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Highly scab-resistant transgenic apple lines achieved by introgression of *HcrVf2* controlled by different native promoter lengths

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Abstract Apple scab, caused by the ascomycete *Venturia inaequalis*, is the most damaging fungal disease of commercial apple orchards. Functional scab resistance genes are present in some wild *Malus* species. The *HcrVf2* gene, derived from the *Vf*-region of the wild apple *Malus floribunda* 821 and encoding a receptor-like protein, has proved to confer scab resistance in a transgenic susceptible cultivar. In order to minimize nonplant DNA in genetically modified apple and to go a step toward the development of cisgenic apples, we have studied the capability of the *HcrVf2* gene to confer apple scab resistance when it is controlled by its own promoter. Three promoter deletion constructs containing 115, 288, and 779 bp of the 5′ untranslated region and the *HcrVf2* gene were used to transform the scab susceptible apple cvs. 'Gala' and 'Elstar.' The influence of the promoter length on both the *HcrVf2* expression level and the response to *V. inaequalis* was analyzed in different transgenic lines. Promoter length was found to influence both the constitutive transcription levels of *HcrVf2* in transgenic lines and the resistance level. Highly scab resistant 'Elstar' and 'Gala' plants were obtained, proving that the *HcrVf2* gene controlled by its native promoter is effective in conferring resistance to *V. inaequalis* similarly as *Vf* introgressed in apple cvs. through classical breeding.

Keywords Apple scab · *HcrVf* resistance gene · *Malus* · *Venturia inaequalis*

Abbreviations

CaMV	Cauliflower mosaic virus
gus	β-Glucoronidase
IBA	Indole-3-butyric acid
MS	Murashige and Skoog
NAA	1-Naphthalene acetic acid
TDZ	Thidiazuron
YEP	Yeast extract broth
QTL	Quantitative trait locus

Introduction

Apple scab, caused by the ascomycete *Venturia inaequalis*, is the most destructive fungal diseases in commercial apple

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production. The disease is controlled by multiple fungicide applications. Alternative approaches include the breeding of resistant apple cvs. by conventional crossing using major resistance genes and QTLs or the use of genetic engineering for cv. improvement (Bolar et al. 2000 and 2001, Belfanti et al. 2004, Faize et al. 2004, Malnoy et al. 2007). In conventional breeding programs, one of the most used sources of resistance derives from the wild apple accession *Malus floribunda* 821, the resistance gene *Vf*, which forms a gene-for-gene relationship with races of *V. inaequalis* (MacHardy 1996). Recently, a cluster of three constitutively expressed resistance genes encoding putative receptor-like proteins (*HcrVf1, 2* and *4*) was identified within the *Vf*-region (Vinatzer et al. 2001, Xu and Korban 2002). Sequence analyses indicated that the genes encode, similarly to the *Cladosporium fulvum (Cf)* resistance genes of tomato, transmembrane proteins with an extracellular leucine-rich repeat domain, and for this reason they were called "homologues of *Cladosporium fulvum* resistance genes of the *Vf* region" (*HcrVf*s) (Vinatzer et al. 2001). Although constitutively expressed, differential expression has been observed among the paralogs during leaf development (Xu and Korban 2002). *HcrVf1* and *HcrVf2* (syn. *Vfa1* and *Vfa2*) are highly expressed in immature leaves, whereas *HcrVf4* (syn. *Vfa4*) is highly expressed in older leaves (Xu and Korban 2002). The role in scab resistance of these genes was confirmed using a transgenic approach. *HcrVf2* controlled by the Cauliflower mosaic virus 35S (CaMV35S) promoter has been shown to confer scab resistance to the susceptible apple cv. 'Gala' (Belfanti et al. 2004). Malnoy et al. (2008) reported that both *HcrVf1* and *HcrVf2*, controlled by about 2 kb of their own promoter, induce partial scab resistance in the apple cvs. 'Galaxy' and 'McIntosh,' while no resistance was conferred by *HcrVf4*.

Resistance based on only one gene is often not durable and be can be overcome by the pathogen. A way to develop new cultivars with durable resistance against scab is the combination of different resistance genes in the same background (pyramiding). Besides the *HcrVf* gene family, several other scab resistance genes from apple have been mapped recently (reviewed by Gessler et al. 2006), and some are targets of positional cloning projects. The use of constitutive promoters such as the CaMV35S is helpful to understand the roles of genes, but it is not desirable in gene stacking because of the risk of homology-dependent transgene silencing (Vaucheret et al. 1998). Thus, expression of resistance genes under the control their own promoter is preferred.

Moreover, there is considerable concern about the use of genetically modified plants. One of the main criticisms is gene transfer across very wide taxonomic boundaries. In an attempt to develop genetically modified plants with increased acceptability, the concept of cisgenesis has been developed. Cisgenic plants, at the end of the transformation process, contain only genes (in a sense orientation) derived from a sexual compatible species controlled by their own promoters and terminators (Schouten et al. 2006a and b). Therefore, the prerequisite of cisgenesis is the isolation and characterization of genes of interest, their promoter and terminator sequences, as well as a method which allows removing (if used) the selection markers from the final products. Currently, only a technology has been tested to develop cisgenic apples (Krens et al. 2004). As this method requires the initial insertion of the large T-DNA, and this is generally associated with a reduction of the transformation efficiency, any kind of reduction of the length of the T-DNA is highly desirable.

Recently, the functional promoters of the three members of the *HcrVf* gene family have been isolated and used for qualitative and quantitative analyses in apple and tobacco. Silfverberg-Dilworth et al. (2005) used different promoter deletion constructs along with the *gus* gene in transient expression assays. Construct design was based upon differences observed between promoter sequences of the three transcribed genes of the *HcrVf* cluster *HcrVf1*, *HcrVf2*, and *HcrVf4*. From the *HcrVf2* promoter, two constructs were designed with 288 and 779 bp of the sequence. The lengths of 288 and 779 bp were chosen because they cover regions of greatest sequence discrepancy between −229 and −667 (Silfverberg-Dilworth et al. 2005). Sequences between −1 and −115 bp of all three genes have 95% identity; therefore, it was assumed that results obtained from the promoter region −1 and −115 from *HcrVf1* are true for *HcrVf2* as well. Within the 115-bp section, a TATA box between −70 and −64 as well as the transcription start estimated at −36 was identified (Vinatzer et al. 2001). The region from −155 to −288 contains many *cis*-acting elements, among them regions which are also found in the promoter regions of light regulated as well as of tissue-specific genes (Silfverberg-Dilworth et al. 2005). Between −289 and −799 bp, binding sites for the transcription repressor protein S1F and W boxes as binding sites for WRKY proteins are present (Silfverberg-Dilworth et al. 2005). W boxes are especially found in promoter regions of many plant defense genes (reviewed by Singh et al. 2002). Results of transient expression analyses in tobacco with the different promoter deletion constructs, along with the *gus* gene, indicated that the expression driven by the 115-bp promoter was significantly reduced compared to the longer promoter sequences. A promoter length of 288 bp resulted in highest expression level compared to the 115 and 779 bp length, but about half of the strength of the 35S promoter from cauliflower mosaic virus (Silfverberg-Dilworth et al. 2005).

In this study, we used the previously identified promoter sequences (Silfverberg-Dilworth et al. 2005) along with the *HcrVf2* gene. Three gene constructs containing the *HcrVf2* gene along with different lengths (115, 288, and 779 bp) of

the own promoter region were used in stable transformations of the scab-susceptible apple cvs. 'Gala' and 'Elstar.' The influence of the length of the promoter region on transcription levels and scab resistance was assessed.

Material and methods

Plant material

Plant material consisted of *in vitro* cultivated shoots of apple (*Malus* × *domestica* Borkh.) of the scab-susceptible cvs. 'Elstar' and 'Gala' and the scab-resistant cv. 'Florina,' heterozygous for the *Vf* gene. Shoot cultures were maintained on propagation medium containing MS salts and vitamins (Murashige and Skoog 1962), 3% (*w*/*v*) sucrose, and 0.8% (*w*/*v*) plant agar. For 'Elstar,' the medium was supplemented with 3.1 μM BAP, 0.5 μM NAA and 2.8 μM GA3, for 'Gala' and 'Florina' with a combination of 4.4 μM BAP and 0.5 μM IBA. Before autoclaving, the pH was adjusted to 5.7. Cultures were incubated at 24±1°C under a 16/8 h photoperiod.

For quantitative real time polymerase chain reaction (PCR) analyses, transgenic plants regenerated in this study, as well as the respective controls were used. In addition, 'Florina' and four progeny plants of a cross between 'Florina' and 'NovaEasygro' (two heterozygous for the *Vf* gene and two homozygous for the *Vf* gene) were used.

Agrobacterium strain and binary vectors

The *HcrVf2* gene was identified in the BAC clone M18-5 (Vinatzer et al. 2001) of the library developed from the apple cv. 'Florina' (Vinatzer et al. 1998). The entire ORF of *HcrVf2* (GenBank accession no. AJ297740) along with 5`UTR fragments of the lengths of 115, 288, and 779 bp were PCR amplified by *Pfu Taq* polymerase (Promega, Madison, WI, USA), using reverse primer HcrVf2-R2 and forward primer HcrVf2-1F (Table 1), HcrVf2-3F and HcrVf2-8F (Silfverberg-Dilworth et al. 2005) from the BAC clone to introduce restriction sites for *Bst*EII and *Sal*I for cloning. PCR products were introduced into the binary vector pCambia2301 (GenBank accession no. AF234316) by replacing the *gus* (β-glucuronidase) gene by using the same restriction sites. Clones were verified by sequencing. Constructs were named as follows: P2-115::HcrVf2, P2-288::HcrVf2, P2-779::HcrVf2 (two indicates the *HcrVf* number, and then, separated by hyphen, the length of the 5` region). Additionally, pCambia2301 carries on its T-DNA the *nptII* gene coding for neomycin phosphotransferase II driven by the constitutive cauliflower mosaic virus 35S (CaMV35S) promoter conferring kanamycin resistance in transformed plant cells. Plasmids were introduced into *Agrobacterium tumefaciens* EHA105 (Hood et al. 1993) through electroporation.

Transformation and regeneration

Agrobacterium tumefaciens-mediated transformation of the apple cvs. 'Elstar' and 'Gala' was done as described by Szankowski et al. (2003). In brief, the middle part of the four youngest unfolded leaves from 4-week-old micropropagated shoots was excised and inoculated with agrobacteria (grown overnight in YEP medium, centrifuged, and resuspended in liquid MS medium to an OD of 0.8) and co-cultivated in the

Table 1 Primers used for vector construction, probe preparation as well as for RT- and quantitative real-time PCR

Primer	Sequence 5′→3′	PCR efficiency %	Correlation Coefficient
HcrVf2-R2	ATCTAGATGGTCACCCTAGACATATTCAACAATTACATG	–	–
HcrVf2–1F	GCTAACCGATGTCGAT*GTCGAC*TCTCATGCCGTAGAGGATGG	–	–
167nptII-for	CCACAGTCGATGAATCCAGA		–
367nptII-rev	AGCACGTACTCGGATGGAAG	–	–
HcrVf2-for	GCCTGGATCAGTCGAGCTTC	–	–
HcrVf2-rev	ACCAAGCACAATCCAAAACC	–	–
HcrVf2-for2	ATGGAGAGAACCATGAGAGTTG	–	–
HcrVf2-rev2	AAAATTGTGGAAGCATCTCGG	–	–
EF1for	TACTGGAACATCACAGGCTGAC	–	–
EF1rev	TGGACCTCTCATCATGTTGT	–	–
RT1-for	CAATGCCTTACGTGGTGAAA	102.1	0.959
RT2-rev	CAGGGATTCCAGCCAATCTA		
RNAPOL II for	ATATGCCACCCCGTTCTCTACT	73.3	0.998
RNAPOL II rev	CACGTTCCATTTGTCCAAACTT		
RUBISCO for	GCTTGTCCAAGAGCAAGAGAAT	85.2	0.993
RUBISCO rev	CTCCCTCCCCTCAATTATAACC		

Underlined: *Bst*EII restriction site; underlined and italic: *Sal*I restriction site. For those used for quantitative real time PCR analysis, PCR efficiency and correlation coefficient is given

dark on regeneration medium [MS salts and vitamins (Murashige and Skoog 1962), 3% (*w*/*v*) sorbitol; for 'Elstar' 3 μM TDZ and 1 μM IBA, for 'Gala': 22.7 μM TDZ and 2.6 μM NAA, 0.3% (*w*/*v*) gelrite; pH 5.7] for 3 days. After co-culture, explants were placed on regeneration medium supplemented with 100 mg l^{-1} ticarcillin and 150 mg l^{-1} cefotaxime (to eliminate the agrobacteria) and 50 mg l^{-1} kanamycin for selection of transgenic cells. Explants were cultivated in the dark for 2 weeks at 24±1°C, then under 16/8 h photoperiod at the same temperature. The medium was changed every 2 weeks, and after 6 weeks on regeneration medium, they were cultivated on elongation medium (MS salts and vitamins, 3% (*w*/*v*) sucrose, 4.4 μM BAP, 0.28 μM GA_3, and 0.8% (*w*/*v*) plant agar; pH 5.7).

Transgenic shoots and *in vitro* control plants of 'Gala' and 'Elstar' were micropropagated and afterwards rooted on rooting medium (Puite and Schaart 1996) consisting of MS salts and vitamins, 3% (*w*/*v*) sucrose, 7.3 μM IBA, and 0.7% (*w*/*v*) plant agar, 100 mg l^{-1} ticarcillin and 150 mg l^{-1} cefotaxime. The rooted plants were acclimatized to greenhouse conditions.

Verification of the integration of the transgenes

Genomic plant DNA was isolated using the CTAB-extraction method of Doyle and Doyle (1990). All regenerated plants were analyzed by PCR and Southern blot. The PCR reactions contained 0.2 U of *Taq* polymerase (Genecraft, Lüdingshausen, Germany), 16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl (pH 8.8), 1.5 mM $MgCl_2$, 200 μM of each dNTP, and 0.4 μM of each primer in a total volume of 25 μl.

The primers RT1-for and RT2-rev (Vinatzer et al. 2001) were used to amplify a specific *HcrVf2*-fragment with the size of 856 bp under the following conditions: 94°C for 3 min, followed by 30 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and with a final extension at 72°C for 10 min. The presence of the *nptII* gene was confirmed by using the primers 167nptII-for and 367nptII-rev (Table 1), which amplify a 200-bp *nptII*-specific fragment. The PCR reaction conditions were the same as for *HcrVf2*.

To verify the integration of the transgenes, Southern blot analyses were performed. Twenty micrograms of genomic DNA were restricted with 20 U *Hin*dIII at 37°C overnight followed by the addition of another 10 U of the enzyme. After 4 h, the DNA was fractionated in a 1% agarose gel and blotted on a positively charged nylon membrane (Roche, Mannheim, Germany). Membranes were hybridized with the digoxygenin-labeled PCR probes for *HcrVf2* and *nptII*, incubated with alkaline phosphate-conjugated antidigoxygenin followed by the chemiluminescent substrate CDP-Star and finally exposed to a chemiluminescent detection film (Roche, Mannheim, Germany). All steps were performed following the supplier's instructions (Roche). For the *HcrVf2* probe primers *HcrVf2*-for and *HcrVf2*-rev (Table 1), amplifying a 194-bp long specific fragment (Fig. 1), were used. For the detection of the *HcrVf2* gene in the transgenic line E04-5, a longer probe of 2237 bp, generated by using the primer *HcrVf2*-for2 and *HcrVf2*-rev2 (Table 1), was used. This was necessary because the shorter probe did not give an additional signal despite PCR-positive results; probably bands were hidden behind those obtained from endogenous homologous sequences. PCR program was the same as mentioned above. The *nptII* probe (Fig. 1) was constructed using the same primers and PCR program as for transgene detection. The binary plasmid P2-779::HcrVf2 was used as a template for probe generation.

Reverse transcriptase quantitative real-time PCR

Total RNA was isolated using PureLink™ Plant RNA Reagent (Invitrogen, Paisley, Scotland) from 100 mg of young leaves harvested from greenhouse-grown transgenic lines and control 'Florina,' 'Gala,' and 'Elstar' plants and from budwood kept in water to induce bud break of two homozygote (*VfVf*) and two heterozygote (*Vfvf*) progeny plants of the cross 'Florina' (*Vfvf*) × 'Nova Easygro' (*Vfvf*). One microgram RNA was treated with *DNase I* (MBI Fermentas, St. Leon-Rot, Germany) to eliminate genomic DNA contaminations. Specific primers (EF1for and EF1rev; Table 1) for an apple gene encoding the elongation factor EF1 alpha (*EF1-α,* GenBank accession no. DQ341381) were used to screen for DNA contaminations. The PCR reaction was done as mentioned above with 1 μl of total RNA as template and the primer pairs mentioned in Table 1. The amplified fragments were separated on a 0.8–1% (*w*/*v*) agarose gel containing 0.5 μg ml^{-1} ethidium bromide in 1×TAE buffer.

The remaining RNA was reversed transcribed with oligo$(dT)_{18}$ primers and RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas), and the cDNA was purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). All steps were performed according to the manufacturer's instructions. The success of reverse

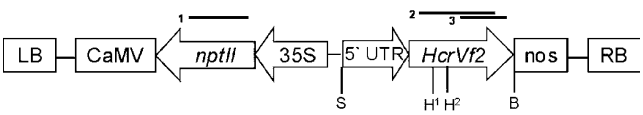

Fig. 1 Schematic representation of T-DNA region of the binary vectors used in the transformation experiments. *nptII* is controlled by the cauliflower mosaic virus 35S promoter and the *HcrVf2* gene is controlled by different promoter regions of the lengths of 115, 288 and 779 bp, respectively. *S Sal*I, *B Bst*EII, *H Hin*dIII, *LB* left border, *RB* right border, *CaMV* cauliflower mosaic virus 35S poly(A) signal, *Nos* Nos poly(A) signal. H1 and H2 are *Hin*dIII restriction sites 1 and 2. *Thick bars* represent Southern blot probes for the detection of *nptII* (1) and *HcrVf2* (2 and 3)

transcription was tested by PCR with 1 µl of cDNA and the primers Ef1forw and EF1rev as described. Quantitative real-time PCR was performed with iQ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) and first strand cDNA on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). For each experiment, the cDNA sample was measured in three different wells.

The specific amplification was evaluated by melt curve analysis and agarose gel electrophoresis. No primer dimers were obtained, and only one product was amplified from each analyzed gene. To determine the amplification efficiencies and correlation efficiencies of each PCR reaction, a serial dilution series of cDNA of all samples was analyzed. The efficiencies and the calculation of the expression level were estimated using the iQ5 Optical System Software 2.0 (Bio-Rad) according to Vandesompele et al. (2002). For each line, the *HcrVf2* transcription level was quantified relatively using the primers RT1-for and RT2-rev (Table 1). All samples were normalized using ribulose-1,5-bisphosphate carboxylase/oxygenase activase mRNA and mRNA of the *M. domestica* cDNA clone Mdfw2033f21.y1 (similar to the RNA polymerase subunit II) as internal control samples for each line (Table 1, Li et al. 2007). The scaling of the *HcrVf2* gene expression in the transgenic lines was performed relative to the mRNA expression level of the scab resistant cv. 'Florina' (heterozygous for the *Vf* gene) thus harboring the complete native 5`UTR (promoter).

Scab resistance evaluation

Random numbers were given to all plants to be tested, so that plants could not be directly identified. From that moment on, plants were handled by others to avoid any possibly bias. The plants were inoculated with a mixture of *V. inaequalis* conidia obtained from sporulating lesions of an apple scab susceptible seedling of the Agroscope Changins-Wädenswil breeding program.

Plants were actively growing, producing young leaves and having a height between 40 and 60 cm. Five plants per tested line together with a few 'Golden Delicious' seedlings were sprayed with a hand-operated mist blower with a conidial (6×10^4 conidia/ml in the first inoculation round and 10×10^4 conidia/ml in the second) suspension until visibly wetted; however, before coalescence of the microdrops, they were immediately placed into a plastic tent with 100% rH and constant temperature of 20°C in an experimental greenhouse with day length of ca. 16 h. The plastic tent was removed after 48 h and the plants incubated at ca. 18–21°C, 60–70% rH for additional 10 days and than re-subjected to high humidity for 2 days. Disease symptoms were assessed 21 days after inoculation. Response to scab inoculation was evaluated after the scale of Chevalier et al. (1991) by two independent evaluators with score 0 no visible symptoms, 1 pinpoint pits, 2 yellow-chlorotic flecks, 3a yellow-chlorotic to necrotic flecks without sporulation, 3b necrotic flecks with sparse sporulation, and 4 abundant sporulation and no necroses (considered as susceptible). The highest score of any leaf (overwhelmingly the youngest unfolded leaf at the inoculation time) of the plant was retained. Plants showing symptoms different than class 4 were considered as apple scab resistant. After the first scab assessment round, the plants were cut back and allowed to grow a new shoot, which was inoculated in the second round of evaluation.

Results

Regeneration of transgenic 'Gala' and 'Elstar' plants and verification of the plants

In order to assess the influence of functional regulatory sequences of the *HcrVf2* gene on transcription levels of *HcrVf2* and scab resistance, the ORF with increasing lengths of the upstream region (115, 288, and 799 bp) were transferred via *Agrobacterium tumefaciens* to the apple scab-susceptible cvs. 'Gala' and 'Elstar.' In total, four independent transgenic lines of 'Elstar' (two with P2-115::HcrVf2 and two with P2-288::HcrVf2) and three independent lines from 'Gala' (one with P2-288::HcrVf2, two with P2-799::HcrVf2) were regenerated (Table 2) on kanamycin-selective medium, acclimated, and tested for scab resistance. No shoots of 'Gala' P2-115::HcrVf2 and 'Elstar' P2-799::HcrVf2 have been regenerated. Integration of the T-DNA was confirmed by PCR (data not shown) and Southern blot analyses using primers and probes specific for the *HcrVf2* and the *nptII* gene. In untransformed control DNA samples restricted with *Hin*dIII, no fragment hybridized to the *nptII* probe (Fig. 2a), and 13 fragments were detected by the *HcrVf2* probe 3 (Fig. 1 and 2b) in Southern blot analyses. Additional transgene-specific fragments hybridizing to the *HcrVf2* probe were obtained by one *Hin*dIII cutting inside the T-DNA and an additional one in the plant genome (Figs. 1 and 2b). These, as well as the fragments hybridizing to the *nptII* probe (Fig. 2a), had a specific size for each transgenic line and indicated the copy number of transgenes stably integrated into the genome. One or two integrations of the transgenes were detected in the transgenic lines (Fig. 2 and Table 2), and both transgenes, *nptII* and *HcrVf2*, were found. In case of the transgenic line, E04-5 detection of the *HcrVf2* specific bands was only possible by using *HcrVf2* probe 2, covering a longer part of the ORF (Figs. 1, 2b). Using that probe, seven fragments were detected in the untransformed 'Elstar' control, while two additional fragments were obtained from DNA of the transformed line.

Table 2 Scab susceptibility of seven independent transgenic lines transformed with *HcrVf2* with deletion of the 5′ regulatory sequences and nontransformed "Gala" and "Elstar" plants 21 days postinoculation

Line or cvs	Presence *HcrVf2*	Promoter length (bp)	Copy nr	Plant					Line/control considered as
				1	2	3	4	5	
Elstar	–	–	–	4/4	3b/4	4/4	4/4	4/4	Susceptible
E04-5	+	115	2	4/4	3a(3b[a])/4	4/4	2/4	3a/4	Susceptible
E06-1a	+	115	2	3b/3b	2/3b	2/3b	3a/4	2/3b	Resistant
E05-2a	+	288	1	0/0	0/0	0/0	–	–	Resistant
E05-2b	+	288	1	0(2[a])/0	0/0	0	–	–	Resistant
Gala	–	–	–	4/4	4/4	4/4	–	–	Susceptible
G05-8	+	288	2	0/0	0/0	0/0	0/0	0/0	Resistant
G06-9	+	779	1	1/0	1/2	–	–	–	Resistant
G06-14	+	779	1	0(2[a])/0	0(2[a])/2	–	–	–	Resistant

Symptoms were assessed after Chevalier *et al.* (1991) with 0 = no macroscopically visible symptom, 1 = hypersensitive pinpoints, 2 = yellow/chlorotic areas with no sporulation, 3a = chlorotic and necrotic areas with no sporulation, 3b = chlorotic and necrotic areas with sparse sporulation, and 4 = clear sporulation without other necrotic reaction. A Score of 4 is considered susceptibility

[a] In brackets are indicated the different scoring assigned to the plant by the two evaluators. Values of symptoms from two independent scab inoculations and two evaluators are given

The acclimated greenhouse-grown plants exhibit *ex vitro* growth behavior similar to that of the controls and were undistinguishable to the control plants except for some of the plants of the two 'Gala' lines with P2-799::HcrVf2 which stopped active growing at a height of about 30 cm which coincided with a reallocation to another greenhouse environment. Cutting back however induced new growth.

Expression analyses

RT-PCR and quantitative real-time PCR were performed to confirm transcription of the transgenes and to determine the influence of the lengths of the upstream regions on the transcription level of *HcrVf2*. Using *HcrVf2*-specific primers in RT-PCR, the expected fragment with the size of 855 bp was amplified from the *Vf*-resistant cv. 'Florina,' as well as from the regenerated transgenic lines. The two untransformed non-*Vf* cvs. 'Gala' and 'Elstar' gave only a minimal signal which was considered as background. The *nptII* gene transcription was confirmed in all transgenic lines but not in untransformed control cvs. 'Elstar,' 'Gala,' and 'Florina.' PCR carried out with primers specifically binding to *EF1-α* and spanning an intron confirmed that no DNA contaminations were present in the cDNA samples (data not shown).

Real-time PCR analyses were used to quantify *HcrVf2* mRNA. The cv. 'Florina,' heterozygous for the *Vf* gene, harbors the complete 5`UTR and was the source of the *HcrVf2* gene used in this study; therefore, the amount of *HcrVf2* mRNA of the transgenic lines was measured in relation to *HcrVf2* expression of that cv. All transgenic lines harboring *HcrVf2* accumulated higher amounts of *HcrVf2* mRNA than the *Vf* cv. 'Florina' (*Vfvf*), independently of the promoter length (Fig. 3). Within the transgenic lines of a cv., there was a correlation between the length of the promoter and the amount of accumulating *HcrVf2* mRNA. In transgenic 'Gala' values varied from 16-fold (line G05-8) in relation to 'Florina' for the 288-bp promoter and from 108 (line G06-9) to 115 (line G06-14) fold for the 779-bp promoter. In the genetic background of 'Elstar,' the shortest promoter, which is 115 bp in length, led to an increase in transcription from 1 (line E04-5) to 1.7-fold (E06-1a) in transgenic plants compared to the expression in "Florina," while 56 (line E05-2A) to 30-fold (E05-2B) increased expression was detected in plants expressing *HcrVf2* under the control of the 288-bp promoter (Fig. 3).

In order to compare the *HcrVf2* transcription levels of transgenic plants with those of plants carrying *Vf* resistance introduced by classical breeding approaches, we additionally analyzed *HcrVf2* transcription of four progeny plants of a cross between 'Florina' (*Vfvf*) and 'NovaEasygro' (*Vfvf*) (two heterozygous for the *Vf* gene and two homozygous for the *Vf* gene) in relation to 'Florina.' The cv. 'Nova Easygro' is reported in the literature as carrying the apple scab-resistance gene *Vr*; however, Gianfranceschi et al. (1996) proved that 'Nova Easygro' carries a single apple scab resistance gene, and this is *Vf*. The two plants homozygous for the *Vf* gene showed six- and eightfold higher *HcrVf2* transcription levels than 'Florina' and the other two heterozygous progenies (0.3- and 1.5-fold), but all (homo- and heterozygous) exhibited a lower expression than plants transformed with *HcrVf2* controlled by 288 and 779 bp of the promoter.

Scab resistance evaluation

The scores of the scab symptoms assigned by the two independent evaluators and between the two inoculation

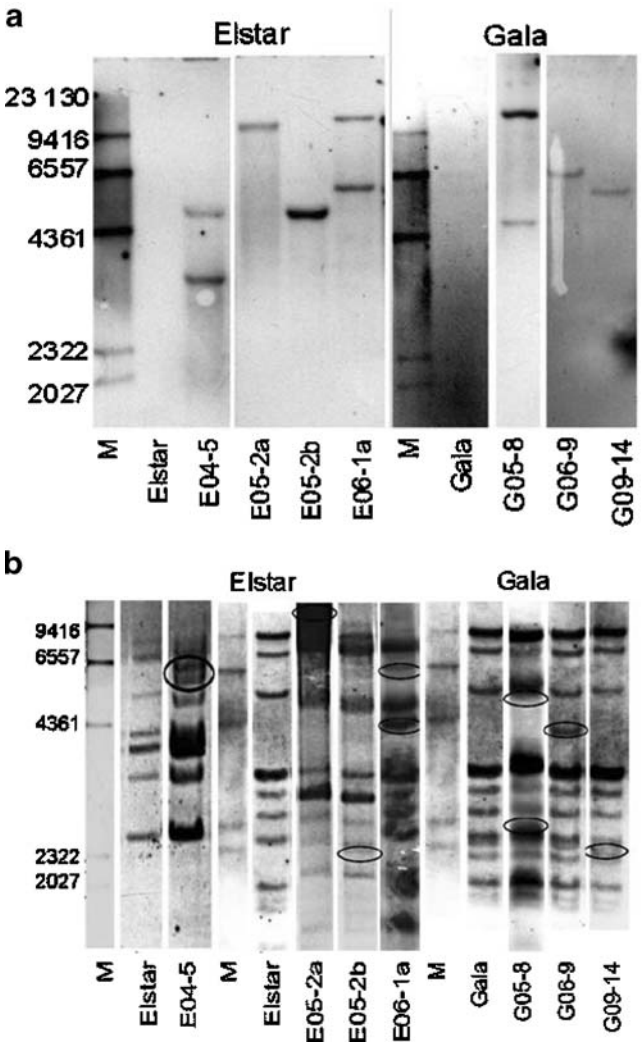

Fig. 2 Southern blot of *Hin*dIII digested genomic DNA isolated from transgenic apple plants and respective control plants hybridized to *nptII* probe (**a**) and *HcrVf2*-specific probe 3 (**b**). For the transgenic line E04-5, *HcrVf2*-specific probe 2 (Fig. 1) was used. *M* molecular weight markers II, DIG labelled (Roche)

rounds overwhelmingly concurred (Table 2). In a few cases, a plant was scored as "no symptoms" (0) by one evaluator and as presenting yellow chlorotic flecks (2) by the other. In a single case, a plant was scored as 3a and 3b as the second evaluator detected also sparse sporulation. Generally, the symptoms were more severe in the second round (see, e.g., lines E04-5 and E06-1a).

The untransformed cvs. 'Elstar,' 'Gala,' and the 'Golden' seedlings (seedling data not shown) as well as one (E04-5) of the two 'Elstar' lines transformed with *HcrVf2* controlled by the shortest promoter length of 115 bp showed clear symptoms of susceptibility with sporulating lesions and absence of any type of resistance reaction (Fig. 4). On the leaves of the other line, E06-1a harboring *HcrVf2* controlled by the 115-bp of the promoter region, light sporulation was also observed. However, in this case, typical resistance reactions like chlorosis and necrosis were observed (class 3b of Chevalier et al. 1991). Line E06-1a has been therefore considered as resistant.

No symptoms of any kind could be detected in ten of 11 tested plants transgenic 'Gala' and 'Elstar' plants harboring the *HcrVf2* gene along with 288 bp of the promoter region, while only one plant was grouped into class 2 by one of the evaluators and into class 0 by the other. These lines therefore exhibited a high level of resistance.

From the two independent transgenic lines transformed with P2-799::HcrVf2, nine plants were inoculated with *V. inaequalis* spores. Only four (two per line) were taken into consideration for evaluation because all others did not exhibit an actively growing shoot tip. These not growing plants were free of symptoms, too, but excluded from the evaluation because it was not clear whether the absence of symptoms was due to resistance caused by *HcrVf2* or by ontogenic resistance because of the leaf age.

During the first inoculation, both plants of G06-9 (P2-799::HcrVf2) were scored as showing hypersensitive pinpoints (score 1), however, this resistance reaction atypical for *Vf* was no longer present in the second inoculation round. Lines E05-2a, E05-2b, G06-9, and G06-14 all exhibited a high level of resistance.

Discussion

This study confirmed that *HcrVf2* can confer full resistance to scab in GM apple plants (Belfanti et al. 2004) independently from the genetic background. It shows that using an appropriate length of the promoter region sequence of *HcrVf2* instead of the CaMV 35S constitutive promoter, a high resistance level can be obtained. Three gene-constructs containing progressive 5′ deletions of the promoter region (115, 288, and 779 bp, Silfverberg-Dilworth et al. 2005) and the *HcrVf2* gene were used to transform the scab-susceptible apple cvs. 'Gala' and 'Elstar.' Six transgenic lines out of seven exhibited resistance against *V. inaequalis*.

The shortest promoter of 115 bp induced expression of the *HcrVf2* mRNA similar of those observed in 'Florina' in both P2-115::HcrVf2 lines (Fig. 3), but this was below the expression obtained with the 288- and 799-bp fragments. Although the result is consistent with those previously obtained by Silfverberg-Dilworth et al. (2005) using quantitative GUS assessments, we cannot exclude that the integration site and the number of integrations play a role. Both P2-115::HcrVf2 lines contain two copies possibly resulting in posttranscriptional gene silencing. While Silfverberg-Dilworth et al. (2005) observed reduced expression from longer sequences than 288 bp, we measured equal or even higher levels of *HcrVf2* mRNA when *HcrVf2*

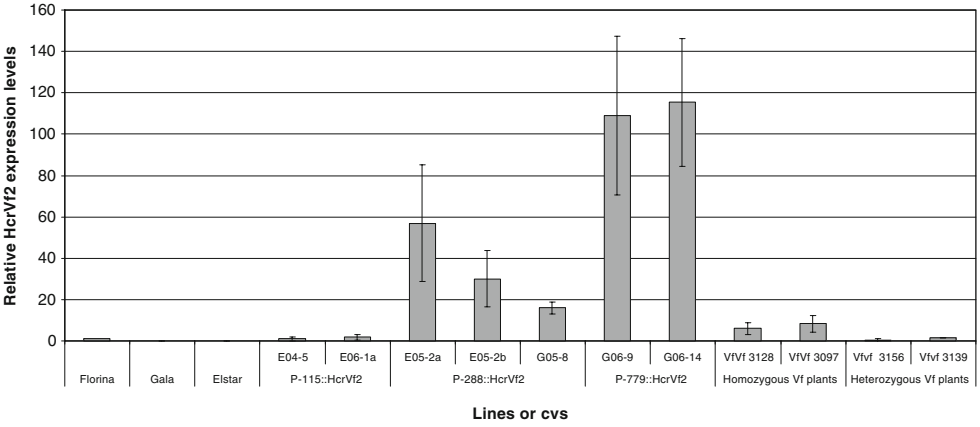

Fig. 3 Determination of transcription of *HcrVf2* controlled by different lengths of upstream regions in transgenic 'Elstar' and 'Gala' lines by real-time PCR in relation to the level of mRNA transcript in *Vf*-resistant cv. 'Florina' (*Vfvf*). Values of the transgenic lines, as well as of 'Elstar' (0.0004), 'Florina' and 'Gala' (0.025) are the mean±SD of three independent experiments (each using different RNA extractions). Values of the homo- and heterozygous plants are the mean±SD of two independent experiments

was controlled by 799 bp of the promoter. However, Silfverberg-Dilworth et al. (2005) measured GUS protein activity while we quantified mRNA. Therefore, direct comparison is not possible.

The 288-bp fragment appears to be sufficient to confer high resistance. The short fragment of 115 bp seams to be not reliable, since one transgenic line (E04-5) showed clear symptoms of susceptibility, while the other one (E06-1a) is the only resistant line which allowed some degree of sporulation (weak resistance).

Correlations between the *HcrVf2* mRNA expression level and resistance of transgenic lines are not possible. Excluding the 115-bp promoter lines (discussed above), all the lines exhibited expression levels well above those of the cv. 'Florina' and showed practically the same resistance reaction (no symptoms).

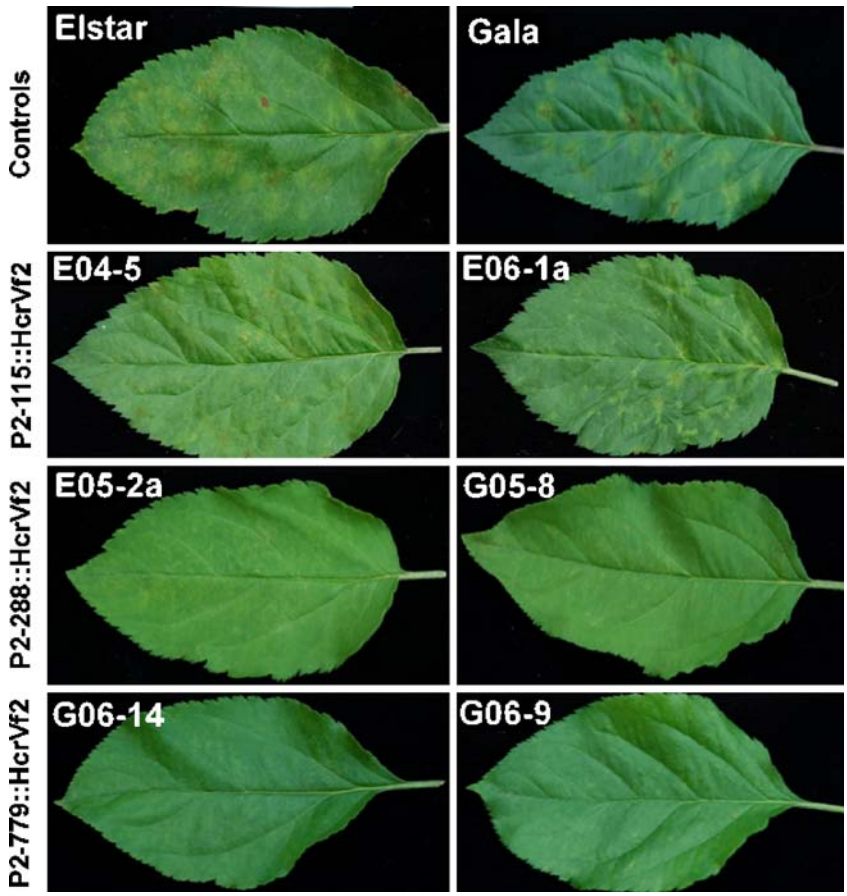

Fig. 4 Representative symptoms observed on transgenic lines harboring *HcrVf2* with different promoter lengths and controls 'Gala' and 'Elstar' 21 days after inoculation with *V. inaequalis* conidia. Untransformed 'Gala' and 'Elstar' were highly susceptible as well as 'Elstar' line E04-5 transformed with P2-115::HcrVf2. Leaves of transgenic 'Gala' and 'Elstar' plants harboring *HcrVf2* controlled by 288 or 779 bp of the promoter had in most cases no symptoms or yellow/chlorotic areas with no sporulation. The second line carrying the P2-115::HcrVf2 construct (E06-1a) is the only line considered as resistant that showed (reduced) sporulation with however typical resistance reaction like chlorosis and necrosis

In order to analyze the possibility of a dose effect of *Vf* more in detail, we additionally analyzed transcription levels in genotypes homo- and heterozygous for *Vf* obtained from a cross between 'Florina' (*Vfvf*) and 'Nova Easygro' (*Vfvf*). Homozygous *Vf* genotypes are generally more resistant against scab than genotypes having only one copy of *Vf* (Gessler et al. 1997). Indeed, the amount of *HcrVf2* mRNA was higher in homozygous genotypes than in heterozygous genotypes, but were below that of mRNA in plants transformed with P2-288::HcrVf2 and P2-799::HcrVf2. Thus, it can be concluded that in the promoter region, up to −779 bp binding sites for activators (up-regulating transcription factors) might be present, while reduced expression in *Vf* genotypes obtained by classical breeding might be due to the presence of binding sites from transcriptional repressors in further upstream regions. The increased mRNA levels in regenerated transgenic lines might also be due to the fact that these cvs. do not additionally harbor the other two transcribed genes of the *HcrVf* cluster. Expression of all the genes with high nucleotide sequence similarity in the *Vf* cvs. 'Florina' and the homo- and heterozygous progeny plants might also result in posttranscriptional gene silencing affecting the amount of mRNA. However, it is not known whether there is a direct correlation between mRNA levels and expression of the active mature HcrVf2 protein.

HcrVf2 has been shown to be constitutively expressed (Vinatzer et al. 2001; Xu and Korban 2002). All used promoter sequences used in this study also led to constitutive expression of *HcrVf2*, being above the native expression level of the *Vf* cv. 'Florina.' In a similar study, Malnoy et al. (2008) used the *HcrVf2* gene (renamed by Xu and Korban [2002] in *Vfa2*) along with at least 2 kb of the promoter region (and the own terminator). They could detect increased transcription compared to the untransformed non-*Vf* control cvs. only after inoculation with *V. inaequalis.* Plants used in our expression study were not previously inoculated with scab and however expressed *HcrVf2*. In the same way, *HcrVf2* mRNA was detected in uninoculated 'Florina' plants. In order to exclude the possibility that there was a gene induction due to other biotic stress factors present in the greenhouse, real-time PCR analyses were also performed with RNA isolated from *in vitro* plants grown under sterile conditions. Results were similar to those obtained from greenhouse-grown plants (data not shown), thus the expression of *HcrVf2* is constitutive but might be further up-regulated by a variety of stimuli. Constitutive expression is also likely because it is proposed that HcrVf2 acts as a receptor involved in recognition of the fungus *V. inaequalis*, therefore the protein should be present before pathogen attack. The fact that constitutive expression of *HcrVf2* leads to high resistance against *V. inaequalis* was already demonstrated by Belfanti et al. (2004), who used the CaMV 35S promoter to induce constitutive expression of *HcrVf2* in 'Gala.' In the study of Malnoy et al. (2008), the *HcrVf2* along with at least 2 kb of the promoter was introduced into 'Galaxy' (a mutant of 'Gala') and 'McIntosh' leading to partial scab resistance in the apple cvs. 'Galaxy' and 'McIntosh,' but did not provide the high level of resistance usually seen in *Vf* cvs. obtained by classical breeding. In contrast, the introduction of *HcrVf2* along with 288 or 799 bp of the promoter sequence leads to the development of an incompatible interaction between *V. inaequalis* and the otherwise susceptible cvs. 'Gala' and 'Elstar.' The plants were highly resistant, showing in most cases no symptoms after inoculation with *V. inaequalis*. The differences in the scab resistance response might be either due to the higher inoculum pressure or to the fact that, in the case of Malnoy et al. (2008), transformed noninoculated plants showed expression levels similar to those of untransformed non-*Vf* control plants. We observed high expression levels also in non-inoculated plants, probably allowing faster recognition of the elicitor and triggering of resistance responses.

As the plants of the various lines show no visible deviation from the habitus of their wild origin, this overexpression apparently causes no harm. These results aid further to implement the concept of cisgenic plants (Schouten et al. 2006a), as they show that using an apple own gene with its own promoter, it is possible to change a popular apple cv. such as 'Gala' and 'Elstar' from scab susceptible to scab resistant.

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