

RNAi-mediated oncogene silencing confers resistance to crown gall tumorigenesis

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Crown gall disease, caused by the soil bacterium *Agrobacterium tumefaciens*, results in significant economic losses in perennial crops worldwide. *A. tumefaciens* is one of the few organisms with a well characterized horizontal gene transfer system, possessing a suite of oncogenes that, when integrated into the plant genome, orchestrate *de novo* auxin and cytokinin biosynthesis to generate tumors. Specifically, the *iaaM* and *ipt* oncogenes, which show ~90% DNA sequence identity across studied *A. tumefaciens* strains, are required for tumor formation. By expressing two self-complementary RNA constructions designed to initiate RNA interference (RNAi) of *iaaM* and *ipt*, we generated transgenic *Arabidopsis thaliana* and *Lycopersicon esculentum* plants that are highly resistant to crown gall disease development. In *in vitro* root inoculation bioassays with two biovar I strains of *A. tumefaciens*, transgenic *Arabidopsis* lines averaged 0.0–1.5% tumorigenesis, whereas wild-type controls averaged 97.5% tumorigenesis. Similarly, several transformed tomato lines that were challenged by stem inoculation with three biovar I strains, one biovar II strain, and one biovar III strain of *A. tumefaciens* displayed between 0.0% and 24.2% tumorigenesis, whereas controls averaged 100% tumorigenesis. This mechanism of resistance, which is based on mRNA sequence homology rather than the highly specific receptor–ligand binding interactions characteristic of traditional plant resistance genes, should be highly durable. If successful and durable under field conditions, RNAi-mediated oncogene silencing may find broad applicability in the improvement of tree crop and ornamental rootstocks.

Crown gall disease is a chronic and resurgent disease problem that affects many perennial fruit, nut, and ornamental crops. Growers and nursery industries suffer significant annual losses worldwide to crown gall disease in the form of unsalable nursery stock, lowered productivity from galled trees, and increased susceptibility of infected plants to pathogens and environmental stress (1). Although extensive research efforts have studied the epidemiology of crown gall throughout this century, prevention strategies have, for the most part, remained focused on phytosanitation, such as the “careful cultural methods,” “strict inspection of nursery stock,” and “abandonment of infected soils” prescribed by Smith in 1911 (2). More recently, the biocontrol strain *Agrobacterium radiobacter* K84 has been widely used for crown gall disease management; however, effective control is limited to a subset of all virulent *Agrobacterium tumefaciens* strains (3). Traditional breeding strategies for crown gall disease resistance are possible in some perennial crop species where resistant germplasm is present, but breeding requires decades to achieve resistance in commercially available perennial crop rootstocks (1). Overall, the prevalence of crown gall disease in the field is a testament to the limited success of traditional control strategies.

Although control of crown gall disease has proved elusive, the molecular biology and genetics of *A. tumefaciens*–plant interactions has become well characterized, in part because of the pivotal role of *Agrobacterium* as a vector for the introduction of foreign genes into plants (4). *A. tumefaciens* pathogenesis can be considered a two-stage process: (i) bacterial responses facilitat-

ing horizontal gene transfer and integration into the plant genome (transformation) and (ii) postintegration events occurring *in planta* (tumorigenesis). *A. tumefaciens* transformation occurs at plant wound sites, where phenolic compounds released by wounded plant cells trigger induction of a set of virulence (*vir*) genes, one of which is responsible for the cleavage of a discrete single strand of DNA (T-DNA) from the large tumor-inducing (Ti) plasmid. The T-DNA is transferred from *Agrobacterium* into the plant cell through a complex *vir* gene-encoded protein and nucleic acid translocator, which is homologous to a plasmid conjugation pilus (5). Inside the plant cell, the T-DNA is localized to the nucleus where it becomes integrated into the genome (6). Despite their prokaryotic origin, genes in the T-DNA are expressed in the plant’s nucleus and produce proteins that initiate tumorigenesis and create an environment suitable for *A. tumefaciens* growth and interbacterial Ti plasmid conjugation (7).

Two main classes of T-DNA genes are expressed in infected plant cells: opine synthesis genes and oncogenes. Opine synthesis genes direct the production of novel metabolites termed opiines, most of which are simple derivatives of amino acids conjugated with sugars or diketo acids (8). Opiines can serve as the sole carbon and/or nitrogen source for the infecting strain of *A. tumefaciens*. Expression of oncogenes causes overproduction of the plant hormones auxin and cytokinin and may alter phytohormone sensitivity, resulting in the initiation of uncontrolled cell growth/division and gall formation (7). The oncogene region of the T-DNA has classically been referred to as “core sequence” because the region shares high DNA sequence homology across all virulent *A. tumefaciens* strains (9, 10).

We have developed a crown gall disease control strategy that targets the process of gall formation (tumorigenesis) *in planta* by initiating RNA interference (RNAi) of the *iaaM* and *ipt* oncogenes. The *iaaM* gene codes for a tryptophan monooxygenase that converts tryptophan to the auxin precursor indoleacetamide (11). The *ipt* gene product catalyzes the condensation of AMP and isopentenyl pyrophosphate to form the cytokinin zeatin (12). Expression of both of these oncogenes is required for wild-type tumor formation (13). RNAi is a form of homology-dependent gene silencing common to fungi, animals, and plants (14). Although some specifics of the silencing mechanism may differ between kingdoms, double-stranded RNA seems to be a universal initiator of RNAi (14). In plants, expression of self-complementary RNAs from introduced transgene constructions has proved a rapid and consistent initiator of RNAi for several genes in *Arabidopsis thaliana* and *Nicotiana tabacum* (tobacco) (15–17). We transformed *A. thaliana* and *Lycopersicon esculentum* (tomato) with a transgene construction designed to generate

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Abbreviations: RNAi, RNA interference; T-DNA, *Agrobacterium*-transferred DNA; Ti, tumor inducing; GUS, β -glucuronidase; dpi, days postinoculation.

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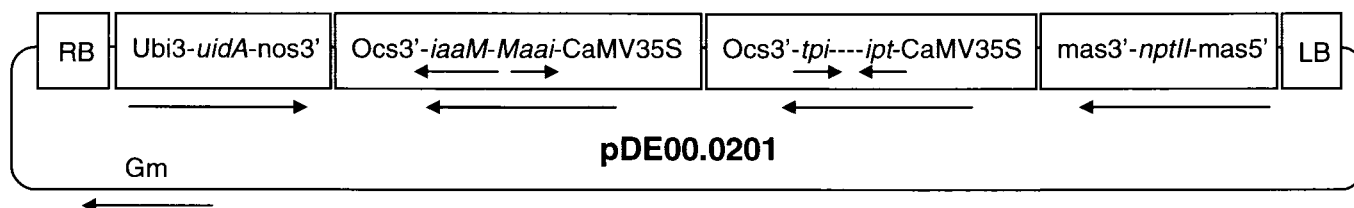


Fig. 1. The *Agrobacterium* binary vector pDE00.0201 for the expression of self-complementary *iaaM* and *ipt* oncogenes. This vector contains an *nptII*-selectable marker gene driven by the mannopine synthase 2' promoter (*mas5'*), a *uidA* scorable marker gene driven by the *ubi3* promoter (*ubi3*), and self-complementary *iaaM* and *ipt* genes driven by 35S cauliflower mosaic virus promoters. Arrows indicate the direction of transcription. LB and RB indicate the left and right T-DNA border sequences.

self-complementary *iaaM* and *ipt* transcripts. Resultant transgenic lines retained susceptibility to *Agrobacterium* transformation but were in some cases highly refractory to tumorigenesis, providing functional resistance to crown gall disease.

Materials and Methods

Oncogene Sequence Analysis. The MEGALIGN multiple sequence alignment program (DNASTar, Madison, WI) was used to align the *iaaM* and *ipt* DNA sequences from biovar I and biovar II *A. tumefaciens* present in GenBank (data not shown). Based on cross-strain oncogene sequence homology ($\approx 90\%$), we designed two sets of PCR primers with minimal degeneracy for the amplification of *iaaM* and *ipt* from other *A. tumefaciens* strains. We PCR amplified and cloned an internal 2,224-bp fragment of the *iaaM* gene from the virulent biovar 1 *A. tumefaciens* strain 20W-5A, which had previously been isolated from a *Juglans regia* (English walnut) crown gall. Similarly, a fragment corresponding to the first 627 bp of the *ipt* coding sequence was cloned from *A. tumefaciens* 20W-5A.

Binary Vector Construction and Plant Transformation. A self-complementary *ipt* construct was created by fusing one sense copy of *ipt*-20W-5A bp 45–627 to an identical fragment in the antisense orientation. The two complementary *ipt* fragments were separated by a 1,000-bp region of noncoding linker DNA derived from the cloning vector. A self-complementary *iaaM* construction was generated by fusing one antisense copy of *iaaM*-20W-5A bp 1,274–2,224 to the 5' end of a full-length sense *iaaM*-20W-5A fragment. This created an inverted repeat of a 950-bp region corresponding to the 3' end of *iaaM*, interrupted by a noncomplementary linker region corresponding to bp 1–1,273 of *iaaM*. The self-complementary constructions were individually ligated into a plant expression vector containing the 35S cauliflower mosaic virus promoter and the octopine synthase terminator. The resultant expression cassettes were then ligated into the binary vector pDU99.2215, which contains an *nptII*-selectable marker gene driven by the mannopine synthase promoter and a *uidA* (β -glucuronidase, GUS) scorable marker gene driven by the *ubi3* promoter from potato. The resultant binary

vector, pDE00.0201, was transformed into the disarmed *A. tumefaciens* strain EHA101 by electroporation (Fig. 1).

Agrobacterium-mediated transformation of *A. thaliana* ecotype Wassilewskija was performed by the whole-plant infiltration method described by Bechtold *et al.* (18). Transformants were identified by selecting seed on half-strength Murashige–Skoog medium containing 75 mg/liter kanamycin. *Agrobacterium*-mediated transformation of *L. esculentum* cultivar Moneymaker was performed as described by Fillatti *et al.* (18). Transformed shoots were selected on 2Z medium containing 50–100 mg/liter kanamycin (19).

Molecular Characterization of Transformants. Southern hybridization of total genomic DNA and fluorimetric GUS analysis was performed essentially as described by Sambrook *et al.* and Jefferson, respectively (20, 21). Total leaf and stem RNA was isolated by using a Qiagen plant RNeasy kit. Isolated RNA was treated with RNase free DNase, and first-strand cDNA synthesis was primed with a poly T primer by using Stratagene's ProSTAR first-strand cDNA kit. First-strand synthesis reactions lacking reverse transcriptase were included as a control for DNA contamination.

For competitive PCR amplification of *ipt* cDNA, reactions included 25 pmol of each primer, 200 μ M dNTPs, 1 \times CLONTECH cDNA PCR reaction buffer, 1 \times CLONTECH Advantage cDNA polymerase, and 0.005, 0.05, or 0.5 pg of serially diluted internal standard (22). Thirty thermocycles were performed with 94°C denaturation (30 s), 60°C annealing (1 min), and 68°C extension (2 min). Amplification of *iaaM* cDNA was as described above, except 0.002, 0.01, or 0.05 pg of internal standard was included and 32 thermocycles were performed with a 63°C annealing temperature. PCR products were electrophoresed through 1.6% agarose gels and analyzed by spot densitometry using an Alpha Innotech IS-1000 digital imaging system. Relative quantification of amplified cDNA bands was performed as described by Kermouni *et al.* (22).

Transformation and Tumorigenesis Bioassays. Roots from *Arabidopsis* transformants were harvested from plants grown in culture and diced into ≈ 0.5 -cm pieces. Four to five individual root pieces

Table 1. Genotypic properties of *A. tumefaciens* strains used in disease-challenge bioassays of transgenic plants

Strain	Biovar	K84 sensitivity	Opine(s) produced	Homology to <i>ipt</i> , % [†]	Homology to <i>iaaM</i> , % [†]
20W-5A*	I	R	Mannopine/agropine	100.0	100.0
15955	I	R	Octopine/mannopine/agrocinopine	91.1	90.8
A208	I	S	Nopaline	99.0	93.0
C58	I	S	Nopaline	99.0	95.7
127A*	II	S	Nopaline	Unknown	Unknown
CG49	III	R	Nopaline	Unknown	Unknown
EHA101	I	R	—	—	—

*Strain isolated from walnut crown gall, not previously reported in the literature.

[†]Homology to oncogene-silencing construct pDE00.0201.

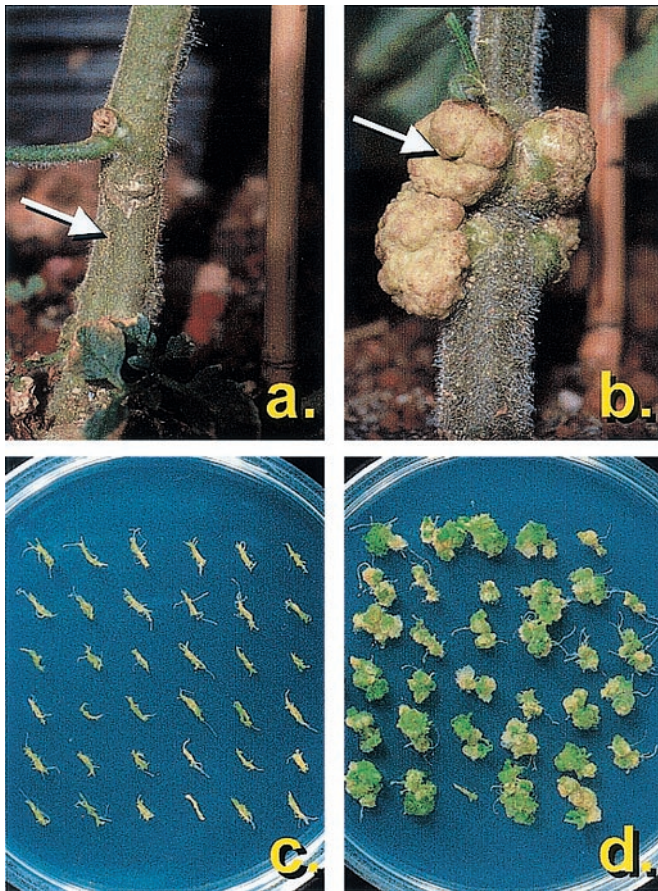


Fig. 2. *A. tumefaciens* tumorigenesis on tomato and *Arabidopsis*. Transgenic (a) and wild-type (b) tomatoes were infected with *A. tumefaciens* 20W-5A by piercing the stem with a syringe and extruding a small amount of bacterial suspension into the wound. Tumorigenesis was scored 5 weeks after inoculation. Large tumors are evident at the inoculation site (marked with an arrow) on the wild-type plant but not the transgenic plant, indicating suppression of tumorigenesis. In the lower panels, *in vitro* grown roots were harvested from transgenic (c) and wild-type (d) *Arabidopsis* lines and inoculated with *A. tumefaciens* A208. Tumorigenesis was scored 5 weeks after inoculation. Large teratoma-type tumors are present on wild-type root bundles but not on transgenic root bundles, again indicating suppression of tumorigenesis.

were gathered into a bundle and inoculated with a 2×10^9 CFU/ml suspension of the virulent *A. tumefaciens* strains A208 or 20W-5A to assay tumorigenesis or with the avirulent strain EHA101 containing the glufosinate resistance binary vector pMLBART to assay transformation efficiency (23, 24). *In vitro* culture of inoculated root bundles was performed essentially as described by Nam *et al.*, with the exception of the use of cefotaxime (250 mg/liter) as a bacteriostatic agent (24). Tumorigenesis (A208, 20W-5A) or the formation of glufosinate-resistant calli (EHA101/pMLBART) was scored 5 weeks after inoculation. A minimum of 60 root bundle replicates was performed for tumorigenesis assays and a minimum of 30 replicates was performed for transformation assays.

The stem of tomato seedlings was inoculated with an 8×10^7 CFU/ml suspension of various *A. tumefaciens* (Table 1) or *Agrobacterium rhizogenes* strains. A syringe equipped with a 25-gauge needle was loaded with bacterial suspension, the needle was pushed through the stem, and a small droplet of suspension was extruded as the needle was pulled back through the stem. Tumorigenesis or rhizogenesis was scored 5 weeks after inoculation. A minimum of 12 seedlings was inoculated for each

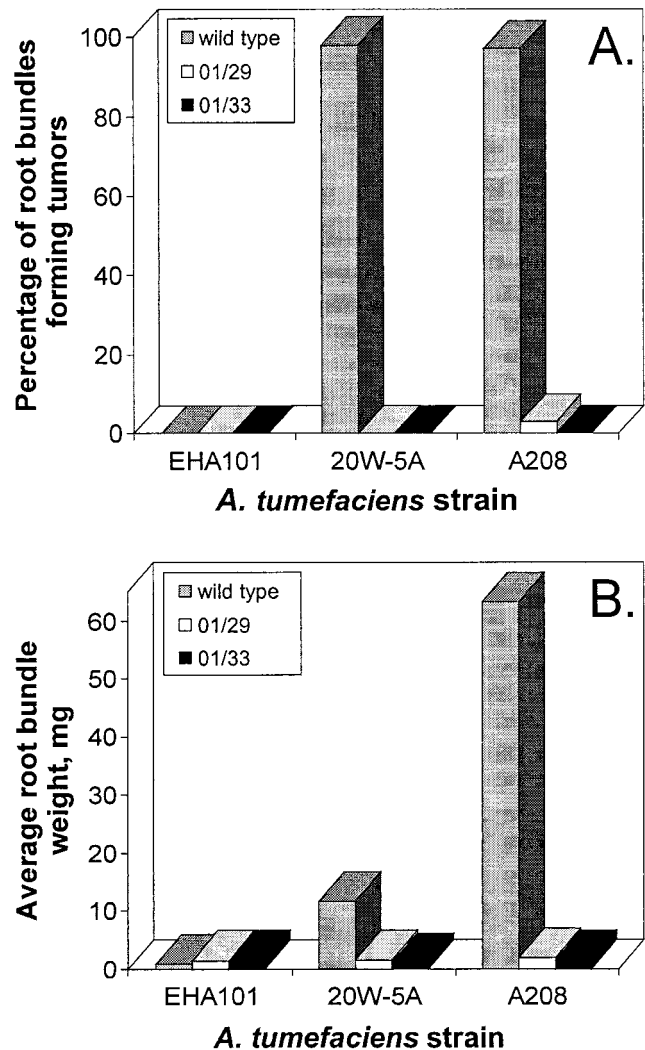


Fig. 3. Comparison of tumor incidence (A) and biomass (B) in wild-type and transgenic *Arabidopsis* lines. (A) Summary of tumor incidence among 60 *A. thaliana* root bundles obtained from the wild-type or the transgenic lines 01/29 and 01/33 when inoculated with three different *A. tumefaciens* strains. The percentage of root bundles displaying tumors was assayed 5 weeks after inoculation. A208 and 20W-5A are virulent strains, and EHA101 is an avirulent strain that serves as a control. (B) Summary of the average root bundle weight from 60 root bundles 5 weeks after *Agrobacterium* inoculation. Any increase in average root bundle weight over the basal level of the EHA101 control is because of the accumulation of tumor biomass. The 01/29 and 01/33 lines are significantly different from the wild type in percentage tumor incidence and average root bundle biomass at the $P = 0.05$ level.

Agrobacterium strain (20W-5A, 15955, C58, 127A, CG49, and A4).

Results and Discussion

Although *A. tumefaciens* is highly diverse in terms of genomic DNA structure and Ti plasmid organization, all virulent strains share highly homologous oncogenes that are required for tumor formation. We have attempted to exploit this commonality to generate resistance to crown gall tumorigenesis by targeting the *iaaM* and *ipt* oncogenes for RNAi-mediated gene silencing. Endogenous plant phytohormone biosynthesis pathways may contain reactions that are biochemically analogous to those catalyzed by *iaaM* and *ipt*, but no plant genes with significant sequence similarity to these oncogenes have ever been reported (25, 26). Thus, *iaaM* and *ipt* could be silenced in plant cells

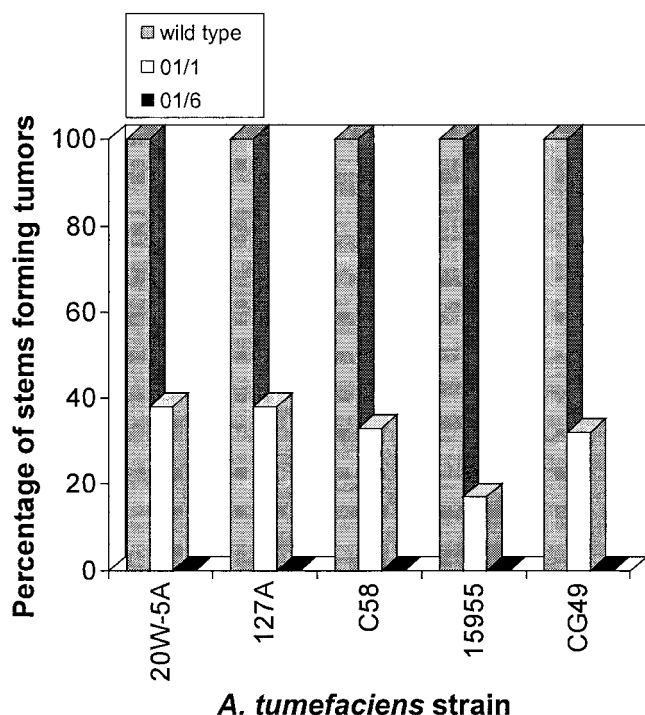


Fig. 4. Comparison of tumor incidence in wild-type and transgenic tomato lines. The figure shows an analysis of tumor incidence among wild-type and transgenic (01/1, 01/6) tomato seedlings inoculated with five different virulent *A. tumefaciens* strains (20W-5A, 127A, C58, 15955, and CG49). The percentage of stems displaying tumors was assayed 5 weeks after inoculation. The 01/1 and 01/6 lines are significantly different from the wild-type in percentage tumor incidence at the $P = 0.05$ level.

infected by *A. tumefaciens* without otherwise altering plant hormone metabolism. Using *iaaM* and *ipt* sequences cloned from the biovar I *A. tumefaciens* strain 20W-5A, we constructed two expression cassettes designed to generate self-complementary *iaaM* and *ipt* transcripts. These cassettes were cloned into an *Agrobacterium* binary vector, generating pDE00.0201 (Fig. 1). Tomato and *Arabidopsis*, two plants that are highly susceptible to crown gall disease, were transformed with pDE00.0201, and putative transformants were regenerated under kanamycin selection.

Plant transformation and transgene expression were confirmed by Southern hybridization of total genomic DNA, fluorimetric GUS analysis, and reverse transcriptase-PCR (data not shown). Transgenic plants were generally indistinguishable from the wild type in gross morphology and development. To identify putatively resistant individuals, several primary transformant lines were challenged with biovar I strains of *A. tumefaciens* in small-scale disease bioassays. *Arabidopsis* transformants were

infected with *A. tumefaciens* A208 in *in vitro* root inoculation bioassays, and primary tomato transformants were stem inoculated with *A. tumefaciens* 20W-5A. Sixty percent (3 of 5) of tested tomato transformants and 50% (3 of 6) of tested *Arabidopsis* transformants displayed a complete abolition of tumorigenesis in these initial primary screens (Fig. 2). A high percentage of transformants displaying the silenced phenotype is characteristic of the expression of self-complementary RNA; however, it is interesting to note that two different RNA targets were simultaneously silenced with such high efficiency (15–17). No consistent tumor morphology phenotypes indicative of the silencing of a single oncogene were observed (e.g., rooty tumors, shooty tumors, attenuation; ref. 13).

Two putatively resistant tomato lines (01/1, 01/6) and two putatively resistant *Arabidopsis* lines (01/29, 01/33) were selected for large-scale disease screening with various biovar I, II, and III strains of *A. tumefaciens* (Table 1). Based on genomic Southern hybridization analysis, lines 01/6 and 01/29 have a single T-DNA insert, line 01/33 has two T-DNA inserts, and line 01/1 has three T-DNA inserts. Transgenic individuals selected from segregating T_1 populations were used for large-scale screening to discern any phenotypic effects of hemizygosity vs. homozygosity of the transgene locus.

A minimum of 60 root bundles from the *A. thaliana* lines 01/29, 01/33, and wild type were separately inoculated with *A. tumefaciens* 20W-5A and A208. On average, 97.5% of wild-type root bundles generated tumors, whereas the two transgenic lines averaged 0.0% and 1.5% tumorigenesis (Fig. 3). Further, the average root bundle biomass from the wild-type root bundles infected with virulent *A. tumefaciens* was at least one order of magnitude greater than the mass of comparable root bundles from the transgenic 01/29 and 01/33 lines (Fig. 3).

Analyzing tomato, a minimum of 12 seedlings from the lines 01/1, 01/6, and wild type were separately inoculated with the *A. tumefaciens* strains 20W-5A, 15955, C58, 127A, and CG49. For each inoculum, 100% of the wild-type seedlings developed undifferentiated tumors (Fig. 4). The 01/6 line was completely resistant to all tested *A. tumefaciens* strains, with 0% tumorigenesis (Fig. 4). The 01/6 line also displayed perfect cosegregation between GUS activity and resistance to disease development (52 of 52) and lack of GUS activity and disease susceptibility (14 of 14). Interestingly, 100% (66 of 66) of T_1 01/1 individuals displayed GUS activity, but the line averaged 25.3% tumorigenesis. This line possesses three independent T-DNA insertion events, and it is possible that one or more of these inserts is capable of GUS expression but is incapable of initiating oncogene silencing. Alternatively, high transgene copy number in specific T_1 individuals could lead to transcriptional gene silencing of the 35S cauliflower mosaic virus promoter that drives the silencing constructs (27). For both *Arabidopsis* and tomato, no phenotypic differences in resistance were apparent between hemizygous and homozygous individuals, which were subsequently identified by segregation analysis.

Table 2. Comparison of *Agrobacterium* transformation efficiencies in wild-type and transgenic plants

Plant-line	Tumorigenesis susceptibility	Inoculum	Transformation frequency, % [†]
<i>Arabidopsis</i> -wt	S	<i>A. tumefaciens</i> EHA101*	73.3 (22/30)
<i>Arabidopsis</i> -01/33	R	<i>A. tumefaciens</i> EHA101*	87.5 (28/32)
Tomato-wt	S	<i>A. rhizogenes</i> A4	83.3 (10/12)
Tomato-01/6	R	<i>A. rhizogenes</i> A4	76.9 (10/13)

**A. tumefaciens* EHA101 containing a glufosinate resistance binary vector.

[†]Transformation frequency determined by percentage of root bundles forming glufosinate-resistant calli (*Arabidopsis*) or percentage tumor/root formation at site of inoculation (tomato).

In a previous study, Nam *et al.* demonstrated that a relatively high percentage (0.7%) of randomly mutagenized *A. thaliana* lines display novel crown gall disease resistance phenotypes (28). Thus, it is possible, although unlikely given the resistance frequencies in primary transformants, that resistance to crown gall disease development is a result of random mutations inherent in the transformation system. If resistance were conferred by RNAi-mediated silencing of the *iaaM* and *ipt* genes, we would expect a highly specific block in tumorigenesis but no hindrance in the process of *Agrobacterium* transformation. Alternatively, all other forms of crown gall resistance characterized to date interfere in processes of transformation such as bacterial attachment, T-DNA transfer, or T-DNA integration (24, 28). To define the mechanism of resistance as a specific block in tumorigenesis, we inoculated tomato with *A. rhizogenes* A4, a hairy root-inducing species that possesses a transformation machinery that is functionally identical to *A. tumefaciens* but which does not require *iaaM* and *ipt* for rhizogenesis (29). Similarly, we performed *A. tumefaciens*-mediated transformation of *Arabidopsis* root bundles with the avirulent strain EHA101 containing an herbicide (glufosinate) resistance binary vector. Transformation was assayed by hairy root/tumor formation in tomato and by formation of glufosinate-resistant calli in *Arabidopsis* (24). As summarized in Table 2, transformation efficiencies were highly similar between wild-type and highly resistant plant lines, demonstrating that differences in transformation efficiency cannot account for resistance to disease development. Thus, the resistant transgenic lines possess a unique and highly specific uncoupling of the processes of transformation and tumorigenesis.

Because the process of transformation is unhindered in resistant plant lines, we would expect that individual cells from resistant plants are transformed by *A. tumefaciens* at a rate comparable with the wild type, but that these cells do not accumulate *iaaM* and *ipt* transcripts and therefore do not initiate tumorigenesis. To demonstrate directly a decrease in abundance of *iaaM* and *ipt* mRNAs in plant tissue inoculated with *A. tumefaciens*, we compared *iaaM* and *ipt* transcript abundance in 01/6 and wild-type tomato lines 3, 10, and 20 days after inoculation with *A. tumefaciens* 20W-5A. There are potentially three populations of *iaaM/ipt* mRNAs in infected plant tissue: self-complementary transcripts derived from the integrated oncogene-silencing constructs (present only in transgenic plants), transcripts produced in living *A. tumefaciens* by expression of the oncogene region of the Ti plasmid (produced at very low levels), and transcripts produced *in planta* from integrated virulent T-DNA (30). To isolate the last population, we performed first-strand cDNA synthesis using a poly T primer (elimination of nonpolyadenylated bacterial transcripts) and subsequently PCR-amplified regions of each oncogene that were not present in the oncogene-silencing constructions. To obtain a relative quantification of *iaaM* and *ipt* mRNA abundance, competitive PCR was performed on first-strand cDNA by using serial dilutions of a coamplified heterologous DNA internal standard (22). As shown in Fig. 5, the accumulation of both *iaaM* and *ipt* transcripts is significantly reduced in the transgenic line as compared with the wild type, demonstrating a positive correlation between oncogene mRNA abundance and disease susceptibility. This highly specific oncogene silencing efficiently targets mRNAs transcribed from both primarily nonintegrated [3 days postinoculation (dpi)] and integrated (10, 20 dpi) T-DNAs and is apparent even before phenotypic indications of resistance (≈ 15 dpi).

Conclusions

Using an RNAi-mediated oncogene-silencing strategy, *A. tumefaciens* tumorigenesis was severely impaired in several transgenic lines of the highly susceptible model species *Arabidopsis* and tomato. The high level of cross-strain DNA sequence conserva-

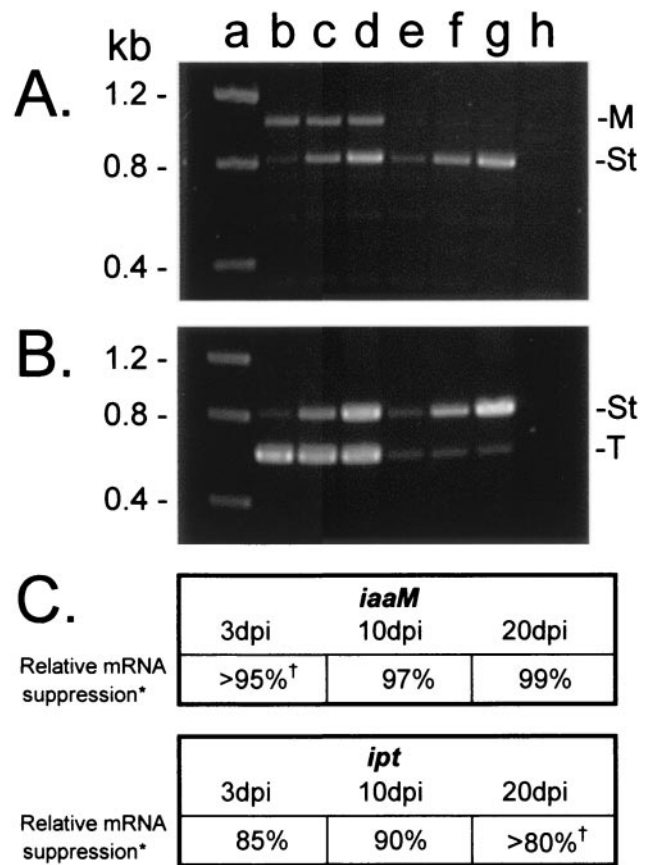


Fig. 5. Comparison of *ipt* and *iaaM* transcript abundance in wild-type and transgenic tomato by using competitive reverse transcriptase-PCR. Wild-type plants and plants from the crown gall-resistant transgenic line 01/6 were stem inoculated with *A. tumefaciens* 20W-5A, and RNA was extracted from inoculated stem tissue 3, 10, and 20 dpi. First-strand cDNA synthesis was primed with a polyT primer, and cDNA was subsequently PCR amplified in the presence of a known concentration of serially diluted competitive standard. (A) Agarose gel electrophoresis of 20 dpi *iaaM* competitive PCR reactions. Note the presence of an 815-bp internal standard amplification product (St) and a 1,035-bp *iaaM* cDNA amplification product (M). (B) Agarose gel electrophoresis of 20 dpi *ipt* competitive PCR reactions. Note the presence of an 815-bp internal standard amplification product (St) and a 609-bp *ipt* cDNA amplification product (T). Lane markers apply to both panels. Lane a, molecular weight standard; lanes b–d, PCR amplification of wild-type cDNA in the presence of 0.002 pg (lane b), 0.01 pg (lane c), and 0.05 pg (lane d) of *iaaM* internal standard (A) or 0.005 pg (lane b), 0.05 pg (lane c), and 0.5 pg (lane d) of *ipt* internal standard (B). Lanes e–g, PCR amplification of cDNA from the transgenic line 01/6 in the presence of 0.002 pg (lane e), 0.01 pg (lane f), and 0.05 pg (lane g) of *iaaM* internal standard (A) or 0.005 pg (lane e), 0.05 pg (lane f), and 0.5 pg (lane g) of *ipt* internal standard (B). Lane h, PCR amplification of cDNA from a noninoculated control plant. (C) Comparison of oncogene mRNA abundance in wild-type and 01/6 plants at 3, 10, and 20 dpi. *, relative mRNA suppression refers to the abundance of 01/6 cDNA oncogene PCR amplification product as compared with the wild-type cDNA oncogene product (1 – [conc. of 01/6 product/conc. of wild-type product]). †, examples in which the 01/6 cDNA oncogene product was below the lower limit of detection are noted; the concentration of these products was recorded as less than the lowest concentration of internal standard.

tion in the *iaaM* and *ipt* oncogenes ($\approx 90\%$ identity in all sequenced genes) suggests that this strategy can provide broad spectrum resistance to crown gall tumorigenesis. Assuming that *A. tumefaciens* does not possess viral-type suppressors of gene silencing, we hypothesize that resistance could also be highly durable because specificity is mediated by RNA hybridization rather than protein–ligand binding. For many plant resistance

gene systems, minor mutations in pathogen avirulence genes can disrupt the protein–protein interactions required to initiate a defense response (31). However, in an RNAi-mediated resistance system, mutation is unlikely to rapidly alter the target gene sequence to a level at which homology-dependent gene silencing no longer operates ($\approx 70\%$ homology; ref. 32).

In addition to their application in plant protection, oncogene-silenced plants may provide a unique opportunity to study the mechanism of posttranscriptional gene silencing in plants. As in plants engineered with posttranscriptional gene silencing-mediated viral resistance, the mRNA species targeted for silencing is present only subsequent to pathogen infection. However, unlike a virus, *A. tumefaciens* integrates virulent DNA into the plant genome, and this integration event is unique in organization and copy number in every infected plant cell. Thus, at any *A. tumefaciens* infection site, there are potentially tens to hundreds of different T-DNA integration events, any one of which, if not silenced, could generate a crown gall. The fact that tumorigenesis is uniformly inhibited in transgenic lines suggests that the silencing of *iaaM* and *ipt* transcripts is independent of the structure, location, and copy number of the target locus in genomic DNA. Thus, our results suggest that the silencing locus alone, not a specific interaction between the silencing locus and the target locus, determines the efficiency of gene silencing.

Several factors lend this oncogene-silencing strategy to application in susceptible ornamental and horticultural plants. The

resistance phenotype is generated with a single integrated copy of the silencing locus, eliminating the lengthy step of selecting homozygous progeny in slow-maturing perennials. Further, the prevalence of graft propagation allows transformation of rootstocks alone, providing resistance without altering the genetic composition of the harvested crop and without the possibility of undesired transgene flow through pollen. However, although oncogene-silenced plants are highly resistant to tumorigenesis, it should be noted that there is no evidence that they are deficient as carriers of rhizoplane populations of *A. tumefaciens* (33). As such, good nursery practices would need to be maintained in the cultivation of these plants to minimize the dissemination of large populations of virulent *A. tumefaciens* into growers' fields. Cocultivation of susceptible indicator plants with resistant plants in the nursery could provide a useful visual check of *A. tumefaciens* abundance and virulence. We are currently applying this technology to English walnut rootstocks, a crop in which crown gall disease can severely limit productivity.

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