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Abstract

Key message*:*
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Abstract:
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Keywords (separated by '-') Crown gall - Transgenic grapevine - *Agrobacterium tumefaciens* - *Agrobacterium vitis* - *Vitis berlandieri* × *V. rupestris* cv. 'Richter 110'

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ORIGINAL PAPER

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

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Keywords Crown gall · Transgenic grapevine · *Agrobacterium tumefaciens* · *Agrobacterium vitis* · *Vitis berlandieri* × *V. rupestris* cv. 'Richter 110'

Introduction

Agrobacterium vitis and *Agrobacterium tumefaciens* induce uncontrolled cell division, called crown gall disease, on dicotyledonous plants. In tumorigenic agrobacteria, genes responsible for virulence are located on a large tumor-inducing plasmid. During infection, the bacterium genetically transforms host cells using a type IV secretion system encoded by the *virB* operon. Virulence genes mediate the transport of a segment of the Ti plasmid, called T-DNA, into the plant cell. The T-DNA becomes integrated into the host genome leading to abnormal auxin and cytokinin production and opine synthesis. The auxin (*iaaH*, *iaaM*) and cytokinin (*ipt*) genes cause tumor formation and thus are called oncogenes (for reviews see Gelvin 2009, 2010; Pitzschke and Hirt 2010; Tzfira and Citovsky 2008).

Crown gall causes serious economic losses both in grapevine nurseries and plantations (Burr et al. 1998). Several strategies may reduce the damage caused by *Agrobacterium* spp. on grapevines. These include production of pathogen-free stocks (Bisztray et al. 2012), biological control of the pathogen (Kawaguchi 2012; Toklikishvili et al.

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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). Alburquerque 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 GAACCAAGCGGTTGATAACAGCC 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 [α-^{32}P] dCTP using a Pharmacia Ready-to-go labeling kit.

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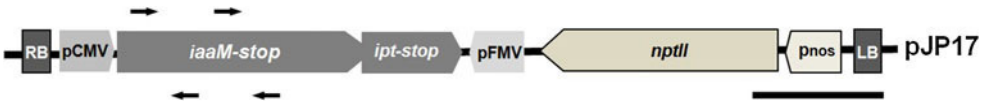

Fig. 1 Oncogene-silencing T-DNA in pJP17. LB and RB: *left* and *right* borders. p*nos*, pFMV and pCMV are nopaline synthase, figwort mosaic virus and cauliflower mosaic virus 35S promoters, respectively. *nptII*, *ipt* and *iaaM* are neomycin-phosphotransferase, isopentenyl acetyl-transferase and indol-acetamide-monooxydase sequences, respectively. *Arrows* represent primer pairs used for qPCR. *Solid line* below p*nos* shows the probe used to determine T-DNA copy number

Table 1 Tumorigenic *Agrobacterium* strains used

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

[a] 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)

[b] Complete genome sequences are available (Slater et al. 2009)

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

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

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

To isolate *iaaM* sequences from *A. vitis* AT1, all *iaaM* sequences from GenBank were aligned by Clustal W (www.ebi.ac.uk/Tools/msa/clustalw2/) or EMBOSS Needle (www.ebi.ac.uk/Tools/psa/), and primers were designed for two conserved regions inside the coding sequence (GGGGCGATGCGATTTCCTC and GCGCCCTCCACCCATCC). The sequence of this fragment showed 97 % identity to the *iaaM* gene of *A. vitis* Tm4; therefore, two additional primer pairs identical to the Tm4 sequence were designed to amplify the 5′ end (GCACAGTATTCCCCGATTCTCAAC and CACATGTATCGGCAACCCTCGTAG) and the 3′ end (CAAGCGCTGGACATGACTAATGA and AGACGCCAAAATAAGGGTGACGAT) of the *iaaM* coding region from *A. vitis* AT1. DNA sequence of the *A. vitis* AT1 *iaaM* gene was assembled from the sequences of the above PCR products and registered in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession number FN669137.

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

Leaf or stem samples (0.2 g) were ground in liquid nitrogen, and total RNA was extracted as described (Hamiduzzaman et al. 2005). After treatment with DNaseI (Fermentas, Vilnius, Lithuania), cDNA was synthesized from 300 ng of total RNA in 20 µl using the RevertAid Premium First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) following the manufacturer's protocol. In the first experiments, random primers were used to synthesize cDNA representing the entire transcriptome. To distinguish sense and antisense transcripts of the *iaaM-ipt* transgene, we used a single primer iam3R (CCAGATCCTATTCCCATTAG) or iam3F (CCTTGAAATCAGGAGACATTAG) to prime cDNA synthesis from the sense or from the antisense strand, respectively.

After cDNA synthesis, qPCR was performed using a Step OneTM Real-Time PCR System (Applied Biosystems, USA) in 20 µl reactions containing 1× MaximaTM SYBRGreen/ROX qPCR Master Mix (Fermentas, Vilnius, Lithuania), 2 µl of cDNA diluted fivefold and 1.0 mm each of two *iaaM*-specific primers (ATCTGACAATGGTCGATAAG and ACTGCTACCTTTCCACCA) to amplify a 184 bp product. Samples were measured in triplicate, and

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relative quantification was performed by the ΔΔCT method using Step OneTM 2.0 Software (Applied Biosystems).

The qPCR program was 95 °C for 10 min followed by 40 cycles of 94 °C for 30 s; 60 °C for 30 s; and 72 °C for 40 s. Transcript levels were calculated by normalization relative to elongation factor EF-1α mRNA (GenBank accession: XM_002284928) because it produces a stable transcript level in grapevine (Szalontai et al. 2012). The 238 bp EF-1α sequence was amplified using GATTGACAGGCGATCTGGCAAG and CTTTGCTGCAGAC TTGGTGAC primers.

Results and discussion

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

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

To test whether resistance to *A. tumefaciens* A348 correlated with elevated expression of the oncogene-silencing construction, qPCR experiments were performed on RNA isolated from five A348-resistant transgenic lines, four susceptible transgenic lines, and the non-transgenic parental 'Richter 110' line. The A348-resistant lines contained 6–13-fold more transgene-encoded RNA than susceptible line # 61, which contained more oncogene-silencing RNA than the other fully susceptible lines tested (Table 2). Among the five A348-resistant lines, levels of oncogene-silencing RNA did not correlate with resistance to *A. vitis* AT1 (Table 2). A348-resistant line # 57 was sensitive to *A. vitis* AT1 even though this line contained tenfold more oncogene-silencing RNA than susceptible line # 61. In contrast, line # 58 was resistant to both *A. tumefaciens* A348 and *A. vitis* AT1, although this line contained sevenfold more oncogene-silencing RNA than susceptible line # 61 (Table 2).

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

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

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

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

Transgenic line	Plants in crown gall test		T-DNA insertions	RNA level[1]	
	A. tumefaciens A348	*A. vitis* 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[3]	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[2]	s	s	2	1	0.4/1.8
R110[4]	s	s	0	0	0

R resistant, *s* susceptible

[1] 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

[2] The levels of transgene-encoded RNA in the other plants were normalized to RNA levels in this line

[3] One or two of three plants were resistant

[4] 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

sequences and 89 % identity between the A348 and AT1 sequences (electronic supplemental figure) as well as between A348 and Tm4 sequences (not shown). In contrast, less than 50 % identity was detected between the *iaaM* coding sequences of *A. tumefaciens* A348 and *A. vitis* S4. We expected that the silencing transgene might not be effective against the *iaaM* gene of *A. vitis* S4 due to low sequence identity. However, the result that the transgene did not silence some highly similar *iaaM* genes (from C58 and Tm4) but was effective on others (from AT1 and 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

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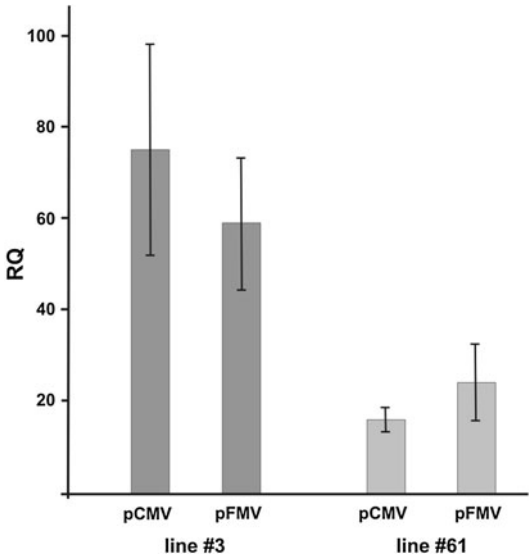

Fig. 4 Expression of *iaaM-ipt* silencing construct from sense (pCMV) and antisense (pFMV) promoters in transgenic plants. *Line* # 3 showed resistance to *A. tumefaciens* A348 and *A. vitis* AT1 while *line* # 61 was susceptible to crown gall formation (Table 2). Primers iam3R and iam3F were used to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

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

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

Here we have shown that crown gall resistance induced by the oncogene-silencing transgene from pJP17 is highly specific to the strain from which the *iaaM* gene was derived. Similar variability in the susceptibility pattern was observed when grapevines were transformed with a truncated *virE2* gene (Krastanova et al. 2010). Our observations are not in agreement with the results of Dandekar's group, which achieved resistance to a wide range of various agrobacteria on transgenic tomato (Escobar et al. 2003). These differences may be explained by the different oncogene-silencing transgenes or by different agrobacteria used for inoculation or by different susceptibilities of the host plants.

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