Progress of Map-based Cloning of the *Vf*-resistance Gene and Functional Verification: Preliminary Results from Expression Studies in Transformed Apple

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Additional index words. Venturia inaequalis, *Malus* ×*domestica*, *Vf*-resistance gene cloning, genetic transformation, GM-apples

Host resistance against disease has been widely used in many crops with success. For more than 50 years, apple breeding programs in the United States, Europe, and elsewhere throughout the world have attempted to develop cultivars resistant to scab (*Venturia inaequalis*) via the introgression of major genes originating from wild *Malus* species. Indeed, today more than 100 cultivars have been released, most of which carry the *Vf* gene from *Malus floribunda* 821 and a few other resistance genes. Yet these accomplishments have not translated into commercial success, as growers have largely ignored the new varieties and continued to cultivate scab-susceptible varieties despite the costly outlays for fungicide treatments such cultivars entail. The reasons behind this reluctance to adopt the new cultivars reside in weak marketing campaigns, unsatisfactory fruit and storage quality, and the fact that races 6 and 7 of the pathogen appear to have overcome *Vf* resistance in some European districts.

The optimal approach would be to employ an already successful cultivar and add resistance to it. It was thus a logical step to attempt to transfer resistance genes to the most widely grown and marketed, but scab-susceptible, apple cultivars. Some attempts in this direction have been reported using heterologous genes, i.e., not of the apple species itself, known for their antifungal resistance, the most noteworthy among these transformations being the endochitinase gene of *Trichoderma* (Aldwinckle et al., 2000). While the results achieved so far have been important, these experimental efforts are perhaps best viewed as preliminary steps towards a more complete and effective transformation with homologous genes, i.e., those of apple itself. Success in achieving a transformant cultivar to replace its original may best be approached if the transformant has no apple-foreign DNA and the probability of maintaining the durability of the resistance for at least the life of an orchard is high.

Malus ×*domestica* has a large array of resistance genes, although their effect is no longer effective as the pathogen population has adapted to an extent proportional to the selection pressure exerted by the various cultivars (Gessler, 1989; Koch et al., 2000; MacHardy et al., 2001). Even in 'Golden Delicious' a resistance gene (*Vg*) has been identified (Bénaouf and Parisi, 1997; Durel et al., 2000), but it is not effective in all areas because where this cultivar is popular, the pathogen population carries the corresponding virulence gene. It is thus reasonable to view *Vf* transformation as a first step in a "gene therapy" aimed at correcting the susceptibility of apple genotypes to scab and restoring resistance to the plant. Clearly, the adaptation of the pathogen population will follow and new genes will have to be added. In other words, this approach attempts to achieve the same results as the decades-old traditional breeding programs but in a much shorter time, with the additional advantage of safeguarding the prized traits of the best cultivars while introducing resistance to *Venturia*.

The present paper summarizes a long series of molecular studies on apple, largely conducted as a joint effort by the DCA, University of Bologna, and the Phytopathology research group of the Swiss Federal Institute of Technology, Zurich (ETH). Section 1 is based on published literature and sections 2 to 5 provide preliminary results, as yet unpublished, and can be taken as indicating the current state of the art. The findings recorded to date are encouraging, although they are not yet final proof of identification of the *Vf* resistance gene.

1. Positional cloning of HcrVf candidate genes. Three RAPD markers associated with the *Vf* gene were found by Koller et al. (1994) and Tartarini (1996) and were transformed into more reliable PCR markers. Marker M18 was transformed into a CAPS marker (Gianfranceschi et al., 1996), while AM19 and AL07 were transformed into SCAR markers (Tartarini et al., 1999). M18 mapped to one side of the *Vf* locus and AM19 and AL07 to the other. Patocchi et al. (1999a) determined via physical mapping that the maximum distance between the markers M18 and AL07 was less than 870 kb. This meant it was feasible to cover the distance between these markers by chromosome walking using a large-insert DNA library. Vinatzer et al. (1998) constructed a BAC library of the scab-resistant cultivar Florina, heterozygous for the *Vf* gene. This library has an average insert size of 120 kb and consists of ≈36,000 clones, thus representing more than five times the haploid genome of apple and meaning that there is a greater than 99% chance of finding any apple gene in this library. A chromosome walk starting from two molecular markers (M18 and AM19) flanking *Vf* was performed using the apple BAC library. Nine steps of chromosome walking were necessary to identify the 13 BAC clones that span the region between the two markers. The size of the resulting "resistant" contig (contig in coupling with the *Vf* gene) is about 550 kb. A gap on the "susceptible" chromosome could not be closed, probably because no clones corresponding to this gap exist in the apple BAC library (Patocchi et al.,1999b).

Markers were then derived from the identified BAC clones and the possible location of the *Vf* gene was restricted to seven BAC clones covering 350 kb. Only five BAC clones were needed for a minimum tiling path spanning the *Vf* region. These five BAC clones were then used to screen a cDNA library of 'Florina' leaves, the same variety used to construct the BAC library. Healthy and scab-infected leaves were used as starting material. More than 50 different cDNA clones hybridized to the five BAC clones (Vinatzer et al., 2001). Some of these cDNA clones had homology to the *Cladosporium fulvum* resistance genes of tomato and thus represented a class of candidate *Vf* genes. Hybridizing these cDNAs to the five BAC clones covering the *Vf* locus made it possible to identify several members of this class of homologues of the *Cf* resistance genes. These genes were called *HcrVf* genes (homologues of *C. fulvum* resistance genes of the *Vf* region). Sequencing of DNA fragments derived from the *Vf* BAC clones and subsequent assembly of these sequences led to the identification of three open reading frames (ORFs), called *HcrVf1*, *HcrVf2*, and *HcrVf3*. The expression of *HcrVf1* and *HcrVf2* was determined to be constitutive in leaves by RT-PCR and the completeness of the respective ORFs was verified by 5´ RACE. Expression of *HcrVf3* could not be demonstrated in leaves, making it an unlikely *Vf* gene candidate, although a very similar gene, *HcrVf4*, was found to be expressed in leaves. As the *HcrVf1*, *HcrVf2,* and *HcrVf4* are expressed in leaves and cosegregate with the resistance, they were considered the candidate *Vf* genes. The presence of additional *HcrVf* genes at the *Vf* locus can be predicted from as yet unassembled sequence fragments from the *Vf* BAC clones with homology to the other *HcrVf* genes.

2. Construction of pCORF2. Sequences isolated in the *Vf* region were used to produce different constructs. In this paper we report

Received for publication 7 Mar. 2003. Accepted for publication 17 Mar. 2003.

preliminary data about a plasmid construct, suitable for *Agrobacterium*-mediated transformation of *HcrVf2,* which was developed using the binary vector pCambia2301 (Gene Bank AF234316). The section of pCambia2301 between the *Eco*RI and *Bst*EII restriction sites, which contains a GUS gene, was removed and replaced by the complete ORF of *HcrVf2* and a CaMV35S promoter. The ORF of *HcrVf2* was PCR amplified using *Pfu Taq* Polymerase from BAC clone M18-5 and simultaneously ligated into pCAMBIA2301 with the CaMV35S promoter. The construct was transformed into *E. coli* and the resulting clone, pCORF2, was verified via sequencing.

Sequencing of pCORF2 revealed a base pair mutation at the five prime end of *HcrVf2*. The sixth base in the *HcrVf2* ORF, a guanine, is a cytosine in pCORF2. This alteration converts the amino acid coded from glutamic to aspartic acid. The side chain of both amino acids is positively charged and, hence, their interchange should have little effect on the structure or function of the protein. In addition, the mutation is in the signal peptide, which is not present in the mature protein. Therefore, despite this base pair mutation, pCORF2 was further transferred into *A. tumefaciens* EHA105 containing helper plasmid pCH32.

3. Transformation and regeneration. The transformation experiments were run at Bologna's DCA in 2000–01 and produced thousands of explants (5200) infected by *A. tumefaciens* with plasmids carrying various constructs for *Venturia* resistance. Note, however, that only 1000 explants of 'Gala' were infected with the construct pCORF2 developed at Zurich's ETH. The remaining explants were infected with *HcrVf4*, and the non-mutated *HcrVf2* constructs (data not shown); transformations with *HcrVf1* are under way. The transformation and regeneration processes required a number of complex phases that were backed up by years of fine-tuning the proper protocols. These steps can be briefly encapsulated in preconditioning, explant samplings, inoculum preparation, infection, decontamination, selection-regeneration, proliferation of regenerants, all involving tests of various treatment combinations to optimize the infection and regeneration processes. A detailed description of these protocols will be the focus of an upcoming publication.

Of the 1000 explants grown on selective medium for 15 months, 390 survived, producing calli and nearly 200 independent regenerations. Of the latter, 77 were isolated and transferred to proliferation media: 15 of these have been selected because they have lost their vitreous aspect, started to proliferate and have become plantlets suitable for molecular assays and in vitro infection. The best results in terms of regeneration rate have been recorded using TN505 medium [sorbitol 30 g·L^{-1}; MS salts 4.3 g·L^{-1}; modified MS vitamins; mioinositol 100 mg·L^{-1}; NAA 0.5 mg·L^{-1}; TDZ 5.1 mg·L^{-1}, phytagel 2.3 g·L^{-1}; pH 5.7 (E. Chevreau, personal communication)] in pre-conditioning and regeneration starting from leaf or internode explants. Leaf explants regenerated faster than internode explants: 20% one month after co-culture enriched with kanamycin (50 mg·L^{-1}). While internode explants had a 10% regeneration rate after 6 months and regenerated better if kanamycin is added with a gradient (7 d at 15 mg·L^{-1}; 4 weeks at 30 mg·L^{-1}; then 50 mg·L^{-1}) these regenerants were less vitreous and proliferated better than leaf regenerants.

4. Molecular assays. Validation of the effective transformation of the regenerated shoots was carried out via PCR assays designed to detect the *HcrVf2* and *nptII* transgenes using their specific primers (Vinatzer et al., 2001; Yao et al., 1995). While the *HcrVf2* specific markers (Fig. 1) yielded only one band of ≈1050 bp in the negative control (nontransformed 'Gala'), in the positive control ('Enterprise') there appeared a second band of the expected size for *HcrVf2* (≈880 bp).

The molecular assays run to detect *HcrVf2* in 48 regenerants that developed most fully over the 15 months of infection showed that 87.5% had a profile analogous to that of the resistant cultivars. All these regenerants also showed the presence of the *nptII* gene. Of the six remaining regenerants negative for *HcrVf2*, four tested positive and two tested negative for *nptII*. The two best-growing independent regenerant lines L1 and L2 were selected for further analysis. Transcription of the introduced *HcrVf2* gene was confirmed by RT-PCR (OneStep RT-PCR kit, Qiagen, Germany), following manufacturer's protocol.

5. In vitro scab-resistance tests. An early scab-resistance screening approach using young leaves from in vitro plantlets was developed (Silfverberg-Dilworth, unpublished data). One leaflet per plant was detached and kept for 2 d on water and agar and then inoculated with 20–40 µL/leaf *Venturia inaequalis* conidia in suspension (at least 150,000 conidia/mL) taken from 'Golden Delicious' and 'Red Chief' naturally infected in open field. After 4–5 d, the leaflets were cleared and stained with aniline blue. Fungal development was microscopically assessed in a blind evaluation by three independent readers. The following classification scheme was used:

A) secondary expanding running hyphae, often star-shaped, going out from a central plate of primary stroma;

B) absence of the typical running stroma hyphae, primary stroma as plate with a typical lobed structure;

C) single hypha, clearly larger than a germination tube, extending straight out from the appressoria for 20–100 and more µm; hyphal borders at several sites clearly lobed;

D) as stage C but with straight hyphal borders;

E) no fungal development on appressoria and penetration peg.

Samples were first checked over the whole surface to enable immediate detection of the presence of development stage A. If no stage A was detected, conidia with appressoria were observed and counted until the most developed stage found more than once was determined. A maximum of 100 conidia were observed in stage E. A single observation, except for a stage A, was not considered.

Determining at which development stage of the pathogen the threshold line between resistance and susceptibility has to be drawn is unclear (either between A and B, or B and

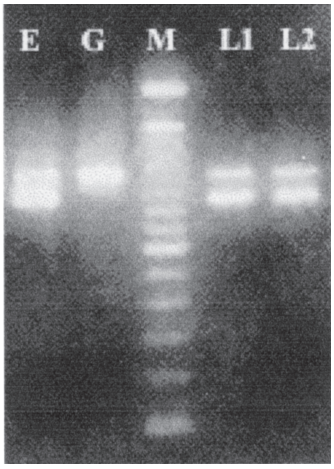

Fig. 1. PCR amplification with *HcrVf2* primers. E = Enterprise; G = not transformed Gala; L1 and L2 = GM-Gala lines 1 and 2; and M = molecular weight marker.

Table 1. Results of scab in vitro inoculum on 'Gala' (susceptible and GM lines) and 'Enterprise' (*Vf*-resistant). Microscopic evaluation of scab infection led to the positing of two thresholds for resistance/susceptibility.

			Threshold 1		Threshold 2	
Leaves from cv/GM line	PCR analysis of *HcrVf2* gene	Total no. of leaves	Resistant classes = B+C+D+E	Susceptible class = A	Resistant classes = C+D+E	Susceptible classes = A+B
Gala	–	8	4	4	1	7
Enterprise	+	10	8	2	5	5
GM-Gala line 1	+	11	10	1	8	3
GM-Gala line 2	+	4	4	0	4	0

Table 2. Statistical analysis of the inoculum results presented in Table 1 (chi-square test).

		P	
	Degree of freedom	Threshold 1 (Class A = susceptible)	Threshold 2 (Classes A+B = susceptible)
Total χ^2	2	0.05*	0.01**
R^{z} vs. T	1	0.42^{NS}	0.14^{NS}
S vs. R + T	1	0.02*	0.01**

[z]R = resistant cultivar Enterprise; S = nontransformed 'Gala'; T = 'Gala' *HcrVf2* transformed lines.
$^{NS, *, **}$Nonsignificant or significant at $P \leq 0.05$ or 0.01, respectively.

C) and influences the results (see the two thresholds and their effects in Table 1).

Of the 10 'Enterprise' (resistant) samples, two were classified untypically as A and three as B. In the susceptible 'Gala', four of eight samples did not show infection stage A or B. Clearly, some individual samples did not classify as expected. Indeed, no matter what the threshold, some samples of the susceptible cultivar did not show any signs of stroma while some of the resistant control did. Nor did threshold have an effect on the outcome of the statistical analysis, which led to the same results (Table 2): scab resistance of the *HcrVf2* transformant lines 1 and 2 did not differ from that of the resistant Enterprise while the susceptible Gala is clearly different from the resistant ones. These data indicate that the transformation with the *HcrVf2* gene appears to increase scab resistance in the resulting transformed lines.

Additional samples (≈30% of all leaves assayed) are not reported as they could not be scored due to insufficient conidia numbers or the presence of secondary infections. It is evident that fine-tuning of the methodology is needed, especially in regard to certain biological parameters like stroma development.

CONCLUSIONS

The transformation of 'Gala' suggests that the candidate *HcrVf2* gene is in all likelihood associated with scab resistance. Indeed, the pCORF2 construct containing *HcrVf2* transferred via *A. tumefaciens* to leaf explants induced resistance to *V. inaequalis* in a sufficient enough number of leaves to prove the transfer of resistance. It is evident that definitive proof of transformation and acquired resistance to *Venturia* must come from in-vivo artificial inocula of greenhouse plants and from *Vf* gene transfer to progeny.

We are not sure that *HcrVf2* is the only or even the most effective gene for this purpose; it remains to be seen whether constructs carrying the original, non-mutated *HcrVf2*, *HcrVf1* or *HcrVf4* will perform better and whether resistance is due to a cluster of genes or only one major *Vf* gene. Each transgenic line must be phenotypically characterised to identify the one with the best resistance expression. It is also important to ascertain at the genotype level the number of transgene copies that have been integrated into the apple genome since plants homozygous for the *Vf* gene seem to be more resistant than the heterozygous ones (Gessler et al., 1997; Tartarini et al., 2000).

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