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Functional analysis and expression profiling of *HcrVf1* and *HcrVf2* for development of scab resistant cisgenic and intragenic apples

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Abstract Apple scab resistance genes, *HcrVf1* and *HcrVf2*, were isolated including their native promoter, coding and terminator sequences. Two fragment lengths (short and long) of the native gene promoters and the strong apple rubisco gene promoter (P_{MdRbc}) were used for both *HcrVf* genes to test their effect on expression and phenotype. The scab susceptible cultivar 'Gala' was used for plant transformations and after selection of transformants, they were micrografted onto apple seedling rootstocks for scab disease tests. Apple transformants were also tested for *HcrVf* expression by quantitative RT-PCR (qRT-PCR). For *HcrVf1* the long native promoter gave significantly higher expression that the short one; in case of *HcrVf2* the difference between the two was not significant. The apple rubisco gene promoter proved to give the highest expression of both *HcrVf1* and *HcrVf2*. The top four expanding leaves were used initially for inoculation with monoconidial isolate EU-B05 which belongs to race 1 of *V. inaequalis*. Later six other *V. inaequalis* isolates were used to study the resistance spectra of the individual *HcrVf* genes. The scab disease assays showed that *HcrVf1* did not give resistance against any of the isolates tested regardless of the expression level. The *HcrVf2* gene appeared to be the only functional gene for resistance against *Vf* avirulent isolates of *V. inaequalis*. *HcrVf2* did not provide any resistance to *Vf* virulent strains, even not in case of over-expression. In conclusion, transformants carrying the apple-derived *HcrVf2* gene in a cisgenic as well as in an intragenic configuration were able to reach scab resistance levels comparable to the *Vf* resistant control cultivar obtained by classical breeding, cv. 'Santana'.

Keywords *Venturia inaequalis* · *Malus* × *domestica* · Resistance · Cisgenesis · GMO

Introduction

Most of the present day apple (*Malus* × *domestica*) cultivars are susceptible to apple scab which is caused by the fungus *Venturia inaequalis*. Fruit growers spray on average 15 times in one season to control the disease (Patocchi et al. 2004).

Most of the conventionally bred resistant cultivars can give resistance against race 1 to race 5 (Szankowski et al. 2009) and against race 8 of *V. inaequalis*. The first report of breaking down of *Vf* resistance by a new race of *V. inaequalis* was made by Parisi et al. (1993) and they called this race 6. Later, again an isolate was identified which had overcome the resistance of *Vf* which was subsequently named as race 7 (Parisi et al. 2004).

Several apple scab resistance genes such as *HcrVf* (**H**omologues of ***C****ladosporium fulvum* **r**esistance genes of ***Vf*** region) (Patocchi et al. 1999), *Vr2* (Patocchi et al. 2004), *Vd3* (Soriano et al. 2009), *Vb* (Erdin et al. 2006), *Va* (Hemmat et al. 2003), *Vbj* (Gygax et al. 2004) have been identified and were mapped on different linkage groups of the apple genome. However, only the *Vf* locus has been positionally cloned (Vinatzer et al. 2001) and proved to consists of a gene cluster with four paralogs, namely

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HcrVf1, *HcrVf2*, *HcrVf3* and *HcrVf4* (Xu and Korban 2002). Initially, only the *HcrVf2* gene was tested by overexpression using the *CaMV35S* promoter and *nos* terminator, in scab susceptible apple plants (Belfanti et al. 2004) and the transgenic apple plants overexpressing *HcrVf2* proved to be resistant to apple scab.

Later, Malnoy et al. (2008) transformed *Vfa1* (which is the same as *HcrVf1*) and *Vfa2* (which is the same as *HcrVf2*) with their native promoters and terminators, to susceptible cultivars 'Galaxy' and 'McIntosh' and showed partial resistance of *HcrVf1* and *HcrVf2* to a mixture of isolates (Race 1–Race 5) of *V. inaequalis*. They used a 2 kb promoter for both *Vfa1* and *Vfa2* and they observed a reduction in sporulation by 50 and 38% in plants with good expression for *Vfa1* and *Vfa2* respectively. Szankowski et al. (2009) showed complete resistance by inserting only *HcrVf2* under the regulation of different lengths of native promoters and the *nos* terminator into susceptible cv. 'Gala'. They used also a mixture of *V. inaequalis* isolates for scab inoculation. This conflict in results prompted us to investigate this in more detail.

Ribulose-1, 5-bisphosphate carboxylase/oxygenase (rubisco) is an abundant protein in plants (Ellis 1979) and rubisco protein contents were correlated with total rubisco small subunit (RbcS) mRNA levels in rice (Suzuki et al. 2009). Using the promoter and terminator of rubisco small subunit gene (*rbcS1*) of chrysanthemum, expression levels up to sevenfold to eightfold those provided by the constitutively expressed *CaMV35S* promoter were obtained (Outchkourov et al. 2003). The combination of the apple rubisco promoter (P_{MdRbc}) and terminator (T_{MdRbc}) with the *gus* reporter gene resulted in expression levels similar to the one provided by the *CaMV35S* promoter in combination with the *nos* terminator (Schaart et al. 2011).

Transgenes, consisting of foreign genes or parts of foreign genes, are frequently used in GM plants and are a new gene pool for plant breeding. Nowadays, cisgenes defined as genes from the crop plant itself or from crossable species, with their native regulatory elements including introns in normal sense orientation (Schouten et al. 2006a) receive increasing interest. Cisgenic plants do not contain foreign genes by definition. Cisgenes are already present in the species or in cross-compatible relatives and therefore cisgenesis does not alter the gene pool used in breeding a crop (Schouten et al. 2006b). Intragenes are composed of different genetic elements originating from the crop species itself or from crossable plant species (Rommens et al. 2007). Genetic elements include promoters, coding sequence, terminators and DNA sequences that are similar to T-DNA borders from *Agrobacterium tumefaciens* (Jacobsen and Schouten 2008).

In this study we report on the role and resistance spectrum of the cisgenes *HcrVf1* and *HcrVf2* together with their native promoters in two lengths (short and long) and their native terminator in conferring resistance to apple scab. As a reference, the two *HcrVf* genes were combined with regulatory sequences of the apple rubisco gene and are therefore referred to as intragenes. The correlations between inserted gene copy number and gene expression and between gene expression and fungal sporulation were investigated. To our knowledge this is first detailed study of expression profiling of two individual *Vf* resistance genes using different isolates of *V. inaequalis* identifying their resistance level and spectrum.

Materials and methods

Gene amplification

Preparation of HcrVf constructs with native promoters

An apple BAC library was constructed from the genomic DNA of the breeding line 1980-015-025 which harbors several scab resistance genes such as *HcrVf*, *V25*, *Vd3* (Soriano et al. 2009). The BAC library construction was carried out as previously described by Rouppe van der Voort et al. (1999). Primers described by Xu and Korban (2002) (Table 1) were used to identify individual BAC clones from BAC pools positive for *HcrVf1* and *HcrVf2*. One BAC clone containing *HcrVf1* and one containing *HcrVf2* were sequenced at GATC Biotech AG (Konstanz, Germany). Sequenced contigs were aligned with the published *HcrVf1* (Gene bank accession number AY397723) and *HcrVf2* (Gene bank accession number AJ297740) sequences using the software SeqMan in DNASTAR® version 7.0. On the basis of the recommendations by Silfverberg-Dilworth et al. (2005) sequences up to 312 and 288 bp upstream of the transcription start site were used as short promoters (SP) for *HcrVf1* and *HcrVf2*, respectively. Similarly, sequences up to 480 and 437 bp downstream of the stop codon were used as terminators for *HcrVf1* and *HcrVf2*, respectively. Sequences of 1,990 and 2,000 bp upstream of the transcription start site were used as long promoters (LP) for *HcrVf1* and *HcrVf2*, respectively, and terminators were the same as in case of SP*HcrVf1* and SP*HcrVf2*. The details of gene sequences are shown in Fig. 1.

The fragments were amplified using Phusion® DNA polymerase (Finnzymes, Espoo, Finland) and BAC DNA as template. The primers used were extended with *Asc*I and *Mlu*I restriction site sequences for *HcrVf1* at 5′ and 3′ ends respectively, and *Asc*I and *Pac*I restriction site sequences for *HcrVf2* at 5′ and 3′ ends respectively. The primers are listed in Table 1. The PCR reaction included 5× Phusion® HF buffer, 5 mM dNTPs, 10 μM forward and reverse

Table 1 Primers used in the cloning of *HcrVf* fragments and in molecular analysis of *HcrVf* transformants

Primers	Sequence	Fragment length (bp)
HcrVf1-Forward	5′-TCTATCTCAGTAGTTTCTATAATTCC-3′	505
HcrVf1-Reverse	5′-GTAGTTACTCTCAAGATTAAGAACTT-3′	
HcrVf2-Forward	5′-CTCAATCTCAGTAGTTTCTATGGA-3′	505
HcrVf2-Reverse	5′-CCCCCGAGATTAAGAGTTG-3′	
SP*HcrVf1*-Forward	5′-***GGCGCGCCGCGATCGC***GGGTCTTAAATTCCACACGTA-3′	3,840
SP*HcrVf1*-Reverse	5′-***ACGCGT***TCACACATTTCTCTTGTCATTC-3′	
LP*HcrVf1*-Forward	5′-***GGCGCGCCGCGATCGC***TCTCTCCCAATTTCTTTAGGGTTA-3′	5,537
LP*HcrVf1*-Reverse	5′-***ACGCGT***CCATTTTCACACATTTCTCTTGTC-3′	
SP*HcrVf2*-Forward	5′-***GGCGCGCCGCGATCGC***TTCCAAGTGGGGTCTTAGATTAAC-3′	3,668
SP*HcrVf2*-Reverse	5′-TTAATTAAACGCGTAATCCCTAAACCATTTTCACACAT-3′	
LP*HcrVf2*-Forward	5′-***GGCGCGCCGCGATCGC***CGATTCGTTACAACAGAAGTGAAC-3′	5,390
LP*HcrVf2*-Reverse	5-TTAATTAAACGCGTATCCCTAAACCATTTTCACACATT-3′	
NptII-Forward	5′-TCGGCTATGACTGGGCACAACAGA-3′	721
NptII-Reverse	5′-AAGAAGGCGATAGAAGGCGATGCG-3′	
TrfA-Froward	5′-CGAGGAACTATGACGACCA-3′	345
TrfA-Reverse	5′-CCACACCAGTTCGTCATCGT-3′	
NptIII-Forward	5′-CATGATGGCTGGAGCAATCT-3′	475
NptIII-Reverse	5′-AGCTCGACATACTGTTCTTC-3′	

Primers	Gene bank accession	Sequence	Fragment length (bp)	Efficiency
Primers used in quantitative RT-PCR				
HcrVf1-qForward	AY397723	5′-CTGTTTAACCAAAAAGACCTTGCC-3′	117	1.90
HcrVf1-qReverse		5′-GTAGTTACTCTCAAGATTAAGAACTT-3′		
HcrVf2-qForward	AJ297740	5′-CTTGATCCGATTCCCAAATTGT-3′	131	1.92
HcrVf2-qReverse		5′-CCCCCGAGATTAAGAGTTG-3′		
MdActin-qForward	DT002474	5′-CTATGTTCCCTGGTATTGCAGACC-3′	82	1.96
MdActin-qReverse		5′-GCCACAACCTTGTTTTTCATGC-3′		

Bold and *italics* indicate *Asc*I and *Asi*SI restriction sites, Bold, *italics* and underline indicate *Mlu*I restriction site

Underline indicates *Pac*I and *Mlu*I restriction sites

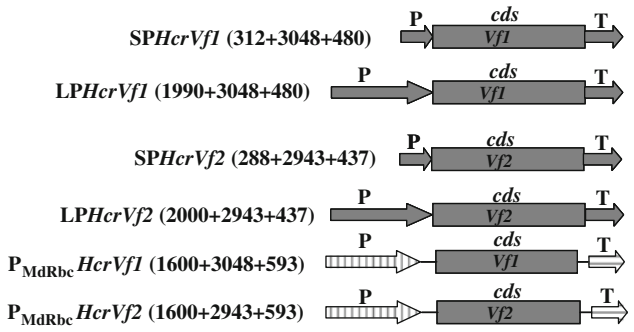

Fig. 1 Constructs used for plant transformation. *P* Promoter, *cds* coding sequence, *T* terminator. The numbers in *parentheses* indicate the lengths of promoter, coding sequence and terminator in basepairs. *Vertical stripes* represent apple rubisco promoter and *horizontal stripes* represent apple rubisco terminator. All LP and SP constructs represent stretches cloned as a whole; the P_{MdRbc} constructs represent new combinations

primers (each), 0.2 U Phusion® DNA polymerase making a total volume of 20 µl. The PCR conditions were as follows: 98°C for 30 s, followed by 35 cycles of 98°C for 10 s, 56°C for 30 s and 72°C for 4 min and final extension at 72°C for 10 min to generate whole gene amplification of *HcrVf1* and *HcrVf2*. The PCR fragments were cloned in pGEMT-Easy (Promega, WI, USA) and the cloned genes were sequenced to confirm that the complete gene sequences were free of PCR-errors. Subsequently, these fragments were subcloned into the binary, marker-free vector pMF1 (Schaart et al. 2010) in the multiple cloning site using *Asc*I and *Pac*I restriction sites.

Preparation of HcrVf constructs with apple rubisco gene regulatory elements

P_{MdRbc} and T_{MdRbc} sequences were amplified using sequence specific primers (Schaart et al. 2011). Sequence

amplification was done using primers with restriction site sequences of *Pac*I and *Xma*I at their 5′ and 3′ ends respectively for P_{MdRbc} and *Kpn*I and *Asc*I at 5′ and 3′ ends respectively for T_{MdRbc}. P_{MdRbc} and T_{MdRbc} sequences were cloned in pGEMT-Easy and subsequently combined so that they were separated by *Xma*I and *Kpn*I and flanked by *Pac*I and *Asc*I. *HcrVf1* and *HcrVf2* genes were amplified using gene specific primers (Table 1) giving restriction sites *Xma*I at the 5′ end and *Kpn*I at 3′ end and cloned in a vector. These gene fragments were cloned in pGEMT-Easy and confirmed by sequencing to make sure that the sequences are PCR-error-free. Both *HcrVf* sequences were excised using *Xma*I and *Kpn*I, and subsequently subcloned into the P_{MdRbc} and T_{MdRbc} construct. Then the P_{MdRbc}–*HcrVf*–T_{MdRbc} fragments were subcloned as *Pac*I-*Asc*I fragment into the binary vector pMF1 (Schaart et al. 2010). The gene constructs used in plant transformation are given in Fig. 1.

Plant transformation and regeneration

The resulting pMF1 with *HcrVf* genes under control of native or rubisco gene regulatory elements were transformed to the supervirulent *Agrobacterium tumefaciens* strain AGL0 (Lazo et al. 1991). Transformation of apple was basically performed as described by Puite and Schaart (1996) with some minor modifications. The top four leaves of 4-week-old, in vitro propagated 'Gala' shoots were used as explants and transferred to a sterile Petri dish filled with 15 ml *A. tumefaciens* suspension for inoculation. The leaves were cut into 0.5 cm^2 pieces and incubated for 30 min. Then, the explants were dried on a sterile filter paper and transferred for co-cultivation to shoot induction medium (SIM) consisting of MS medium with vitamins, micro and macro elements (Murashige and Skoog 1962), 3% (w/v) sorbitol, 9.9 μM thidiazuron (TDZ), 0.5 μM NAA, 13.3 μM BAP, 0.3% (w/v) Gelrite, pH 5.8 for 4 days. After 4 days of cocultivation the explants were transferred to selective regeneration medium, i.e. SIM with 100 mg/l kanamycin for selection of transformants and 250 mg/l cefotaxim to eliminate *A. tumefaciens*. Then, the leaf explants were cultured in the dark at 24°C and subcultured at 4-weekly intervals on fresh selective regeneration medium. After culturing in the dark for 12 weeks, when callus had been produced on the explants and shoot-like structures started emerging from the calli, the explants were exposed to diffused light and later gradually to full light conditions of 7,000 lux (16 h/day) to avoid direct light stress (Espley et al. 2007). When the shoots were big enough, they were isolated and transferred to shoot elongation medium (SEM) containing MS with vitamins, micro and macro elements, 1% (w/v) galactose, 2% (w/v) sucrose, 0.5 μM NAA, 4.4 μM BAP, 0.9% (w/v) Daishin agar, pH 5.8 for 4 weeks with 16 h light/8 h dark at 24°C; normal developing shoots, i.e. putative transformants, were subsequently multiplied on shoot propagation medium (SPM) containing MS with vitamins, micro and macro elements, 3% (w/v) sucrose, 96 mg/l FeEDDHA, 3.1 μM BAP, 0.9% (w/v/) Daishin agar, pH 5.8.

Molecular analysis of transformants

Genomic DNA from putative transformants was isolated as described by Jaccoud et al. (2001). All the putative transformants were analyzed for the presence of inserted genes using *HcrVf1* and *HcrVf2* gene specific primers and with *nptIII*, and *trfA* primers for the presence of pMF1 binary vector backbone (primers mentioned in Table 1). The PCR reaction included 10× Supertaq^{TM} buffer, 5 mM dNTPs, 10 μM each of forward and reverse primers, 0.5 U Supertaq^{TM} DNA polymerase making a total volume of 20 μl. The PCR conditions were as follows: 96°C for 5 min, followed by 30 cycles of 96°C for 30 s, 55°C for 45 s and 72°C for 1 min and 30 s and final extension at 72°C for 10 min to generate an internal fragment of 505 bp for both *HcrVf1* and *HcrVf2*. Similar PCR conditions were used for *nptIII* and *trfA* to generate a fragment of 475 and 345 bp, respectively.

T-DNA integration was analyzed by Southern hybridization, as described by Southern (1975). For this, 20 μg of genomic DNA was digested overnight at 37°C with the restriction enzyme *Bgl*II, separated on a 1% (w/v) agarose gel, and finally blotted onto a positively charged Hybond N+ nylon membrane (Amersham, little Chalfont, UK). The *nptII* probe was prepared using primers (mentioned in the Table 1) amplifying a fragment of 721 bp. The *nptII* probe was labeled with ^{32}P dCTP (Amersham, Little Chalfont, UK) using Rediprime II random prime labelling system (Amersham) and used for hybridization. Untransformed cv. 'Gala' was used as negative control and a plasmid containing the *nptII* gene was used as positive control.

Micrografting

For each construct, six independent transformation events (unless stated otherwise) were selected and multiplied in vitro to provide six replicates per event for *HcfVf* gene expression and scab resistance studies. Apple seedlings derived from a cross between Golden Delicious × Baskatong or from a cross between Elstar × Baskatong were obtained from the NAKTuinbouw (Horst, The Netherlands) and were used as rootstock in micrografting experiments. The 'in vitro' grown plants were grafted directly onto these rootstocks as described by Lane et al. (2003) and grown in the greenhouse. Four to six weeks after micrografting, the grafted plants had developed at least four, new, young

leaves and were ready for scab inoculations. At this stage non-infected plant material was harvested for DNA and RNA extraction.

RNA isolation and quantitative RT-PCR

Total RNA was isolated from young leaves of the micro-grafted plants using the RNeasy mini kit (Qiagen, Carlsbad, CA, USA). From all replicates per event, leaves were harvested and pooled to get sufficient amounts of plant material for RNA isolation and for further quantification; hence, each RNA sample constituted the average of the six biological repeats for each event. The RNA samples were run on a 1% (w/v) agarose gel to determine the RNA quality. An aliquot total RNA of 2 µg was used to treat with *DNaseI* (Invitrogen Carlsbad, CA, USA) and subsequently reverse transcribed with a blend of oligo (dT) and random primers to synthesis cDNA using iScript first strand cDNA synthesis kit (Bio-rad Hercules, CA, USA). The reactions were performed according to the manufactures guidelines. Quantitative RT-PCR (qRT-PCR) was carried out to check the expression levels of the *HcrVf* genes and to correlate the expression with the gene copy number and also to correlate inserted gene expression with fungal sporulation. Cv. 'Santana' is a scab resistant variety carrying both *HcrVf1* and *HcrVf2* genes introduced by conventional breeding and the expression of *HcrVf1* and *HcrVf2* in transgenic cv. Gala plants was measured as fold change by comparing with expression in 'Santana'. Untransformed 'Gala' does not harbour functional *HcrVf* scab resistance genes.

The qRT-PCR was performed with iQ SYBR® green super mix (Bio-rad) with MyiQ Single Color Real time detection system. As endogenous reference the *β*-actin gene (Accession number DT002474) was used. Each extract was checked twice (technical repeats). The primer sequences used for qRT-PCR are presented in Table 1. Primer efficiencies were calculated through different dilution series of cDNA (Rebrikov and Trofimov 2006). All the PCR reactions were carried out in duplicates. *HcrVf* gene expression levels were analyzed by using relative quantification method i.e. $2^{-\Delta\Delta Ct}$ method through qRT-PCR (Li et al. 2004). The threshold cycle (Ct) is the PCR cycle at which a statistically significant increase in the transcript is first detected.

The normalized Ct value difference (ΔCt) was calculated from Ct (*HcrVf*)-Ct (*β-actin*) for all the reactions. The ΔCt value of 'Santana' which has one copy of the natural *HcrVf* genes was chosen as reference sample and for all samples relative ΔΔCt values were calculated using ΔCt (reference sample 'Santana') − ΔCt (target sample). Finally the transgene expression levels were determined as fold change using the formula $2^{-\Delta\Delta Ct}$.

Scab resistance evaluation against EU-B05

Scab disease tests were conducted in a temperature and humidity controlled greenhouse. A conidial suspension of *V. inaequalis* isolate EU-B05 (Bus et al. 2005) containing 5×10^5 conidia/ml was prepared. During the inoculations, the suspensions were checked for germination in vitro and this was generally found to be more than 90%. The top four young, expanding leaves of the scions were used for inoculation with the well-characterized, monoconidial isolate EU-B05. For each transgenic event six replicates (one plant per replicate) were used unless stated otherwise. Inoculated plants were kept in a plastic tunnel in the dark for 48 h at 20°C and 100% relative humidity. After this period, the plants were transferred outside the tunnel, but still kept in the same greenhouse compartment with the temperature set at 19°C during day and 16°C during night, day length of 16 h and relative humidity of 85%.

Disease symptoms were assessed macroscopically 17 days post inoculation and classified in eight classes as reported by Durel et al. (2003), indicative for the amount of sporulation as follows: class 0, 0% of sporulation; class 1, 1–2% sporulation; class 2, 2–5% sporulation; class 3, 5–10% sporulation; class 4, 10–25% sporulation; class 5, 25–50% sporulation; class 6, 50–75% sporulation; class 7, 75–100% sporulation. This scale was adapted from Croxall et al. (1952).

Scab resistance evaluation against different isolates

Based on the results of scab resistance evaluation against the EU-B05 scab experiment, plant transformants were selected from all the representatives of the gene constructs to check the resistance spectrum of the *HcrVf* genes. Six isolates, of which EU-B05, 1639, US-3 and NZ 188 (Bus et al. 2005) are avirulent isolates and EU-D42 (Bus et al. 2005) and EU-NL05 (Parisi et al. 2004) are virulent isolates of *V. inaequalis* for cv. 'Santana' and other *Vf* based resistant varieties, were used to study the resistance spectrum.

Both quantitative (as described above) and qualitative (Chevalier et al. 1991; Szankowski et al. 2009) scales were used for scoring 21 days after inoculation.

Statistical analysis

For statistical analysis of the relative expression, each event was taken as experimental unit with the leaves of the six replicates (biological repeats) pooled before RNA isolation. Here, the experimental design was unbalanced due to variation in number of replications. The data were analyzed through Student's *t* test (unpaired) by comparing two promoters at a time.

For the statistical analysis of scab resistance, each plant was taken as experimental unit. The scab resistance evaluation against EU-B05 was conducted with Randomized Complete Block Design (RCBD). Six replicates were used for each transformation event; for each construct 3–6 independent lines were taken. In total, 31 events and 198 plants were investigated. All the statistical analyses were performed using Genstat® 12 (Genstat® 2009). Since the treatments were not balanced, an ANOVA could not be used. In stead we used the linear mixed model (LMM) procedure. The expression data were log transformed and correlation was studied between phenotypic data and log transformed expression data using Spearman's correlation.

The scab resistance evaluation against different isolates was also performed as a Randomized Complete Block Design (RCBD). The genotypes and isolates were used as the experimental units. The number of replicates used in this experiment was four and in total 201 plants were used in this analysis. In some genotypes there were not equal number of plants per replication and there were not equal number of plants for inoculation with some isolates. Therefore the experimental setup became unbalanced, so a linear mixed model (LMM) was fitted and used for analysis.

Results

Production of transformation vectors carrying *HcrVf* genes

A BAC library of the genotype 1980-015-025, containing the *Vf* resistance, was screened for clones, harboring *HcrVf1* and *HcrVf2*. Four independent BAC pools namely #12 and #199 for *HcrVf1*, #105 and #228 for *HcrVf2* were identified by PCR and the BAC clones harboring these genes were isolated from the pools. The complete gene sequences including promoters of two different lengths, coding sequences and terminators as single stretches for both genes were amplified (Figs. 1, 2) using the BAC clone DNA as template and equipped with the appropriate restriction sites for further cloning. The fragments with the expected sizes (boxed fragments in Fig. 2) were isolated for further processing. Short promoter (SP) *HcrVf1*, long promoter (LP) *HcrVf1*, short promoter (SP) *HcrVf2* and long promoter (LP) *HcrVf2* were then cloned into the transformation vector for production of marker-free plants, pMF1 (Schaart et al. 2004). In addition to this, the coding regions of both *HcrVf* genes were combined in two constructs with the apple rubisco gene promoter (P_{MdRbc}) and terminator (T_{MdRbc}) and introduced into pMF1 as *AscI-PacI* fragments. The individual binary vectors were introduced into *Agrobacterium* strain AGL0 and used for plant transformation.

Production and molecular analysis of transformants

Six independent transgenic lines were selected for SP*HcrVf1*, LP*HcrVf1*, P_{MdRbc}*HcrVf1* and P_{MdRbc}*HcrVf2* and four and three independent transgenic lines were selected for SP*HcrVf2* and LP*HcrVf2*, respectively. All the *HcrVf1* and *HcrVf2* plant transformants were checked for the presence of the respective genes using *HcrVf1*- and *HcrVf2*-specific primers and proved to be positive (data not shown). Twenty six out of 31 transformants showed the absence of the pMF1 vector backbone which was checked using the primers for *nptIII* and *trfA* (primer details given in Table 1, data not shown). T-DNA integration and copy number determination were analyzed by Southern hybridization (Table 2, Fig. 3). Out of 26 transformants taken for estimation of inserted gene copies, 17 have a single T-DNA insert, seven were having two inserts, while two transformants could not be analyzed.

Quantitative RT-PCR

There was a wide variation in the expression of the *HcrVf* genes, controlled by different promoters. The relative expression of the genes is given in the Table 2. The expression levels of SP*HcrVf1*, LP*HcrVf1* and P_{MdRbc}*HcrVf1* were in the range of 0.4–2.3, 5.5–20, and 223–762-fold respectively, compared to *HcrVf1* expression in the resistant control cv. 'Santana' obtained by conventional breeding. The expression levels of SP*HcrVf2*,

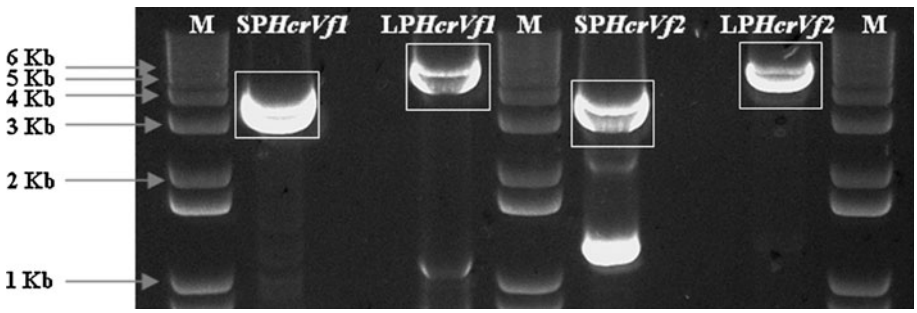

Fig. 2 Amplification of full length *HcrVf* genes by PCR from BAC clones 12 (*HcrVf1*) and 105 (*HcrVf2*). M = 1 Kb + DNA ladder, SP*HcrVf1* = Short promoter *HcrVf1*, LP*HcrVf1* = Long promoter *HcrVf1*, SP*HcrVf2* = Short promoter *HcrVf2*, LP*HcrVf2* = Long promoter *HcrVf2*

Table 2 The relative expression in *HcrVf* transformants compared to expression in cv 'Santana' that contains the *HcrVf* genes because of classical breeding

Transformants	Relative expression of *HcrVf1*	Inserted gene copy number
'Gala'	0.12	–
'Santana'	1.00	–
SP*HcrVf1*-1	0.43	1
SP*HcrVf1*-3	0.94	1
SP*HcrVf1*-6	1.35	1
SP*HcrVf1*-8	0.90	1
SP*HcrVf1*-11	0.79	ND
SP*HcrVf1*-12	2.4	1
LP*HcrVf1*-1	5.9	ND
LP*HcrVf1*-3	7.1	ND
LP*HcrVf1*-4	9.6	2
LP*HcrVf1*-6	5.5	ND
LP*HcrVf1*-7	13	1
LP*HcrVf1*-8	20	ND
P_{MdRbc}*HcrVf1*-4	762	2
P_{MdRbc}*HcrVf1*-7	238	1
P_{MdRbc}*HcrVf1*-8a	249	1
P_{MdRbc}*HcrVf1*-8b	223	ND
P_{MdRbc}*HcrVf1*-9	421	2
P_{MdRbc}*HcrVf1*-10	240	1
Transformants	**Relative expression of *HcrVf2***	**Inserted gene copy number**
'Gala'	0.00	–
'Santana'	1.00	–
SP*HcrVf2*-1	0.23	1
SP*HcrVf2*-2	0.30	1
SP*HcrVf2*-11	0.84	2
SP*HcrVf2*-15	1.2	1
LP*HcrVf2*-1	1.6	2
LP*HcrVf2*-4	7.1	1
LP*HcrVf2*-16	1.1	2
P_{MdRbc}*HcrVf2*-1	66	ND
P_{MdRbc}*HcrVf2*-2	57	1
P_{MdRbc}*HcrVf2*-3	81	1
P_{MdRbc}*HcrVf2*-10	121	1
P_{MdRbc}*HcrVf2*-11	78	1
P_{MdRbc}*HcrVf2*-12	163	2

ND not determined

LP*HcrVf2* and P_{MdRbc}*HcrVf2* were in the range of 0.23–1.2, 1.1–7.1, and 57–163 respectively compared to *HcrVf2* expression in 'Santana'.

Statistical analysis of the data showed that *HcrVf1* under control of its LP showed a significantly higher expression than in case of the SP. The apple rubisco promoter gave the highest (355 times *HcrVf1* expression in 'Santana') expression levels (Fig. 4a). In case of *HcrVf2*, the performances of SP and LP were not significantly different, although LP generally gave a slightly higher expression. Still, expression by P_{MdRbc} was significantly higher (94 times the *HcrVf2* expression in 'Santana') than by SP and LP for *HcrVf2* (Fig. 4b).

Resistance to the *Vf* avirulent strain EU-B05

All the 18 *HcrVf1* transformants, i.e. six independent transgenic lines of each SP*HcrVf1*, LP*HcrVf1*, and P_{MdRbc}*HcrVf1*, showed heavy sporulation after inoculation with the *Vf* avirulent strain EU-B05. Sporulation was statistically similar to the level found in non-transgenic 'Gala' (susceptible control) (Supplement 1a). The high sporulation levels observed were independent of promoter type or promoter length. In some transgenic lines the sporulation was even more than in 'Gala'.

On the other hand, 10 out of 13 *HcrVf2* transformants showed less or no sporulation and were statistically similar to 'Santana' (resistant control) (Supplement 1b). All six independent transgenic lines of P_{MdRbc}*HcrVf2* showed no sporulation at all after inoculation with the *Vf* avirulent strain EU-B05. Among the plants carrying the constructs with the native promoters, three out of four independent transgenic lines of SP*HcrVf2* showed less or no sporulation, compared to untransformed 'Gala'. Only one of the three independent transgenic lines of LP*HcrVf2* showed less or no sporulation. Two transformants viz., LP*HcrVf2*-4 and P_{MdRbc}*HcrVf2*-12 showed the lowest level of sporulation among all transformants. A view of the symptoms of sporulation as an indication of susceptibility and resistance offered by the respective promoters and genes used in this study is given in Fig. 5.

Spectrum of resistance

The complete sporulation pattern of different genotypes and transformants after inoculation with six different fungal monoconidial isolates is summarized in the Table 3. All the *HcrVf1* transformants showed heavy sporulation for all isolates tested. The sporulation levels of the *HcrVf1* transformant did not differ significantly from the sporulation level of the susceptible control 'Gala'.

In *HcrVf2* all transformants, but one showed a sporulation pattern similar to 'Santana' (resistant control). A reduced sporulation was observed for the *Vf* virulent isolate EU-NL05 and heavy sporulation was found for the *Vf* virulent isolate EU-D42. The exception was LP*HcrVf2*-16, which a showed similar sporulation level as 'Gala' for all the tested fungal isolates. LP*HcrVf2*-4 was tested only against isolates EU-D42 and NZ 188, and showed a sporulation of isolate EU-D42 (2.8), and complete resistance

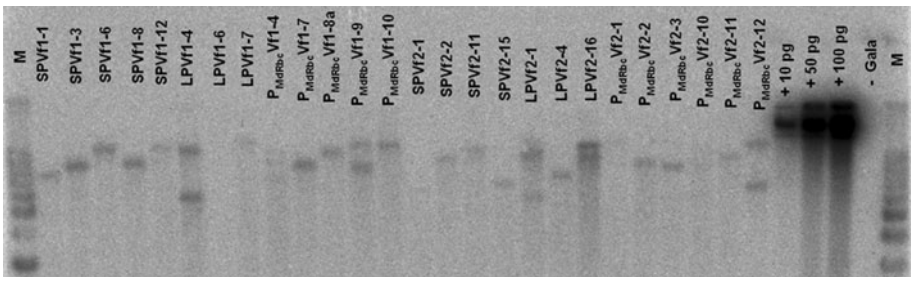

Fig. 3 Estimation of transgene copy number in apple transformants through Southern hybridization. Probe: *nptII*; digestion by *Bgl*II. M = 1 kb + DNA ladder, + = positive control (plasmid), − = negative control (untransformed 'Gala')

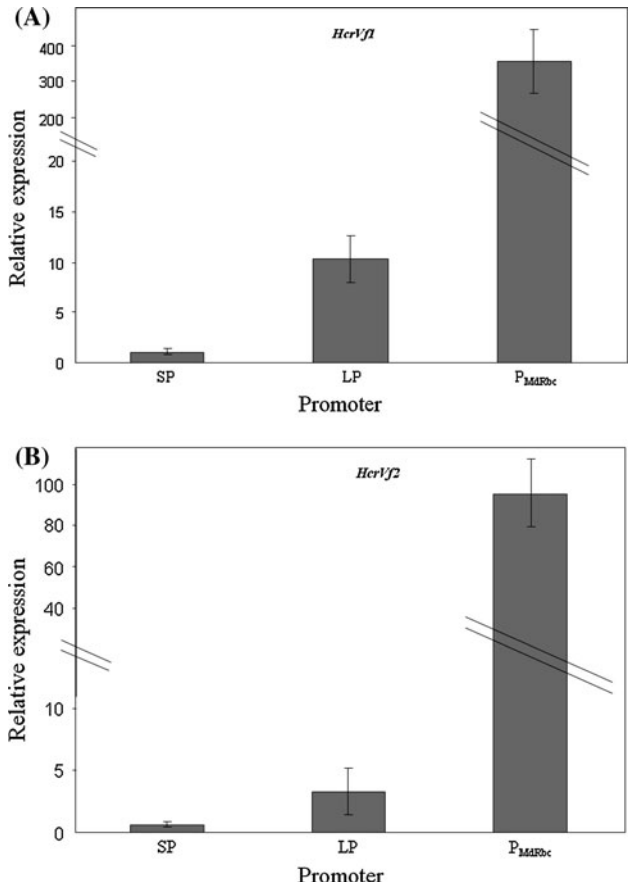

Fig. 4 **a** and **b** Relative expression of *HcrVf* genes under control of different gene promoters. *SP* short native promoter, *LP* long native promoter, P_{MdRbc} apple rubisco promoter. Santana as reference was set at 1 (see also Table 2). For visual convenience two scales have been plotted together. *Two oblique lines* indicate change in the scale in the vertical axis

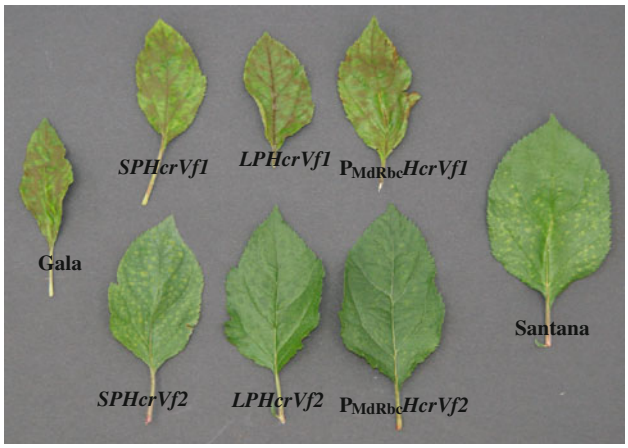

Fig. 5 Sporulation of *Vf* avirulent monoconidial isolate EU-B05 of *V. inaequalis* as observed on leaves of *HcrVf* transgenic lines and of 'Santana' (resistant control) and of 'Gala' (susceptible control). The difference in the size of the leaves among the different plants with or without sporulation may be attributed to the improper development of leaves due to *V. inaequalis* growth

against isolate NZ 188 (0.0). Transformant P_{MdRbc}*HcrVf2*-12 was tested against isolates EU-B05, EU-D42 and NZ 188 and showed resistance against EU-B05 (0.0), and NZ 188 (0.0), and sporulation with EU-D42 (2.1). For comparison: the scab resistant cultivar 'Santana' showed a sporulation level of 0.37, 0.37 and 3.37 when inoculated with EU-B05, NZ 188 and EU-D42, respectively.

In a graphic presentation of the data of Table 3, including means and standard errors, two distinct groups of plants could be observed for all the isolates except EU-D42 in case of *HcrVf2* transformants and cv. 'Santana'. One group of plants showed sporulation levels similar to (not significantly different from) the sporulation of the susceptible control. The other group of plants showed similar sporulation as the *Vf*-cultivar 'Santana'. These two groups of plants were significantly different from each other for all the *Vf* avirulent isolates used. These two groups can be regarded as susceptible and resistant group respectively. Among the cultivars and transformants there was no significant difference observed for their response to the *Vf* virulent isolate EU-D42.

Correlation between sporulation and gene expression

The sporulation data obtained from the greenhouse experiment and the gene expression data from the qRT-PCR analysis were used to study their correlation. In *HcrVf1* transformants, where no resistance whatsoever was found and all showed high levels of sporulation, no correlation could be established. When all *HcrVf2* transformants were taken together, an overall negative correlation was observed i.e. the higher the expression, the lower the sporulation. The

Table 3 Means and their standard deviations of sporulation of different isolates on genotypes and transgenic lines

Genotypes	Race						
	Expression of inserted gene[a]	EU-B05	1639	US-3	EU-D42	EU-NL05	NZ 188
'Gala'	0.00	2.87 ± 1.97	4.16 ± 2.02	4.00 ± 0.81	3.62 ± 1.75	3.16 ± 1.04	5.00 ± 0.40
'Santana'	1.00	0.37 ± 0.25	0.16 ± 0.28	0.25 ± 0.50	3.37 ± 1.79	1.00 ± 0.00	0.37 ± 0.47
SP*HcrVf1*-12	2.4	3.50 ± 1.77	5.0	5.00 ± 0.57	2.87 ± 1.65	3.50 ± 0.40	4.87 ± 0.62
LP*HcrVf1*-7	13	2.37 ± 0.85	4.16 ± 0.76	4.50 ± 0.70	2.87 ± 1.88	3.87 ± 0.85	5.25 ± 0.28
P_{MdRbc}*HcrVf1*-9	421	2.62 ± 0.85	NA	4.50 ± 1.22	2.62 ± 0.62	3.12 ± 0.94	4.00 ± 1.08
SP*HcrVf2*-11	0.84	0.25 ± 0.28	1.00 ± 1.41	0.62 ± 0.94	3.62 ± 0.85	0.62 ± 0.25	0.75 ± 0.28
LP*HcrVf2*-16	1.1	4.00 ± 1.22	4.30 ± 0.76	4.37 ± 1.31	3.62 ± 1.03	2.37 ± 0.47	3.62 ± 1.65
P_{MdRbc}*HcrVf2*-3	81	0.00 ± 0.00	1.50 ± 0.91	0.62 ± 0.75	3.37 ± 1.97	0.62 ± 0.47	0.41 ± 0.58
P_{MdRbc}*HcrVf2*-12	163	0.00 ± 0.00	n.d.	n.d.	2.12 ± 1.03	n.d.	0.00 ± 0.00
LP*HcrVf2*-4	7.1	n.d.	n.d.	n.d.	2.83 ± 1.60	n.d.	0.00 ± 0.00

Races are represented in horizontal direction, Genotypes are represented vertically, SP-Short Promoter, *LP* Long Promoter, P_{MdRbc}-Apple rubisco gene promoter, *'Santana'* resistant cultivar, *'Gala'* susceptible cultivar, *n.d.* not determined, [a] data from Table 2; expression of inserted genes measured in relation to Santana which is considered as 1.00, Means represent average of two leaves of four plants (eight values), ± represents the standard deviation of the mean. Sporulation were scored based on scale as described by Durel et al. (2003) which is class 0, 0% sporulation, class 1, 1–2% sporulation, class 2, 2–5% sporulation, class 3, 5–10% sporulation, class 4, 10–25% sporulation, class 5, 25–50% sporulation, class 6, 50–75% sporulation, class 7, 75–100% sporulation

correlation co-efficient observed was −0.57 ($P = 0.007$) between the expression and sporulation. Looking at the *HcrVf2* transformants in more detail, it was found that all the P_{MdRbc}*HcrVf2* transformants were statistically similar to 'Santana' in resistance and were highest in the gene expression. One of the LP*HcrVf2* transformants, LP*HcrVf2-4*, was the highest in expression among the native promoters, and also proved to be statistically similar to 'Santana' in resistance. However two SP*HcrVf2* transformants (SP*HcrVf2*-2 and SP*HcrVf2*-11) were statistically similar to 'Santana' in resistance while having lower level of expression of *HcrVf2* compared to 'Santana' (0.30 and 0.84 times 'Santana' respectively). On the other hand SP*HcrVf2*-15, LP*HcrVf2*-1, and LP*HcrVf2*-16 that also had expression levels close to 'Santana', showed complete sporulation like untransformed, susceptible cv. 'Gala'. Nevertheless, the overall correlation was significant.

Discussion

We intended to develop cisgenic cultivars with resistance to apple scab. For the *Vf* resistance locus, candidate genes had already been isolated and functionally analyzed. The *Vf* locus comprises of four paralogs namely *HcrVf1*, *HcrVf2*, *HcrVf3* and *HcrVf4* (Vinatzer et al. 2001). *HcrVf3* and *HcrVf4* were predicted to be truncated and non-functional genes (Xu and Korban 2002). The roles of *HcrVf1* and *HcrVf2* were investigated first by Malnoy et al. (2008). They transformed *Vfa1* (which is the same as *HcrVf1*) and *Vfa2* (which is the same as *HcrVf2*) with their native promoters of 2 kb in length and their native terminators, to the susceptible cultivars 'Galaxy' and 'McIntosh'. In their hands, *Vfa1* and *Vfa2* both gave partial resistance to a mixture of isolates (Race 1–Race 5) of *V. inaequalis* with a reduction in sporulation by 50 and 38% in plants with *Vfa1* and *Vfa2* respectively. Later, Szankowski et al. (2009) showed complete resistance by inserting *HcrVf2* under the regulation of native promoters of 288 and 799 bp length and the *nos* terminator into susceptible cvs. 'Gala' (both 288 and 799 bp promoters) and 'Elstar' (only 288 bp promoter). However, using a 115 bp promoter giving an *HcrVf2* expression in an 'Elstar' transformant similar to the expression in the *Vf* resistant control cv. 'Florina', they found no or only a low level of resistance. They used also a mixture of *V. inaequalis* isolates for scab inoculation. Here, we checked for both *HcrVf1* and *HcrVf2* individually their effects on sporulation of apple scab, using promoter lengths at both size classes reported to be functional by Malnoy et al. (2008) and by Szankowski et al. (2009). Moreover in an intragenic approach, a promoter of apple origin, i.e. the small subunit rubisco gene promoter, capable of giving expression at high levels (Schaart et al. 2011) was used. Instead of a mixture of isolates, we used at first a monoconidial isolate and later we determined the effect of the individual *HcrVf* genes on representatives of the spectrum of *Vf* virulent and *Vf* avirulent *Venturia* isolates.

Regulating *HcrVf* gene expression

In *HcrVf1* transformants, the relative expression of the different events showed considerable variation. In spite of

this variation, the long promoter proved to give a significantly higher expression than the short promoter. Expression of LP*HcrVf1*, regulated by the long promoter of 1,990 bp length, was on average ten times higher than that of SP*HcrVf1*, representing the short promoter of 312 bp (Supplement 2). This could be an indication for the presence of 5′upstream cis-acting elements having a positive effect on driving expression of *HcrVf1*. Similar results have been obtained in other crops, e.g. in kidney bean (*Phaseolus vulgaris* L.), where seed-specific Unknown Seed Protein (USP) promoters, short (637 bp) and long (1,149 bp) were used to direct reporter gene expression (Zakharov et al. 2004). They showed that the long promoter led to three times stronger expression than the short promoter. In a similar study, expression of reporter gene *gus* in tobacco was studied with a series of deleted promoter fragments of the *legumin* gene (Bäumelein et al. 1991). They showed that major expression-enhancing cis-elements are present beyond 200 bp upstream from the transcription start site of the *legumin* gene. In apple, Silfverberg-Dilworth et al. (2005) studied different *Vf*-promoter fragments by combining them with the *gus* reporter gene in tobacco and they observed results that seem to be in contrast to the findings reported here in apple, although they also met with considerable variability within their samples. They found that an *HcrVf1*-derived promoter with a length of 1,200 bp showed lower *gus* expression than shorter *HcrVf1* promoters. They concluded that use of promoter fragments greater than 1 kb should be avoided because of reduced activity in tobacco in their experiment. They obtained similar results by combining the *gus* reporter gene with the *HcrVf2* promoter at different lengths and showed that *HcrVf2* with a promoter length of 779 bp showed less expression compared to the gene controlled by a short promoter of 288 bp. However, the same research group found in apple that the *HcrVf2* gene with a native promoter of 779 bp was expressed at lower levels as compared to a short promoter of 288 bp (Szankowski et al. 2009). Also here, especially within the short promoter group, variability was high.

In our study, as a consequence of the variability observed among the *HcrVf2* events, statistical analysis could not demonstrate a significant difference in expression between LP*HcrVf2* and SP*HcrVf2*, even though expression of LP*HcrVf2* was on an average five times higher than that of SP*HcrVf2* (Supplement 2).

A striking difference was found in the expression of SP*HcrVf2* when we compare our results with those of Szankowski et al. (2009). Our results with a short promoter of 288 bp showed expression levels comparable to 'Santana', while they found an approximately 20-fold increase with the 288 bp promoter over their resistant control cultivar 'Florina'. This could be attributed to the different resistant cultivar that was used as reference but also to the use of a native terminator sequence by us and the *nos* terminator sequence by Szankowski et al. (2009). The significance of terminator sequences in directing expression was first reported by Dean et al. (1989) and confirmed by Ingelbrecht et al. (1989). Schaart et al. (2011) demonstrated in *gus* expression studies in tobacco that the apple rubisco terminator gave significantly higher expression than the *nos* terminator when combined with both the apple rubisco promoter or the *CaMV35S* promoter.

Scab resistance evaluation and correlation with *HcrVf* gene expression

Since in the natural situation *HcrVf1* and *HcrVf2* inherit as one locus, it has not been possible to study the role of the individual *HcrVf1* and *HcrVf2* genes in conferring resistance to different isolates of apple scab when classically bred *Vf* cultivars are used. We have developed apple transformants with *HcrVf1* under the regulation of their short promoter (SP), long promoter (LP) or the apple rubisco gene promoter (P_{MdRbc}) and *HcrVf2* under the influence of SP, LP, or P_{MdRbc}. As far as we know, this is the first study on the resistance spectra of *HcrVf1* and *HcrVf2* separately. Since cv. 'Gala' (susceptible control) does not harbor any known effective resistance gene to *V. inaequalis* it showed high levels of sporulation by all isolates used (Belfanti et al. 2004; Szankowski et al. 2009). All *HcrVf1* transformants also showed similar high sporulation with all isolates, demonstrating complete susceptibility, and behaving similar to untransformed cv. 'Gala' (Table 3). This result with *HcrVf1* is however in contrast to the result obtained by Malnoy et al. (2008). They inserted *Vfa1* (which is the same as *HcrVf1*) and *Vfa2* (which is the same as *HcrVf2*) under their native promoters of 2 kb length and their 1 kb of native terminator into cvs. 'Galaxy' and 'McIntosh'. They used a mixed inoculum representing races 1–5 and they observed only partial resistance in both cases of *HcrVf1* and *HcrVf2*. The partial resistance was explained by either the high concentration (2.7×10^7 conidia/ml) of fungal inoculum used or to a mutation occurring in the *HcrVf1* and *HcrVf2* genes during the different steps of the transformation process. As a third possible explanation, the physical separation of *HcrVf1* and *HcrVf2*, which in the natural situation exist together, was suggested as possible explanation for the observed partial resistance.

However, we found that *HcrVf1*, regardless of the promoter and of the expression level, did not provide any resistance to race 1–5, when used separately as monoconidial isolates and therefore, we conclude that *HcrVf1* is not functional against *V.inaequalis.*

HcrVf2 transformants, also regardless of the promoter used and the expression levels obtained, behaved like the *Vf* cultivar 'Santana' (Table 3) with respect to their response to exposure to the different virulent and avirulent isolates. Similar results were obtained by Szankowski et al. (2009) for the short promoter. Together, this indicated that the resistance from the *Vf* cluster is caused by *HcrVf2*, and not by *HcrVf1*. However, we cannot exclude that the partial resistance observed by Malnoy et al. (2008) could be due to inoculation with a mixture of *Venturia* isolates.

From the *HcrVf2* transformants, LP*HcrVf2*-16 was completely susceptible to all isolates confirming the results of the previous experiment with EU-B05 only. Presence of the *HcrVf2* gene was demonstrated in this line and expression was at a similar level to the expression in cv. 'Santana'. Possibly, in this line the *HcrVf2* insert was mutated in its promoter region, resulting in possible failure of inoculation-induced *HcrVf2* upregulation, or, alternatively, a mutation in the coding region may have affected translation or functionality of the *HcrVf2* protein.

The two best performing apple transformants which gave very high resistance and showed a relatively high *HcrVf2* gene expression are LP*HcrVf2*-4 and P_{MdRbc}*HcrVf2*-12. They showed only sporulation with the *Vf* virulent isolate EU-D42. This indicates that the isolate EU-D42 lacks the avirulence (*AVR*) protein that is putatively recognized by the *HcrVf2* gene. The reason for non-recognition of *AVR* genes may be either due to mutation or silencing of *Vf-AVR* gene in the isolate EU-D42. Another explanation could be that the isolate has such *AVR* genes, but is able to block the downstream defense reaction. Still, the *Vf* virulent isolate EU-D42 was confirmed to be virulent, also in case of overexpression of *HcrVf2*. Even the 7- and 163-fold increase in *HcrVf2* gene expression as compared to cv. 'Santana' could not help in providing resistance against this *Vf* virulent isolate.

Among all lines and cultivars checked with the spectrum of scab isolates, two clear groups could be distinguished except for isolate EU-D42 (Table 3). It is evident from Table 3 that in general *HcrVf1* transformants followed the pattern of untransformed cv 'Gala' and *HcrVf2* transformants followed the pattern of cv. 'Santana'. Table 3 also shows the relation between expression of *HcrVf* genes and sporulation of different isolates. In 'Gala' due to the absence of scab resistance genes there was no expression and the sporulation was high irrespective of the isolates. In spite of the high expression of *HcrVf1* in transformants SP*HcrVf1*-12 LP*HcrVf1*-7 and P_{MdRbc}*HcrVf1*-9 these plants showed sporulation levels similar to cv. 'Gala' irrespective of the isolates tested. So in conclusion, there was no correlation between expression of *HcrVf1* and sporulation for any of the isolates tested. For *HcrVf2*, the picture was also clear, except for transformant LP*HcrVf2*-16. In cv. 'Santana', SP*HcrVf2*-11, P_{MdRbc}*HcrVf2*-3, P_{MdRbc}*HcrVf2*-12, and LP*HcrVf2*-4 expression of *HcrVf2* provided resistance against the *Vf* avirulent isolates (EU-B05, 1639, US-3 and NZ 188), led to reduced sporulation of *Vf* virulent isolate EU-NL05 and could not prevent complete sporulation of *Vf* virulent isolate EU-D42. This is a strong indication that the resistance coming from the *Vf* cluster is entirely and solely due to *HcrVf2* and not due to *HcrVf1*.

Correlation between T-DNA insert copy number and gene expression

Twenty-six apple transformants were analyzed for transgene copy number through Southern hybridization. Seventeen of them were found to have a single copy inserted and seven carried two T-DNA inserts. No individuals carrying more than two T-DNA copies were obtained. Both types (single copy and two copies) of transformants showed a wide variation in transgene expression and neither a positive nor a negative correlation could be found between copy number and gene expression. In literature, negative correlations between T-DNA insert copy number and expression have been reported due to co-suppression, e.g. in Citrus where a negative correlation between *gus* gene copy number and GUS activity was found by Cervera et al. (2000). In other cases, no clear correlations could be found (Jones et al. 1985; Zanek et al. 2009; Zeng et al. 2009), as was the case in this study. A correlation did exist between expression and sporulation in *HcrVf2* transformants but, as can be seen in supplement 3, expression beyond a certain level did not result in even higher resistance nor in an extension of the resistance spectrum to *Vf* virulent isolates.

Most variation in the observed expression among different transformation events with the same number of copies is thought to be due to the position effect of the inserted T-DNA (Dean et al. 1988). From two of the LP*HcrVf1* transformants, LP*HcrVf1*-7 and LP*HcrVf1*-4, LP*HcrVf1*-7 has a single copy insert and showed a fourfold higher gene expression level than LP*HcrVf1*-4 which has two transgene copies. In the case of the two P_{MdRbc}*HcrVf2* transformants, P_{MdRbc}*HcrVf2*-11 and P_{MdRbc}*HcrVf2*-12, P_{MdRbc}*HcrVf2*-11 has one transgene copy and showed more than twofold lower gene expression than P_{MdRbc}*HcrVf2*-12 that has two copies.

In conclusion, two genes *HcVf1* and *HcrVf2* under the regulation of their native promoter (SP and LP cisgenic approach) and the apple rubisco promoter (intragenic approach) were studied for expression and resistance against six isolates of *V. inaequalis*. It was proven that the resistance provided by the *Vf* cluster is from *HcrVf2*. *HcrVf1* does not confer resistance against any used isolate.

Increasing the *HcrVf2* gene expression to high levels did not help in conferring resistance against *Vf* virulent isolates, but is helpful for resistance against *Vf* avirulent isolates. From this perspective, the *HcrVf2* gene is presently the best choice for development of good resistance against *Vf* avirulent isolates and can certainly be a good choice for generating both cisgenic and intragenic plants. In order to get even better and more durable resistance we need more resistance genes against apple scab giving a broad spectrum resistance against *Vf* avirulent and virulent isolates and ways to combine them efficiently and rapidly.

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