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ORIGINAL ARTICLE

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 h after inoculation. Most were significantly up- or downregulated 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

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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 (Parlevliet 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 et al. 2003, 2004; Liebhard et al. 2003; 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 gene 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 h 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 h 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 Center 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 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 *V. 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 3-l plastic pots with a potting mix in the greenhouse at 20 °C under 16 h 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-l 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 1). 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 to 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 1). 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 2).

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 ($\log2^{\Delta\Delta Ct}$) was tested using a single factor ANOVA. The significance level was fixed at $P<0.05$, and the proportion of variation of $\log2^{\Delta\Delta Ct}$ 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 $\log2^{\Delta\Delta Ct}$ 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.

Table 1 List of the candidate defense genes (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	Apple–*V. inaequalis*
			Song et al. 1995	Rice–*Xanthomonas oryzae*
Signal transduction				
2EU181	JZ719320.1	Putative MAP kinase	Zhang and Klessig 2001	*Arabidopsis*–several pathogens
			Pedley and Martin 2004	Tomato–*Pseudomonas syringae*
			Eckey et al. 2004	Barley–*Blumeria graminis*
Transcription				
53HU89	JZ719483.1	Zinc finger homeodomain protein1	Korfhage et al. 1994	Parsley–various fungal and bacterial elicitors
			Park et al. 2007	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				
44EU122/ 44EU118	JZ719578.1 JZ719577.1	Cysteine protease	Krüger et al. 2002	Tomato–*Cladosporium fulvum*
			Avrova et al. 1999	Potato–*Phytophthora infestans*
			Hao et al. 2006	Tomato–*Pseudomonas syringae*
			D'Silva et al. 1998	Cowpea–*Uromyces vignae*
			Solomon et al. 1999	Soybean–*Pseudomonas syringae*
37DU41	JZ719360.1	Cysteine protease inhibitor	Gutierrez-Campos et al. 1999	Tobacco–potyvirus
			Solomon et al. 1999	Soybean–*Pseudomonas syringae* *Arabidopsis*–*Alternaria brassicicola*
44GU182	JZ719427.1	Lysosomal Pro-X carboxypeptidase	Liu et al. 2008	Rice–*Magnaporthe grisea*
				Arabidopsis–*Pseudomonas syringae*
			Moura et al. 2001	Tomato–insects
Defense response				
56AU29	JZ719503.1	Chitinase	Collinge et al. 1993	Numerous plant–fungi interactions
			Métraux and Boller 1986	Cucumber–numerous pathogens
			Jongedijk et al. 1995	Tomato–*Fusarium oxysporum*
			Vögeli-Lange et al. 1988	Tobacco–*TMV*
			Krishnaveni et al. 1999	Sorghum–*Fusarium moniliforme*
			Sahai and Manocha 1993	Numerous plant–fungi interactions
Cell wall organization				
44GU173	JZ719426.1	Pectin methylesterase inhibitor	Lionetti et al. 2007	*Arabidopsis*–*Botrytis cinerea*
			An et al. 2008	Pepper–*Xanthomonas campestris*

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 (Fig. 1). 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 1), 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).

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 2. '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 2 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 2): 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 ($P>0.05$).

From our pathological tests (Table 3), 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.

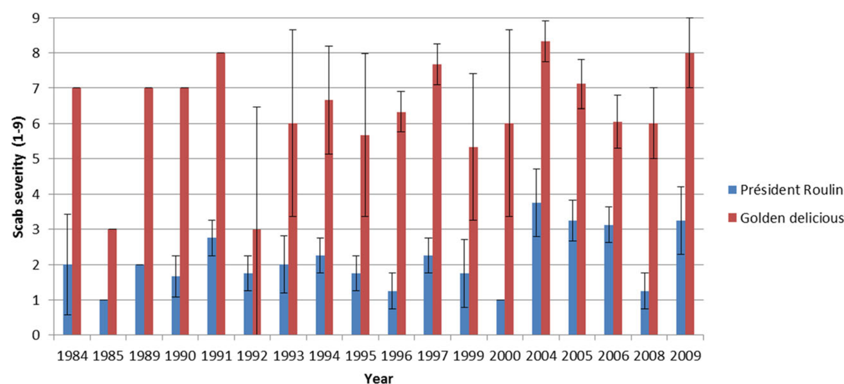


Fig. 1 Scab incidence recorded on 'Président Roulin' (partially resistant) and 'Golden Delicious' (susceptible) over some 25 years in different orchards in Gembloux, Belgium. Scab incidence was evaluated under natural infection and no fungicide treatments using the scale proposed by Lateur and Populer (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

Table 2 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	120	1:1	0.3	0.584
EU-NL24	Ch/N/2	4/5	22	98	120	1:3	2.8	0.092
EU-B04	Ch/N/3	4/5	34	86	120	1:3	0.7	0.399
1066	Ch/N/3	4/5	34	86	120	1:3	0.7	0.399
EU-D42	Ch/N/2	4/5	42	78	120	1:2	6.4	0.699
EU-NL05	HR/Ch/2	4/5	46	74	120	1:2	6.5	0.245
EU-B05	HR/Ch/2	4/5	63	57	120	1:1	0.3	0.584
EU-D49	Ch/N/3	4/5	63	57	120	1:1	0.3	0.584
Inoculum mix[c]	Ch/N/2	4/5	63	57	120	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

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 microsatellite 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 (Fig. 2).

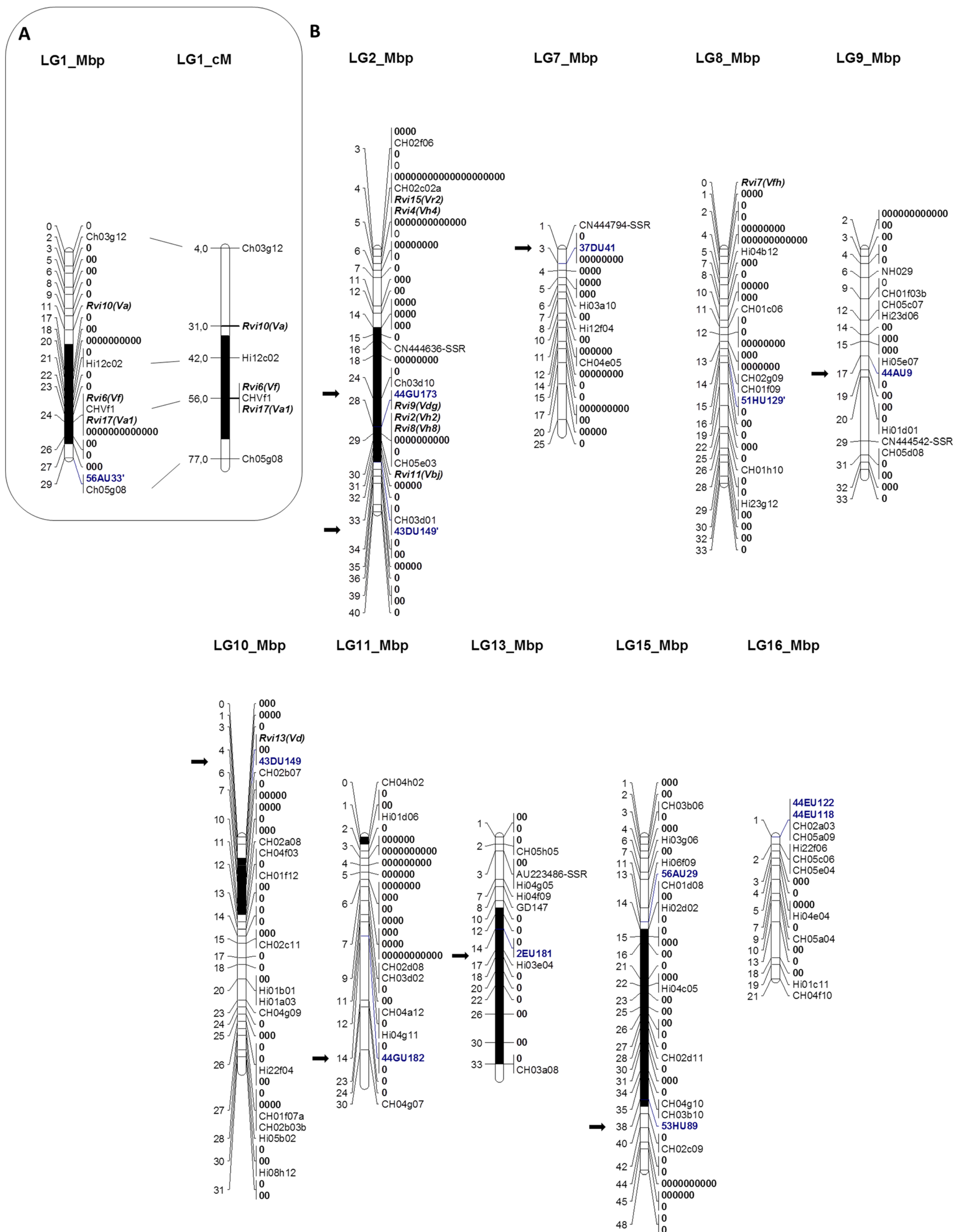
Investigation of the time-course expression during *V. inaequalis–Malus* interactions

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 overexpression). 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 Fig. 3, 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 4. 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 R^2 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


A
LG1_Mbp
LG1_cM
Ch03g12
Rvi10(Va)
Hi12c02
Rvi6(Vf)
CHVf1
Rvi17(Va1)
56AU33'
Ch05g08
4,0
31,0
42,0
56,0
77,0
B
LG2_Mbp
LG7_Mbp
LG8_Mbp
LG9_Mbp
CH02f06
CH02c02a
Rvi15(Vr2)
Rvi4(Vh4)
CN444636-SSR
Ch03d10
44GU173
Rvi9(Vdg)
Rvi2(Vh2)
Rvi8(Vh8)
CH05e03
Rvi11(Vbj)
CH03d01
43DU149'
CN444794-SSR
37DU41
Hi03a10
Hi12f04
CH04e05
Rvi7(Vfh)
Hi04b12
CH01c06
CH02g09
CH01f09
51HU129'
CH01h10
Hi23g12
NH029
CH01f03b
CH05c07
Hi23d06
Hi05e07
44AU9
Hi01d01
CN444542-SSR
CH05d08
LG10_Mbp
LG11_Mbp
LG13_Mbp
LG15_Mbp
LG16_Mbp
Rvi13(Vd)
43DU149
CH02b07
CH02a08
CH04f03
CH01f12
CH02c11
Hi01b01
Hi01a03
CH04g09
Hi22f04
CH01f07a
CH02b03b
Hi05b02
Hi08h12
CH04h02
Hi01d06
CH02d08
CH03d02
CH04a12
Hi04g11
44GU182
CH04g07
CH05h05
AU223486-SSR
Hi04g05
Hi04f09
GD147
2EU181
Hi03e04
CH03a08
CH03b06
Hi03g06
Hi06f09
56AU29
CH01d08
Hi02d02
Hi04c05
CH02d11
CH04g10
CH03b10
53HU89
CH02c09
44EU122
44EU118
CH02a03
CH05a09
Hi22f06
CH05c06
CH05e04
Hi04e04
CH05a04
Hi01c11
CH04f10

◀ **Fig. 2** 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

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).

Discussion

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

The resistance in 'Président Roulin' corresponded with partial resistance as defined by Parlevliet and van Ommeren (1975). Under natural infection in the orchard (Additional Figure 1) or when inoculated with various *V. inaequalis* isolates (Table 2), 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 3). 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 2). 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

Table 3 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

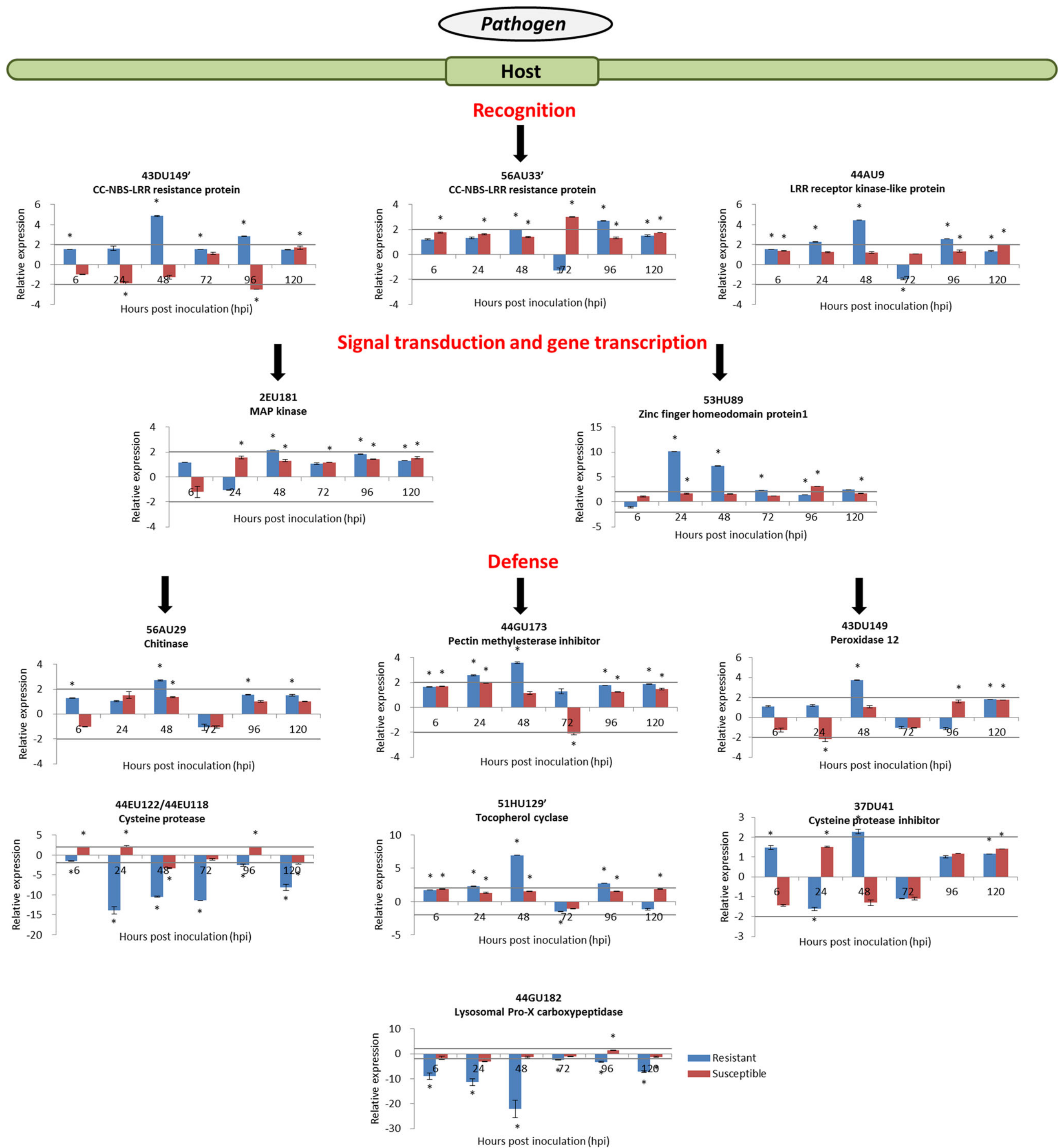


Fig. 3 Time-course expression analysis of CDGs analyzed by qRT-PCR during *V. inaequalis*–*Malus* interaction. The possible involvement in the recognition, the transduction of the signal, and the plant defense responses of specific genes was inferred from their involvement in other plant–pathogen interactions 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 ($\Delta\Delta$Ct) 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

'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 1971), we suggested that the partial resistance of 'Président Roulin' would be governed by at least five 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 3). 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, and 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 relationship model in 'Président Roulin' (Table 5) 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

Table 4 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) of the segregating population 'Gala' (susceptible) x 'Président Roulin'(partially resistant). 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. For each CDG, correlations associated with the highest probabilities and R^2 were indicated in italic

Isolates	1639		EU-NL24		EU-B04/ 1066		EU-D42		EU-NL05		EU-05/EU-D49/mix	
CDGs	*P*	*R*² %[a]	*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*
Percent of phenotypic variation of MSPOR explained by the CDG[b]	19.1 %		16.1 %		23.6 %		35.9 %		30.7 %		46.0 %	

[a] 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 $2^{\Delta\Delta Ct}$ method, taking the glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) as internal reference

[b] Total % of phenotypic variance accounted by the set of CDGs was calculated by performing a multiple regression of MSPOR (as the explained variable) on CDGs $\log 2^{\Delta\Delta Ct}$ (multiple explanatory variables)

Table 5 Model of gene-for-gene relationships between 'Président Roulin' and various *V. inaequalis* isolates (− indicates incompatible interaction, + compatible interaction). 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

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

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

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).

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 h 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 1). 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; Alignan 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 signaling 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; González 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) (Fig. 2). 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 eQTL 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 (Fig. 3). 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 transcriptomic 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 Fig. 3 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 terms 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 4). 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 are 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).

Conclusions 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, 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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