

Characterization of oncogene-silenced transgenic plants: implications for *Agrobacterium* biology and post-transcriptional gene silencing

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SUMMARY

Agrobacterium tumefaciens tumorigenesis is initiated by the horizontal transfer of a suite of oncogenes that alter hormone synthesis and sensitivity in infected plant cells. Transgenic plants silenced for the *iaaM* and *ipt* oncogenes are highly recalcitrant to tumorigenesis, and present a unique resource to elucidate fundamental questions related to *Agrobacterium* biology and post-transcriptional gene silencing (PTGS). The oncogene-silenced transgenic tomato line 01/6 was used to characterize *A. tumefaciens* growth *in planta* and to screen for *iaaM* and *ipt* sequence variants. Even in the absence of macroscopic and microscopic indications of tumorigenesis, *A. tumefaciens* is capable of long-term survival in the hypocotyl tissues of the 01/6 line. *A. tumefaciens* growth, however, is significantly reduced in the 01/6 line, with populations decreased by 96% relative to wild-type at 52 days post-inoculation. In addition, the 01/6 line displayed suppression of tumorigenesis against all 35 tested strains of *A. tumefaciens*. High target homology is an absolute requirement of PTGS, therefore this result suggests that regions of the *iaaM* and *ipt* oncogenes are very highly conserved across most *A. tumefaciens* strains. Finally, graft transmissibility of oncogene silencing was assessed by grafting various non-silenced tomato genotypes on to the 01/6 line. Phenotypic and molecular evidence (tumorigenesis and absence of small interfering RNAs, respectively) suggest that oncogene silencing is not graft-transmissible, at least to wild-type and antisense *iaaM*-over-expressing genotypes.

INTRODUCTION

The causal agent of crown gall disease, *Agrobacterium tumefaciens*, has been a subject of intensive scientific inquiry for nearly 100 years (Smith and Townsend, 1907). Many early *A. tumefaciens* research efforts were driven by apparent similarities between plant crown galls and animal cancers, but the unique significance

of this common soil bacterium was revealed 25 years ago with the discovery that *A. tumefaciens* is an agent of horizontal gene transfer, capable of genetically transforming plant cells with a discrete segment of its own DNA (Chilton *et al.*, 1977). *A. tumefaciens* generally survives saprophytically on decaying plant matter or root exudates (Agrios, 1997). Under inductive conditions characteristic of a plant wound, however (e.g. low pH, presence of phenolic compounds), *A. tumefaciens* is thought to generate a single stranded DNA copy of a discrete region of its large tumour inducing plasmid (Ti plasmid). Complexed with virulence (*vir*) proteins, this single stranded transferred-DNA (T-DNA) is exported to the plant cell in a process similar to bacterial conjugation, and is localized to the nucleus where it may be integrated into the cell's genome (reviewed by Zupan *et al.*, 2000).

Crown gall formation (tumorigenesis) is initiated by oncogenes present on the T-DNA that influence plant hormone synthesis and sensitivity (reviewed by Binns and Costantino, 1998). The *iaaM* and *iaaH* oncogene products catalyse the conversion of tryptophan to the plant hormone indole acetic acid (IAA), and the *ipt* oncogene product catalyses the rate-limiting step in the synthesis of the hormone zeatin from adenosine monophosphate and a terpenoid precursor (Astot *et al.*, 2000; Klee *et al.*, 1984; Lichtenstein *et al.*, 1984). The uncontrolled synthesis of IAA and zeatin in infected cells causes cell expansion and division, generating a mass of rapidly cycling cells derived from one or more *A. tumefaciens* transformation events. These cells, which are capable of hormone-independent growth in the absence of the infecting bacteria, generate the neoplastic crown gall.

Crown gall disease is an agricultural concern primarily in perennial fruit, nut, ornamental, and vine crops, although nearly one-thousand species of dicotyledonous plants have been characterized as crown gall-susceptible (DeCleene and DeLey, 1976). We have previously described a novel oncogene-silencing strategy for the generation of crown gall disease resistance in plants (Escobar *et al.*, 2001). *Arabidopsis thaliana*: *Lycopersicon esculentum* (tomato) and *Juglans regia* (walnut) plants transformed with self-complementary transgene constructs designed to initiate post-transcriptional gene silencing (PTGS) of the *iaaM* and *ipt* oncogenes exhibit robust resistance to crown gall tumorigenesis (Escobar *et al.*, 2001, 2002).

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PTGS is a sequence-specific RNA degradation pathway that is highly conserved among animals, fungi, and plants (Sharp, 2001). In plants, PTGS can be initiated by sense RNA (cosuppression), antisense RNA (antisense-mediated gene silencing), self-complementary RNA (RNA interference, RNAi), and viruses (virus induced gene silencing, VIGS). All of these molecules are thought to directly or indirectly produce double-stranded RNA, the primary inducer of PTGS (Vaucheret *et al.*, 2001). This double-stranded RNA is digested to unique 21–25 bp small-interfering RNAs (siRNAs), which are thought to mediate sequence-specific mRNA degradation and the catalytic amplification of further double-stranded RNA from homologous mRNA templates (Hamilton and Baulcombe, 1999; Sijen *et al.*, 2001). In oncogene-silenced plants, crown gall resistance is correlated with the accumulation of high levels of *iaaM* and *ipt* homologous siRNAs and a substantial reduction in *iaaM* and *ipt* mRNA accumulation (Escobar *et al.*, 2001, 2002).

While the agricultural applications of oncogene-silenced plants are apparent, these plants also provide a unique resource for the investigation of fundamental questions related to *A. tumefaciens* biology and PTGS. The objectives of the studies presented here were to determine: (i) how suppression of tumorigenesis affects *A. tumefaciens* growth *in planta*, (ii) if oncogene silencing-mediated resistance is graft-transmissible to crown gall-susceptible genotypes, and (iii) if DNA sequence variation in the *iaaM* and/or *ipt* oncogenes is sufficient to overcome oncogene-silencing mediated resistance.

RESULTS AND DISCUSSION

Effect of tumorigenesis suppression on *A. tumefaciens* growth *in planta*

Suppression of crown gall disease development is not necessarily correlated with decreased growth of *A. tumefaciens in planta* (Robbs *et al.*, 1991; Sule *et al.*, 1994). Presumably, different mechanisms of resistance [e.g. antimicrobial metabolites (Sahi *et al.*, 1990), inefficient T-DNA integration (Nam *et al.*, 1997), low phytohormone sensitivity (Beneddra *et al.*, 1996)] have varying effects on *A. tumefaciens* growth. Oncogene-silenced plants possess a unique resistance mechanism that acts late in the process of disease development, specifically degrading *iaaM* and *ipt* mRNAs transcribed from *Agrobacterium* T-DNA in the plant cell. In order to characterize *A. tumefaciens* growth in oncogene-silenced plants, the hypocotyls of wild-type and oncogene-silenced (transgenic line 01/6) tomato seedlings were inoculated with the virulent *A. tumefaciens* strain 20W-5A, and *Agrobacterium* populations were determined at various times post-inoculation.

As expected, wild-type hypocotyl tissue inoculated with *A. tumefaciens* grew significantly heavier than corresponding 01/6 tissue over the course of the experiment. As illustrated in Fig. 1A,

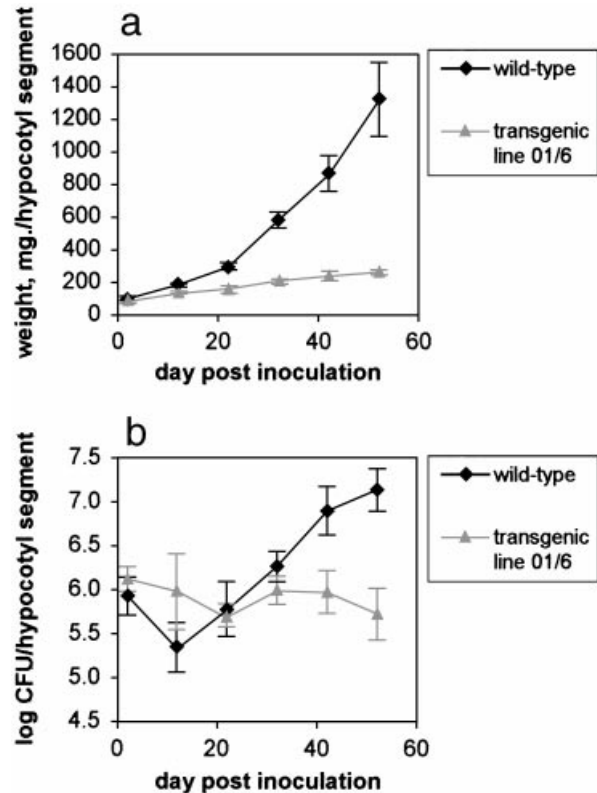


Fig. 1. Comparative tissue weight (a) and *A. tumefaciens* growth (b) in wild-type and oncogene-silenced (01/6) plants. The hypocotyls of tomato seedlings were inoculated with the virulent *A. tumefaciens* strain 20W-5A and assayed at various times following inoculation. Bacterial populations were determined by dilution plating. Each data point represents six total seedlings from two separate experimental replicates. Error bars indicate ± 1 s.e.m. Averaged over the course of the experiment, both weight and bacterial populations are significantly greater in wild-type tissue than 01/6 tissue ($P < 0.05$).

the wild-type line displayed near-exponential accumulation of tumour biomass, while the 01/6 line displayed modest linear tissue growth without visible tumour development. Similarly, on average wild-type tissue contained significantly higher populations of *A. tumefaciens* than 01/6 tissue, with wild-type harbouring > 20-fold more *A. tumefaciens* cells than 01/6 at the final sampling date (52 days post-inoculation [dpi]) (Fig. 1B). Examining each sampling date individually, *Agrobacterium* populations were not significantly different between the wild-type and 01/6 genotypes at initial time points (2, 12, 22 dpi), similar to the pattern of *Agrobacterium* growth previously reported in the crown gall-resistant grapevine cultivar 'Gloire de Montpellier', which also possesses a late block in disease development (presumptive T-DNA-integration deficient) (Sule *et al.*, 1994). After 22 dpi, however, *Agrobacterium* multiplies at a near-exponential rate in wild-type tissue, with population growth closely mirroring tumour growth (Fig. 1B). Alternatively, a low but stable population of *Agrobacterium* is supported in the 01/6 line despite the absence of visible tumour development.

These results are fully consistent with the fact that synthesis and secretion of nutritive opines occurs only in *Agrobacterium*-transformed cells, and that the proliferation of these transformed cells is highly suppressed in oncogene-silenced plants.

We previously described the substantial ($\approx 90\%$), but not complete, suppression of *iaaM* and *ipt* mRNA accumulation in tomato line 01/6 (Escobar *et al.*, 2001). The presence of detectable levels of oncogene mRNA associated with *A. tumefaciens* survival *in planta* could indicate that tumorigenesis is initiated in the 01/6 line, but that tumour development is slowed to the extent that no gall formation was apparent over the assay period. In order to investigate this possibility, we examined plastic-embedded hypocotyl sections at various time points following *Agrobacterium* inoculation. Wild-type tissue inoculated with *A. tumefaciens* developed foci of localized cell division activity in the hypocotyl cortex beginning at 6 dpi. These foci gave rise to numerous cortical tumours by 30 dpi (Fig. 2). In the 01/6 line, no anomalous cell organization developed in the hypocotyl cortex over the course of the experiment (30 days). This suggests that the *in planta* survival of *Agrobacterium* in line 01/6 is fully independent of tumour development. Opine synthesis genes are not silenced in the 01/6 line, thus it is possible that individual transformed cells produce sufficient opines to support the observed populations of *Agrobacterium*. In addition, the recent identification of putative tannase, xylanase, polygalacturonase, and β -endoglucanase genes in the genome sequence of *A. tumefaciens* strain C58 suggests that *Agrobacteria* may be able to utilize a variety of simple and complex carbohydrates *in planta* (Goodner *et al.*, 2001).

Oncogene silencing-mediated resistance is not graft-transmitted to crown gall-susceptible genotypes

A unique aspect of PTGS-mediated resistance is the potential for transmission of silencing (resistance) to unmodified genotypes through grafting. This graft transmission of silencing could have significant applications in woody crop species, in which transgenic rootstocks might be used to modify the transcriptome

of existing scion cultivars. For example, Palauqui *et al.* (1997) demonstrated that PTGS of endogenous genes and transgenes could be transmitted from silenced rootstocks to non-silenced scions through a graft union (see also Crete *et al.*, 2001; Palauqui and Vaucheret, 1998; Sonoda and Nishiguchi, 2000). However, reciprocal grafting experiments utilizing our oncogene-silenced transgenic line 01/6 suggest that oncogene-silencing is not transmitted to wild-type tomato plants in either the rootstock to scion or the scion to rootstock direction (Table 1). *Agrobacterium*-inoculated wild-type rootstocks or scions grafted to line 01/6 developed tumours similar in size and morphology to tumours on control (wt : wt) grafts along the length of the stem. Even at the graft union, where 01/6 cell layers lie directly adjacent to wild-type cell layers, there was a clear genotype-specific fate of *Agrobacterium*-inoculated plant cells (Fig. 3). The formation of tumours in wild-type tissue clearly indicates that the targeted *iaaM* and *ipt* oncogenes are expressed, but that silencing is not initiated. Similar results were obtained when the high-expressing antisense *iaaM* transgenic line 326/4 was grafted on to 01/6 plants. Despite the presence of abundant *iaaM* target RNA, transmission of *iaaM* silencing was not initiated in grafted 326/4 tissues, as determined by wild-type tumour development at *A. tumefaciens* inoculation sites (Table 1). (Tissues silenced for *iaaM* should generate a tumour phenocopy of an *iaaM* mutant strain, such as *A. tumefaciens* A328.)

In addition to examining crown gall morphology and development, silencing was characterized by investigating the presence and abundance of oncogene-homologous siRNAs (Hamilton and Baulcombe, 1999). In concert with tumorigenesis susceptibility phenotypes, *iaaM* and *ipt* siRNAs are highly abundant in the 01/6 line (silenced), and absent in the wild-type and 326/4 transgenic line (non-silenced) (Fig. 4). Grafting of wild-type or 326/4 scions to a 01/6 rootstock does not induce siRNA production, confirming that silencing is not propagated through the graft union (Fig. 4). It is interesting that the presence of a single-stranded mRNA target (*iaaM* and *ipt* transcripts derived from integrated *Agrobacterium* T-DNA) is not necessary for siRNA

Table 1. Grafting studies to investigate systemic transmission of oncogene silencing

Rootstock genotype ^a	Rootstock state ^b	Scion genotype	Scion state	Number of grafted plants	Inoculated tissue	Percentage tumorigenesis
WT	WT	WT	WT	10	rootstock/scion	100
01/6	S	01/6	S	4	rootstock/scion	0
WT	WT	01/6	S	17	rootstock	100
01/6	S	WT	WT	21	scion	100
326/4	NS	326/4	NS	2	rootstock/scion	100
326/4	NS	01/6	S	8	rootstock	100
01/6	S	326/4	NS	10	scion	100

^aWT = wild-type; 01/6 = *iaaM/ipt* silenced, tumorigenesis-resistant transgenic line; 326/4 = antisense *iaaM*-expressing, tumorigenesis-susceptible transgenic line.

^bWT = wild-type; S = silenced; NS = not silenced.

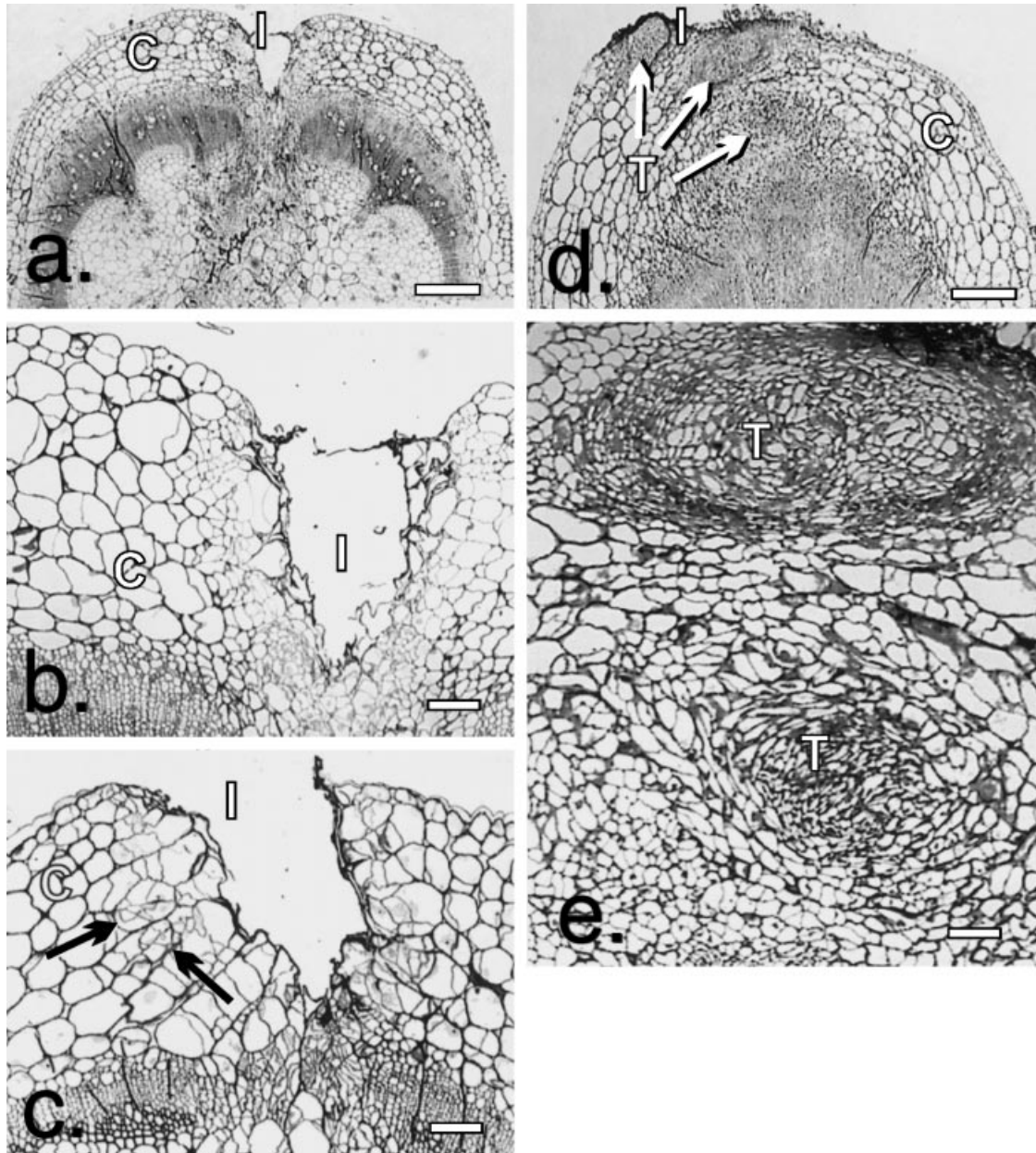


Fig. 2. Hypocotyl sections from oncogene-silenced and wild-type tomato plants following inoculation with *A. tumefaciens* 20W-5A. (a and b) Transgenic line 01/6 at 30 days post-inoculation (dpi) displaying no cellular indications of tumorigenesis. The files of cells formed parallel to the inoculation site are a typical wound-healing response also seen in tissue inoculated with avirulent *A. tumefaciens* (not shown). (c) Wild-type tomato at 6 dpi. Tumorigenic activity is evident as nests of dividing cells in the hypocotyl cortex (arrows). Note the irregular organization of new cell walls that form at these sites. (d and e) Wild-type tomato at 30 dpi displaying well-developed tumours in the cortex, as evidenced by small, cytoplasmically dense cell aggregates lacking an organized pattern of cell division (arrows). C, cortex; I, inoculation site; T, tumour. Scale bars = 200 μm (a, d) and 100 μm (b, c, e).

accumulation in the 01/6 line. This result contrasts with recent observations in *Caenorhabditis elegans* individuals fed double-stranded RNA expressing bacteria, in which enzymatic, target mRNA-dependent amplification of double-stranded RNA is required for significant siRNA accumulation and PTGS (Sijen *et al.*, 2001). Presumably, target mRNA mediated enzymatic amplification

of double-stranded RNA is not necessary for high-level siRNA synthesis and silencing in plants that constitutively express self-complementary transgenes.

Why is oncogene silencing graft autonomous? One possibility is that the grafting technique itself influences the propagation of silencing, as suggested by Crete *et al.* (2001). This explanation



Fig. 3. Oncogene silencing-mediated resistance to tumorigenesis is not graft transmitted to wild-type tomato. Wild-type (a) or O1/6 (b) scions were cleft-grafted to a O1/6 rootstock. Three weeks after grafting a shallow incision was made through stock and scion tissues at the graft union and the wound was inoculated with virulent *A. tumefaciens*. Tumour formation is apparent in the wild-type scion (a, top), but abruptly terminates at the graft union with the O1/6 rootstock. No tumour development is apparent in O1/6-self grafts. Note that the adventitious roots visible on the surface of the rootstocks develop independently of *Agrobacterium* inoculation.

seems unlikely, however, because our cleft grafting technique is very similar to that employed in the successful experiments of Sonoda and Nishiguchi (2000). It also seems unlikely that the abundance of target RNA is limiting (Palauqui and Vaucheret, 1998; Palauqui *et al.*, 1997), at least in the case of 326/4, in which *iaaM* antisense mRNA is abundant (data not shown). Although antisense RNA is certainly a target for silencing (Di Serio *et al.*, 2001), and is presumably quantitatively sufficient in line 326/4, it is possible that antisense RNA targets are qualitatively incapable of propagating transmission of PTGS in a manner similar to a sense RNA. In this scenario, the 'receiving' genotype (326/4) would be incompetent to propagate graft transmissible silencing signals produced by the oncogene-silenced line O1/6. Alternatively, it is possible that PTGS induced by expression of double-stranded RNA (RNAi) does not elaborate high levels of

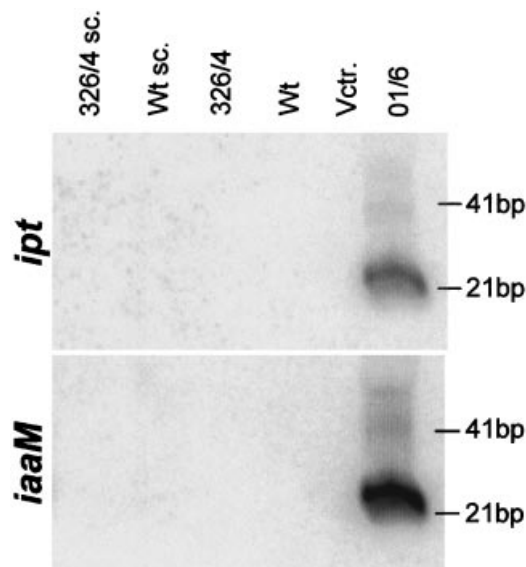


Fig. 4. Oncogene-homologous siRNA accumulation in various tomato genotypes. Small RNA fractions from young tomato leaves were electrophoresed through a denaturing polyacrylamide gel, blotted to nylon membranes, and probed with *iaaM* and *ipt*-specific radioactive probes. *iaaM* and *ipt*-homologous siRNAs are apparent in the silenced O1/6 line, but not wild-type (Wt), the antisense *iaaM*-expressing line 326/4, or a vector control (Vctr.). siRNA is also absent in wild-type and 326/4 scions grafted to O1/6 rootstocks (Wt sc., 326/4 sc.). Single-stranded DNA oligonucleotides were utilized as size standards.

graft-transmissible silencing signals (i.e. the O1/6 'transmitter' genotype O1/6 does not generate sufficient signal for graft transmission of silencing). The data presented here are insufficient to differentiate between these scenarios. It is interesting, however, that all successful demonstrations of graft-transmissible silencing have utilized cosuppressed (sense-suppressed) transmitter genotypes (Palauqui and Vaucheret, 1998; Palauqui *et al.*, 1997; Sonoda and Nishiguchi, 2000), and that antisense-mediated silencing transmitters do not appear to generate graft-transmissible silencing (Crete *et al.*, 2001). In addition, the presence of fundamental genetic differences between cosuppression and RNAi in the establishment phase of PTGS (Beclin *et al.*, 2002) suggests that different inducers of silencing (double-stranded RNA, virus, antisense RNA, or sense RNA) could elaborate different patterns of local and systemic transmission (see also Crete *et al.*, 2001; Voynet *et al.*, 2000).

Broad spectrum suppression of tumorigenesis suggests regions of high sequence conservation among the *iaaM* and *ipt* oncogenes

Oncogene-silencing mediated resistance is highly dependent upon homology between the double-stranded initiator of silencing

(self-complementary *iaaM* and *ipt* sequences derived from *A. tumefaciens* strain 20W-5A), and the target oncogene mRNA. Elomaa *et al.* (1996) demonstrated an inverse relationship between initiator/target sequence homology and target mRNA abundance, with ≈ 70 – 80% overall sequence identity an approximate minimum requirement for silencing. More recently very limited regions of absolute identity (23–98 bp, depending on the specific silencing vector), have been shown to be sufficient to mediate silencing of target mRNAs (Thomas *et al.*, 2001; Wesley *et al.*, 2001). Although the oncogene region has traditionally been called the 'core sequence' of *Agrobacterium* T-DNA due to high nucleotide sequence conservation (Paulus *et al.*, 1991;

Schroder *et al.*, 1983), the *iaaM* and *ipt* genes of a relatively small number of primarily Biovar 1 strains have been well characterized. Thus, we were interested in assessing the spectrum of oncogene-silencing mediated resistance through challenge with a large and diverse population of *A. tumefaciens* strains.

Wild-type and line 01/6 tomato plants were inoculated with 35 *A. tumefaciens* strains of diverse origin, chromosomal background, and Ti plasmid characteristics (Table 2). All strains except 1D141 generated tumours, of variable size and morphology, on wild-type tomato. The oncogene-silenced line 01/6 was consistently resistant to tumorigenesis induced by all tested *A. tumefaciens* and *A. vitis* strains. These results suggest that the sequences of

Table 2. *A. tumefaciens* strains utilized in disease challenge bioassays

Strain	Biovar	K84 sensitivity	Ti plasmid type	Reference/source
A208	I	S	nopaline	Sciaky <i>et al.</i> (1978)
A281	I	R	mannopine/agropine	Sciaky <i>et al.</i> (1978)
A348	I	R	octopine	Garfinkel <i>et al.</i> (1981)
A356	I	R	octopine	Garfinkel <i>et al.</i> (1981)
A328	I	R	octopine	Garfinkel <i>et al.</i> (1981)
Ach5	I	unknown	octopine	Hamilton and Fall (1971)
B6S3 \times 200	I	unknown	octopine	Fraley <i>et al.</i> (1985)
C58	I	S	nopaline	Sciaky <i>et al.</i> (1978)
CG49	III	R	nopaline	Burr and Reid (1994)
Ec1	I	unknown	unknown	This study
K12	I	unknown	octopine	Dandekar <i>et al.</i> (1987)
Tm4	III	R	unknown	Bonnard <i>et al.</i> (1989)
2516	I	S	unknown	This study ^a
15955	I	R	octopine	Barker <i>et al.</i> (1983)
1D135	I	unknown	nopaline	This study ^b
1D141	I	unknown	unknown	This study ^b
1D1130	I	unknown	unknown	This study ^b
1D1248	II	unknown	unknown	This study ^b
1D1144	I	unknown	unknown	This study ^b
1D1299	I	unknown	unknown	This study ^b
18W-7A	I	R	mannopine/agropine	This study ^a
20W-5A	I	R	mannopine/agropine	Escobar <i>et al.</i> (2001)
53-1B	II	unknown	nopaline	This study ^c
68-3A	II	R	unknown	This study ^c
69-2B1	I	R	unknown	This study ^c
85-1A	I	S	unknown	This study ^c
92A	I	R	unknown	This study ^c
93A	II	R	unknown	This study ^c
99	I	R	unknown	This study ^c
105-1A	I	S	unknown	This study ^c
127A	II	S	unknown	Escobar <i>et al.</i> (2001)
132A	I	S	unknown	This study ^c
142-4A	II	R	unknown	This study ^c
143-1A	I	R	unknown	This study ^c
149-1A	II	R	unknown	This study ^c

^aStrain courtesy of M. Schroth, University of California, Berkeley.

^bStrain courtesy of C. Kado, University of California, Davis.

^cStrain courtesy of L. Epstein, University of California, Davis.

the *iaaM* and *ipt* oncogenes are highly conserved, either locally or throughout, over an exceptionally broad range of *Agrobacterium* strains. This broad conservation of *iaaM* and *ipt* sequence is fully consistent with a model of oncogene evolution by horizontal gene transfer, as proposed by Otten *et al.* (1992). From the standpoint of disease control, the broad spectrum of oncogene-silencing mediated resistance is significant, and contrasts with the relative specificity of the commonly utilized crown gall biocontrol, *A. radiobacter* K84 (see Table 2) (Shim *et al.*, 1987).

EXPERIMENTAL PROCEDURES

Plant material

All described studies were performed on wild-type and transformed individuals of the tomato cultivar Moneymaker. The 01/6 transgenic line displays PTGS of the *iaaM* and *ipt* *Agrobacterium* oncogenes as a result of the expression of two self-complementary oncogene constructs (Escobar *et al.*, 2001). Oncogene silencing in the 01/6 line suppresses crown gall tumour development (Escobar *et al.*, 2001). The 326/4 transgenic line possesses an antisense *iaaM* transgene driven by a 35S Cauliflower mosaic virus promoter (35S CaMV). Although antisense *iaaM* transcript accumulates in 326/4 (data not shown), no oncogene silencing is apparent in these plants, which produce crown galls that are indistinguishable from wild-type.

A. *tumefaciens* strains and inoculation techniques

The *A. tumefaciens* strains utilized in tumorigenesis studies are listed in Table 2. The strains are primarily classical isolates (e.g. A208, A281, 15955) and previously undescribed isolates from crown galls of fruit and nut trees (e.g. 20W-5A, 92A, 53-1B). The T-DNA mutant strains A356 (*ipt*⁻) and A328 (*iaaM*⁻) (Garfinkel *et al.*, 1981) were utilized to determine single oncogene silencing phenotypes.

For general tumorigenesis screening, inoculations were performed using a 25 gauge needle attached to a 5 mL syringe containing a suspension of *A. tumefaciens* in water (OD₆₀₀ = 0.1). The hypocotyl and first internode of two wild-type and two 01/6 seedlings were inoculated with each individual *A. tumefaciens* strain. Tumorigenesis was assayed 5 weeks after inoculation. For histological studies and *in planta* *Agrobacterium* growth analysis, a 25 µL Hamilton syringe was used to pierce the hypocotyl and deliver a defined amount of inoculum into the wound site (see below).

Grafting

In order to investigate graft transmission of oncogene silencing, the crown gall-susceptible wild-type and 326/4 lines were grafted

to the oncogene-silenced line 01/6. Grafting was performed by the cleft grafting method, essentially as described by Sonoda and Nishiguchi (2000), using seedlings with approximately four to five true leaves and a stem diameter of ≈ 3–4 mm. The base of the scion was trimmed to a wedge and inserted into the decapitated stem of a rootstock that had been cut vertically to create a cleft. The graft junction was secured with cut segments of 3/16-inch Tygon tubing. Plants were maintained in clear plastic boxes for the first 3 days after grafting (to reduce dehydration) and then transferred to a greenhouse.

Three weeks after grafting, the non-silenced genotype (wild-type or 326/4) was inoculated with the virulent *A. tumefaciens* strains 20W-5A or A356 (*ipt*⁻) at three sites along the stem (1 cm and 3 cm above/below the graft union and at the most apical/basal internode). Alternatively, a shallow incision was made along the stem through the graft union and inoculum was delivered into the incision using a micropipettor. Tumorigenesis was scored 5 weeks after inoculation.

Growth of *A. tumefaciens* in planta

Wild-type and 01/6 tomato seedlings were inoculated just below the cotyledonary node with 1.0 µL of a suspension of *A. tumefaciens* 20W-5A in water (OD₆₀₀ = 0.05). Bacterial populations were sampled from three wild-type and three 01/6 individuals at 2, 12, 22, 32, 42 and 52 days post-inoculation (dpi). Excised stems were surface-disinfested for 1 min in 20% commercial bleach/0.1% Tween-20 and rinsed three times in sterile distilled water. An 8 mm section (4 mm above and below the inoculation site) was excised from the sterilized stem, diced and crushed. Crushed tissue was incubated in sterile water (1 mL/300 mg tissue) for 30 min, and then a 50 µL aliquot was serially diluted in sterile water. A 75 µL aliquot of each serial dilution was plated on *Agrobacterium* biovar 1 selective medium 1A (Moore *et al.*, 1988) and incubated for 48 h at 28 °C. Two experimental replicates were performed (six total samples/genotype/time point) and a similar group of control seedlings was mock-inoculated with sterile water.

A. tumefaciens population level data were first log transformed and then analysed using three-way ANOVA models, examining the effects of genotype, sampling date, and experiment. Following these analyses, *post hoc* ANOVA models were run to examine the effects of genotype and experiment within each sampling date. In the text, significance refers to $P < 0.05$.

Small RNA analysis

Total RNA was isolated and size-fractionated from young tomato leaves as described by Johansen and Carrington (2001). Low molecular weight RNA was subjected to electrophoresis through a 19% polyacrylamide–7 M Urea–0.5 × TBE gel and electroblotted

to a Hybond-NX membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). Hybridization of small RNA blots was performed as described by Johansen and Carrington (2001), and image analysis of autoradiograms was carried out using a phosphorimager.

Histology

The hypocotyls of wild-type and 01/6 tomato seedlings were inoculated with 2.5 µL of *A. tumefaciens* strain 20W-5A (virulent) or EHA101 (avirulent control). Plants were harvested at 3, 6, 9, 12, 15 and 30 dpi and hypocotyl segments were fixed in 3% glutaraldehyde in 0.25 M phosphate buffer, pH 6.8. The fixative solution was infiltrated into the tissue under slight vacuum. The sections were stored in fixative at 4 °C for 7 days. The hypocotyl segments were rinsed three times in buffer and then dehydrated in an ethanol series beginning at 10% ethanol and increasing in 10% ethanol increments for 1 h each through 90%, 95% and two changes of 100% ethanol. The segments were infiltrated with Historesin (Leitz Instruments GmbH, Heidelberg, Germany) glycolmethacrylate basic resin (GMA) through a dilution series of 1 : 1 and 2 : 1 GMA : 100% ethanol for 8 h each at room temperature, followed by daily changes of 100% GMA until the tissue became translucent at 3 weeks. The resin was then polymerized according to the manufacturer's directions. Sections were cut at a nominal thickness of 3 µm on a Leitz microtome and stained with toluidine blue O (O'Brien and McCully, 1981). Images were recorded using an Optronics 750 digital camera (Optronics Inc., Goleta, Calif.) on a Nikon E600 microscope (Nikon USA, Stamford, CT).

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