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Article Sub-Title		
Article CopyRight	Springer-Verlag Berlin Heidelberg (This will be the copyright line in the final PDF)	
Journal Name	Plant Cell Reports	
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Schedule	Received	15 May 2013
	Revised	16 July 2013
	Accepted	17 July 2013
Abstract	<p><b>Key message:</b>  <b>Grapevine rootstock transformed with an <i>Agrobacterium</i> oncogene-silencing transgene was resistant to certain <i>Agrobacterium</i> strains but sensitive to others. Thus, genetic diversity of <i>Agrobacterium</i> oncogenes may limit engineering crown gall resistance.</b></p> <p><i>Abstract:</i>  Crown gall disease of grapevine induced by <i>Agrobacterium vitis</i> or <i>Agrobacterium tumefaciens</i> causes serious economic losses in viticulture. To establish crown gall-resistant lines, somatic proembryos of <i>Vitis berlandieri</i> × <i>V. rupestris</i> cv. ‘Richter 110’ rootstock were transformed with an oncogene-silencing transgene based on <i>iaaM</i> and <i>ipt</i> oncogene sequences from octopine-type, tumor-inducing (Ti) plasmid pTiA6. Twenty-one transgenic lines were selected, and their transgenic nature was confirmed by polymerase chain reaction (PCR). These lines were inoculated with two <i>A. tumefaciens</i> and three <i>A. vitis</i> strains. Eight lines showed resistance to octopine-type <i>A. tumefaciens</i> A348. Resistance correlated with the expression of the silencing genes. However, oncogene silencing was mostly sequence specific because these lines did not abolish tumorigenesis by <i>A. vitis</i> strains or nopaline-type <i>A. tumefaciens</i> C58.</p>	
Keywords (separated by '-')	Crown gall - Transgenic grapevine - <i>Agrobacterium tumefaciens</i> - <i>Agrobacterium vitis</i> - <i>Vitis berlandieri</i> × <i>V. rupestris</i> cv. ‘Richter 110’	
Footnote Information	Communicated by A. Feher. <b>Electronic supplementary material</b> The online version of this article (doi:10.1007/s00299-013-1488-0) contains supplementary material, which is available to authorized users.	

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2 **Silencing *Agrobacterium* oncogenes in transgenic grapevine results**  
3 **in strain-specific crown gall resistance**

4 A. Galambos · A. Zok · A. Kuczmog · R. Oláh ·  
5 P. Putnoky · W. Ream · E. Szegedi

6 Received: 15 May 2013 / Revised: 16 July 2013 / Accepted: 17 July 2013  
7 © Springer-Verlag Berlin Heidelberg 2013

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10 **an *Agrobacterium* oncogene-silencing transgene was**  
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12 **to others. Thus, genetic diversity of *Agrobacterium***  
13 **oncogenes may limit engineering crown gall resistance.**

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30  
31 **Keywords** Crown gall · Transgenic grapevine ·  
32 *Agrobacterium tumefaciens* · *Agrobacterium vitis* ·  
33 *Vitis berlandieri* × *V. rupestris* cv. ‘Richter 110’

34 **Introduction**

35 *Agrobacterium vitis* and *Agrobacterium tumefaciens* 35  
induce uncontrolled cell division, called crown gall dis- 36  
ease, on dicotyledonous plants. In tumorigenic agrobacte- 37  
ria, genes responsible for virulence are located on a large 38  
tumor-inducing plasmid. During infection, the bacterium 39  
genetically transforms host cells using a type IV secretion 40  
system encoded by the *virB* operon. Virulence genes 41  
mediate the transport of a segment of the Ti plasmid, called 42  
T-DNA, into the plant cell. The T-DNA becomes inte- 43  
grated into the host genome leading to abnormal auxin and 44  
cytokinin production and opine synthesis. The auxin (*iaaH*, 45  
*iaaM*) and cytokinin (*ipt*) genes cause tumor formation and 46  
thus are called oncogenes (for reviews see Gelvin 2009, 47  
2010; Pitzschke and Hirt 2010; Tzfira and Citovsky 2008). 48

49 Crown gall causes serious economic losses both in  
50 grapevine nurseries and plantations (Burr et al. 1998).  
51 Several strategies may reduce the damage caused by *Agro-*  
52 *bacterium* spp. on grapevines. These include production of  
53 pathogen-free stocks (Bisztray et al. 2012), biological con-  
54 trol of the pathogen (Kawaguchi 2012; Toklikishvili et al.

A1 Communicated by A. Feher.

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2010; Zäuner et al. 2006), selection for resistance among wild *Vitis* spp. (Kuczmog et al. 2012; Roh et al. 2003; Süle et al. 1994; Szegedi et al. 1984) and genetic manipulation of grapevine for crown gall resistance (Krastanova et al. 2010; Rosenfield et al. 2010; Vidal et al. 2006).

Control of crown gall through transgenic technology can be achieved by inhibiting the bacteria or by blocking T-DNA transfer, integration or expression. Reisch and co-workers used the magainin genes to produce antimicrobial peptides in transgenic *Vitis vinifera* cv. 'Chardonnay' grapevines. Such plants showed significant reduction of tumor development (Rosenfield et al. 2010; Vidal et al. 2006). The expression of a truncated *virE2* gene in transgenic *V. vinifera* results in the production of non-functional VirE2 protein that may compete with intact VirE2, thereby preventing T-DNA transport into the plant cell nucleus. This may also lead to resistance of the transgenic grapevines to *Agrobacterium* (Krastanova et al. 2010).

Silencing T-DNA oncogenes by RNAi provides a novel alternative. To silence *Agrobacterium* oncogenes, three types of constructs were designed. The first one contains the *iaaM* and *ipt* genes under the control of separate promoters/terminators fused to each other in sense and antisense orientation to produce self-complementary mRNAs (Escobar et al. 2001). Such constructs efficiently silenced the *Agrobacterium* oncogenes in walnut (Escobar et al. 2001, 2002, 2003). Ream and co-workers cloned oncogene sequences (*iaaM*, *ipt*), each carrying a premature STOP codon, between two promoters in opposite orientations. This plasmid, called pJP17, directs sense and anti-sense transcription of the cloned *iaaM* and *ipt* sequences which silenced the *iaaM* oncogene in tobacco and apple, leading to crown gall resistance (Lee et al. 2003; Viss et al. 2003). Albuquerque et al. (2012) fused *iaaM* and *ipt* fragments in sense and antisense orientation to the left and right ends of an intron to produce hairpin mRNA. Transformation of *Nicotiana tabacum* with this vector efficiently yielded crown-gall-resistant transgenic plants.

To test the suitability of oncogene silencing in the prevention of tumor formation on grape, transgenic plants of *Vitis berlandieri* × *V. rupestris* cv. 'Richter 110' rootstock were produced and tested with various agrobacteria for resistance to crown gall. Our results showed that oncogene silencing in grapevine is highly strain specific and thus has limited effectiveness in engineering crown gall resistance.

## Materials and methods

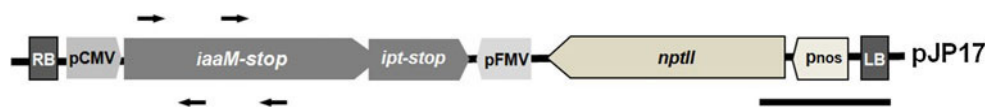
Grapevine transformation and analysis of putative transgenic plants

*A. tumefaciens* EHA101 (pJP17) was used for genetic transformation. The oncogene-silencing plasmid pJP17

contained oncogene sequences derived from the octopine-type plasmid pTiA6. Plasmid pJP17 was designed to express complementary sense and antisense RNAs corresponding to the first 1,797 base pairs of *iaaM* and the entire *ipt* coding sequence. The third codon of each gene was converted to a stop codon, and a frameshift mutation was introduced into each oncogene (Viss et al. 2003). The *iaaM* and *ipt* sequences were fused and situated between opposing cauliflower mosaic virus 35S (CMV 35S) and figwort mosaic virus (FMV) promoters (Fig. 1; Viss et al. 2003). Transformation of the rootstock variety *V. berlandieri* × *V. rupestris* cv. 'Richter 110' embryogenic calli and regeneration of transgenic plants were carried out as previously described (Oláh et al. 2003).

DNA was isolated from young grape leaves using Qiagen Easy Plant DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA samples were analyzed for the presence of the *nptII* gene by PCR using primers described by Hoffmann et al. (1997). The *iaaM* gene was detected by PCR with primers GA-ACCAAGCGGTTGATAACAGCC and CTGCGACTCATAGTCCAGGAATAC (Viss et al. 2003), which amplify a 150 bp fragment of the *iaaM* gene. PCR with *iaaM*-specific primers began with an initial denaturation at 94 °C for 2 min, followed by denaturation at 94 °C for 1 min, annealing at 50 °C for 30 s, and elongation at 72 °C for 1 min. After 35 cycles, the amplification ended with a final elongation step at 72 °C for 5 min. All steps were carried out in a PTC 200 thermocycler (MJ Research, USA). Samples were separated by electrophoresis through a 1.5 % (w/v) agarose gel, and the DNA bands were visualized after staining with ethidium-bromide. To test for contaminating *Agrobacterium* in the plant tissue, all samples were analyzed by PCR using the VCF/VCR primers (Sawada et al. 1995). These primers are designed to detect the *virC* gene, which is present on the Ti plasmid outside the T-DNA. Transgenic grape plants grown in vitro were acclimatized for greenhouse growth and vegetatively propagated for further studies.

To determine the number of T-DNA insertions in the transgenic grape plants, DNA samples (3 µg) were digested with restriction enzymes *PvuII* or *PaeI* (Fermentas, Vilnius, Lithuania), and restriction fragments were separated by electrophoresis through a 1 % agarose gel. Samples were transferred onto nylon membranes (Hybond-N+, Amersham) by the capillary method, and DNA hybridization was performed as described (Sambrook et al. 1989). Blots were probed with a 692 bp amplicon carrying pJP17 T-DNA sequences extending from the left border through the *nptII* gene. This probe was amplified using primers ATTCAATTGTAAATGGCTTCATG and CATAGCCG AATAGCCTCTC; the amplicon was labeled with [ $\alpha$ -<sup>32</sup>P] dCTP using a Pharmacia Ready-to-go labeling kit.



**Fig. 1** Oncogene-silencing T-DNA in pJP17. LB and RB: left and right borders. pnos, pFMV and pCMV are nopaline synthase, figwort mosaic virus and cauliflower mosaic virus 35S promoters, respectively. nptII, ipt and iaaM are neomycin-phosphotransferase,

isopentenyl acetyl-transferase and indol-acetamide-monoxydase sequences, respectively. Arrows represent primer pairs used for qPCR. Solid line below pnos shows the probe used to determine T-DNA copy number

**Table 1** Tumorigenic *Agrobacterium* strains used

Strain	Ti plasmid type	Original host plant	Reference
<i>A. tumefaciens</i> A348 <sup>a</sup>	Octopine/agropine	Black raspberry	Sciaky et al. (1978), Garfinkel et al. (1981), Tempé and Petit (1983)
<i>A. tumefaciens</i> C58 <sup>b</sup>	Nopaline/agrocinopine A and B	Cherry	Sciaky et al. (1978), Tempé and Petit (1983), Slater et al. (2009)
<i>A. vitis</i> Tm4	Octopine/cucumopine	Grapevine	Szegedi et al. (1988), Paulus et al. (1989)
<i>A. vitis</i> AT1	Nopaline	Grapevine	Szegedi et al. (1988), Paulus et al. (1989)
<i>A. vitis</i> S4 <sup>b</sup>	Vitopine	Grapevine	Szegedi et al. (1988), Paulus et al. (1989), Slater et al. (2009)

<sup>a</sup> A348 contains *A. tumefaciens* pTiA6 in C58 chromosomal background. All of the other strains are wild type. *A. tumefaciens* correspond to biotype/biovar 1, *A. vitis* to biotype/biovar 3 (Young et al. 2005)

<sup>b</sup> Complete genome sequences are available (Slater et al. 2009)

157 Susceptibility of the pJP17-transformed 'Richter 110'  
158 grape rootstock lines to agrobacteria

159 Transgenic grape rootstock lines were infected with the  
160 tumorigenic *A. tumefaciens* A348, the strain from which  
161 the silencing construct was derived, and *A. tumefaciens*  
162 C58. These grape rootstocks were also infected with *A.*  
163 *vitis* Tm4, *A. vitis* AT1, and *A. vitis* S4 (Table 1). Bacterial  
164 suspension of 2 µl ( $5 \times 10^8$  cfu/ml) in 0.9 % NaCl (w/v)  
165 was inoculated into wounds made by a sterile needle on the  
166 stems. Tumor formation was evaluated after 6 weeks  
167 incubation in the greenhouse at 23–28 °C.

168 Sequence determination of *iaaM* gene from *A. vitis*  
169 AT1 and alignment of *iaaM* sequences

170 To isolate *iaaM* sequences from *A. vitis* AT1, all *iaaM*  
171 sequences from GenBank were aligned by Clustal W  
172 ([www.ebi.ac.uk/Tools/msa/clustalw2/](http://www.ebi.ac.uk/Tools/msa/clustalw2/)) or EMBOSS Needle  
173 ([www.ebi.ac.uk/Tools/psa/](http://www.ebi.ac.uk/Tools/psa/)), and primers were designed  
174 for two conserved regions inside the coding sequence  
175 (GGGGCGATGCGATTTCCTC and GCGCCCTCCACC-  
176 CATCC). The sequence of this fragment showed 97 %  
177 identity to the *iaaM* gene of *A. vitis* Tm4; therefore, two  
178 additional primer pairs identical to the Tm4 sequence were  
179 designed to amplify the 5' end (GCACAGTATTCCTCGA  
180 TTCTCAAC and CACATGTATCGGCAACCCTCGTAG)  
181 and the 3' end (CAAGCGCTGGACATGACTAATGA and  
182 AGACGCCAAAATAAGGGTGACGAT) of the *iaaM*  
183 coding region from *A. vitis* AT1. DNA sequence of the *A.*

*vitis* AT1 *iaaM* gene was assembled from the sequences of  
184 the above PCR products and registered in the EMBL,  
185 GenBank, and DDBJ nucleotide sequence databases under  
186 accession number FN669137.  
187

Quantitative PCR (qPCR) analysis of oncogene-  
188 silencing RNA in susceptible and resistant plants  
189

Leaf or stem samples (0.2 g) were ground in liquid nitro-  
190 gen, and total RNA was extracted as described (Ham-  
191 iduzzaman et al. 2005). After treatment with DNaseI  
192 (Fermentas, Vilnius, Lithuania), cDNA was synthesized  
193 from 300 ng of total RNA in 20 µl using the RevertAid  
194 Premium First Strand cDNA Synthesis Kit (Fermentas,  
195 Vilnius, Lithuania) following the manufacturer's protocol.  
196 In the first experiments, random primers were used to  
197 synthesize cDNA representing the entire transcriptome. To  
198 distinguish sense and antisense transcripts of the *iaaM-ipt*  
199 transgene, we used a single primer iam3R (CCAGATCCT  
200 ATTCCCATTAG) or iam3F (CCTTGAAATCAGGAGAC  
201 ATTAG) to prime cDNA synthesis from the sense or from  
202 the antisense strand, respectively.  
203

After cDNA synthesis, qPCR was performed using a  
204 Step One™ Real-Time PCR System (Applied Biosys-  
205 tems, USA) in 20 µl reactions containing 1× Maxima™  
206 SYBRGreen/ROX qPCR Master Mix (Fermentas, Vilnius,  
207 Lithuania), 2 µl of cDNA diluted fivefold and 1.0 mm each  
208 of two *iaaM*-specific primers (ATCTGACAATGGTCTGA  
209 TAAG and ACTGCTACCTTTCCACCA) to amplify a  
210 184 bp product. Samples were measured in triplicate, and  
211

212 relative quantification was performed by the  $\Delta\Delta CT$  method  
213 using Step One™ 2.0 Software (Applied Biosystems).

214 The qPCR program was 95 °C for 10 min followed by  
215 40 cycles of 94 °C for 30 s; 60 °C for 30 s; and 72 °C for  
216 40 s. Transcript levels were calculated by normalization  
217 relative to elongation factor EF-1 $\alpha$  mRNA (GenBank  
218 accession: XM\_002284928) because it produces a stable  
219 transcript level in grapevine (Szalontai et al. 2012). The  
220 238 bp EF-1 $\alpha$  sequence was amplified using GAT-  
221 TGACAGGCGATCTGGCAAG and CTTTGCTGCAGAC  
222 TTGGTGAC primers.

## 223 Results and discussion

224 Twenty-one ‘Richter 110’ grapevine lines that had true-to-  
225 type morphology were regenerated. Their transgenic nature  
226 was confirmed by PCR analysis. All lines contained the  
227 *iaaM* (Fig. 2) and *nptII* (data not shown) genes carried on  
228 the T-DNA, but these lines lacked *virC*-specific sequences  
229 (data not shown), indicating that the positive results with  
230 *nptII*- and *iaaM*-specific primers did not arise from con-  
231 taminating bacteria. Southern analysis of ten transgenic  
232 lines showed that nine contained a single T-DNA insert  
233 (Table 2).

234 Vegetatively propagated progenies of these 21 lines  
235 were inoculated with *A. tumefaciens* strains A348 and C58  
236 and with *A. vitis* strains Tm4, AT1, and S4 to test their  
237 susceptibility to crown gall disease. Eight lines showed  
238 resistance (no tumor formation) to *A. tumefaciens* A348  
239 from which the oncogene-silencing construction was  
240 derived. Three of these lines showed resistance to *A. vitis*  
241 AT1 as well. All lines were susceptible to *A. tumefaciens*  
242 C58 and *A. vitis* strains Tm4 and S4 (Fig. 3). No line  
243 showed resistance to all of the agrobacteria tested.

244 To test whether resistance to *A. tumefaciens* A348 cor-  
245 related with elevated expression of the oncogene-silencing  
246 construction, qPCR experiments were performed on RNA  
247 isolated from five A348-resistant transgenic lines, four  
248 susceptible transgenic lines, and the non-transgenic  
249 parental ‘Richter 110’ line. The A348-resistant lines

250 contained 6–13-fold more transgene-encoded RNA than  
251 susceptible line # 61, which contained more oncogene-  
252 silencing RNA than the other fully susceptible lines tested  
253 (Table 2). Among the five A348-resistant lines, levels of  
254 oncogene-silencing RNA did not correlate with resistance  
255 to *A. vitis* AT1 (Table 2). A348-resistant line # 57 was  
256 sensitive to *A. vitis* AT1 even though this line contained  
257 tenfold more oncogene-silencing RNA than susceptible  
258 line # 61. In contrast, line # 58 was resistant to both *A.*  
259 *tumefaciens* A348 and *A. vitis* AT1, although this line  
260 contained sevenfold more oncogene-silencing RNA than  
261 susceptible line # 61 (Table 2).

262 We used strand-specific primers to examine whether the  
263 different resistance spectra of the transgenic lines result  
264 from different ratios of the sense and antisense strands  
265 encoded by the oncogene-silencing transgene. Figure 4  
266 shows that the sense and antisense transcript levels were  
267 comparable in resistant line 3, suggesting that both CMV  
268 and FMV promoters possess similar activity. Sense and  
269 antisense transcript levels were also equivalent in sensitive  
270 line 61, although the transcript levels in this susceptible  
271 line were significantly lower than in resistant line 3  
272 (Fig. 4).

273 In grapevine, only auxin synthesis, which is mediated by  
274 *iaaM* and *iaaH*, contributes to tumor formation (Huss et al.  
275 1990); the cytokinin gene (*ipt*) is not essential (Bonnard  
276 et al. 1989). Apple roots showed a similar response to  
277 oncogenes (Viss et al. 2003). Therefore, silencing only  
278 *iaaM* may be sufficient to generate crown-gall-resistant  
279 transgenic grapevines.

280 Agrobacteria infecting grapevines show a high genetic  
281 diversity that include several *A. tumefaciens* (octopine and  
282 nopaline) and *A. vitis* (octopine, nopaline and vitopine)  
283 strains (Momol et al. 1998; Palacio-Bielsa et al. 2009).  
284 Sequence differences among the *iaaM* genes may explain  
285 the strain-specific nature of silencing. To carry out  
286 sequence comparisons, we established the coding sequence  
287 of *iaaM* from *A. vitis* AT1 (see Materials and methods).  
288 The *iaaM* sequences of the other strains we used were  
289 retrieved from DNA databases. We found 94 % identity  
290 between the silencing (A348) and C58 *iaaM* coding



**Fig. 2** Detection of *iaaM* sequences by PCR from DNA samples of putative pJP17-transformed transgenic *V. berlandieri*  $\times$  *V. rupestris* Richter 110 plants. Lane 1: *A. tumefaciens* EHA101 (pJP17) used as

positive control, lane 2: DNA-free negative control, lane 3: non-transformed Richter 110 DNA and lanes 4–20: 16 independent transgenic lines



**Table 2** Crown gall resistance and transgene RNA levels in transgenic grapevine

Transgenic line	Plants in crown gall test		T-DNA insertions	RNA level <sup>1</sup>	
	<i>A. tumefaciens</i> A348	<i>A. vitis</i> AT1		RQ	RQ min/max
# 3	R	R	1	119.2	107.0/132.8
# 57	R	s	1	93.7	83.5/105.3
# 23	R	R	1	78.9	69.7/89.4
# 58	R	R	1	61.4	57.2/65.9
# 19	R	s	1	51.5	47.8/55.5
# 43 <sup>3</sup>	R? (1/3)	R? (2/3)	1	18.8	17.5/20.3
# 61	s	s	1	9	7.9/10.3
# 35	s	s	1	2.9	2.7/3.0
# 5	s	s	1	2.2	1.9/2.6
# 38 <sup>2</sup>	s	s	2	1	0.4/1.8
R110 <sup>4</sup>	s	s	0	0	0

R resistant, s susceptible

<sup>1</sup> Expression of the sense-antisense *iaaM* sequences from the integrated T-DNA of pJP17. RQ values are given as means with 95 % confidence intervals RQ min/max column shows the minimum and maximum values measured in three independent experiments

<sup>2</sup> The levels of transgene-encoded RNA in the other plants were normalized to RNA levels in this line

<sup>3</sup> One or two of three plants were resistant

<sup>4</sup> Non-transgenic parent line



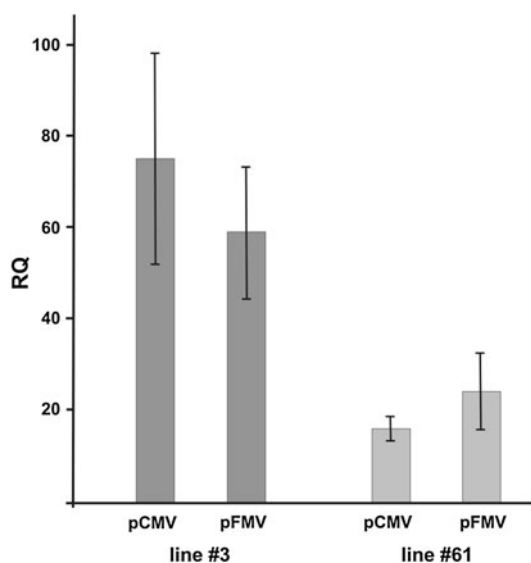
**Fig. 3** Virulence assays on pJP17-transformed transgenic *V. berlandieri* × *V. rupestris* Richter 110 grapevines. **a** Non-transgenic Richter 110 plant inoculated with *A. tumefaciens* A348, **b** mock-

inoculated Richter 110, **c** A348-resistant line # 23 inoculated with *A. tumefaciens* A348, **d** line # 23 inoculated with *A. vitis* S4

291 sequences and 89 % identity between the A348 and AT1  
 292 sequences (electronic supplemental figure) as well as  
 293 between A348 and Tm4 sequences (not shown). In con-  
 294 trast, less than 50 % identity was detected between the  
 295 *iaaM* coding sequences of *A. tumefaciens* A348 and *A. vitis*  
 296 S4. We expected that the silencing transgene might not be  
 297 effective against the *iaaM* gene of *A. vitis* S4 due to low  
 298 sequence identity. However, the result that the transgene  
 299 did not silence some highly similar *iaaM* genes (from C58  
 300 and Tm4) but was effective on others (from AT1 and  
 301 A348) was unexpected. To determine whether differences

in the distribution of sequence identity in the *iaaM* genes  
 may explain differences in silencing, we examined the  
 sequence alignments from this point of view.

RNA-induced silencing complexes (RISCs) contain  
 21 bp RNA sequences that mediate recognition of mRNAs  
 carrying complementary sequences (Pratt and MacRae  
 2009; Rana 2007). In pairwise sequence alignments, we  
 identified those regions in the *iaaM* genes of strains AT1  
 and C58 that show at least 21 contiguous base pairs of  
 identity to the 1,797 bp silencing sequence from A348. We  
 found 47 regions of identity ranging from 21 to 41 bp in



**Fig. 4** Expression of *iaaM-ipt* silencing construct from sense (pCMV) and antisense (pFMV) promoters in transgenic plants. Line # 3 showed resistance to *A. tumefaciens* A348 and *A. vitis* AT1 while line # 61 was susceptible to crown gall formation (Table 2). Primers iam3R and iam3F were used to detect sense and antisense transcripts, respectively (see “Materials and methods”). Error bars correspond to technical repeats

313 the C58 sequence, whereas the *iaaM* gene from AT1  
314 contained only 28 such regions (electronic supplementary  
315 figure). Four transgenic lines blocked tumor formation by  
316 *A. vitis* AT1, but none of our transgenic lines showed  
317 resistance against *A. tumefaciens* C58, even though the  
318 silencing sequence shows a higher identity to the *iaaM*  
319 gene of C58. Thus, the extent of sequence identity did not  
320 correlate with the strain-specific nature of silencing.

321 Beside DNA homology, other factors may influence the  
322 success of silencing. Elevated phytohormone levels can  
323 suppress gene silencing. Some *Agrobacterium* strains may  
324 overproduce phytohormones rapidly enough to prevent  
325 oncogene-silencing (Dunoyer et al. 2006). This could result  
326 from more robust delivery of T-DNA or from stronger  
327 expression of the oncogenes. Alternatively, some *Agro-*  
328 *bacterium* strains may deliver anti-silencing proteins  
329 analogous to those made by some viruses.

330 Here we have shown that crown gall resistance induced  
331 by the oncogene-silencing transgene from pJP17 is highly  
332 specific to the strain from which the *iaaM* gene was  
333 derived. Similar variability in the susceptibility pattern was  
334 observed when grapevines were transformed with a trun-  
335 cated *virE2* gene (Krastanova et al. 2010). Our observa-  
336 tions are not in agreement with the results of Dandekar’s  
337 group, which achieved resistance to a wide range of various  
338 agrobacteria on transgenic tomato (Escobar et al. 2003).  
339 These differences may be explained by the different  
340 oncogene-silencing transgenes or by different agrobacteria

used for inoculation or by different susceptibilities of the  
host plants.

**Acknowledgments** The authors are grateful to Gabriella Endre for her help in the course of the work and to Sabouran Zaheri and Vera Tóth for technical assistance. We thank Monsanto (St. Louis, MO) for their kind permission concerning the use of pCGN5927, from which pJP17 was constructed. This work was supported by the Hungarian National Science Found (OTKA) grant no. K68053 and K83121 and by TÁMOP 4.2.1./B-09/01/KMR/2010-0005 and 4.2.2/B-10/1-2010-0029.

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