3% to 23% of the seedlings in the F1 and F2 progenies were fully incompatible with the set of monoconidial *V. inaequalis* tested, whereas from 16% to 58% were fully compatible (**Additional Table 10** see Supporting Information). The remaining seedlings showed complex differential interactions with *V. inaequalis*, with some isolates showing very similar differential interactions. For example, in the F1 progenies, isolate 1774-1 was similar to NZ188B in both F1 families, and to EU-B05 in the 'Geneva' × BB family (except for three plants). Across the various progenies, some seedlings were incompatible with only one isolate among the set of isolates tested (the other isolates were fully compatible with these particular seedlings). This was notably the case for isolates EU-NL19 (in E × 'Geneva', G × Q71, E × Q35, but not in GD × M33), 1639 (in E × 'Geneva', 'Geneva' × BB, GD × M33, but not in G × Q71 or E × Q35), EU-B04 (in 'Geneva' × BB and G × Q71, but not in E × Q35) and, finally, EU-D42 (in GD × M33 and E × Q35). Some of the differential interactions and symptoms observed in various progenies are illustrated in **Additional Figure 4** (see Supporting Information).

Table 15: Host-pathogen interactions observed between 12 monoconidial isolates of *Venturia inaequalis* on clonal trees of 'Geneva' and selected derivatives.

Accession	Isolate											
	1639[a]	**EU-NL19**	**EU-B04**	1066	**EU-D42**	**1770-3**	174	**1774-1**	**NZ188B**	**EU-B05**	EU-NL05	**EU-NL24**
'Geneva'	R 0/Chl	R 0	R 0/1	R 1/Chl	R 1	R 1	R 1/2	R 0/Chl	R 1	R 0/1	R 2/3A	S 4
M95	R 0/1 2	R 0 2/Chl	R 0/1	R 0/Chl	R 0/SC	R 0/1 SC	R 0/SC	R 0/SC Chl	R 0/1 Chl	R 0/Chl	R 2/3A	S 4
Q27	R 0 2	R 0/Chl	R 0/1 1	R 0/1	R 1	R 1	R 2/3A	R 1/Chl 3B	R 1 2+	R 0/1 2	R 2/Chl	S 4
M19	R 0 2	R 0/1 2	R 0/1	R 2/3A	R 1	R 1 1/2	R 3A/3B	R 0/Chl 2	R 1 1/2	R 1/2	(S) 3B/4	S 4
M42	R 0/Chl 2/Chl	R 0/Chl 2/Chl	R 0/SC	R SC/Chl	R Chl/3A	(R) 2/3A 4	R Chl/3B	R SC/Chl SC/Chl+	R SC/3A Chl	R SC/Chl	S 4	S 4
M33	R SC Chl+	R Chl/SC Chl+	R Chl/SC	R 3A	R Chl/3A	(R) 3A 4	(S) 3B/4	(R) Chl 4	(R) 3A 4	R Chl	S 4	S 4
M45	R 2/Chl 2	R 0/1 2	R 0/2	R 2/Chl	R Chl/3B	S 4	R 2/3A	(S) 3B/4- 4	(R) 2/Chl+ 4	R 3A/3B	(S) 3B/4	S 4
M40	R SC/Chl 2/SC/Chl	(R) SC/Chl 4	R 1/Chl	R Chl/3A	(S) 3B/4	R Chl Chl+	S 4	(R) 3A/3B Chl>4-	(S) Chl/3B 4	R SC/3A	S 4	S 4
Q35	R 2/3A 2	R 2/Chl	R 1/Chl 2	R 2/Chl	R 2/3A	(S) 3B	S 4	(R) 3A 4	(R) 2/3A 4	S 4	(S) 4-	S 4
Q71	R 2/Chl	R 0	R Chl/SC	R Chl/3A	R Chl/3A	S 4	S 4	S 4	S 4	(S) Chl/3B 4	S 4	S 4
Q49	R 2/Chl 2	S 4	S 4	S 4	S 4	R 2/Chl	R 2/Chl	S 4	S 4	S 4	(S) 3B/4	S 4

[a] The isolates and accessions in bold were used to study the genetics of scab resistance in 'Geneva'. Symptoms were recorded 21 days after inoculation according to the classification of Chevalier et al. (1991), with additional descriptions of resistance reactions when observed, e.g. necrosis (N), stellate necrosis (SN), stellate chlorosis (SC), or chlorosis (Chl). R = resistant; S = susceptible; ( ) = moderately

Based on these observations and considering all the differential interactions among the isolates and the segregation ratios observed, we hypothesized a model for the GfG relationships between the *V. inaequalis* isolates and the resistance loci (**Table 16**), the interpretation of which will be developed in the Discussion section.

Table 16: Model for gene-for-gene interactions between the various *Venturia inaequalis* isolates and the scab resistance loci in 'Geneva'

R-locus name[a]	1639	EU-NL19	EU-B04	EU-D42	1770-3	1774-1	NZ 188B	EU-B05	EU-NL24
Rvh3.1	-	+	+	+	+	+	+	+	+
Rvh3.2	+	-	-	+	+	+	+	+	+
Rvh3.3	+	+	-	-	+	+	+	+	+
rvh3.4	-	-	-	-	+	-	-	-	+
rvh3.5	-	-	-	-	-	+	+	+	+

[a] Our data suggest dominant genetic control for *Rvh3.1*, *Rvh3.2* and *Rvh3.3*, and more complex (recessive) genetic control for *rvh3.4* and *rvh3.5*.

GBS and linkage analysis

In total, 23 585 GBS-derived SNPs polymorphic for the resistant parent Q71 were detected. Of these, 10 631 grouped into 17 LGs with a logarithm of odds (LOD) score of 5, which corresponded to an average of one mapped SNP for every 56 kb (**Additional Table 11**, see Supporting Information). Loci for resistance to the isolates EU-NL19, EU-B04, 1774-1 and 1639 all mapped to LG4. **Figure 21** presents a genetic linkage map of a subset of markers around these loci. No linkage was detected for the locus for resistance to the 1770-3 isolate.

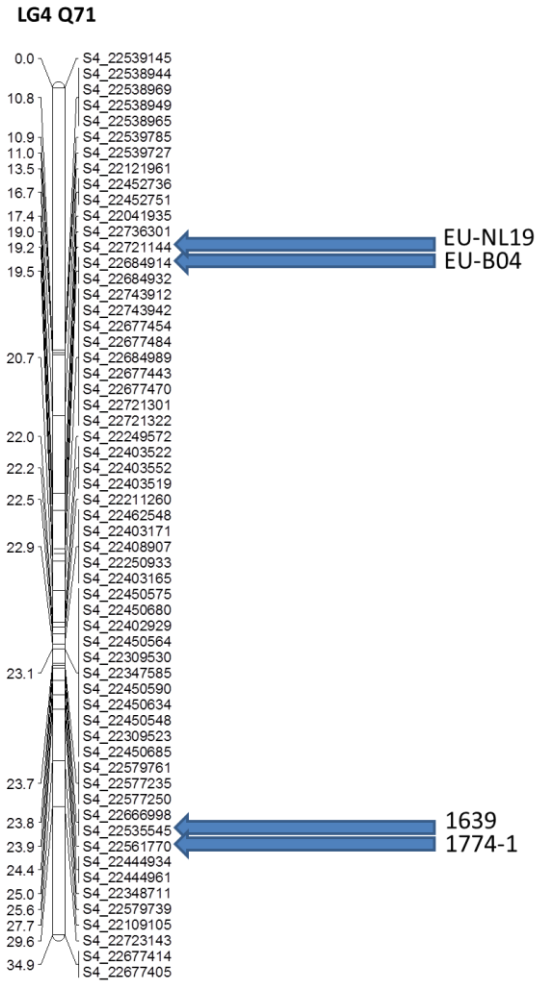

Figure 21: Development of a high-density linkage map using GBS sequence tags and phenotypic data for reaction to the isolates EU-NL19, EU-B04, 1639 and 1774-1 in 'Gala' x Q71 progeny. Marker names indicate the physical location of the SNPs at the lower end of chromosome 4 of 'Golden Delicious' genome assembly.

Fine mapping of the resistance loci on LG4

Genetic maps for apple LG4 were constructed for the resistant parents 'Geneva', Q71, M33, Q35 and Q49 using between 9 and 17 SNP markers in each population (Figs 2 and 3). Single sequence repeat (SSR) markers mapped to similar positions compared with previous genetic maps (Liebhard *et al*., 2002; Silfverberg-Dilworth *et al*., 2006). The marker order was consistent between families, indicating the robustness of the genetic maps. No significant segregation distortion was observed for any of the mapped markers on LG4. Before the mapping of the resistance loci commenced, genotype–phenotype incongruent (GPI) plants (Gygax *et al*., 2004) were identified (ranging from 0 to 16 per family) and excluded from the mapping population. Moreover, in population E × Q35, the R : S = 3 : 1 segregation ratio did not allow us to fine map the *R* genes interacting with isolates EU-NL19 and EU-D42. In agreement with our proposed GfG relationships model, we combined the phenotypes of the isolates EU-NL19 + EU-B04 and EU-D42 + EU-B04 to map the two resistance loci. In cases of contrasting phenotypes among the pair of isolates, the plants were scored towards susceptibility. The positions of the various *R* loci identified by the isolate phenotypes used to map them are given in the six parental genetic maps (**Figure 22** and **Figure 23**).

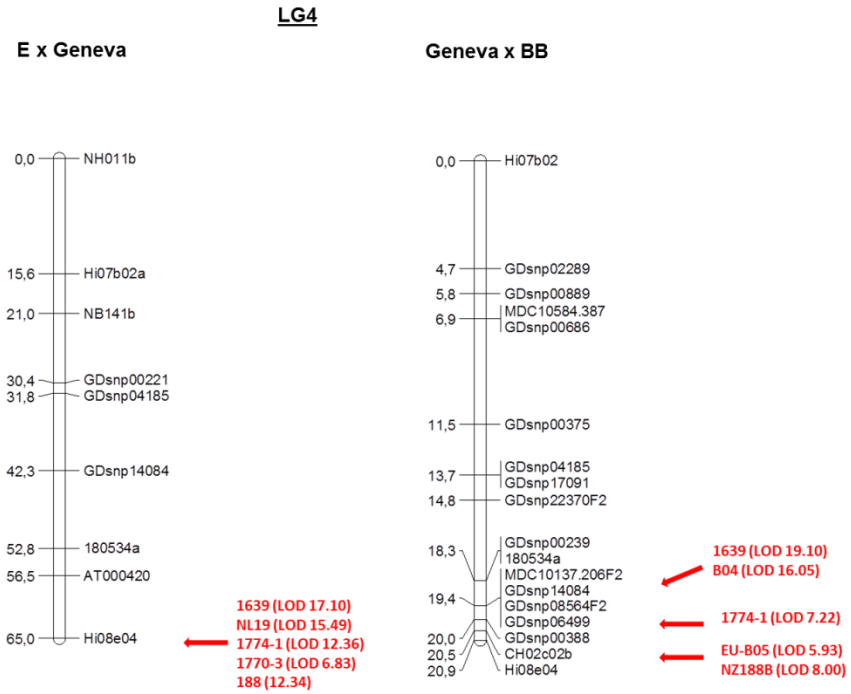

Figure 22: Genetic linkage analysis for the resistant parents 'Geneva' among SNP and SSR molecular markers on linkage group 4 (LG4) of F1 families 'Elstar' x 'Geneva' and 'Geneva' x 'Braeburn', and resistance towards various monoconidial *Venturia inaequalis* isolates.

In the F1 progenies, resistance to all the isolates mapped to the lower end of LG4 (**Figure 22**). In the F2 families (**Figure 23**), resistance to the various isolates was resolved to three distinct genomic regions of the denser maps: (1) resistance to isolate 1639 was linked to MDC10137.206 and GDsnp00239 in Q71 and M33, respectively, which corresponds to a position located around 22 Mbp on LG4 of the reference GD assembly; (2) resistance to isolates 1774-1 and 1770-3 was linked to Rbsnp23382430, MDC10137.206, GDsnp14084, GDsnp00388 and GDsnp04185 in Q71, M33, Q35 and Q49, respectively, which corresponds to 21.5 Mbp of GD LG4; (3) resistance to isolates EU-NL19, EU-B04 and EU-D42 was linked to GDsnp04185, GDsnp01026 and GDsnp00221 in Q71, M33 and Q35, respectively, which corresponds to 21 Mbp in the GD LG4 assembly. The resistance loci for isolates 1639 and EU-NL19 co-located with 1774-1 and 1770-3 when mapped in the E × Q35 and GD × M33 families, respectively. All resistance loci were in coupling phase.

Co-segregation between fully informative markers that were heterozygous in both parents ('ef × eg' segregation type in JoinMap format) and resistance to isolates that segregated 1 : 3 was examined (**Additional Table 12**, see Supporting Information). In family G × Q71 and E × Q35, most of the resistant seedlings for isolates 1774-1, 1639 and 1770-3 co-segregated with only one class of allelotypic data, among the four possible classes 'ef', 'eg', 'ee' and 'fg', whereas susceptible plants mostly co-segregated with the remaining three classes. None of the 'ef × eg' markers deviated significantly from the expected Mendelian segregation.

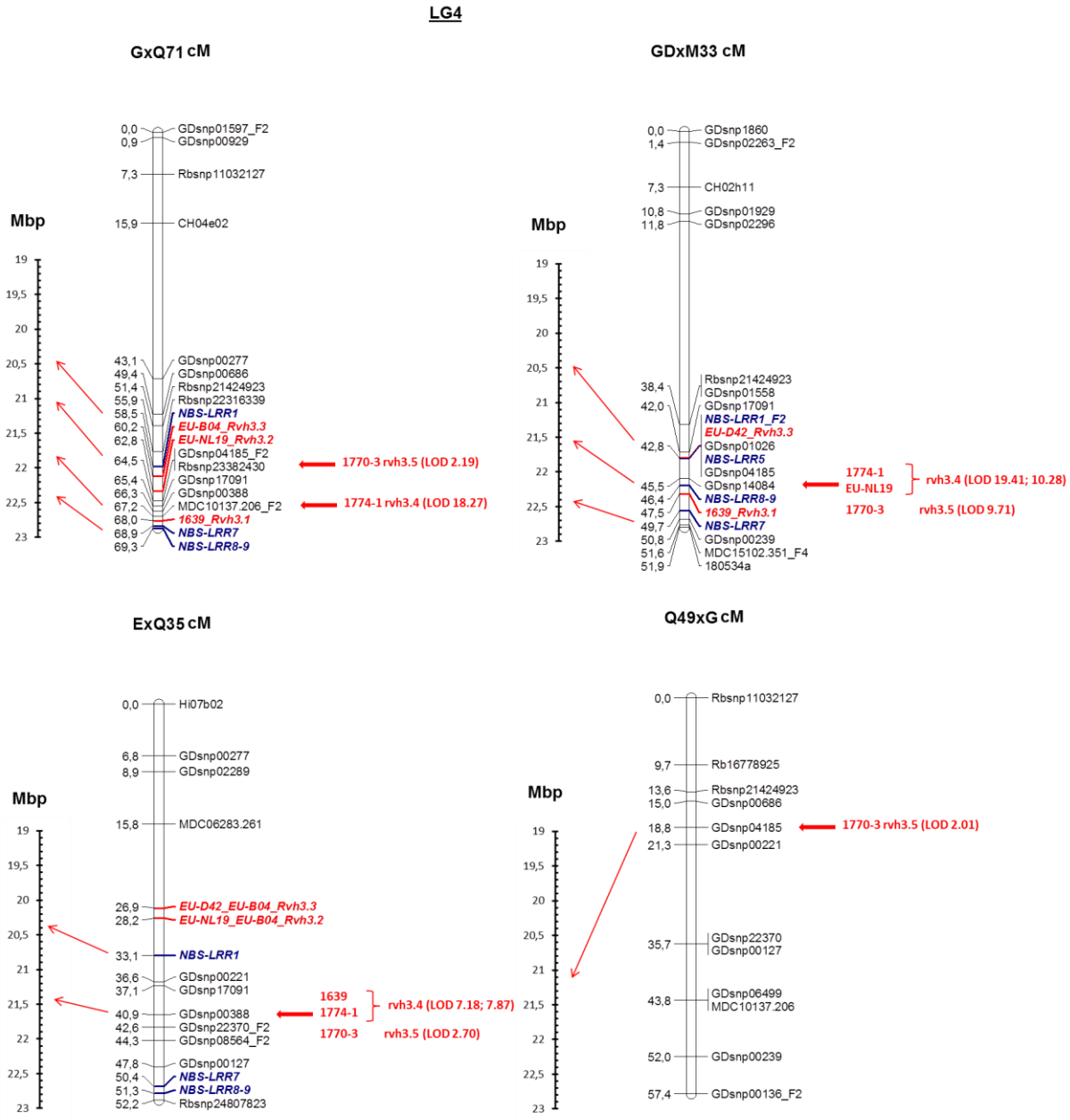

Figure 23: Genetic maps of linkage group 4 (in cM) of F1 resistant parents Q71, M33, Q35 crossed with 'Gala' (G), 'Golden Delicious' (GD) and 'Elstar' (E), and resistance towards various monoconidial *Venturia inaequalis* isolates. On the left of each map, arrows indicate the position on the reference 'Golden Delicious' physical map (in Mbp) of the closest molecular markers flanking each resistance locus.

Co-localization of disease resistance loci with analogues of disease R genes

Based on the GD LG4 assembly (Velasco *et al.*, 2010), nine predicted genes encoding proteins belonging to the NBS-LRR disease *R* gene family (NBS-LRR 1–9, Additional Table :9, see Supporting Information) were identified within a 2.2-Mbp genomic region (from 20.3 to 22.5 Mbp) between the markers flanking the resistance loci in the various families (**Figure 24**). As two NBS-LRRs had the same DNA sequence (NBS-LRR 8 and 9), a single primer set was designed for these two analogues. Five candidate genes (NBS-LRR 1, 5, 7, 8 and 9) mapped to LG4 of 'Geneva' derivatives and were linked to the resistance loci (**Figure 23**), enabling a reduction of

the intervals between markers and the three resistance loci. These loci mapped to the homologous region of GD at 20.5, 21.5 and 22.5 Mbp, respectively. NBS-LRR 2, 3, 4 and 6 did not map to LG4. No analogues of *R* genes showing homology to the *Cladiosporium fulvum R* gene family of tomato (and *Rvi6* scab *R* gene) were identified in this region.

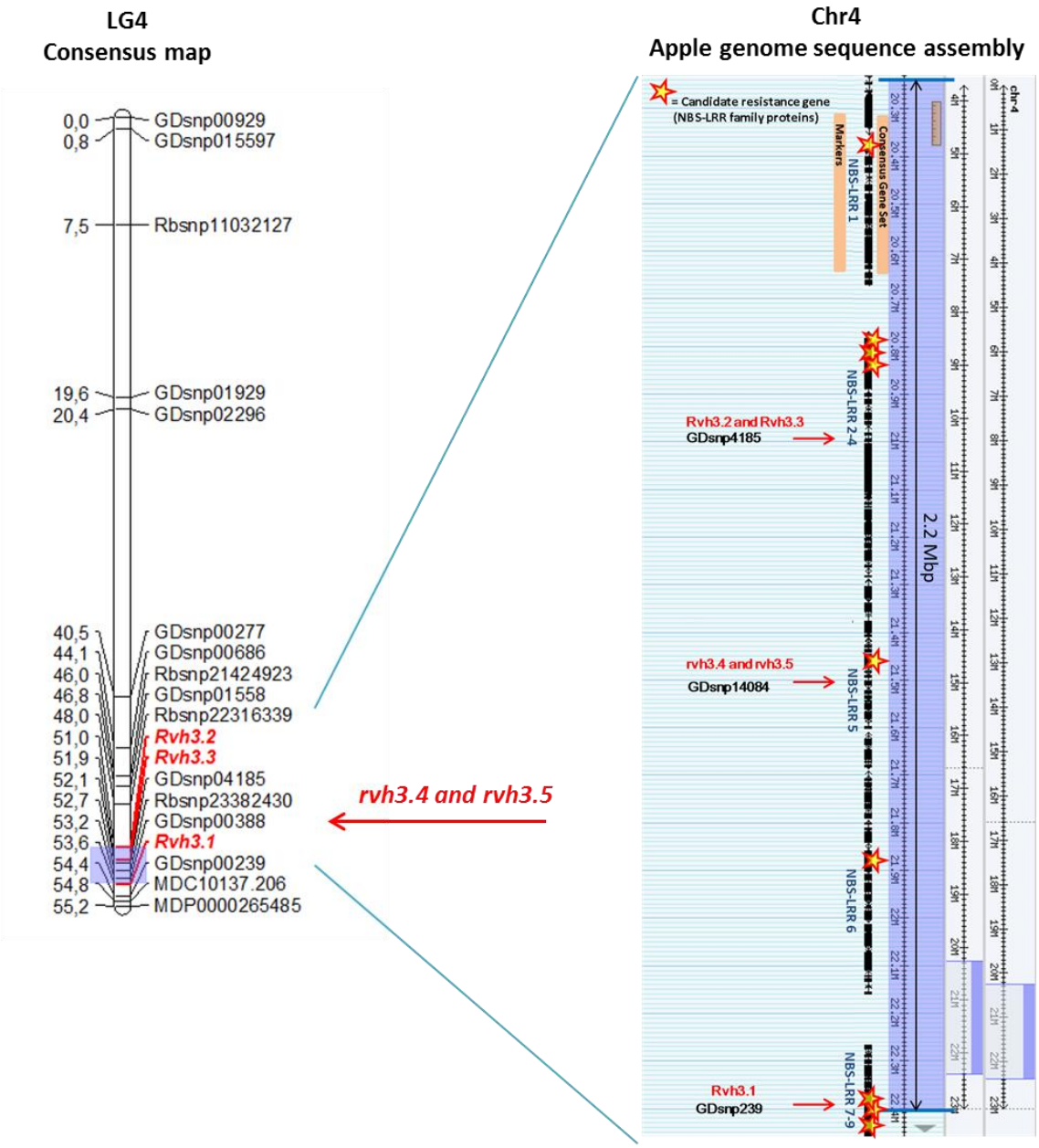

Figure 24: Consensus map of linkage group 4 (LG4) of 'Geneva' derived from the populations GD x M33 and G x Q71, aligned with the homologous region of the reference apple genome (Velasco et al. 2010) showing the relative positions of the mapped disease resistance loci (Rvh3.1-3 and rvh3.4-5) to candidate disease resistance genes (NBS-LRR family protein) on the genome assembly.

Discussion

The hypothesis from previous research (Shay and Williams, 1956; Williams and Shay, 1957) was that the apple scab resistance of 'Geneva' was conditioned by a spontaneous pyramided complex involving three major *R* genes (Bus *et al*., 2011).We now propose that at least five genes underlie the 'Geneva' resistance. Our conclusions were drawn from the differential interactions of nine monoconidial *V. inaequalis* isolates on six F1 and F2 progenies and were based on GfG theory, which postulates that, for each *R* gene in a plant, there is a corresponding avirulence gene in the pathogen (Flor, 1971). Some resistant seedlings were incompatible with only one isolate (1639, EU-NL19 or EU-D42/EU-B04) among the set of isolates tested, suggesting the existence of at least three independent resistance loci, the first incompatible with isolate 1639, the second incompatible with EU-NL19, and the third incompatible with both EU-B04 and EU-D42. As such specific GfG relationships with isolates 1639 and EU-NL19 were not observed in the F2 families GD × M33 and E × Q35, we suggest that the first two loci probably did not segregate in the resistant M33 and Q35 families. In contrast, no differential interactions of this type were observed for the other isolates tested (1770-3, 1774-1, NZ188B and EU-B05). However, our data indicated that these isolates were able to overcome all of these first three resistance loci, suggesting the presence of two additional resistance loci. The number of resistance loci segregating in Q49 × G remains hypothetical as inoculation failed with isolates 1639 and EU-B04 in this population. Nevertheless, we did observe segregation for resistance with isolate 1770-3, whereas isolate 1639 was shown to be incompatible with clonal Q49 (Tables 2 and 3). This suggested the presence of at least one resistance locus in this progeny, which is in agreement with earlier findings of one of the *Avr* genes of 1639 showing a GfG relationship with a gene in Q49 (Broggini *et al*., 2011).

Our data suggest both dominant control for isolates fitting a 1 : 1 segregation ratio (1639, EU-NL19, EU-B04 and EU-D42), and more complex genetic control for resistance to isolates 1774-1, 1770-3, NZ188B and EU-B05. With these isolates, the following observations were made: (i) incompatibility with less than onehalf of the progenies and segregation ratios approximating R : S = 1 : 3 in some families; (ii) the single genotype obtained for resistant plants with fully informative markers (segregating 1 : 1 : 1 : 1); and (iii) the observation that the resistance could 'skip' one generation (segregation for resistance was found in progenies resulting from the cross of two susceptible parents). At first glance, these results could be interpreted as a genetic recessive control of the 'Geneva' resistance against the subset of *V. inaequalis* isolates, with resistance to isolates conferred in homozygous plants. Two susceptible heterozygous parents for the recessive *R* gene (Sr × Sr) would lead to the R : S = 1 : 3 segregation ratio observed in the progeny (e.g. interaction of 1774-1 and 1770-3 with G × Q71). In contrast, a resistant homozygous (rr) parent would give an R : S = 1 : 1 segregation ratio when crossed with a susceptible heterozygous (Sr) parent (e.g. interaction of 1774-1 and 1770-3 with E × Q35). Finally, two susceptible homozygous parents (Ss) would result in the whole progeny being susceptible (e.g. interaction of 1774-1 with Q49 × G). Such recessive genetic control has been found in many plant–pathogen relationships, particularly in the resistance of plants to viruses, with about 100 recessive *R* genes identified (Diaz-Pendon *et al*., 2004), but also to bacteria (Deslandes *et al*., 2002; Iyer and McCouch, 2004; Li *et al*., 2001) and fungi (Büschges *et al*., 1997; Calvo *et al*., 2008; Hua *et al*., 2009; Peng *et al*., 2014; Perchepied *et al*., 2005;). In apple, recessive genetic control of resistance was proposed for bitter rot (*Glomerella cingulata*) (Thompson and Taylor, 1971), blotch (*Phyllosticta solitaria*) (Mowry, 1964) and *Alternaria*

blotch (*Alternaria alternata*) (Moriya *et al*., 2011), and could also form the basis of segregation observed for the partial resistance to race (6) isolates of *V. inaequalis* in the cross 'Prima' × 'Fiesta' (Durel *et al*., 2003).

Various biological mechanisms have been proposed to explain a recessive resistance. Either a mutation or loss of a susceptibility allele that facilitates infection of the plant by the pathogen (e.g. the negative regulator of the defence response *mlo* gene in barley) and hence causes an abnormal defence response in the host (Büschges *et al*., 1997; Devoto *et al*., 1999; van Schie and Takken, 2014), or a dosage-dependent effect of each allele of the recessive *R* gene that might accumulate until a sufficient resistance is reached to be effective (e.g. *xa5* gene in rice) (Iyer and McCouch, 2004; Vanderplank, 1984). Nevertheless, a homozygous recessive nature of *R* genes in 'Geneva' would be remarkable from a genetics point of view, given the fact that the segregation of resistance to isolates 1774-1, 1770-3, NZ188B and EU-B05 has been found in almost all families under investigation. It suggests that the same resistance allele is present in all four susceptible genotypes used to develop the F1 and F2 families and has conferred the homozygous resistant status in part of the subsequent progenies. In addition, this theory cannot explain the segregation ratios observed with some isolates in F1: crossing the homozygous resistant parent 'Geneva' (rr) with a heterozygous susceptible cultivar E of BB (Sr) should have led to an R : S = 1 : 1 segregation ratio instead of the 1 : 2, 1 : 3 or 1 : 7 observed.

In the *Arabidopsis* plant model, the recessive resistance locus *RRS1* (*Resistant to Ralstonia solanacearum 1*) has received increasing attention from researchers. Although genetically defined as recessive in segregating populations (R : S = 1 : 3), this *R* gene encodes a protein whose structure combines the Tollinterleukin- like receptor (TIR)-NBS-LRR domains found in several R proteins with an additional WRKY DNA-binding motif characteristic of some plant transcriptional factors, and behaves as a dominant gene in transgenic susceptible plants (Deslandes *et al*., 2002, 2003). More recently, it has been suggested that *RRS1* alone would not be sufficient to confer resistance, but its protein has been found to physically associate with an R protein from another gene, *RPS4* (*Resistant to Pseudomonas syringae 4*), to cooperatively trigger immunity against a range of pathogens (Narusaka *et al*., 2009; Sohn *et al*., 2014; Williams *et al*., 2014). The *R* gene pairs *RRS1* and *RPS4* would function as a heterocomplex receptor with each partner having a different role: *RPS4* acting as an inducer of disease resistance signalling and *RRS1* acting as an effector-binding receptor and repressor of *RPS4* TIR signaling activity. The action of complementary *R* gene pairs, in which both proteins are required to confer resistance against a pathogen isolate, has been found to function in the recognition of bacterial, viral, oomycete and fungal pathogens in both monocotyledonous and dicotyledonous plants, suggesting that this is a common and widespread mechanism in plant immunity (Cesari *et al*., 2014; Griebel *et al*., 2014). Moreover, as there is increasing structural evidence for both coiled coil (CC) and TIR domains of NBS-LRR proteins being able to form dimers (Maekawa *et al*., 2011; Williams *et al*., 2014), and also because of the lack of polymorphism within this family protein that might hide such dimerizations, *R* gene action in pairs may be even more general than already demonstrated. It might also form the basis of the complex resistance of 'Geneva' against *V. inaequalis*.

Interestingly, all of the R protein pairs identified in the literature are encoded by two tightly linked resistance loci, most of which are transcribed in opposite directions with a relatively short intergenic region. This conserved genomic organization could be important for the co-regulation of these *R* genes or to prevent recombination events leading to the separation or inappropriate

pairing of *R* genes, which could cause a loss of resistance or spontaneous necrosis (Cesari *et al*., 2014). However, it cannot explain the R : S = 1 : 3 segregation observed in some *Arabidopsis* progenies deriving from the Nd-0 and Ws-2 resistant RRS1/RPS4 parents and the Col-0 susceptible parent. One possible interpretation of this recessive nature of *RRS1* is that the Col-0 susceptible allele encoding a protein would interfere *in trans* with the pathogen effector responsiveness and thus act as a 'poison subunit' of the *RRS1*/*RPS4 R* gene complex (Sohn *et al*., 2014). Such a model, as well as potential recombination events between the *R* gene pair loci if not tightly linked, could be at the origin of the distorted segregation ratio R : S = 1 : 2, 1 : 3 or 1 : 7 observed with some *V. inaequalis* isolates across the various F1 and F2 'Geneva' families. Further genetic studies, including the creation of new segregating populations involving backcrosses or the cloning of the resistance loci, could help confirm the status of the genetic control of the complex resistance of 'Geneva' against *V. inaequalis*. Moreover, we are currently proceeding with the genome resequencing of the 'Geneva' resistance loci to investigate the potential presence of pairs of NBS-LRR genes.

Based on these observations and considering all the differential interactions among the isolates and the segregation ratios observed, we hypothesized a model of the GfG relationships between the *V. inaequalis* isolates and the resistance loci (**Table 16**). In this model, we propose a dominant genetic control for the first three loci revealed by the isolates 1639, EU-NL19 and EU-B04/EU-D42, and more complex (recessive) genetic control for the last two loci revealed by the isolates 1774-1, 1770-3, NZ188B and EU-B05. We have accordingly assigned temporary names to the resistance alleles: *Rvh3.1*, *Rvh3.2* and *Rvh3.3* for the dominant loci, and *rvh3.4* and *rvh3.5* for the other two alleles to indicate that their control does not fit the classic dominant model (**Table 16**). Nevertheless, some interactions remain unexplained. For example, the proposed model does not explain why, in GD × M33, EU-NL19 was compatible with some seedlings for which incompatibility was shown with 1774-1 and 1770-3. Such interactions were not observed in the other families and were not included in our GfG relationships model. Such unexplained interactions could be caused by mis-phenotyping of some genotypes, either as 'escapes' during the inoculation procedures or of plants being erroneously scored as susceptible whilst carrying resistance factors. This is possible as some resistant symptoms allowed considerable sporulation when exposed to a longer period of humidity, as shown in previous studies (symptoms 2→4; Shay and Williams, 1956). This could also explain some inconsistencies in the phenotypes of the replicated clonal trees of the selected F1 progeny. Finally, we cannot exclude the possibility of additional *R* genes governing the resistance of 'Geneva'. In this regard, we noticed the presence of resistant seedlings carrying the marker alleles for susceptibility for all markers tested. These plants were classified as GPI plants (Gygax *et al*., 2004), and their genetic data were discarded from the JoinMap analysis; however, they could suggest the involvement of a sixth *R* gene independently inherited from another LG. Finally, epistatic interactions among the *R* genes were not considered in our GfG relationships model. Epistasis leading to increased resistance has already been shown in pyramided dominant resistances against apple scab (Bus *et al*., 2002), as well as in recessive resistance of rice against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) races, for example, between the recessive *R* genes *xa5* and *xa13*, or among these two recessive genes and a dominant one (Li *et al*., 2001).

To further elucidate the genetic organization of 'Geneva' resistance loci related to specific strains of *V. inaequalis*, we performed fine mapping of resistance to nine isolates in six segregating F1 and F2 populations derived from 'Geneva'. In order to construct a particularly dense genetic map, we employed GBS to obtain 23 585 SNPs polymorphic in the resistant parent

Q71. The resulting map revealed the location of resistances to isolates EU-NL19, EU-B04, 1639 and 1774-1 at the distal end of LG4 (**Figure 21**). Fine mapping of the individual resistance loci was then carried out in six families derived from 'Geneva'. Our genetic linkage analysis identified three close but distinct chromosomal regions on LG4 where resistance loci were detected. These regions are located on the homologous GD sequence assembly at about: (1) 22.5 Mbp for isolate 1639; (2) 21.5 Mbp for 1774-1 and 1770-3, and (3) 20.5 Mbp for EU-NL19, EU-B04 and EU-D42 (Figs 2 and 3). These three chromosomal regions would encompass the five resistance loci governing the resistance of 'Geneva' against *V. inaequalis* that were indicated by our initial analysis of phenotypic segregation by family and isolate (**Table 16**). Region (1) would carry the dominant *Rvhi3.1* locus, region (2) the *rhvi3.4* and *rhvi3.5* loci harbouring a more complex (recessive) genetic control, and region (3) the dominant *Rvhi3.2* and *Rvhi3.3* loci. We noted that resistance to isolates 1639 and EU-NL19 mapped at the same position as 1774-1 in families E × Q35 and GD × M33, respectively, suggesting that *Rvhi3.1* might be missing in Q35 and the dominant *Rvhi3.2* in M33.

Our in-depth analysis of the 'Geneva' scab resistance enables us to clarify the preliminary findings suggesting two linked genes, temporarily named *Vh3.1* and *Vh3.2*, which were mapped to LG4 in the E × 'Geneva' (using the isolates EU-NL19 and 1770-3, respectively) and 'Geneva' × BB (using the isolates 1639 and EU-B05, respectively) progenies (Bus *et al*., 2011). Considering our GfG relationships model (**Table 16**) and the isolate phenotypes used to map the resistance loci, it is most likely that *Vh3.1* corresponds to *Rvh3.1* and/or *Rvh3.2* and *Vh3.2* to *rvh3.4* and/or *rvh3.5* loci in our study. However, in the study by Broggini *et al*. (2011), *Vh3.2* corresponds to *Rvh3.1* as the other *V. inaequalis* parent, EU-B04, is compatible with Q49 (**Table 15**); hence, avirulence gene *AvrVh3.2* in *V. inaequalis* isolate 1639 (Broggini *et al*., 2011) should now be named *AvrRvh3.1*. Alternatively, the differential interaction in Q49 also involves the *rvh3.5* locus as suggested by the segregation of resistance to the 1770-3 isolate in the Q49 × G family, but the location of the corresponding avirulence loci in *V. inaequalis* could not be mapped employing the EU-B04 × 1639 progeny (Broggini *et al*., 2011) as both isolates were incompatible with hosts carrying this gene (**Table 16**).

One of the criteria for assigning a single *Rvik* name to a locus is that it has a sufficiently wide spectrum against *V. inaequalis* isolates to be of value for breeding (Bus *et al*., 2011). Only *rvh3.4* appears to meet this criterion from the small sample of *V. inaequalis* isolates in our study, and hence is the best candidate for *Rvi3*, the name assigned in the international nomenclature system to the main scab *R* gene in the 'Geneva' host (Bus *et al*., 2011). However, extensive evaluations need to be carried out to confirm the resistance spectrum of this gene in a host only carrying this gene, which, at the same time, would become the differential host used for pathotype monitoring (Patocchi *et al*., 2009; www.vinquest.ch). With 'Geneva' having been reported as considerably infected under field infections in multiple years (Beckerman *et al*., 2009; Bengtsson *et al*., 2000), races may readily develop in orchard populations of *V. inaequalis*, despite the presence of a spontaneous pyramided complex of at least five *R* genes. This seems in contradiction with previous theories suggesting durable resistance in cultivars pyramided with various disease *R* genes with complementary modes of action (Joshi and Nayak, 2010; Mundt, 1990). Further investigation of the GfG relationships at the molecular level should be performed to understand why *V. inaequalis* was able to overcome all five genes. Nevertheless, based on the considerable sporulation on 'Geneva' by, for example, EU-NL24, a race (1,3,6,7) isolate (Caffier *et al*., 2015), we can hypothesize that the *R* genes do not impose a high penalty to the pathogen for adaptation (Leach *et al*., 2001). Nevertheless, (part of) the 'Geneva' *R* gene complex has

demonstrated field resistance in New Zealand (V. Bus *et al*., unpublished data), and hence will still be useful for gene pyramiding with other scab genes by breeders applying MAS enabled by our fine mapping of five 'Geneva' resistance loci.

We have demonstrated that five NBS-LRR candidate genes are tightly linked to the dominant *R* genes *Rvh3.1*, *Rvh3.2* and *Rvh3.3*, and these were anchored on our 'Geneva' genetic maps. This co-localization indicates the potential involvement of NBS-LRR family proteins in the resistance of 'Geneva' to *V. inaequalis*. In contrast, no *HcrVf* paralogues were identified on LG4 of GD. In future studies, complemented by effector gene research in *V. inaequalis* (Bowen *et al*., 2009, 2010), it would be of interest to investigate the possible complementary mode of action of pairs of NBS-LRR genes among the 'Geneva' resistance loci, or the co-localization of our recessive resistance loci with genes that do not conform to typical *R* gene structural classes, as cloning studies indicate that recessive plant *R* genes usually encode proteins with no previously known role in disease resistance (Antony *et al*., 2010; Büschges *et al*., 1997; Deslandes *et al*., 2002; Devoto *et al*., 1999; Diaz-Pendon *et al*., 2004; Iyer and McCouch, 2004; Iyer-Pascuzzi and McCouch, 2007).

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Additional tables and figures

Additional Table 6: Origin and race status of monoconidial *Venturia inaequalis* isolates used in this study.

Isolates	Origin	Race
1770-3	USA	1
EU-B05	Belgium	1
1774-1	USA	1,7
EU-B04	Belgium	1,10
EU-NL19	The Netherlands	1,10
NZ188B	New Zealand	1,7,8
EU-D42	Germany	1,6,10
EU-NL24	The Netherlands	1,3,6,7
1639	France	1,2,8,9
1066	France	6,7,8,10
EU-NL05	The Netherlands	6,7,8,10,13
174	France	1,5,7,8,10,13

Additional Table 7: Composition of the CTAB-based buffer for extraction of genomic DNA from apple leaf samples

Buffer constituents	pH	Amount
tris (hydroxymethyl) aminomethane (Tris)	pH 8.0 with HCl	100mM
CTAB[a]		2%
n-lauroyl sarcosine		1%
Sorbitol		350 mM
NaCl		1.5 M
EDTA[b]		50 mM
EGTA[c]		10 mM
L-ascorbic acid		10 mM
sodium metabisulphite		10 mM
DIECA [d]		5 mM
PVP-40[e]		1%
RNase A		100 µg/ mL

[a] cetyltrimethylammonium bromide

[b] ethylene diamine tetra-acetic acid-di sodium

[c] ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetra-acetic acid

[d] diethyldithio carbamic acid

[e] polyvinylpyrrolidone

Additional Table 8: List of primers for SSR and SNP molecular markers used in this study (Yamamoto et al., 2002; Liebhard et al., 2002; Silverberg-Dilworth et al., 2006)

Name	Type	Forward	Reverse
GDsnp01597_F2	SNP	TCTCAAACCCCTGTCCAAAA	CTAAGGTGGGAGTGGTGGAA
GDsnp00929	SNP	TGGGACCAAAATAGGACAAACT	AGCTCAACCCAAAAACTCAAAG
GDsnp00277	SNP	TAGCTGGGGGCCTTTTTATT	GGGAAGAAGCAACTACATTTACCA
GDsnp00686	SNP	TACCTTGGGGGAATCACCTT	ACTTACTGGGCAGCGTGTG
GDsnp04185	SNP	TGCTCAATAGGCAACGGTTT	TCGTATTACAAAGACAATTCACAGA
GDsnp04185_F2	SNP	GCCCAAGTCGAGAGATTGAC	AGTACCGGCCTCTTTCGAG
GDsnp00388	SNP	AAGCGTCAGCTTTGATCCAT	TGAGGAACTCGAAGGGAAGA
GDsnp01860	SNP	GAACTGGCCTAACCGCTATCT	GATCTCTGTCGGCAACAATTC
GDsnp02263_F2	SNP	CGGCAATAAATCATCCTCCA	TGCAACAGTCCAAAAGGTAGC
GDsnp01929	SNP	TTATTCCATCCTCCCATCCA	GCGCATAACCGAATTTTTGA
GDsnp02296	SNP	TGGGTCGAAACCTCCACTAA	AGCTTGGGAGAAGCAGAACC
GDsnp01558	SNP	CCTAAAACGTCATTGCGTCTAGTT	TTCAAAGGGACTTCACGATG
GDsnp01026	SNP	AAATGTCGATTCTCCTGTTGAG	GACACGACGAGAACATATCAGC
GDsnp14084	SNP	CACGTGCGGAAGAATTAACA	GGTATGCCACATCCCTGTAG
GDsnp00239	SNP	AGGGCTTTCGTTTTCTGACA	ACTGTGCAAGAAACCCCATC
GDsnp00768	SNP	CCACTTGCAGAACCCAAACT	ACGTCACCATGCCTTCAAAT
GDsnp02289	SNP	TCGTGCAACTTCCTCTACCA	GCATCCTTACTCTCAGAACAGCA
GDsnp00221	SNP	GGATACGAATCGGTATAGTGTTGTG	CAATCCCGATCAAACTGAAA
GDsnp11916	SNP	AAGTCGCATTCTGGTCTCGT	GGAAAAACTGAGGTGTGCAAG
GDsnp22370_F2	SNP	CATGAATTACTGTCGCAACGA	TTTTGGAAATGATGGGTTTGA
GDsnp08564_F2	SNP	GGTGTGGCCTCTCAACTCTG	CCACAAACAGAAAGACACTTCTC
GDsnp00127	SNP	TCTCCAGATATGCAAAGAAATTGA	TCTCTAGGCAATAAAAATTGGAAA
GDsnp00375	SNP	AATTTGCAGGGCCACACTT	GTCCCCAGCAGTGATGTTTC
GDsnp00388	SNP	AAGCGTCAGCTTTGATCCAT	TGAGGAACTCGAAGGGAAGA
GDsnp00127	SNP	TCTCCAGATATGCAAAGAAATTGA	TCTCTAGGCAATAAAAATTGGAAA
GDsnp06499	SNP	TGCAACATGCAGATGAAGAAG	AATTCTACTGGCCACACCCTA
GDsnp00136_F2	SNP	TTGCCTCTTGAAGCTGTTGA	ATGAATCCAACGTGCTTGCT
GDsnp17091	SNP	GGGTGATGAGAGGATGTTGG	CCTCGCTAAAAGTCGGAGAA

GDsnp00889	SNP	CCGTCTACTAGGGTTCATCCA	CGGGGTCGAAGTCATACTCTT
Rbsnp24807823	SNP	CATTATCTACAGACCTCACATCGTC	GGTTTCTTCTGCTCATCCTTG
Rbsnp23382430	SNP	TCCCACCGTCTTGGATAAAC	CTCCTTGAGCAGGTAATGTCC
Rbsnp21424923	SNP	TATTCCATCAGGAGGGCAAG	CCCTTTCTGCAAACTTGAGTG
Rbsnp22316339	SNP	TGTTGCACTTCGAATGATGG	CCATGGGTTTCATCCATCTC
Rbsnp11032127	SNP	GGCTTCAAGGGTGTTGTGTT	TGTGTGAGGCTATTGTTTTTCA
MDC10584.387	SNP	TTTGCCCAGTTAAATTCCTTT	TTTCACCCAATGCTTTCTGA
MDC10137.206	SNP	GGGAGGAAGGACCAACTG	GCTATTGCTTGTTACCCAAGTT
MDC10137.206_F2	SNP	ACCTAAAACCGGCCTACCC	AATTGCACCAGTTCTCAGCA
MDC15102.351_F4	SNP	AAGCATAGAGGAAATCATAGAAGGAA	ACTTGTCGCTTGCCCTAAGA
MDC06283.261	SNP	CATCTTAGAATTGGAGTCCTTTCA	CTCTTCCACGAGGGGAGTTT
MDP0000265485	SNP	TCCCTCCCTAACTGCTCCTT	AGGATTGCTATTCCCAACTGA
CH04e02	SSR	GGCGATGACTACCAGGAAAA	ATGTAGCCAAGCCAGCGTAT
CH02h11	SSR	CGTGGCATGCCTATCATTTG	CTGTTTGAACCGCTTCCTTC
Hi07b02	SSR	ATTTGGGGTTTCAACAATGG	GTTTCGGACATCAAACAAATGTGC
180534a	SSR	CCAGTTGGTTATACAAATCGCAAAG	CCTGATCCTCAAAATTACAGCA
NH011b	SSR	GGTTCACATAGAGAGAGAGAG	TTTGCCGTTGGACCGAGC
NB141b	SSR	CAGAGAAAGACAGAGGTAGAGAGAA	GGATTGATCGCCTTATGGTTGT
AT000420	SSR	TTGGACCAATTATCTCTGCTATT	GATGTGGTCAGGGAGAGGAG
Hi08e04	SSR	GCATGGTGGCCTTTCTAAG	GTTTACCCTCTGACTCAACCCAAC
CH02c02b	SSR	TTGCATGCATGGAAACGAC	GTTTGGAAAAAGTCACACTGCTCC
NBS-LRR1	SNP	TGAGCGAGTAGTAGGTTTAAGAAAG	TCGAAAGGTCAATGGGAGTT
NBS-LRR1_F2	SNP	TGAGCGAGTAGTAGGTTTAAGAAAG	TGAGCAGATCGAAAGGTCAA
NBS-LRR2_F2	SNP	GGGTCATCTGCTATTCCCTTC	TGCCAAATGTTTCAAGCTGT
NBS-LRR2_F3	SNP	GCTTGAAAGGCTAACTTTCGTT	TGACGGTCTTGACACTCCAA
NBS-LRR3_F3	SNP	GCATTCAATCTCCCTGCAAT	CCAACTTGATCCAGGCTCTT
NBS-LRR4_F1	SNP	GCTTGAAAGGCTAACTTTTGTTT	TGACGGTCTTGACACTCCAA
NBS-LRR4_F5	SNP	TTGGCATTTCAAAACTTCAGG	GTCTTGAAGGAATGGCCAAA
NBS-LRR5_F2	SNP	ACACGCTAACTGGAATGGTG	TCTGGTCATGTTTTTGCTTG
NBS-LRR6_F6	SNP	AAAAGCCACAACCATCCAAG	ATCGGAATGGACAAGAACGA
NBS-LRR6_F7	SNP	TCGCGTAACTGCTTCACATC	CGCCTTGGTAAGGGAAAGAT

NBS-LRR6_F8	SNP	GTTGATTCCGGCCTTTGTC	AGAGGCGAAGACACACGTAAA
NBS-LRR7_F2	SNP	AAACTTGAGGAAGATCCAATGC	TCTCAAGTTCGGCAGTTTTTC
NBS-LRR7_RbSNP24765902	SNP	CAAGCCAGCACACTTTTTCA	TCAGAAGCAAAGATAAGGAGCA
NBS-LRR7_RbSNP24766750	SNP	GATCCCTCTCCTCTTTCGATG	TCTCAACAATGCAAACCAAG
NBS-LRR8-9_F2	SNP	AAACTTGAGGAAGATCCAATGC	TCTCAAGTTCGGCAGTTTTTC
NBS-LRR8-9_F3	SNP	TCCCTCCCTAACTGCTCCTT	AGGATTGCTATTCCCAACTGA
NBS-LRR8-9_F5	SNP	CTCAGACCCAACATCATAAAGG	TTCACCACCAATTTCCAATC

Additional Table :9 : Resistance gene analogues (NBS-LRR family proteins) identified within the 2.2 Mbp region between the markers flanking the resistance loci, based on the 'Golden Delicious' genome assembly (Velasco et al, 2010)

LG	Position (Mbp)	Gbrowse ID	Name in our study
4	20,39	MDP0000178082	NBS-LRR1
4	20,78	MDP0000312812	NBS-LRR2
4	20,81	MDP0000304428	NBS-LRR3
4	20,81	MDP0000306942	NBS-LRR4
4	21,46	MDP0000279585	NBS-LRR5
4	21,88	MDP0000420871	NBS-LRR6
4	22,40	MDP0000458200	NBS-LRR7
4	22,40	MDP0000265485	NBS-LRR8
4	22,40	MDP0000300921	NBS-LRR9

Additional Table 10 : Differential host-pathogen interactions observed between monoconidial isolates of *V. inaequalis*, and seedlings of the F1 and F2 progenies derived from 'Geneva' and crossed with 'Elstar' (E), 'Braeburn' (BB), 'Gala' (G) and 'Golden Delicious' (GD). Based on the gene-for-gene governing the interaction between the R-gene in the host and the avirulence gene in the pathogen, we hypothesised from these differential interactions the number and organisation of the resistance genes in 'Geneva'.

Family	1639[b]	EU-NL19	EU-B04	EU-D42	1770-3	1774-1	NZ188B	EU-B05	EU-NL24	Nb[a]
E x 'Geneva'	R	R	-	-	R	R	R	-		26
	R	R	-	-	S	R	R	-		2
	R	R	-	-	S	S	S	-		10
	R	S	-	-	S	S	S	-		3
	S	R	-	-	S	S	S	-		1
	S	S	-	-	S	S	S	-		55
	Others (with I phenotype)									28
									Total	125
G x Q71	R	R	R	-	R	R	-	-		8
	R	R	R	-	R	S	-	-		1
	R	R	R	-	S	R	-	-		21
	R	R	R	-	S	S	-	-		3
	S	R	R	-	S	S	-	-		3
	S	R	R	-	S	R	-	-		1
	S	S	R	-	S	S	-	-		2
	S	R	S	-	S	S	-	-		4
	S	S	S	-	S	S	-	-		41
	Others (with I phenotype)									35
									Total	119
E x Q35	R	R	R	R	R	R	-	-		3
	R	R	R	R	R	S	-	-		1
	R	R	R	R	S	R	-	-		2
	R	R	R	R	S	S	-	-		3
	R	R	S	R	S	S	-	-		1
	S	R	R	R	R	S	-	-		2
	S	R	R	R	S	R	-	-		1
	S	R	R	R	S	S	-	-		19
	S	R	R	S	S	S	-	-		1
	S	R	S	R	R	S	-	-		1
	S	R	S	R	S	S	-	-		7
	S	R	S	S	S	S	-	-		1
	S	S	S	R	S	S	-	-		5
	S	S	S	S	S	S	-	-		20
	Others (I phenotypes)									55
									Total	122

Family	1639	EU-NL19	EU-B04	EU-D42	1770-3	1774-1	NZ188B	EU-B05	EU-NL24	Nb
'Geneva' x BB	R	-	R	-	-	R	R	R	S	7
	R	-	R	-	-	R	R	S	S	3
	R	-	R	-	-	S	S	S	S	11
	S		R			S	S	S	S	1
	R	-	S	-	-	S	S	S	S	1
	S	-	S	-	-	S	S	S	S	43
	Others (with I phenotype)									25
									Total	91
GD x M33	R	R	-	R	R	R	-	-		27
	R	R	-	R	S	R	-	-		4
	R	S	-	R	R	R	-	-		7
	R	S	-	R	R	S	-	-		2
	R	S	-	R	S	R	-	-		6
	R	S	-	R	S	S	-	-		3
	R	S	-	S	S	S	-	-		1
	S	S	-	R	S	S	-	-		2
	S	S	-	S	S	S	-	-		31
	Others (with I phenotype)									35
									Total	118
Q49 x G	-	-	-	-	R	R	-	-		1
	-	-	-	-	R	S	-	-		12
	-	-	-	-	S	R	-	-		1
	-	-	-	-	S	S	-	-		23
	Others (with I phenotype)									3
									Total	40

[a] Number of seedlings

[b] R = resistant, S = susceptible, I = indeterminate

Additional Table 11 : Repartition of GBS-derived SNPs on the 17 linkage groups of apple using the *ApeKI* restriction enzyme

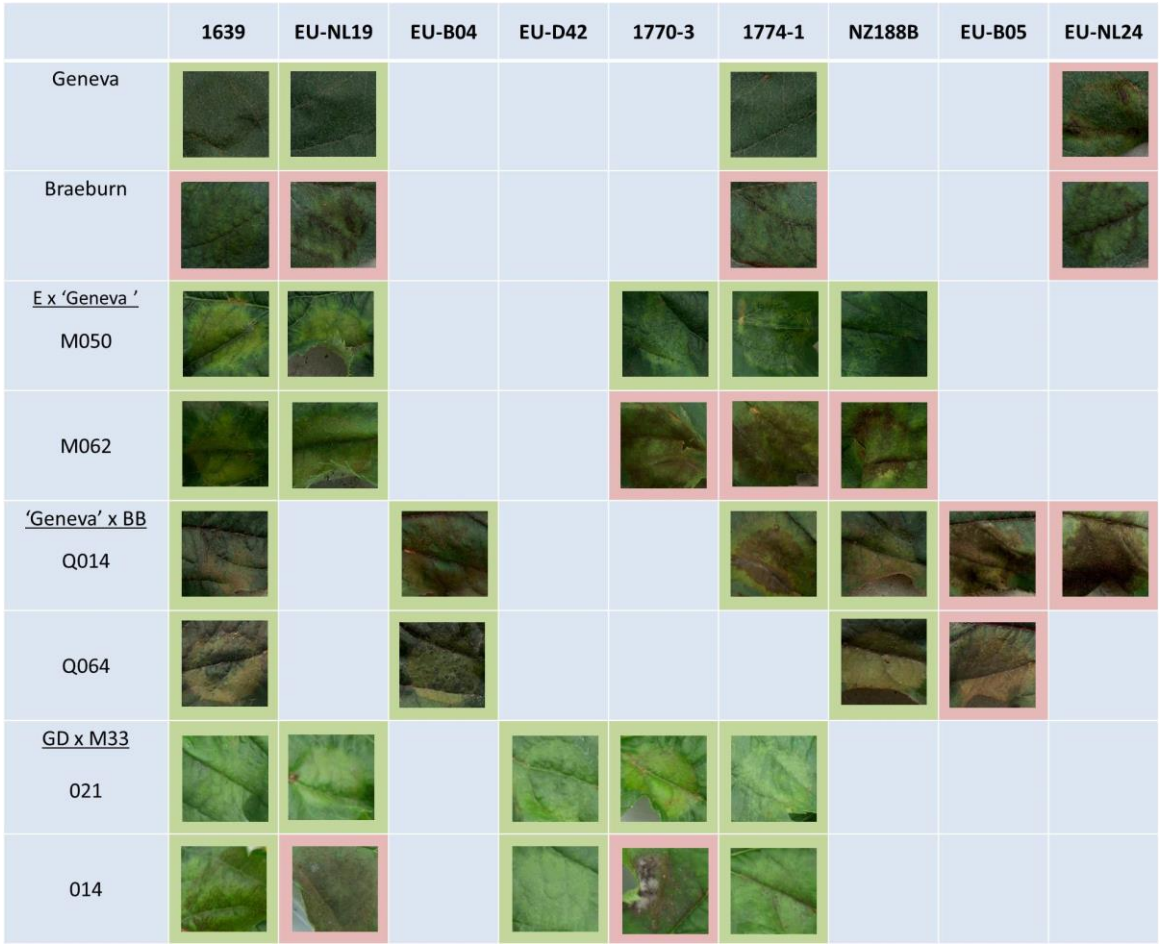
Linkage group	Number of polymorphic markers (backcross type segregation)	Number of markers in largest group with LOD=5
1	1303	598
2	1516	725
3	1182	349
4	1189	545
5	1413	693
6	983	476
7	1189	553
8	1417	717
9	1751	992
10	1506	647
11	1684	901
12	1605	725
13	1714	687
14	1277	646
15	1735	573
16	1072	540
17	1049	264
Total	***23585***	***10631***

Additional Table 12 : Segregation of allelotypes with the phenotype of reaction to *Venturia inaequalis* isolates exhibiting 1:3, 1:2 or 1:7 segregation (R:S) for co-dominant molecular marker data

'Geneva' – derived genotype and R–gene locus	**Isolate**	**Flanking co-dominant marker**		**ef**	**eg**	**fg**	**ee**
Q71 rvh3.4	1774-1	GDsnp00388	**R**	0	3	0	34
			M	0	5	1	2
			S	31	12	26	1
			GPI	0	0	0	0
rvh3.5	1773-0	GDsnp00388	**R**	1	2	1	10
			M	2	3	0	3
			S	28	15	27	24
			GPI	0	0	0	0
M33 rvh3.4	1774-1/ NL19	GDsnp22370	**R**	1	31	0	6
			M	0	0	0	1
			S	26	7	27	17
			GPI	0	0	0	0
rvh3.5	1770-3/ NL19	GDsnp22370	**R**	1	31	0	6
			M	1	0	1	1
			S	25	7	26	17
			GPI	0	0	0	0
Q35 rvh3.4	1774-1 /1639	GDsnp22370	**R**	9	0	2	0
			M	5	0	10	0
			S	3	25	23	37
			GPI	5	0	0	0
rvh3.5	1770-3 /1639	GDsnp22370	**R**	7	0	4	0
			M	6	0	4	0
			S	3	25	27	37
			GPI	6	0	0	0

[a] indetermined genotype

[b] GPI= Genotype-phenotype incongruent plants (Gygax et al. 2004)



Additional Figure 4: Illustrations of various symptoms and differential interaction patterns (incompatible in green background, compatible in red background) between monoconidial isolates of *Venturia inaequalis*, ‘Geneva’ and ‘Braeburn’ cultivars as well as some individuals of various segregating populations (E= ‘Elstar’, BB= ‘Braeburn’, GD= ‘Golden Delicious’).

Addendum

Outline

In this addendum some additional comments and discussion to the chapters 2, 3 and 4 of this thesis are provided.

Genealogy of the resistant accessions used in this study

Little information has been given about the genealogy of the two resistant cultivars investigated in this thesis. 'Président Roulin' (syn. 'Vice Président Roulin') is an old Belgian cultivar created in 1912 by Cyrille Roulin, chairman of the 'Société Royale Horticole de Gembloux'. This cultivar was created by cross pollinating the apple cultivars 'Joseph de Brichy' and 'Bismarck'. 'Geneva' is an open pollinisation of *Malus pumila* 'Niedzwetzkyana' (origin of Central Asia). Knowledge of the genealogy of the various parents used in the families of an apple breeding program is really important since it is informative of resistance genetics of the resulting progenies. It would also be of particular interest for the deployment of genomic selection such as the pedigree genotyping (van de Weg et al., 2003).

Disease evaluation scales used across the various experiments

Various scales have been used for scab resistance assessmenst across the experiments. In the orchard, a global assessment scale taking into account incidence and severity was used (Lateur and Populer, 1996) (**Additional Table 13**).

Additional Table 13: Global assessment scale for scab infection on leaves in the orchard (Lateur and Populer, 1996)

		Rating (%)	
Scale	Field observations	Incidence [a]	Severity [b]
1	No visible symptom	0	-
2	A few small scab spots are detectable on close scrutiny of the tree	≤ 1	-
3	Scab immediately apparent, with lesions very thinly scattered over the tree	≤ 5	-
4	X	X [c]	-
5	Infection widespread over the tree, majority of leaves with at least one lesion	≥ 50	≤ 5
6	X	-	X
7	Heavy infection, multiple lesions or more large surfaces covered by scab on most leaves	-	± 25
8	X	-	X
9	Maximum infection; leaves black with scab	-	≥ 75

[a] Incidence= proportion of infected leaves with at least one lesion

[b] Severity= mean proportion of leaf surface covered by scab

During the phytopathological tests in small inoculation chambers on the various progenies, symptoms were evaluated qualitatively and quantitatively. A description of the resistance symptoms was first given (chlorosis, necrosis, hypersensitive response, stellate chlorosis and stellate necrosis). Then an evaluation of the sporulation level was made according to a scale of 1-5 where: 1= no visible macroscopic sporulation, 2= thinly scattered lesions presenting few sporulation, 3= moderate number of sporulating lesions on at least part of the leaf (on an area delimited by the inoculation chamber), 4= abundant number of sporulating lesions on at least part of the leaf, 5= prolific sporulating lesions developed over most of the leaf (leaf black with scab).

Finally, reference has been made to the scale of Chevalier et al. (1991) where class 1 corresponds to the fast hypersensitive response (HR), which results in the complete inhibition of

pathogen development through a local programmed cell death and the apparition of pinpoint-pit reactions on the epidermal cells. The class 2 of reaction, appearing later during the infection, is characterized by chlorotic lesions (Chl) with irregular edges which center can be slightly necrotic. When the density of symptoms is high, the leaves can take on a crinkled aspect. To the original publication of Chevalier et al. (1991), I added in class 2 of reaction, the distinctive stellate necrotic (SN) and stellate chlorotic (SC) reactions. Within such reactions, conidiophores can sometime differentiate, but are never able to sporulate. In class 3a and 3b, slower resistance reactions are expressed as necrosis (N) and/or chlorosis (Chl) and involve sporulation to different degrees from low (class 3A) to high (class 3B). In these classes, a mix of aborted and sporulating conidiophores are found. Finally, class 4 symptom includes the abundantly sporulating lesions with no host reaction.

In the genetic analysis, plants were classified as resistant in the presence of resistance reactions with low/moderate sporulation (levels 1-3 on the 5-levels scale). Plants were classified as susceptible in the absence of resistance reactions with moderate to high sporulation (levels 4-5 on the 5-levels scale). The choice of such cut off value is quite subjective and probably depends on the breeding program investigating the resistance of the cultivar. There is in fact a continuum between fully resistant to completely susceptible plants, and the attribution of a resistant or susceptible status to moderately R/S plants is not always straightforward (e.g. level 3 on the 5-levels scale could be considered as moderately R/S). To illustrate this fact, in the genetic analysis presented in chapter 4, some moderately R/S phenotypes segregated with a mix of R and S alleles of molecular markers. This phenomenon, also highlighted in the study of Gygax et al. (2004), illustrates the difficulty of a clear cut-off between susceptible and resistant plants.

An alternative to the macroscopic evaluation of the sporulation level of *V. inaequalis* would be the use of real-time quantitative polymerase chain reaction (qPCR) for accurate quantification of fungal development in leaves (Daniëls, 2013).

Phytopathological tests in the various segregating populations

In Chapter 3 and 4, various *V. inaequalis* isolates have been inoculated in F1 and F2 segregating populations derived from 'Geneva' and 'Président Roulin'. The differential interactions observed between the various *V. inaequalis* isolates and the genotypes have been used to construct hypothetical models of gene-for-gene interactions between the hosts and the pathogen isolates.

Little information has been given on the choice of the *V. inaequalis* isolates. The isolates listed in **Additional Table 14** have been chosen among the set of *V. inaequalis* reference isolates characterized for their complementary avirulence alleles to 17 scab resistance genes identified in apple (Bus et al., 2011; Caffier et al., 2015). I selected isolates that were virulent on at least one of the following defined resistance gene hosts: h(1), h(2), h(3), h(4), h(5), h(6), h(7), h(8), h(9), h(10) and h(13), with a wide variety of different virulence patterns. Among the set of isolates tested, ten different virulence patterns were observed. In general, the isolates carried one to three virulences, but some of them were more complex, with up to six virulences. Selecting this set of well-characterized isolates increased the chances of identifying loci being overcome by the pathogen and, hence, the expression of differential interactions between the host and the pathogen. In fact, differential interactions across the various F1 and F2 progenies derived from

'Président Roulin' and 'Geneva' were observed. This permitted to discriminate between the various resistance loci in the segregating populations and to characterize GfG relationships improving our understanding of the host-pathogen interactions in the *V. inaequalis-Malus* pathosystem.

In the F1 segregating population derived from 'Président Roulin', the inoculation of eight isolates and an additional mix of six *V. inaequalis* isolates (14 isolates tested in total) permitted the identification of five resistance loci. In the F1 and F2 segregating populations derived from 'Geneva', the inoculation of nine isolates permitted the identification of five resistance loci. In such analysis, similar differential interactions were obtained for some of the isolates. For example, phenotypes obtained with isolates 1774-1 (race 1,7) in 'Geneva'-derived families were similar to those obtained with NZ188B (race 1,7,8). In the 'Président Roulin'-derived families, isolates belonging to the race 1 of the pathogen also gave similar interactions (EU-B05, EU-D49 and the mix of isolates composed of EU-B04, EU-I09, EU-F11, EU-D49, EU-F05, EU-B16). The observation of similar host-pathogen interactions for isolates belonging to the same race, or having most of the races in common, illustrates the importance of selecting isolates of contrasting virulence patterns in order to differentiate resistance loci in segregating populations. Among the well-characterized set of *V. inaequalis* isolates (Caffier et al., 2015), some isolates present interesting virulence patterns that have not been tested yet in the frame of this PhD (**Additional Table 14**). It would be of particular interest to test them on the various segregating populations in order to differentiate some possible additional resistance loci.

Additional Table 14: Origin of monoconidial isolates of *V. inaequalis* used in this thesis as well as some additional isolates that could be tested in further pathological tests

Race	Isolate	Origin	Tested on parents or families derived from
1	EU-B05	Belgium	'Président Roulin' and 'Geneva'
1	EU-B16	Belgium	'Président Roulin'
1	EU-D49	Germany	'Président Roulin'
1	EU-F05	France	'Président Roulin'
1	EU-F11	France	'Président Roulin'
1	EU-I09	Italy	'Président Roulin'
1	1770-3	USA	'Geneva'
1,7	1774-1	USA	'Geneva'
1,10	EU-B04	Belgium	'Président Roulin' and 'Geneva'
1,10	EU-NL19	The Netherlands	'Geneva'
1,7,8	NZ188B	New Zealand	'Geneva'
1,6,10	EU-D42	Germany	'Président Roulin' and 'Geneva'
1,2,8,9	1639	France	'Président Roulin' and 'Geneva'
1,3,6,7	EU-NL24	The Netherlands	'Président Roulin' and 'Geneva'
6,7,8,10	1066	France	'Président Roulin' and 'Geneva'
6,7,8,10,13	EU-NL05	The Netherlands	'Président Roulin' and 'Geneva'
1,5,7,8,10,13	174	France	'Geneva'
1	104	France	-
1	301	Germany	-
1,4	405	France	-
1,4	1634	France	-
1,4	1638	France	-
1,6	302	Germany	-
1,6	2199	Denmark	-
5,7	147	France	-
1,2,10	413	France	-
1,6,7	1680	France	-
6,7,8,10	1127	The Netherlands	-

During these phytopathological tests, efforts have been made to replicate our phenotypic observations. In the 'Président Roulin'-derived family, I disposed of clonal replications of each

seedling, grafted in triplicate on M9 rootstocks. In the absence of sporulation, such replicates were really important to discriminate some truly resistant phenotypes from potential escapes during the inoculation process. In this regard, the maximum sporulation severity (MSPOR) was computed over the genotype replications, taking into account the most susceptible plant (confer material and methods of Chapter 2). In the 'Geneva' families, I did not dispose of clonal replicates of each seedling from the various families. In that regard, it would be of particular interest to replicate the phytopathological tests, at least on genotypes showing an absence of resistance reactions and sporulation. This would permit to infer the resistance status of those particular genotypes. However, the analysis of our various genetic tests across the F1 and F2 families permitted in some extend to validate our observations. First, the same GfG interaction model was shown to fit the six segregating F1 and F2 populations. Second, the five resistance loci could be mapped at the same location across the various families, as compared to the location of the flanking molecular markers on the reference genome sequence of 'GD'.

However, in both genetic analysis in 'Président Roulin' and 'Geneva', I do not discard the hypothesis that more resistance loci could be revealed by the inoculation of additional *V. inaequalis* isolates showing contrasting patterns of virulence. A replication of the phytopathological tests, as well as a reassessment of some uncertain resistance genotypes showing an absence of reaction, could also certainly permit to narrow down the interval between some molecular markers and the various resistance loci.

Segregating ratios obtained in the various progenies

Segregation ratios deviating from the classical R:S=1:1 ratio, characteristic of the segregation of a major dominant gene, was observed in some progenies derived from 'Président Roulin' and 'Geneva'. These ratios were more complex and approximated a R:S=1:2, 1:3 or 1:7 ratios. Complex interactions between genetic loci, as well as non-dominant genetic controls of the resistance loci might be at the origin of such ratios.

In 'Geneva', some additional information about the genetic control of resistance loci approximating a R:S=1:3 segregation ratio were obtained by investigating the phenotypes obtained in the F1 and F2 families. The following observations have been made: (1) when crossing two susceptible parents for a particular isolate (e.g. the F1 seedling Q71derived from the cross between 'Geneva' and 'Gala'), resistance could be traced back in the resulting F2 progeny and approximated a R:S=1:3 ratio; (2) using fully informative molecular markers (efxeg), resistance was shown to segregate with only one allelo-class of the molecular marker, among the four possible classes 'ef', 'eg', 'ee' and 'fg'.

The hypothesis provided at the Chapter 4 of this PhD is that two resistance alleles would be required and/or gene complementation, for the expression of the resistance to that particular isolate in the genotypes derived from 'Geneva'. This could suggest both recessive (2 alleles at the same locus), but also co-dominant (2 alleles at different loci) nature of the resistance proteins. Such co-dominant or recessive nature of resistance loci has been already debated for the particular *rhg1* candidate gene for the resistance of soybean to nematode (Meksem et al., 2001; Brucker et al., 2005).

cDNA-AFLP analysis

Additional discussion should be addressed regarding some experimental design applied during the cDNA-AFLP analysis. First, for this analysis, choice has been made to pool the RNA samples obtained from two different plants. Such plants were at the same developmental stage and were inoculated at the same time using the same set of *V. inaequalis* isolates. Pooling of the RNA samples has been made in an effort to reduce the effects of biological variation (Chabas et al., 2001; Waring et al., 2001; Enard et al., 2002; Paris et al., 2009; Paris et al., 2012; Biruma et al., 2012). In experiments willing to study the differential expression of genes, the idea behind the latter motivation is that differences due to subject-to-subject variation will be minimized, making substantive features easier to find (Simon and Dobbin, 2003; Churchill, 2002; Kendziorski et al., 2003). Moreover, since the implementation of the cDNA-AFLP is time consuming, such pooling of the samples considerably reduces the time but also the cost, needed to perform the analysis on two separate plants of each cultivar. These are the motivations for pooling the RNA samples from two plants of each cultivars 'Président Roulin' and 'Gala' during the cDNA-AFLP. In the frame of this PhD, this has been judged to be desirable since the primary interest was not on the individual, but rather on characteristics of the cultivar itself (e.g. identifying biomarkers or expression patterns common across individuals of a same cultivar, but different to the other cultivar). However, despite the potential advantages, such experimental procedure also has some important drawbacks. Pooled designs can be discouraged because of concerns regarding the inability to identify and appropriately transform or remove aberrant subjects as well as the inability to estimate within population variation (.Kendziorski et al., 2005). In that regards, it appeared really important to further validate the expression of the candidate defense genes during the cDNA-AFLP using an independent technique. This independent technique was the qRT-PCR. Outcomes of this analysis will be discussed further. For this experiment, RNA was extracted from a single plant for each cultivar, in the absence of any RNA samples pooling.

During the cDNA-AFLP, choice has been made to incubate the control plants, sprayed with water, with the pathogen-inoculated plants in the inoculation chambers. Plants were incubated at 20°C under maximum relative humidity (close to 100% RH) for two days and were then transferred to the greenhouse (20°C, 60-80% RH). Since incubation has been shown to induce a stress to the plants (Daniëls, 2013), choice of applying the same conditions to control and pathogen-inoculated plants has been made in an attempt to normalize the data toward the regulation of genes due to the incubation conditions. In that way, any differential interactions between the water control and the inoculation treatment are more likely to be related to genes induced by the infection of the pathogen, and less likely to the stress induced by the incubation conditions of the plants. Such methods have been employed on other studies willing to identify differential regulation of genes in response to pathogen infection in the apple-*V. inaequalis* interaction (Kondo et al., 1993; Chevalier et al., 2008; Holzapfel et al., 2012; Gusberti et al., 2013), as well as in other plant-pathogen interaction models (Poupard et al., 2003; Eckey et al., 2004; Polesani et al., 2008; Steiner et al., 2009). Nevertheless, I cannot discard the hypothesis that some deviations in the regulation of genes could have been induced, notably by a possible interaction between genes involved in the response to pathogen and genes related to the stress induced by the incubation conditions. For this reason it would be interesting to investigate the regulation of our CDGs identified in this study in response to a possible stress induced by the incubation conditions of the plants.

qRT-PCR analysis of the expression of the candidate defense genes

In Chapters 2 and 3, qRT-PCR analysis were performed to investigate the CDGs expression in 'Président Roulin' in response to *V. inaequalis* infection. In Chapter 2, qRT-PCR was used to confirm the pattern of differential expression observed during the cDNA-AFLP in response to pathogen attack, at 48 hours after inoculation. While the first qRT-PCR amplifications were applied on the same samples used for the cDNA-AFLP (technical replication of the AFLP, using the qRT-PCR as an independent technique), the second amplifications were performed on cDNA extracted from a biological replicate of the experiments. In chapter 3, new plants were inoculated, and a time-course experiment was performed on cDNA samples extracted at 6, 24, 48, 72, 96 and 120 hours after inoculation. For each sample across these various experiments, amplification was estimated on two technical replicates of the same sample loaded on the qRT-PCR plate (technical replicates of the qRT-PCR).

Results from only one biological replicate were presented in Chapter 2. This does not permit to draw definitive conclusions on the pattern of expression of the CDGs. In this addendum, I combine and analyze all the qRT-PCR results obtained in this thesis. The qRT-PCR data of the technical replication of the AFLP was analyzed together with the biological replications presented in Chapter 2 and Chapter 3, at 48 hours after infection. Consistency of the general pattern of differential expression across the various experiments is tested (over expression or repression in 'Gala' and 'Président Roulin' in response to infection, without considering the magnitude of gene regulation), as well as the consistency of level of expression (magnitude of gene regulation, expressed as n-fold regulatation). This new analysis permits to better judge of the stability of expression of the CDGs. Results are presented in the **Additional Table 15**.

Over the 24 CDGs tested, expression pattern (over expression or repression) of 13 of them was fully confirmed across the three independent qRT-PCR analysis (one technical replicate and two biological replicates). This seems to confirm the importance of these particular CDGs in the response of the partially resistant 'Président Roulin' to infection. However, the magnitude of induction or repression was not always consistent across the experiments. In fact for 4 out of the 13 CDGs investigated, significant differences of expression were observed between the technical and biological replicates of the experiment (for 51HU129', the consensus 44EU122/44EU118, 53HU89 and 55HU125'). Such variability in the relative expression of plant genes has been also observed in the literature when comparing samples from different biological replications of the experiments (Hammond et al., 2003; Polesani et al., 2008; Rapaport et al., 2013). This may be due to the interactions of the plant and/or the pathogen with the environment, as demonstrated in the case of quantitative disease resistance (Geiger and Heun, 1989; Young, 1996; Price et al., 2004). In fact, although efforts have been made to control the inoculation of the pathogen and plant growth, slight differences in environmental parameters such as temperature or humidity might give rise to asynchronous or heterogeneous responses of individual plants to *V. inaequalis.* By acknowledging such variability, it appears also important to question in which extend variation in relative RNA expression will influence the final protein level in the plant, and the host response to pathogen. This question seems difficult to address, since attempts to correlate protein abundance with mRNA expression levels had variable success, notably due to the sophisticated programs of gene expression that have evolved in plants, such as the post-transcriptional regulation of mRNA (Taquet et al., 2010).

Additional Table 15: Gene expression analysis of selected TDFs by qRT-PCR in 'Président Roulin' (resistant) and 'Gala' (susceptible). qRT-PCR data confirming the pattern of expression observed in the cDNA-AFLP (>2-fold sur-expression or repression) are highlighted in bold. Then, the statistical differences of level or sur-expression or repression across the various experiments was judged at P<0.5 (*), P<0.01 (**) and P<0.001 (***). NS=Non-significant.

		Resistant cultivar 'Président Roulin'				Susceptible cultivar 'Gala'			
		cDNA-AFLP samples	Biological rep. 1	Biological rep. 2 (time-course exp.)	P value	cDNA-AFLP samples	Biological rep. 1	Biological rep. 2 (time-course exp.)	P value
43DU149'	[a] **IIb +**	**+7.9±2.6*[b]**	**+2.8±1.1**	**+4.8±0.1***	NS	**+1.9±0.0**	**+1.4±0.3**	**-1.3±0.2**	NS
56AU33'	**IIb +**	**+2.6±0.1***	**+2.9±0.5***	**+2.0±0.0***	NS	**+1.5±0.1**	**1.3±0.5**	**+1.4±0.0***	NS
42BUHcrVf	IIb +	+1.04±0.1	-1.2±0.1	-		**-1.9±0.2***	**-1.2±0.2**	-	NS
43CU118	IIb -	+1.6±0.1	-	-		**+1.5±0.2**	**-**	**-**	
44AU9	**IIb +**	**+ 5.1±1.7***	**+2.2±0.4***	**+4.4±0.0***	NS	**+1.4±0.4**	**+1.7±0.0***	**+1.2±0.1**	NS
44GU169	IIb +	+10.3±0.1*	+1.4±0.5	-		+2.2±0.1*	+1.2±0.0	-	
54CU21	IIb +	+3.5±0.0*	+1.1±0.1	-		**-1.2±0.5**	**-1.5±0.1**	-	NS
2EU181	**IIb +**	**+2.2±0.0***	**+2.1±0.3***	**+2.2±0.0***	NS	**+1.4±0.2**	**-1.3±0.0**	**±1.3±0.1***	NS
39AU13	IIb +	+1.4±0.0	-1.0±0.1	-		**+1.1±0.2**	**-1.6±0.0***	-	NS
46EU122	IIb -	-2.4±0.1*	-1.5±0.1	-		+1.3±0.0	+2.4±0.0*	-	
51DU17 (+)	IIb +	+2.0±0.3*	+1.0±0.2	-		**+1.1±0.3**	**+1.1±0.0**	-	*
53DU34 (-)	IIb -	-4.8±0.0*	-1.9±0.0*	-		+1.5±0.2	-2.6±0.1*	-	
56AU5' (+)	IIb +	+6.5±1.3*	-1.0±0.0	-		+3.9±1.1	-1.4±0.2	-	
43DU149 (+)	**IIb +**	**+3.4±0.2***	**+4.0±0.2***	**+3.7±0.0***	NS	**+1.2±0.1**	**+1.9±0.2***	**+1.1±0.1**	NS
51HU129' (+)	**IIb +**	**+8.1±0.0***	**+6.9±0.0***	**+7.0±0.0***	**	**+2.0±0.1***	**+1.5±0.0***	**+1.5±0.0***	*
Consensus 44EU122/ 44EU118 (-)	**IIa -**	**- 12.7±4.5***	**-68.8±0.0***	**-10.5±0.1***	***	**- 4.1±1.2***	**-3.2±0.3**	**-3.3±0.2***	NS
37DU41 (+)	**IIb +**	**+ 2.4±0.1***	**+2.9±0.0***	**+2.3±0.1***	NS	**+1.2±0.1**	**+1.4±0.1**	**-1.3±0.1**	NS
1AU61' (+)	IIb +	+1.5±0.3	-	-		+1.6±0.1	-	-	
56AU29 (+)	**IIb +**	**+2.3±0.1***	**+2.7±0.2***	**+2.7±0.0***	NS	**+1.4±0.1**	**+1.2±0.1***	**+1.4±0.0***	NS
44GU182 (-)	**IIb -**	**- 28.4±4.9***	**-22.0±0.0***	**-22.0±3.4***	NS	**+ 1.4±0.1**	**-1.9±0.1***	**-1.4±0.4**	NS
53HU89 (+)	**IIb +**	**+10.3±0.2***	**+3.3±0.1***	**+7.2±0.1***	***	**+1.7±0.1**	**+1.8±0.1***	**+1.6±0.0**	NS
44GU173 (+)	**IIb +**	**+3.3±0.8***	**+3.6±0.9***	**+3.6±0.1***	NS	**+1.1±0.1**	**+1.2±0.2***	**+1.2±0.1**	NS
55FU102 (+)	**IIa +**	**+3.0±0.1***	**+3.8±0.2***	-	NS	**+1.4±0.5**	**+1.3±0.0**	-	NS
55HU125' (+)	IIb +	+4.8±0.0*	+1.7±0.0	-	**	**- 1.5±0.1**	**+1.5±0.2**	-	NS

[a] Expression pattern according to the cDNA-AFLP. Group IIa represents pathogen-responsive TDFs expressed in common by both genotypes and group IIb pathogen-responsive and genotype specific TDFs. "+"= induced and "–" = repressed TDF

[b] Means and SD of fold induction (+) or repression (-) calculated by the ΔΔCt method applied using qRT-PCR and statistical significance of individual ΔCt values at $P < 0.01$ (*)

The CDGs expression over a time course experiment over infection

In Chapter 3, a time course experiment of the expression of the various CDGs was presented. Gene expression was analyzed at six time points after inoculation: at 6 hours, 24, 48, 72, 96 and 120 hours. According to the gene annotation and the literature, genes might be involved in the recognition of the pathogen (43DU149', 56AU33', 44AU9), the signal transduction and gene transcription (2EU181, 53HU89) as well as in the expression of the final defense products, more downstream of the reaction process (56AU29, 44GU173, 43DU149, 46EU122/44EU118, 51HU129', 37DU41, 44GU182).

Logically, such temporal sequence in the cascade events of the plant response to pathogen should be illustrated by the timing of expression of our CDGs. Apart from one gene (44GU182), this temporal sequence seems to be respected across the various CDGs. Since large time intervals have been chosen, it is unfortunately impossible to discriminate which gene has been activated first between two consecutive intervals. Early after the inoculation, at 24 (44AU9) and 48 hours (43DU149' and 44AU9) after the inoculation of the pathogen, were activated the genes that might act in the recognition of the pathogen. Concomitant to this activation, were expressed the genes acting in the signal transduction and gene transcription at 24 (53HU89) and 48 hours (2EU181) after inoculation. Finally, with the exception of 44GU182, defense response genes were expressed in the time laps ranging from 24 to the late 120 hours after inoculation.

Regulation of expression of the defense-related gene 44GU182, encoding for a lysosomal pro-X carboxypeptidase, was found to precede the induction of genes involved upstream in the plant defense response cascade (24 hours for the earliest activation of genes involved in pathogen recognition). This could suggest that 44GU182 might be involved in other biological process than the onset of resistance to 'Président Roulin', or do not rely on the specific recognition of the pathogen by NBS-LRR resistance proteins to be activated. Another hypothesis would be that 44GU182 could have been activated by other CDGs that are expressed earlier in the plant-pathogen interaction, but not revealed in this study since the time of 48 hours after infection was at the basis of our cDNA-AFLP. A modest functional validation of 44GU182 has been made by finding correlation between the regulation of this particular gene, with the level of resistance to *V. inaequalis* in a F1 segregating population derived from 'Président Roulin' (confer Chapter 3). Further investigations should be made to further validate the action of the CDGs in the resistance of 'Président Roulin'.

About the presence of the cDNA encoding for the apple stem pitting virus (ASPV) in the samples

In the samples used for the cDNA-AFLP, 15 sequences were similar to genes belonging to the apple stem pitting virus (ASPV). ASPV is a widespread pathogen of pome fruit usually transmitted by the rootstock and frequently latent in the host (Mathioudakis et al., 2010). ASPV sequences have been identified in inoculated and control water plants of 'Président Roulin', and no symptoms characteristics of the ASPV were observed, indicating a potential latent status of the virus. However, even if the virus was latent and non-symptomatic, we cannot discard the hypothesis that such virus could have induced gene regulation in plants, as suggested by Aldaghi

et al. (2012) in non symptomatic plants infected by a virus. Expression of the CDGs for the partial resistance of 'Président Roulin' was verified by qRT-PCR in two biological replications of the experiment. Nevertheless, I did not check for the presence of genes derived from the ASPV in these qRT-PCR samples. As a consequence, I cannot discard the hypothesis that the CDGs might be responsive to ASPV infection in apple. This investigation would be really important in future research.

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Chapter 5.

General conclusion and perspectives

Outline

While application of pesticides is harmful to the environment and human health, development and utilization of apple cultivars resistant to scab, one of the main diseases of apple, is an effective, economical, and environmental-friendly approach to disease control. However, durability of resistance is difficult to achieve in commercial apple orchards. In this conclusion, some new insights in the apple-*V. inaequalis* interactions of two apple cultivars with multiple-gene resistances are summarized. The quantitative resistance in the cultivar 'Président Roulin', governed by genes with partial effects, and the less durable resistance in the cultivar 'Geneva' harboring a complex of R genes showing complete but also some partial effects. Resistance in 'Geneva' apple also appears to involve recessive genes, which showed the widest spectrum in the interaction of a limited number of isolates tested. Since achieving durability of resistance is a longstanding challenge of the apple breeder community, we will discuss how this new knowledge could be applied in the creation of new cultivars showing durable apple scab resistance in the orchard.

The literature review of this thesis presented how apple is equipped with complex defense mechanisms to detect and counter attacks by *V. inaequalis*, the fungal pathogen causing scab, one of the most damaging diseases in commercial apple orchards. These include preformed structural and biochemical barriers to prevent pathogen establishment as well as induced resistance responses triggered upon the perception of the invading pathogen (Gessler et al., 2006). The review also described our current knowledge of the genetic basis of the apple-*V. inaequalis* interaction, which is the result of a long-term co-evolution between the host and the pathogen (Gladieux et al., 2008, 2010; Bowen et al., 2011). In natural ecosystems, plant disease outbreaks are usually sporadic and spatially confined, with high genetic diversity among plant and microbial populations (Burdon, 1987; Tack et al., 2012). In commercial apple orchards, however, the extensive use of a limited number of R-genes in cultivars, mainly the *Rvi6* (*Vf*) gene, has been shown to favor selection of virulent pathogen isolates that cause significant yield losses (Parisi et al., 1993). While the application of pesticides is harmful to the environment and human health, development and utilization of resistant cultivars, among other biological control methods and good management practices in the orchard (Andrews et al., 1983; Miedtke and Kennel, 1990; Burr et al., 1996; Cronin et al., 1996; Carisse and Rolland, 2004; Jamar 2011), is the most effective, economical, and environmental-friendly approach to disease control (Parisi et al., 1995). In order to avoid intensive use of pesticides for a sustainable commercial apple production, there is therefore an ongoing need for new apple cultivars with durable apple scab resistance.

Because of their intrinsic interest and agricultural importance, apple scab resistance genes have been investigated from several perspectives and the apple *V. inaequalis* interaction has become the most studied plant-pathogen interaction involving a woody species (Cova et al., 2010.). During these decades of research on the interactions of apple with *V. inaequalis*, the long-standing questions in the apple breeder community were: how do apple cultivars defend themselves against *V. inaequalis*? What is the molecular basis of resistance specificity between the host and the pathogen? What is the genomic organization of apple scab resistance in the host and the effector genes in the pathogen? What drives the pathogen to shift virulence? Today, the first four questions are being answered in increasing detail for the complete apple scab resistance based on single R-genes, which has been shown to be non-durable in apple orchards. In contrast, quantitative scab resistance seems to be more durable (Parlevliet, 2002), but its molecular determinants remain largely unknown.

In that context, the objective of this thesis was to extend our understanding of the plant-pathogens interactions between apple and *V. inaequalis* for two particular apple cultivars carrying contrasting forms of resistance: the quantitative resistance in the cultivar ‘Président Roulin’ governed by multiple genes of partial effect, and the resistance in ‘Geneva’ governed by a complex of R-genes with complete but also some partial effects. This objective was apprehended using a candidate gene approach based on transcriptomics and genomics data, the results of which will be summarized below. Finally, some future prospects of this research will be presented along with a discussion of how these new insights in the understanding of the plant pathogen-interaction could facilitate the creation of new cultivars with durable scab resistances.

Characterization of the genetics of scab resistance to *V. inaequalis* by performing phytopathological tests

Basal plant defense against pathogens is constituted of preformed anti-microbial compounds, as well as non-specific perception of relatively conserved pathogen-associated molecular patterns (PAMPs) governing the PAMP-triggered immunity (PTI). In order to defend against isolates being able to bypass this primary resistance, plants have evolved a more complex immunity based on the specific recognition of pathogen effectors through cell surface receptors (effector triggered immunity, ETI). Such resistance involves a gene-for-gene interaction between the *Avr* gene in the pathogen, and the R gene in the host (Jones and Dangl, 2006). Based on this hypothesis of a gene-for-gene interaction between the host and the pathogen, in Chapter 3 and 4, I used various *V. inaequalis* races to differentiate scab R loci in segregating populations based on crosses of 'Président Roulin' or 'Geneva' with susceptible apple cultivars. Each differential interaction observed between the various isolates and the hosts in F1 and F2 progenies was informative of the number of R loci present in the populations. For example, incompatible reactions for some particular isolate(s) along with compatible interactions with other isolates was indicative of the presence of an R locus effective against that particular set of isolate(s), and overcome by all the other one. If incompatible interactions were observed with any of these other isolates, this suggested the presence of at least another R locus.

The analysis of the whole set of differential interactions observed permitted the elaboration of hypothetic gene-for-gene interaction models between the isolates and the R genes present in the population under examination. In both cultivars 'Président Roulin' and 'Geneva', gene-for-gene interaction models comprising a minimum of five loci governing the resistance were hypothesized. Gene-for-gene relationships involving broad-spectrum genes could form the partial resistance of 'Président Roulin', with possibly 'defeated R-genes' as suggested in the *V. inaequalis*-apple interaction (Durel et al., 2000; Bus et al., 2011) as well as in other plant-pathogen interactions (Nass et al., 1981; Brodny et al., 1986; Li et al., 1999). Such genes would be partially overcome by the pathogen and trigger weak defense response, leading to partially resistant phenotypes. Obviously, such models did not take into account the presence of non-race specific basal defense resistance genes, or the presence of potential additional race specific genes that would have been revealed by the inoculation of other *V. inaequalis* isolates. Using such models I could differentiate between the resistance loci present in each individual of the populations. The pathological tests were also at the basis of the fine mapping of the five resistance loci in 'Geneva'. This fine mapping wouldn't have been possible by inoculating the seedlings with a mix of *V. inaequalis* isolates.

Moreover, pathological test with individual monoconidial isolates allowed the evaluation of the spectrum of the individual genes composing the resistances of the two cultivars under study. While the resistance loci in 'Président Roulin' appeared to be more broad-spectrum (meaning effective against most, if not all of the pathogen isolates tested) based on the limited number of isolates tested, most of the resistance loci in 'Geneva' were narrow-spectrum. Finally, using phytopathological tests on the apple populations, I could determine the resistant to susceptible (R:S) segregation ratio for each gene. In the various F1 and F2 populations derived from 'Président Roulin' and 'Geneva', some dominant (R:S= 1:1 ratio), but also ratios suggesting more complex genetic control were observed (R:S=1:3). In such progenies derived from 'Geneva', resistance was found to be able to 'skip' one generation (resistant seedlings were found in progenies resulting from the cross of two susceptible parents). Finally, resistance was found to

segregate with one allelotype of co-dominant molecular marker data. This could suggest a recessive genetic control of the resistance against these particular *V. inaequalis* isolates, with resistance conferred in homozygous plants only. Such control could notably be explained by the presence of complementary R genes, acting pairwise in effecting resistance as suggested in the literature. Such models are supported by increasing evidence, both at the genetic (molecular markers) and structural levels (evidence of dimers formations between R proteins domains) (Deslandes et al., 2002 and 2003; Narusaka et al., 2009, Sohn et al., 2014; Cesari et al., 2014; Griebel et al., 2014; Maekawa et al., 2011; William et al., 2014).

New insights into the genetic control of scab resistance in 'Président Roulin' and 'Geneva'

In order to identify candidate genes underlying the resistance in 'Président Roulin' and 'Geneva', I used both genetics and transcriptomics approaches. In 'Geneva' (Chapter 4), genotyping-by-sequencing, microsatellite and SNPs markers were employed to fine-map five resistance loci (with dominant and more complex recessive control) in a region of 5 cM located at the distal end of chromosome 4 of 'Geneva'. Resistance phenotypes obtained with the various *V. inaequalis* isolates and interactions between isolates were used to differentiate the resistance loci. This was followed by a candidate gene analysis based on the hypothesis that known-function genes (the candidate genes) could correspond to loci controlling the traits of interest (Pflieger et al., 2001). The 5 cM region in chromosome 4 corresponded to a 2 Mbp region containing nine candidate resistance genes encoding for nucleotide binding site-leucine rich repeat (NBS-LRR) family proteins on the physical map of 'Golden Delicious' (the reference apple genome assembly). Because of the relative small distance of the molecular markers flanking the resistance loci, cloning of the R-genes could be feasible and plant transformation assays would allow confirmation of their role in the resistance. There is some additional interest for the recessive resistance loci, since the nature of the genes governing such kind of resistance remains largely unknown in apple.

In addition to gene sequence variations, modulation of gene expression also shapes the resistance status of plants. In fact, it has been shown that plants respond to pathogen attack through the transcription of a range of genes involved in the recognition of the pathogen effectors, the signal transduction and plant defense (Durrant et al., 2000; Gabriëls et al., 2006; Aligan et al., 2006; Wang et al., 2010; Shi et al., 2011; Li et al., 2012). Based on this observation, a whole genome transcriptomics studies, using the cDNA-AFLP technology, was developed in Chapter 2 to identify some key molecular events, and potential candidate resistance genes leading to the partial apple scab resistance in 'Président Roulin'. The transcriptome analysis revealed specific modulation of genes (156 up- or down-regulated genes, out of 20,500 TDFs) in scab-infected 'Président Roulin' compared with the susceptible 'Gala'. Sequence annotation revealed similarities to several genes encoding for proteins belonging to the NBS-LRR and LRR-RLK classes of plant R genes and to other defense-related proteins. Some specific GO categories appeared to be over-represented in the 'Président Roulin' library compared with other public cDNA apple libraries. GO categories involved in response to stress and photosynthesis were over-represented in 'Président Roulin' compared with an uninfected cDNA apple leaf library. Some common physiological events, and notably differences in the regulation of defense response, the regulation of hydrolase activity and response to DNA damage were revealed when comparing our

partially resistant expression signature with the signature from inoculated leaves of *Rvi6* (*HcrVf2*)-transformed 'Gala' lines (complete disease resistance).

Based on this specific regulation, and on their known role in plant defense according to the literature, thirteen CDGs were selected (Chapter 3). These genes could be involved in the recognition of the pathogen (e.g. NBS-LRR family protein, LRR receptor kinase-like protein), the signal transduction (MAP kinase and zinc finger homedomain protein) or the plant defense system (e.g. chitinase, cysteine protease/protease inhibitor, tocopherol cyclase, peroxidase). In a time-course expression experiment during *V. inaequalis* infection, these genes appeared to be specifically expressed in 'Président Roulin' and not in the susceptible 'Gala', or if they were expressed in 'Gala', a delay in the expression was observed. Co-localization with apple QTLs, major R-genes and RGAs was found, too. Finally, a modest contribution to the functional validation of the candidate defense genes has been made by investigating the extent of the correlation between their expression regulation and the phenotypic variation in the progeny. Induction of nine out of ten CDGs accounted for up to 46% of the phenotypic variance, suggesting their potential involvement in 'Président Roulin' scab defense system.

Making the most of the 'omics' for candidate resistance gene discovery

The approaches used in this thesis contributed to an increase in our understanding of the resistance of 'Geneva' and 'Président Roulin' to *V. inaequalis*, but some questions remained unsolved.

Using the cDNA-AFLP, only a small part of the transcriptome signature of 'Président Roulin' could be visualized. Time- and cost-wise, I could in fact reasonably test a fair number of restriction enzymes and primer pair combinations contributing to the production and visualization of a set of TDFs during the cDNA-AFLP experiments. Then, in order to infer the position of the genomic regions governing the regulation of the genes of interest, cDNA sequences were mapped *in silico* on the 'Golden Delicious' apple reference genome. Such method was not informative of the 'Président Roulin' genetic sequences since I did not have a genetic map at my disposal. As a result, hypothesis of a cis-regulation has been made, without taking into account any possible trans-regulation through distal gene products. In addition to its importance to the understanding of the plant defense response, knowledge of such trans-regulation would constitute a prerequisite for the development of genetic molecular markers based on the cDNA sequence. Finally, in both genomics and transcriptomics methods used to investigate the resistance in 'Président Roulin' and 'Geneva', selection of candidate defense genes was made on an *a priori* knowledge of their role in the establishment of resistance in other plant-pathogen interactions. However, genes of unknown functions could also have an important role in the apple defense system against scab.

Today, the availability of high throughput sequencing technologies at low costs enables the assaying of gene expression and genetic variations simultaneously on a genome-wide basis and in a large number of individuals (Jansen and Nap, 2001; Feltus, 2014), such as segregating populations. These analyses, referred to as expression quantitative trait loci (eQTLs), lead to the identification of genomic loci that correlate with variation of mRNA expression levels. eQTLs that map to the approximate location of their gene-of-origin are referred to as cis eQTLs. In contrast, those that map far from the location of their gene of origin, often on different chromosomes, are referred to as distant trans eQTLs. Alternatively to individual mRNAs regulation, computational biology methods permit the investigation of gene co-expression

networks (Zhang and Horvath, 2005). In such networks, clusters of genes showing similar expression pattern across several samples or experimental conditions are constructed. These novel approaches would be powerful in discovering candidate genes underlying the expressed phenotypes, without any *a priori* knowledge of their function in plant defense. It would also allow the identification of the action of groups of genes instead of individual genes. In fact, phenotypic variations in plants are often governed by complex gene interactions and regulatory pathways. In barley, an eQTL approach led to the identification of strong candidate genes underlying phenotypic QTLs for resistance to leaf rust in barley and for the general pathogen response pathway (Chen et al., 2010). Gene co-expression networks have also been successfully used in other plant-pathogen interactions (Kugler et al., 2013).

Finally, in addition to the nucleotide sequence polymorphisms (investigated in Chapter 4) and variations in gene expression (investigated in Chapter 2 and 3), recent studies suggest that a third source of phenotypic variability would be governed by variations in the allelic expression of genes (Yan et al., 2002; Guo et al., 2004). Mendelian inheritance assumes that genes from maternal and paternal chromosomes contribute equally to the expression of phenotypes in individuals. However, recent studies in human and plants reported significant differences in gene expression between the two alleles (Yan et al., 2002; Lo et al., 2003; Guo et al., 2004). Differences ranged from unequal expression of the two alleles (biallelic) to expression of a single allele (monoallelic) and were associated to some important phenotypic diversity such as the susceptibility to colon cancer in human (Yan et al., 2002) and the plant response to stress (Guo et al., 2004). In future research, it would be interesting to take into account such variations when investigating the influence of expression variations of genes in plant response to pathogens.

Future prospects for durable apple scab resistance breeding

One of the longstanding objectives of the apple breeder community is to achieve durable scab resistances in orchards. This durability is not easy to establish considering the high evolution potential of the pathogen in commercial apple orchards maintained for 10-15 years. Pathogens rapidly evolve virulence against the R gene by mutating or deleting the pathogen gene encoding the elicitor molecule that was recognized by the R gene. However, this risk of resistance breakdown is often believed to be minimal for orchards planted with cultivars cumulating various genes of partial and/or major effects (Parlevliet, 2002).

In the present thesis, two contrasting types of resistance have been studied. A quantitative resistance governed by partial resistance genes in ‘Président Roulin’, and a resistance governed by a complex of major effect, but also some minor effect R genes in ‘Geneva’. Although the resistance in ‘Président Roulin’ has been shown to be durable for more than 25 years of evaluation in various unsprayed orchards in Belgium, resistance in ‘Geneva’ has been demonstrated to be less durable (Bus et al., 2011). Some of the results obtained here could be interpreted in prospect of breeding to achieve more durable apple scab resistance.

Firstly, durability of resistance is a concept that has been extensively discussed in the literature, but which is still difficult to measure and predict. Hence, durability has been defined as a resistance that remains effective over a prolonged period of widespread use under conditions conducive to the disease (Johnson, 1984). So in essence, a resistance cannot be truly durable, but could be qualified as durable, until the demonstration of isolates of the pathogen able to overcome it. In fact, although partial resistance has been considered to be more durable than complete

resistance (Parlevliet, 2002), in the apple-*V. inaequalis* interaction, evidence also show directional selection of isolates (e.g. with an increased rate of infection, a shortened latent period or an increased size of lesions) over generation in pathogen populations infecting cultivars with partial quantitative resistance (Lê Van et al., 2013; Caffier et al., 2014). As with major effect R genes, breakdown of partial resistance might also be encountered. In the context of this thesis, the scab resistance of 'Président Roulin' has been shown to be durable for more than 25 years of evaluation in various orchards, in the absence of any fungicide treatment. Nevertheless, since the use of 'Président Roulin' has been limited to a few small orchards, all located in Belgium, it would of interest to further investigate its resistance durability when planted over larger surfaces. Furthermore, it should be also important to determine the extent of selection pressure exerted by the partial resistance of 'Président Roulin' on the pathogen population (e.g. by monitoring *V. inaequalis* isolate proportions on diseased apple leaves of an F1 progeny.

Secondly, phenotypic distinction between the two categories of resistance, complete and partial resistance, was not always straightforward and it seems to exist a 'great deal of gray area' between the two categories, as suggested by Poland et al. (2009). In the apple-*V. inaequalis* interaction, a continuum of resistance reactions is observed among resistant apple accessions: from the rather strong HR, SN, SC and Chl resistance reactions to the class 3a, 3b and 4 of Chevalier et al. (1991), resistance reactions reduce and the sporulation levels increase at various intensities. Moreover, while 'Président Roulin' demonstrated moderate resistance reactions under natural infection in the orchards as well as by spray inoculating monoconidial isolates of *V. inaequalis* (results not shown in this thesis), droplet inoculation of one particular isolate (isolate 1639) revealed a strong hypersensitive response on 'Président Roulin' with no apparent macroscopic sporulation. However, only a few individuals harbored such strong resistance symptoms with no sporulation in the resulting progeny. Such enhancement of resistance with the droplet inoculation technique has also been observed by Caffier et al. (2015), possibly due to higher concentration of effectors in a limited area of the leaf. However, I cannot discard the hypothesis that 'Président Roulin' could harbor major effect R genes along with partial resistance factors.

Thirdly, it has often been suggested that the basis of resistance durability could notably come from its polygenic nature. A pathogen would require the combination of a larger number of mutations in its genome to overcome polygenic resistance than to overcome monogenic resistance, possibly with a cost to its fitness (Leach et al., 2001; Lindhout, 2002; Poland et al., 2009; Le Vân et al., 2013). Hence, the use of polygenic resistant cultivars carrying partial or complete R genes would permit to avoid, or at least postpone any resistance breakdown. However, this does not seem to hold true for the resistance in 'Geneva'. Despite its complex of five resistance loci, a complete breakdown of the resistance has been found for one particular *V. inaequalis* isolate (EU-NL-24). The reasons of this breakdown are not clear. One hypothesis is that such tightly linked genes could belong to the same allelic series and might share similar mechanisms of action that would be easier to overcome by one particular *V. inaequalis* isolate. In 'Président Roulin' resistance loci seemed to segregate more independently and I failed to demonstrate any complete resistance breakdown using the set of isolates tested. These European isolates were shown to be virulent against various major apple scab R genes (Caffier et al., 2015).

Fourthly, in some host-pathogen interactions, partial resistance has been shown to be governed by proteins differing from the classic NBS-LRR proteins encoded by major resistance genes (Fukuoka et al. 2007; Krattinger et al., 2009). Such resistance mechanisms would be more

general, not based on gene-for-gene interactions between the host and the pathogen, hence would be at the origin of a more durable form of resistance. During the phytopathological tests, differential interactions between the hosts and the isolates were observed in a population derived from 'Président Roulin'. This suggests that race-specific resistance genes could govern the partial resistance. Moreover, the transcriptomics analysis in 'Président Roulin' revealed the regulation of some classic NBS-LRR resistance proteins with significant correlations with the level of resistance in the population. As explained earlier in the text such results could support the hypothesis that 'defeated' R-genes could have a residual effect and act as a QTL against virulent strains of the pathogen as demonstrated in various plant-pathogen interactions including the apple-*V. inaequalis* interaction (Nass et al. 1981; Brodny et al. 1986; Li et al. 1999; Durel et al. 2000; Bus et al. 2011). As far as I know, however, evidence of a direct interaction at the protein levels between the product of a QTL for resistance and the matching *Avr* factor is still lacking. I also do not discard the hypothesis that more basal non race specific resistance and other genes of unknown function could also act in the resistance of 'Président Roulin'.

Finally, beside the distinct phenotypes observed between the two cultivars and the segregation of the resistance genes in cluster or not, one striking difference between 'Geneva' and 'Président Roulin' was the spectrum of action of the resistance loci against the various *V. inaequalis* isolates. In 'Président Roulin', apart from one resistance locus, incompatibility has been shown against most if not all the isolates tested. In 'Geneva', the three dominant loci were shown to be effective against only a few of the isolates tested (narrow-spectrum). The other two loci were more broad-spectrum, but showed a complex recessive genetic control. Hence, the spectrum of action seems to be an important factor in the durability of the resistance of apple against scab. Narrow-spectrum genes are believed to be less durable than broad-spectrum genes, because of the higher pressure of selection that they would exert on the pathogen (Parlevliet, 2002. Kou and Wang, 2010). This selection of virulent isolates of the pathogen would accelerate the breakdown of the resistance. On the other hand, broad-spectrum resistance factors would be more general, and hence more difficult for a pathogen population to evade (Le Vân et al., 2013). This could be the reason why 'Geneva' resistance was less durable in the field. This hypothesis seems to be supported by Le Vân et al. (2013), showing an absence of differential selection pressure on pathogen populations for cultivars carrying broad-spectrum QTLs. With narrow-spectrum QTLs, a decreased frequency of some isolates in the mixed inoculum relative to the susceptible host genotypes were observed.

As a conclusion, results of this thesis suggest the following approaches that could be used in apple breeding programs aiming to extend the durability of apple scab resistance. The effect (major or partial) and the spectrum of action (broad-or narrow-spectrum) of the resistance factors at the basis of the breeding program should be assessed. This could be made by the inoculation of monoconidial isolates of *V. inaequalis* in the various progenies. By using a mixture of races in the inoculum, the presence of individual resistance genes can be masked by compatible gene-for-gene interactions between the host and other races of the pathogen. Moreover, since major effect genes are effective against some races of the pathogen while ineffective against other races, such genes may easily give the impression that quantitative resistance is present when the population pathogen consists of a mixture of inoculum. A preference should be given to broad-spectrum resistance genes, since they were shown to exert less differential selection over the pathogen population than narrow-spectrum genes, and could hence increase their durability (Kou and Wang, 2010; Le Vân et al., 2013). Regarding the effect of the resistance factors, the use of partial resistance and the pyramiding several major effect R genes seem to be two possible strategies

aimed at the minimization of resistance erosion. However, considering the fact that the resistance in 'Geneva' has been overcome while carrying a complex of major effect resistance genes, we might hypothesize two additional strategies to extend the durability of resistances. The first strategy could be to combine in one cultivar R genes governing different, but complementary modes of actions (Le Vân et al., 2013). The idea is that the fitness penalty would be too high for the pathogen to be able to overcome all resistance genes. In breeding programs, such diversity of resistance mechanisms could probably be inferred from the pathological tests performed on the various progenies: diverse resistance mechanisms are more likely to be transmitted in progenies by crossing two parents showing contrasting differential interactions with a set of isolates tested. Parents with contrasting components of resistance (e.g. infection frequency, latent period, lesion size, spore production, infectious period, Parlevliet, 1979) are also more likely to deploy different mechanisms of resistance to the pathogen. The second strategy would be to associate several partial resistance quantitative trait loci with major effect R genes. In such cultivar, resistance was shown to be more durable, probably due to the remaining genetic background surrounding the major gene suppressing the emergence of mutant virulent pathogen isolates (Palloix et al., 2009).

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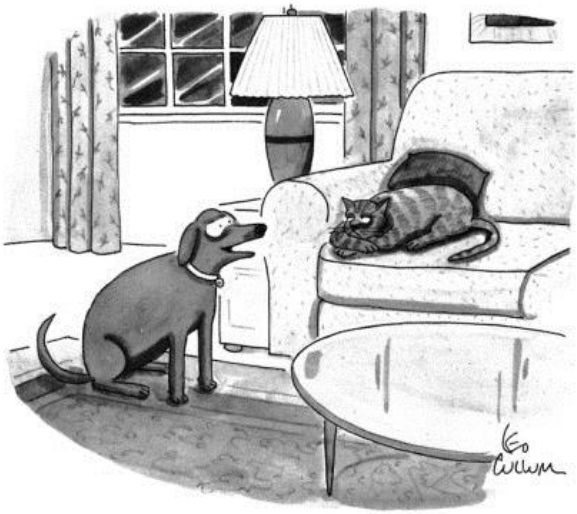

"It's genetic. My father was a dog, and I'm a dog."