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Publication date:
2005-04

Permanent link:
https://doi.org/10.3929/ethz-b-000033099

Originally published in:
Theoretical and Applied Genetics 110(6), https://doi.org/10.1007/s00122-005-1940-9

Theor Appl Genet (2005) 110: 1119–1126
DOI 10.1007/s00122-005-1940-9

ORIGINAL PAPER

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Received: 27 October 2004 / Accepted: 24 January 2005 / Published online: 23 February 2005

Abstract Apple scab (*Venturia inaequalis*) is one of the most damaging diseases affecting commercial apple production. Some wild *Malus* species possess resistance against apple scab. One gene, *HcrVf2*, from a cluster of three genes derived from the wild apple *Malus floribunda* clone 821, has recently been shown to confer resistance to apple scab when transferred into a scab-susceptible apple variety. For this proof-of-function experiment, the use of the 35S promoter from Cauliflower mosaic virus was reliable and appropriate. However, in order to reduce the amount of non-plant DNA in genetically modified apple to a minimum, with the aim of increasing genetically modified organism acceptability, these genes would ideally be regulated by their own promoters. In this study, sequences from the promoter region of the three members of the *HcrVf* gene family were compared. Promoter constructs containing progressive 5′ deletions were prepared and used for functional analyses. Qualitative assessment confirmed promoter activity in apple. Quantitative promoter comparison was carried out in tobacco (*Nicotiana glutinosa*) and led to the identification of several promoter regions with different strengths from a basal level to half the strength of the 35S promoter from Cauliflower mosaic virus.

Keywords Apple scab · *HcrVf* resistance genes · *Venturia inaequalis* · Expression · Agroinfiltration

Communicated by L. Willmitzer

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Introduction

The *Vf* resistance originates from the wild apple accession *Malus floribunda* clone 821 and confers resistance to apple scab, caused by the fungal pathogen *Venturia inaequalis* (Williams and Kuc 1969). The *Vf* resistance forms a gene-for-gene relationship with strains of *V. inaequalis* (MacHardy 1996). Within the *Vf* region, three resistance gene homologues (named *HcrVf1*, *HcrVf2* and *HcrVf4*) were identified. Their sequence reflects leucine-rich repeat and transmembrane proteins domains, similar to the *Cf* resistance genes of tomato (Vinatzer et al. 2001). *HcrVf2* has recently been found to confer resistance to apple scab when transferred to the scab susceptible cultivar Gala (Belfanti et al. 2004; Barbieri et al. 2003). Transcripts from all three *HcrVf* genes are detected in leaves prior to fungal infection (Vinatzer et al. 2001). However, *HcrVf1* and *2* are highly expressed in young leaves, whilst another gene from the *Vf* region appears to have a higher expression level in older leaves (Xu and Korban 2002). This shows that some variation in expression levels of the individual open reading frames within the *HcrVf* gene family may occur.

The transfer of *HcrVf2* to a scab-susceptible apple variety, resulting in scab resistance, has helped to understand the role this gene plays in scab resistance (Belfanti et al. 2003). The role of the other *HcrVf* genes and the way in which these genes are regulated is still to be determined. Full understanding of the resistance mechanism may lead to alternative strategies for disease control of apple scab. For the application of such resistance genes for disease control, the use of a constitutive promoter such as the Cauliflower mosaic virus 35S promoter (CaMV 35S) is not desirable. Firstly, durable disease resistance using major resistance genes could only be achieved through the transfer of several resistance genes (pyramiding) (MacHardy et al. 2001). The use of the same promoter for several gene constructs can lead to homology-dependant transgene silencing (Vaucheret et al.

1998). Secondly, the use of genetic material of non-plant origin is one of the main criticisms of genetically modified plants. Use of tissue specific promoters from other plants could be considered. Promoters from tomato and soybean have been shown to be successful in apple; however, the specificity of the promoter may be reduced (Gittins et al. 2000). Therefore, for further studies and the application of these genes, they would ideally be regulated by their own promoter regions.

Stable genetic transformation of apple, although possible, is a lengthy and tedious process with a low success rate. Copy number and site of integration, in stable transformants, can affect transgene expression (Kumar and Fladung 2001; Iglesias et al. 1997). Therefore, for each construct studied, several transgenic lines have to be analysed in detail. Prior to stable transformation, screening of promoter regions using a transient expression assay, would be advantageous (Yang et al. 2000). Thus, the number of constructs to be studied in stably transformed plants could be reduced to a minimum, by disregarding constructs which perform poorly in the transient assay.

In order to identify functional regulatory sequences, increasing lengths of the upstream region from all three expressed *HcrVf* genes were fused to the *gusA* reporter gene. Qualitative assessment of GUS activity, confirming promoter functionality, was carried out in the apple cultivar Gala. Quantitative measurements via a fluorometric assay proved to be difficult in apple and therefore, for a more detailed comparison, the constructs were agroinfiltrated into *Nicotiana glutinosa*.

Materials and methods

Sequence analysis and plasmid construction

The *HcrVf1*, *HcrVf2* and *HcrVf4* genes were previously identified from BAC clones M18-1, M18-5 and M18-6, respectively, of a BAC library from the apple cultivar Florina (Vinatzer et al. 2001). Promoter sequences (GenBank AY397723, AY397724 and AY397725) for these genes where identified from the same BAC clones, compared and used to design primers for promoter constructs. All sequence alignments were made using Clustal W from the DNA Data Bank of Japan at http://www.ddbj.nig.ac.jp. Putative *cis*-acting elements were identified using the PLACE database (Higo et al. 1999) at http://www.dna.affrc.go.jp/htdocs/PLACE.

To determine the minimal region required for GUS expression, four 5′ deletion constructs from the upstream region of *HcrVf1* were prepared ranging from 115 bp to 1,200 bp in length (Fig. 1). To investigate the significance of sequence variation between the upstream regions of all three genes further 5′ deletion constructs for *HcrVf2* and *4* were made.

The promoter regions, of the defined lengths, were amplified by *Pfu* *Taq* polymerase (Promega, Madison, Wis., USA), using a reverse primer (HcrVf1-4R or R-2R, these primers differ only by the restriction site inserted) starting directly upstream of the ATG start codon and a specific forward primer (Table 1). The BAC clones listed above were used as templates for PCR amplification, except for the *HcrVf2* deletion constructs, which were amplified from a subclone of M18-5. Restriction sites for cloning were introduced via the PCR primer (Table 1). Binary vector pCambia1381Z (AF234306), containing a *gusA* gene with a catalase intron to prevent GUS expression in *Agrobacterium* and convenient cloning sites, was used for all constructs. PCR products and pCambia1381Z were digested with the appropriate enzyme (Table 1) and ligated overnight using T4 DNA ligase (Roche Diagnostics, Switzerland). Plasmids were transferred to MAX Efficiency DH5α competent *Escherichia coli* cells (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Positive colonies were identified by colony PCR and verified by sequencing.

As positive control, a pCambia1381Z construct containing the CaMV 35S promoter (p35SGUS) was prepared. The binary vector pCambia2301 was used as template for amplification of the CaMV 35S promoter

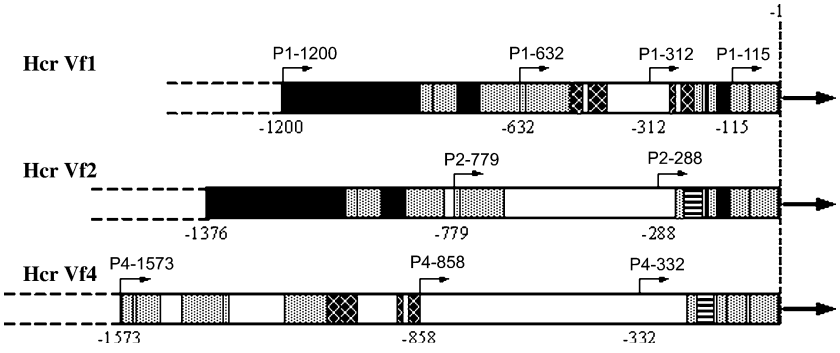

Fig. 1 Sequence identity of 90% or higher between 5′ sequences. *Shaded areas* between all three promoters considered, *black* between *HcrVf1* and *HcrVf2*, *grid* between *HcrVf1* and *HcrVf4*, *stripe* between *HcrVf2* and *HcrVf4*. Regions of high sequence identity appear in the same order for all of the considered promoters. The *labelled arrows* represent the 5′ end of the promoter deletion constructs. All constructs are terminated by a thymine base located in position −1. Distances, shown in base pairs, are relative to the translation start. (Fragments are drawn to scale)

Table 1 Sequences of the primers used for the amplification of the *HcrVf1*, *HcrVf2* and *HcrVf4* promoter regions and length of the generated constructs

Deletion construct[a]	Primer[b]	Sequences[c] (5′ → 3′)	Restriction enzyme
HcrVf1			
P1-1200	HcrVf1-12 F	GACCACGAAATTGGATTTATTG	*Sma*I
	HcrVf1&4 R	GCGTAGCGTGCCAAGCTTAAAGTTCAAGTGTATGAAGC	*Sma*I
P1-632	HcrVf1-6 F	GCTAACCGAT GTCGACCATTCAATGACAGTCTGATC	*Sal*I
	HcrVf2 R	CGCACGGCAT CTGCAGAAAGTTCAAGTGTATGAAGC	*Pst*I
P1-312	HcrVf1-3 F	GATCGATTTAGC GGATCCGGGGTCTTAAATTCCACACG	*Bam*HI
	HcrVf1&4 R	GCGTAGCGTGCC AAGCTTAAAGTTCAAGTGTATGAAGC	*Hin*dIII
P1-115	HcrVf1-1 F	GCTAACCGAT GGATCCTCTCATGCCGTAAAGGATGG	*Bam*HI
	HcrVf1&4 R	GCGTAGCGTGCC AAGCTTAAAGTTCAAGTGTATGAAGC	*Hin*dIII
HcrVf2			
P2-779	HcrVf2-8 F	GCTAACCGAT GTCGACCATTCAATGACAGTCTGATC	*Sal*I
	HcrVf2 R	CGCACGGCAT CTGCAGAAAGTTCAAGTGTATGAAGC	*Pst*I
P2-288	HcrVf2-3 F	TCATACCGAT GTCGACATTCCAAGTGGGGTCTTAGA	*Sal*I
	HcrVf2 R	CGCACGGCAT CTGCAGAAAGTTCAAGTGTATGAAGC	*Pst*I
HcrVf4			
P4-1573	HcrVf4-16 F	TGATCGTGTA GGATCCCTTTAGTCTTAGCTACGACT	*Bam*HI
	HcrVf1&4 R	GCGTAGCGTGCC AAGCTTAAAGTTCAAGTGTATGAAGC	*Hin*dIII
P4-858	HcrVf4-9 F	TGGTGCCATC GGATCCCTCTGAAGGTAAATAGAAAA	*Bam*HI
	HcrVf1&4 R	GCGTAGCGTGCC AAGCTTAAAGTTCAAGTGTATGAAGC	*Hin*dIII
P4-332	HcrVf4-3 F	TATTCTCAGG GGATCCCCCAGCTTAGGTGGGCATATT	*Bam*HI
	HcrVf1&4 R	GCGTAGCGTGCC AAGCTTAAAGTTCAAGTGTATGAAGC	*Hin*dIII

[a]Promoter constructs listed according to the gene from which they originate. Construct names indicate firstly the *HcrVf* gene number and then, separated by the hyphen, the length of the construct. The primer restriction sites are not included in the length

[b]*F* Forward primers, *R* reverse primers

[c]Restrictions sites used for cloning the PCR products into the binary vector pCambia1381Z are *underlined*. The *HcrVf*1−12 fragment was blunt end cloned into *Sma*I

with the following primers: forward, 5′-GATCGATTTAGCGGATCCCATGGAGTCAAAGATTCAAA-3′ and reverse, 5′-GCGTAGCGTGCCAAGCTTAGTCCCCCGTGTTCTCTCCA-3′, containing *Bam*HI and *Hin*dIII restriction sites, respectively. Vector and PCR product were fused and the resulting plasmid (p35SGUS) controlled as above described.

Binary plasmids containing the correct promoter sequences, p35SGUS and an original pCambia1381Z (as negative control) were transferred to *Agrobacterium tumefaciens* EHA105 strain containing helper plasmid pCH32 (Hamilton 1997) according to Höfgen and Willmitzer (1988).

Agrobacterium-mediated infection of apple explants and GUS histochemical assay

Cut leaves, internode sections and petioles, detached from in vitro-cultured shoots of cultivar Gala, were preconditioned overnight on filter paper soaked with liquid TN505 (Barbieri et al. 2003). Explants were infected according to James et al. (1993) with *A. tumefaciens* EHA105 containing the binary plasmids described above. The p35SGUS and pCambia1381Z were used as positive and negative controls, respectively. After 4 days' co-cultivation on solid TN505 medium, the explants were sub-cultured on the same medium containing 200 mg/l cefotaxime (decontamination) and 10 mg/l hygromycin (selection) until the last evaluation 24 days after infection.

Staining of apple explants was carried out 7, 10, 13, 17, 20 and 24 days after infection. The analysis of GUS staining was performed essentially as described by Jefferson et al. (1987). For each construct, a total of 55 samples (five samples from the first evaluation and ten samples from the others) were exposed to 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-GlcA) and incubated overnight at 37°C. The following day, samples were bleached by immersion in a solution containing 50 mg/ml citric acid and 75 mg/ml ascorbic acid and observed for development of blue dye under the microscope and high-resolution scanner.

Agroinfiltration of tobacco leaves and GUS fluorometric assay

Agrobacterium containing the binary vectors described above were grown for 48 h in 10 ml YEB media (DeBondt et al. 1994) containing 50 mg/l kanamycin, 25 mg/l nalidixic acid and 5 mg/l tetracycline. A further 80 ml YEB containing kanamycin and tetracycline at the same concentrations was inoculated with 5 ml from the first culture, and *Agrobacterium* were re-grown overnight. Cells were harvested by centrifugation (2,500 *g*, 10 min) and resuspended to a final OD_{600} of 1.5 in the following solution: (per litre) 4.4 g MS with vitamins (Duchefa, The Netherlands), 30 g sucrose, 1.0 mM L-proline (Sigma-Aldrich, St. Louis, Mo., USA), 0.1 mM acetosyringone (Fluka, Basel, Switzerland), pH 5.2. *Agrobacterium* were left to stand at room temperature for 2 h prior to infiltration. *N. glutinosa* leaves were infiltrated with *Agrobacterium*, using either a 1-ml insulin syringe (U-40, Becton, Dickinson and Company, Dun Laoghaire, Ireland) or a 1-ml syringe with no needle (B-D, Becton,

Dickinson and Company). Four sections of a single leaf were injected for each deletion construct: one section with the p35SGUS, one section with the promoterless pCambia1381Z (negative control) and two sections with one of the pCambia1381Z constructs containing *HcrVf* promoter sequence. Five leaves per *HcrVf* deletion construct were injected for each experiment; all experiments were carried out three times.

Tobacco leaf material, from the infiltrated area, was harvested 72 h after agroinfiltration. Fluorometric GUS assays were carried out according to Fütterer et al. (1995), with the following modifications: leaf material was ground in a 2-ml Eppendorf tube using 2–3 glass beads (1 mm) and a bead mill for 2 min; crude protein extracts were harvested after 10 min centrifugation (15,700 *g*) at 4°C. Fluorescence was measured at 360 nm excitation and 450 nm emission using a Spectra Fluro Plus fluorometer (Tecan AG, Männerdorf, Switzerland). Total protein concentrations were determined according to Bradford (1976), using protein assay dye reagent concentrate (Bio-Rad, Munich, Germany) for the assay and bovine serum albumin as the standard. GUS activity was calculated as nM 4-MU/min/mg protein.

Each deletion construct was injected twice into each leaf therefore an average of these two values was considered. For each leaf studied, the average GUS activity from the deletion construct and GUS activity from pCambia1381Z were normalised to that of the p35SGUS construct. All statistical values were calculated using SYSTAT10 software.

Results

Sequence analysis

Alignment of the three promoter sequences (Fig. 1) revealed 80% identity between *HcrVf1* and *HcrVf2*, 40% between *HcrVf4* and *HcrVf1* and 26% between *HcrVf4* and *HcrVf2*. Local alignments showed various regions of higher homology (Fig. 1). Sequences between −1 and −115 of all three genes have at least 95% identity; apart from single-base pair mutations the only difference, as previously reported by Xu and Korban (2002), is the insertion of a TCCCT sequence at −75 in *HcrVf4*. Other regions of high sequence identity were found within the most distal portions at the 5′ end of the sequence (Fig. 1).

Construct design was based upon differences observed between promoter sequences. Four constructs of 115, 312, 632 and 1,200 bp directly upstream of *HcrVf1* were prepared. Due to the high sequence similarity between the promoter regions of *HcrVf1* and *HcrVf2* only two constructs of 288 bp and 779 bp from *HcrVf2* were designed, which cover regions of greatest sequence discrepancy between −229 and −667 (relative to *HcrVf2*). Due to the lower sequence identity between the *HcrVf4* promoter and the other two sequences, three constructs of 332, 858 and 1,573 bp were prepared for this gene, which cover regions of sequence variation. As the first 115 bp upstream are quite conserved, no construct of this length was made from *HcrVf2* or *HcrVf4*.

For the identification of putative *cis*-acting elements within the *HcrVf* promoter sequences, the publicly available database PLACE was used (Higo et al. 1999). Database analysis identified many *cis*-acting elements within the sequences studied. Among these were several *cis*-acting elements, which occur frequently within the *HcrVf* promoter regions studied and which are also found in the promoter regions of light-regulated genes (Table 2; Terzaghi and Cashmore 1995). *Cis*-acting elements from tissue-specific and stress response genes were also present, as were W boxes, which are found in the promoter regions of many plant defence genes (Table 2; Rushton et al. 1996; Rieping and Schoffl 1992).

Table 2 Frequency and type of some putative *cis*-acting elements found in *HcrVf* promoter sequences

Cis-element	Putative function or gene of origin	*HcrVf1*[a]			*HcrVf2*[a]		*HcrVf4*[a]		
		I	II	III	I	II	I	II	III
CAAT	Tissue specific, enhancement	2	4	5	1	10	2	7	6
CCAATBOX1	Bind enhancers	1	3	1	–	2	2	1	3
DOF	Bind Dof transcription factors	1	3	7	2	7	2	8	8
GATA	Light-regulated and tissue-specific genes	2	5	7	1	6	3	1	12
GT-1 consensus	Light-regulated genes	1	–	8	3	2	1	8	5
GTGA	Late-pollen *g10* gene and tomato *lat59*	–	–	9	1	5	1	5	7
I box	Light-regulated genes	3	4	2	2	2	2	–	5
S1F box	Binds S1F (negative element)	–	1	–	–	1	–	1	1
W box	Defence-related genes	–	1	–	–	3	–	1	2

A selection of putative *cis*-acting elements found in regulatory regions of *HcrVf* genes using the PLACE database (Higo et al. 1999). CCAAT box (Rieping and Schoffl 1992; Haralampidis et al. 2002); Dof (Yanagisawa and Schmidt 1999); GATA (Gidoni et al. 1989; Schindler and Cashmore 1990); GT-1 consensus (Terzaghi and Cashmore 1995); GTGA (Rogers et al. 2001); I box (Terzaghi and Cashmore 1995); S1F binding site, negative regulator (Zhou et al. 1992); W box, defense related (Rushton et al. 1996)

[a]The sequences searched are as follows:
HcrVf1: I: from −115 to −312; II: from −313 to −632; III from −633 to −1200
HcrVf2: I: from −115 to −288; II from −289 to −779
HcrVf4: I: from −115 to −332; II from −333 to −858; III from −859 to −1573

Table 3 GUS staining of apple explants transiently transformed with 5′ deletion promoter constructs

Construct in *Agrobacterium*	Number of explants[a]	Total number of dark-blue spots[b]
Positive control (p35SGUS)	39	98
Negative control (pCambia1381Z)	0	0
P1-115	9	4
P1-312	12	10
P1-632	13	7
P1-1200	3	2
P2-288	10	10
P2-779	16	42
P4-332	13	10
P4-858	24	18
P4-1573	10	19

Number of explants showing either diffuse pale-blue staining or dark-blue spots during the six evaluations. Number of dark-blue spots observed on the leaf explants that reacted to 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-GlcA)

[a]Randomly chosen explants, number showing blue staining (both pale and intense) from a total of 55 observed

[b]On explants which reacted to X-GlcA (i.e. number of explants)

GUS histochemical assay on apple explants

Between 7 days and 24 days after infection, for each of the 5′ deletion constructs, at least one apple leaf explant exhibited some blue staining, following treatment with X-GlcA (Table 3). Such staining was also observed on samples transformed with the positive control (p35SGUS). All samples were easily distinguishable from the samples infected with pCambia1381Z, where blue staining was never observed (Table 3).

Different kinds of staining were revealed (Fig. 2), from diffuse pale-blue areas to dark-blue spots on each type of explant tested (leaves, petioles, internode explants and proliferating calli) (Table 3).

Promoter activity comparison via a GUS fluorometric assay in tobacco

Deletion constructs of the 5′ regulatory sequence of genes *HcrVf1*, *HcrVf2* and *HcrVf4* and control plasmids were successfully agroinfiltrated into tobacco leaves. This transient assay proved to be a good system for the study of promoter sequences as shown by Yang et al. (2000). The p35SGUS positive control drove high levels of GUS synthesis (on average approximately 4,800 nM 4-MU/min/mg total protein) easily distinguishable from the promoterless pCambia1381Z (on average approximately 50 nM 4-MU/min/mg total protein). The promoterless pCambia1381Z alone consistently produced very low GUS activity rarely exceeding 100 nM 4-MU/min/mg total protein. Despite using plants and leaves of a similar age, considerable variation in overall GUS activity was observed between repeats. However, agroinfiltration of a deletion construct and the two controls, into a single tobacco leaf, allowed standardisation

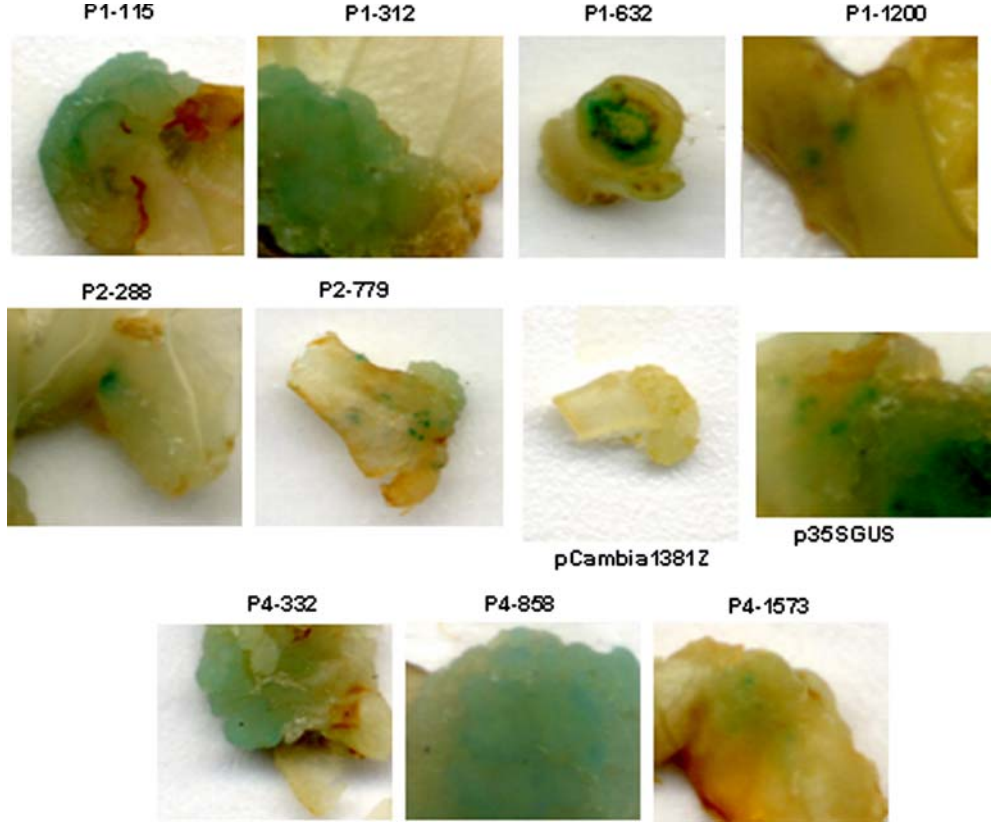

Fig. 2 The GUS staining of apple explants between 7 days and 24 days after infection with individual *HcrVf* promoter:: *gusA* constructs, p35SGUS (*positive control*) or pCAMBIA1381Z (*negative control*)

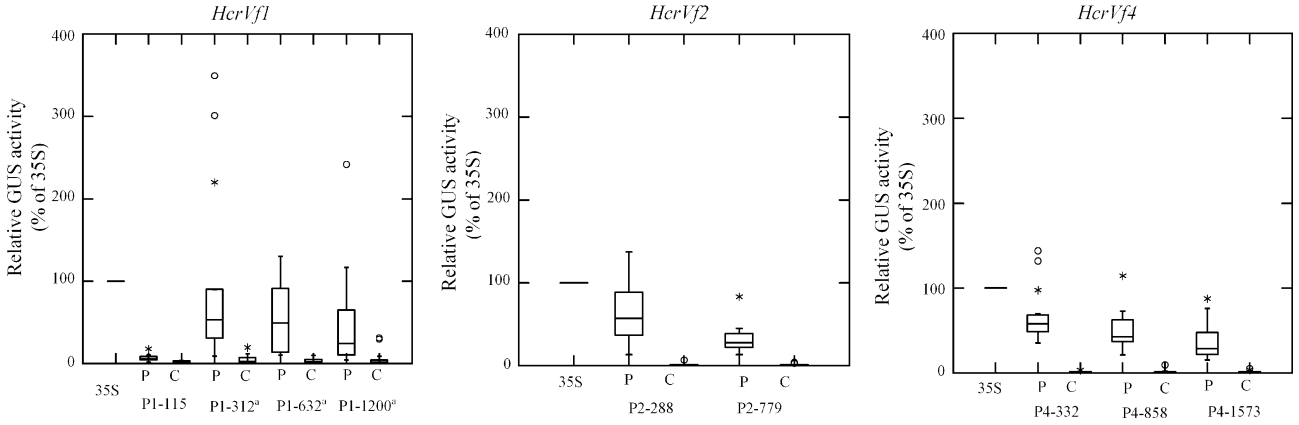

Fig. 3 Relative GUS activity measured in tobacco leaf tissue agroinfiltrated with *HcrVf* promoter:: *gusA* constructs. For each leaf, two sections were infiltration with *HcrVf* promoter constructs; therefore, the average of these two values was taken. For each individual leaf studied, GUS activity, calculated in nM 4-MU/mg protein/min, of the deletion construct (*P*) and promoterless pCambia1381Z (*C*) was normalised to that of p35SGUS (*35S*). *Box plots* show results from 15 individual leaves (three experiments, each of five leaf samples) for each construct; the *line within the box* shows the median and the *upper and lower hinges* represent quartiles. *Stars* and *small circles* represent outlying data. *Numbers* indicate for P, the promoter construct, and for C, the activity from the promoterless pCambia1381Z control infiltrated into the same leaf. For P1-312 only 13 samples, and for P1-632 and P1-1200 only 14 samples were included; other samples were excluded due to low GUS activity from all constructs including the positive control (*35S*)

Table 4 Pair-wise statistical comparisons of promoter construct activity after Agroinfiltration into *Nicotiana glutinosa* (shown in Fig. 3), using the Wilcoxon signed-rank test. Values shown are probabilities (*P*) that there is no statistical difference. All data, including outliers, were included. Only those leaves showing uniformly low GUS activity (two data sets for P1-312 and one each for P1-632 and P1-1200) were excluded

	CAMV35S	P1-115	P1-312	P1-632	P1-1200	P2-288	P2-779	P4-332	P4-858
P1-115	0.001								
P1-312	0.507	0.001							
P1-632	0.008	0.001	0.701						
P1-1200	0.019	0.002	0.382	0.594					
P2-288	0.009	0.001	0.552	0.594	0.221				
P2-779	0.001	0.001	0.055	0.074	0.778	0.015			
P4-332	0.005	0.001	0.917	0.198	0.198	0.650	0.003		
P4-858	0.001	0.001	0.753	0.925	0.124	0.570	0.031	0.078	
P4-1573	0.001	0.001	0.101	0.331	0.778	0.061	0.460	0.031	0.088

between leaves (Fig. 3). The variation between the two sections, from one leaf, agroinfiltrated with the promoter construct was on average 500 nM 4-MU/min/mg total proteins. The average GUS activity from the deletion constructs and the promoterless pCambia1381Z were normalised to that of the p35SGUS value from the same leaf. Comparison of the GUS activity from deletion constructs, with that of the two controls, showed that patterns of expression were consistent (Fig. 3). Although there were a few outlying values, at the most there were three per construct (observed for P1-312 and P4-332) and as all outlying values where higher than any other previous measurement, if anything, the efficiency of the deletion constructs has been underestimated.

A few leaves proved to be unsuitable for agroinfiltration, probably due to leaf age. In such leaves GUS activity from the p35SGUS control was very low (<150 nM 4-MU/mg protein/min) and indistinguishable from the promoterless control. All data from such leaves (four in total) were discarded from the final analysis (from infiltration with P1-312, the data from two leaves and for P1-632 and P1-1200 the data from one leaf were excluded from the final analysis).

Construct P1-115 from *HcrVf1*, which is only 115 bp in length, showed markedly reduced expression compared to other deletion constructs (Fig. 3). Sequences 270–650 bp upstream of the ATG of each gene all showed GUS activity equivalent to approximately 50% of that of the CaMV 35S, being constructs P1-312 (312 bp), P1-632 (632 bp) from *HcrVf1*, P2-288 (288 bp) from *HcrVf2* and P4-332 (332 bp) from *HcrVf4*. Constructs containing longer upstream sequences, P1-1200, P2-779 and P4-1573, appeared to show reduced activity in comparison to shorter deletion constructs. Statistical analysis (using the Wilcoxon signed-rank test) of all the normalised data, including outliers, showed that all deletion constructs (including the shortest sequence in P1-115) were different from the promoterless pCambia1381Z ($P< 0.005$). P1-115 was also different from all other 5′-deletion constructs ($P< 0.005$). All except P1-312 were also statistically different from the p35SGUS construct (for P1-312, $P=0.507$; all others, $P< 0.05$).

Statistical differences ($P < 0.05$) were also found between P2-779 and P2-288 ($P = 0.015$), P2-779 and P4-332 ($P = 0.003$), P2-779 and P4-858 ($P = 0.031$) and between P4-332 and P4-1573 ($P = 0.031$). No other statistically significant differences were observed between other deletion constructs (Table 4).

Discussion

This work, based on an *Agrobacterium*-mediated transient expression assay, has lead to the identification of apple promoter sequences from all three *HcrVf* genes identified in the *Vf* region.

In apple, a GUS histochemical assay, performed after *Agrobacterium*-mediated transient transformation, demonstrated that all promoter sequences function in the tested material. The variation in strength and frequency of GUS staining between individual samples may reflect a change in gene activity but could also be due to transformation rate. Therefore, as the transformation rate and promoter efficiency cannot be separated, the results must be considered qualitative and not quantitative. However, the fact that GUS activity was observed from all promoter constructs proves their functionality in apple.

For a quantitative comparison, agroinfiltration into *N. glutinosa* followed by fluorometric assessment of GUS levels was performed. To analyse each *HcrVf* promoter construct, p35SGUS, the promoterless pCambia1381Z and the promoter construct were agroinfiltrated into a single tobacco leaf. Agroinfiltration of deletion constructs and controls into a single tobacco leaf allows the comparison of promoter efficiency and reduces the chances of physiological differences in the plant influencing the GUS activity as demonstrated by Yang et al. (2000). Due to the morphology of apple leaves agroinfiltration, particularly of four different *Agrobacterium* in one leaf, was not feasible in this species.

In *N. glutinosa*, P1-115, which contains only 115 bp of promoter sequence, exhibited very low levels of GUS activity which were statistically different to all other deletion constructs. This section contains a TATA box between −70 and −64, and the transcription start has been estimated to be at −36 (Vinatzer et al. 2001). Although GUS activity from P1-115 was low, it was still statistically distinguishable from the promoterless control. Furthermore, apple explants transformed with this construct reacted to X-GlcA treatment, showing that this promoter functions in both apple and tobacco cells. However, due to its reduced activity in tobacco, it is mostly likely to be an inefficient promoter and should be considered as being the minimal sequence required for expression.

Promoter regions showing activity equivalent to 50% of the CaMV35S promoter activity were identified for all three genes being P1-312 or P1-632, P2-288 and P4-332 for *HcrVf1*, *HcrVf2* and *HcrVf4*, respectively. These would most likely be suitable candidates for stable apple transformation with the corresponding *HcrVf* genes, for investigations or applications requiring homologous promoters, as they showed the highest rate of expression among the promoter regions tested.

Many putative *cis*-acting elements were found within the *HcrVf* promoter regions. The high number of *cis*-acting elements originating from light-regulated genes indicates that expression of *HcrVf* genes could be light regulated (Terzaghi and Cashmore 1995). This would be consistent with the fact that *HcrVf* genes are apple scab resistance genes and that apple scab is primarily a leaf pathogen. It would also fit observations by Beech and Gessler (1986) that apple calli, incubated in total darkness, of *Vf*-cultivars and scab-susceptible cultivars react to *V. inaequalis* in the same way. The discovery of *cis*-acting elements from defence-related genes is interesting, considering the involvement of *HcrVf2* in plant resistance to a pathogen (Rushton et al. 1996). Investigating the significance of these *cis*-acting elements experimentally by excluding these regions from promoter sequences could help to elucidate the factors, which affect *HcrVf* gene regulation.

In conclusion, at least one promoter region, producing GUS activity equivalent to 50% of the activity from the CaMV 35S promoter, was identified for each gene studied. Quantitative assays showed that promoter sequences of less than 115 bp or greater than 1 kb should be avoided, due to reduced activity in tobacco. The number of potentially useful promoter constructs to be tested in stably transformed apple has therefore been reduced and should ease further studies.

Due to the sequence similarity between the *HcrVf* genes, the accurate comparison of expression levels (via real-time RT-PCR or Northern blotting) is difficult in a *Vf* cultivar (where all three genes are present). The transfer of *HcrVf* genes, under the control of their own promoters, to a scab susceptible apple cultivar (i.e. one containing no resistance genes with high homology to *HcrVf* genes) would ease the comparison of the expression level. The most efficient promoters, found in this study, would be good candidates for this purpose.

The results also provide a further step towards creating a genetically modified scab-resistant apple without cross-species transfer of genetic material. *HcrVf2* has recently been shown to induce scab resistance in a previously susceptible cultivar. The best performing promoter from this gene, together with the open reading frame, should now be tested in stable apple transformation, to see if the high level of resistance observed in CaMV35S:: *HcrVf2* plants is retained.

Acknowledgements We would like to thank Vicente Martinez (Swiss Federal Institute for Technology Zürich) for his technical assistance, Renier van der Hoorn (Wageningen University, The Netherlands) for his helpful advice on agroinfiltration experiments, Serena Maria Granozio (University of Bologna) for help during transformation and Tecan AG, Switzerland for the loan of the spectrofluorometer. This project is supported financially by the Swiss National Foundation grant 31-58914.99 and the Italian Ministry of Agriculture and Forestry (MiPAF).

References

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

Beech I, Gessler C (1986) Interaction between *Venturia inaequalis* and apple callus tissue cultures: an electron microscopic study. J Phytopathol 116:316–324

Belfanti E, Silfverberg-Dilworth E, Tartarini S, Patocchi A, Barbieri M, Zhu J, Vinatzer BA, Gianfranceschi L, Gessler C, Sansavini S (2004) The *HcrVf2* gene from a wild apple confers scab resistance to a transgenic cultivated variety. Proc Natl Acad Sci USA 101:886–890

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Debondt A, Eggermont K, Druart P, Devil M, Goderis I, Vanderleyden J, Broekaert WE (1994) *Agrobacterium*-mediated transformation of apple (*Malus* × *Domestica* Borkh.)—an assessment of factors affecting gene-transfer efficiency during early transformation steps. Plant Cell Rep 13:87–593

Fütterer J, Gisel A, Iglesias V, Klöti A, Kost B, Scheid OM, Neuhaus G, Neuhaus-Url G, Schrott M, Shillito R, Spangenberg G, Wang ZY (1995) Enzyme assay for beta-glucuronidase. In: Potrykus I, Spangenberg G (eds) Gene transfer to plants. Springer, Berlin Heidelberg New York, pp 256–260

Gidoni D, Brosio P, Bondnutter D, Bedbrook J, Dunsmuir P (1989) Novel *cis*-acting elements in petunia cab gene promoters. Mol Gen Genet 215:337–344

Gittins JR, Pellny TK, Hiles ER, Rosa C, Biricolti S, James DJ (2000) Transgene expression driven by heterologous ribulose-1,5-bisphosphate carboxylase/oxygenase small-subunit gene promoters in the vegetative tissues of apple (*Malus pumila* Mill.). Planta 210:232–240

Hamilton CM (1997) A binary-BAC system for plant transformation with high-molecular-weight DNA. Gene 200:107–116

Haralampidis K, Milioni D, Rigas S, Hatzopoulos P (2002) Combinatorial interaction of *cis*-element specifies the expression of the Arabidopsis *AtHsp90-1* gene. Plant Physiol 129:1138–1149

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant *cis*-acting regulatory DNA elements (PLACE) database. Nucleic Acids Res 27:297–300

Hofgen R, Willmitzer L (1988) Storage of competent cells for *Agrobacterium* transformation. Nucleic Acids Res 16:9877–9877

Iglesias VA, Moscone EA, Papp I, Neuhuber F, Michalowski S, Phelan T, Spiker S, Matzke M, Matzke AJM (1997) Molecular and cytogenetic analyses of stably and unstably expressed transgene loci in tobacco. Plant Cell 9:1251–1264

James DJ, Uratsu S, Cheng JS, Negri P, Viss P, Dandekar AM (1993) Acetosyingone and Osmoprotectants like betaine or proline synergistically enhance *Agrobacterium*-mediated transformation of apple. Plant Cell Rep 12:559–563

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucoronidase as a sensitive and versatile genefusion marker in higher plants. EMBO J 6:3901–3907

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

MacHardy WE (1996) Apple scab. APS, St. Paul, pp 29–38

MacHardy WE, Gadoury DM, Gessler C (2001) Parasitic and biological fitness of *Venturia inaequalis*: relationship to disease management strategies. Plant Dis 85:1036–1051

Rieping M, Schoffl F (1992) Synergistic effect of upstream sequences, CCAAT box elements, and Hse sequences for enhanced expression of chimeric heat-shock genes in transgenic tobacco. Mol Gen Genet 231:226–232

Rogers HJ, Bate N, Combe J, Sullivan J, Sweetman J, Swan C, Lonsdale DM, Twell D (2001) Functional analysis of *cis*-regulatory elements within the promoter of the tobacco late pollen gene *g10*. Plant Mol Biol 45:577–585

Rushton PJ, Torres JT, Parniske M, Wernert P, Hahlbrock K, Somssich IE (1996) Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley *PR1* genes. EMBO J 15:5690–5700

Schindler U, Cashmore AR (1990) Photoregulated gene-expression may involve ubiquitous DNA-binding proteins. EMBO J 9:3415–3427

Terzaghi WB, Cashmore AR (1995) Light-regulated transcription. Annu Rev Plant Physiol Plant Mol Biol 46:445–474

Vaucheret H, Beclin C, Elmayan T, Feuerbach F, Godon C, Morel JB, Mourrain P, Palauqui JC, Vernhettes S (1998) Transgene-induced gene silencing in plants. Plant J 16:651–659

Vinatzer BA, Patocchi A, Gianfranceschi L, Tartarini S, Zhang HB, Gessler C, Sansavini S (2001) Apple contains receptor-like genes homologous to the *Cladosporium fulvum* resistance gene family of tomato with a cluster of genes co-segregating with *Vf* apple scab resistance. Mol Plant–Microbe Interact 14:508–515

Williams EB, Kuc J (1969) Resistance in *Malus* to *Venturia inaequalis*. Ann Rev Phytopathol 7:223–246

Xu ML, Korban SS (2002) A cluster of four receptor-like genes resides in the *Vf* locus that confers resistance to apple scab disease. Genetics 162:1995–2006

Yanagisawa S, Schmidt RJ (1999) Diversity and similarity among recognition sequences of Dof transcription factors. Plant J 17:209–214

Yang YN, Li RG, Qi M (2000) In vivo analysis of plant promoters and transcription factors by agroinfiltration of tobacco leaves. Plant J 22:543–551

Zhou DX, Li YF, Rocipon M, Mache R (1992) Sequence-specific interaction between S1f, a spinach nuclear factor, and a negative *cis*-element conserved in plastid-related genes. J Biol Chem 267:23515–23519