AN ABSTRACT OF THE DISSERTATION OF

Jennifer Pitrak for the degree of Doctor of Philosophy in Genetics presented on June 6, 2005.

Title: Silencing the *Agrobacterium tumefaciens* Oncogenes

Abstract approved:

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Crown gall disease is an agricultural problem caused by the soil-borne bacterium, *Agrobacterium tumefaciens*. *A. tumefaciens* oncogenes cause transformed plant cells to overproduce the hormones, auxin and cytokinin. High hormone levels cause unorganized plant cell growth resulting in a gall. Control of crown gall disease is difficult because after plant cell transformation has occurred, the bacterium is no longer required for the disease to progress. Apple trees engineered to express double-stranded RNA of two *A. tumefaciens* oncogenes, *ipt* and *iaaM*, silenced the expression of the wild-type oncogenes and prevented crown gall disease. Only the *iaaM* oncogene was targeted for posttranscriptional gene silencing (PTGS) as measured by biological assays and by quantitative reverse-transcription polymerase chain reaction (q-RTPCR) on transgenic tissue. However, if the translation initiation sequence of the *iaaM* construction was eliminated, gall formation was not prevented, indicating that translatable RNA initiates silencing whereas untranslatable RNA does not. Other data indicate that the *Arabidopsis thaliana* micro-RNA pathway gene is involved in *A. tumefaciens*-mediated tumorigenesis. *A. thaliana* plants with a mutation in *HEN1*, a gene required for micro-RNA maturation, demonstrated a tenfold reduction in tumorigenesis upon *A. tumefaciens* infection compared to wild-type. The same plant line showed no difference in T-DNA transfer and nuclear uptake.

Silencing the *Agrobacterium tumefaciens* Oncogenes

by

Jennifer Pitrak

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Jennifer Pitrak, Author

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CONTRIBUTION OF AUTHORS

Dr. Walt Ream was advisor and editor on all manuscripts included in this work. For Chapter 2, Drs. Walt Viss, Mike Cook, and John Driver, produced and conducted tumorigenesis assays on the transgenic apple trees described. Jodi Humann conducted the RNA extraction and real-time reverse transcriptase PCR of the apple trees, assisted with all figures, and provided help with data interpretation. Josh Cuperus built transgene construction pJC14 (Figure 3.1).

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Chapter 1

Silencing the *Agrobacterium tumefaciens* Oncogenes

Jennifer Pitrak

Agrobacterium tumefaciens and crown gall disease

Agrobacterium tumefaciens biology

Agrobacterium tumefaciens is the causative agent of crown gall disease (Escobar and Dandekar, 2003; Zhu et al., 2000), which is estimated to cause $400,000 in annual losses in the Pacific Northwest alone (Pinkerton et al., 1996). It is a ubiquitous soil bacterium and infects a wide variety of dicotyledonous plant species including fruit and nut trees, grapevines, cane berries, chrysanthemum, rose, and other nursery crops (Bouzar and Moore, 1987; DeCleene and DeLey, 1976; Pinkerton et al., 1996). *A. tumefaciens* genetically engineers its host by transferring a part of its tumor-inducing (Ti) plasmid, transferred DNA (T-DNA), to the plant cell where it integrates into the plant genome, and expression of T-DNA genes ensues (Escobar and Dandekar, 2003; Winans, 1992; Zambryski, 1992) (Figure 1.1A). T-DNA genes encode enzymes for plant hormones (auxin and cytokinin) and opine biosynthesis (Winans, 1992) (Figure 1.1B). Auxin and cytokinin overproduction results from expression of three T-DNA genes: *iaaM* (tryptophan monooxygenase), *iaaH* (indole-3-acetamide hydrolase), and *ipt* (AMP isopentenyl transferase) (Garfinkel et al., 1981; Ream et al., 1983). IaaM converts tryptophan into indole-3-acetamide, which IaaH converts into indole-3-acetic acid (auxin) (Inze et al., 1984; Schroeder et al., 1984; Thomashow et al., 1984; Thomashow et al., 1986; Van Onckelen et al., 1986) (Figure 1.2). Ipt converts adenosine monophosphate into isopentenyl adenosine monophosphate, a cytokinin (Akiyoshi et al., 1984; Barry et al., 1984; Buchmann et al., 1985) (Figure 1.2). Rapid and unorganized plant cell proliferation occurs in response to this auxin and cytokinin production, resulting in a gall. T-DNA also directs the production of amino acid and sugar phosphate derivatives called opines (Winans, 1992). Genes encoded on the *Agrobacterium* Ti-plasmid direct the uptake and catabolism of opines (Escobar and Dandekar, 2003; Guyon et al., 1980; Zhu et al., 2000). Thus, *A. tumefaciens* engineers a nutrient-rich niche for itself at the expense of the host.

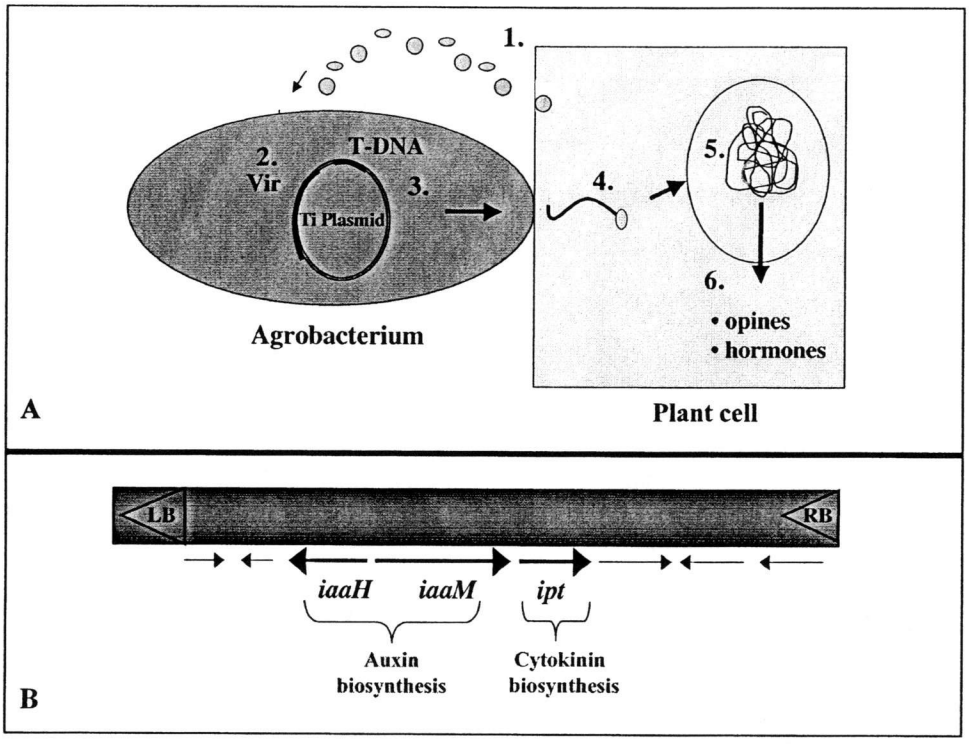

Figure 1.1 Plant cell transformation by *A. tumefaciens.* **A**, Wounded plant cells emit sugars and phenolic compounds (step 1) that induce the expression of virulence (*Vir*) genes on the tumor-inducing plasmid (Ti Plasmid) (step 2); vir proteins process transferred DNA (T-DNA) from another part of the Ti plasmid (step 3) and export the T-DNA to the plant cell (step 4); T-DNA localizes to the plant nucleus and integrates into the plant genome (step 5); T-DNA genes directing opine and hormone biosynthesis are expressed (step 6). **B**, Detail of T-DNA. Left border (LB) and right border (RB) delineate DNA, which is transferred to plant cells; *iaaH* and *iaaM* encode proteins involved in auxin biosynthesis; *ipt* encodes a protein involved in cytokinin biosynthesis (see Figure 1.2).

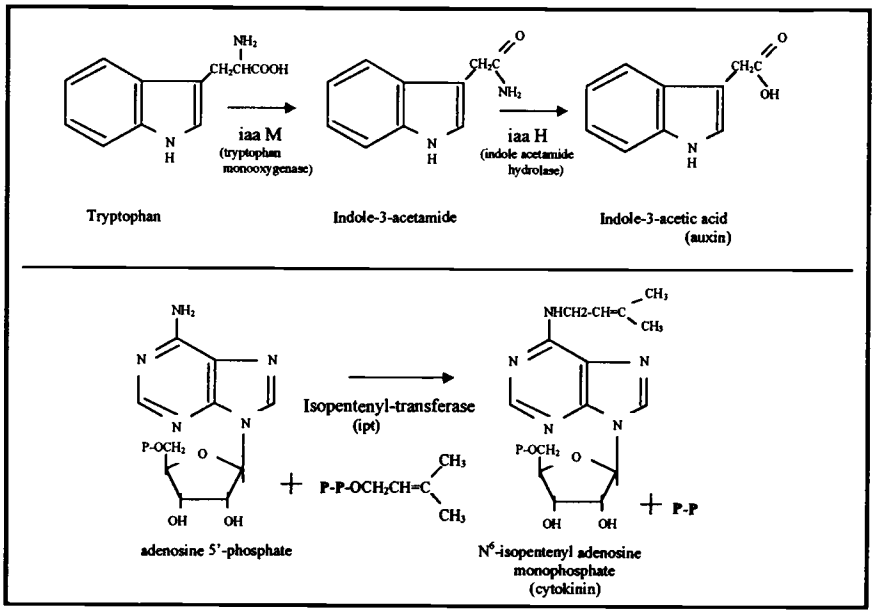

Figure 1.2 Plant hormone biosynthesis. T-DNA genes *iaaM* and *iaaH* encode tryptophan monooxygenase and indole acetamide hydrolase, respectively. T-DNA gene *ipt* encodes isopentenyl transferase.

Control of crown gall disease

Adequate means do not exist to prevent crown gall disease in non-transgenic plants. Current control includes labor-intensive and non-failsafe methods such as the identification and containment of *Agrobacterium* infection, eradication of the bacterium, crop rotation with resistant plant species, and use of crop varieties or cultivars that exhibit reduced or absent susceptibility. For example, standard recommended control procedures for crown gall disease involve the following: sterilization of soil (fumigation), sterilization of horticulture tools using diluted bleach or alcohol solutions, prevention of plant wounding during manipulation, control of plant-wounding insects and worms, destruction of nursery plants exhibiting crown galls, and application of *A. radiobacter* K84 (Mullen and Hagan, 2005). The widely-

used control agent, *Agrobacterium radiobacter* K84, affords some protection against specific strains of *A. tumefaciens* (Jones et al., 1988; Kerr and Panagopoulos, 1977; Kerr and Tate, 1984; Moore, 1979, 1988; Shim et al., 1987). *A. radiobacter* K84 produces a toxin called agrocin 84 (Hayman and Farrand, 1988; Murphy and Roberts, 1979; Ryder et al., 1987), which inhibits growth of bacterial cells (Murphy and Roberts, 1979; Ryder et al., 1987). Agrocin 84 enters *A. tumefaciens* via the active transport system that mediates uptake of the agrocinopine class of opines (Murphy and Roberts, 1979). Only agrocinopine-type *A. tumefaciens* have the permease that confers sensitivity to agrocin 84. Therefore, other *A. tumefaciens* strains are not susceptible to *A. radiobacter* K84 and crown gall disease persists (Pinkerton et al., 1996).

Disease-resistant crop varieties and cultivars have been identified. For example plum (Bliss et al., 1999), rose (Reynders-Aloisi and Pelloli, 1998), aspen (Beneddra et al., 1996), and grape (Sandor and Burr, 1995) demonstrate some resistance to crown gall disease. However, the mechanism of this resistance is not known and plant resistance varies with the inciting strain of *Agrobacterium* (Sandor and Burr, 1995; Sule et al., 1994; Yanofsky et al., 1985). Therefore, crown gall disease remains a problem in these species. Although crown-gall-resistant *Arabidopsis thaliana* mutants have been identified (Nam et al., 1997; Nam et al., 1999), screening of large numbers of mutant woody species is simply not practical.

RNA silencing

RNA silencing is an evolutionarily conserved mechanism of gene regulation and genome protection based on dsRNA (Baulcombe, 2004; Tomari and Zamore, 2005). In plants, dsRNA can arise from various sources including viruses, inverted-repeats, bidirectional transcription, or from the activity of RNA-dependent RNA polymerases (RdRP) (Figure 1.3). dsRNA is processed by silencing machinery to small RNAs (smRNAs), between 21 and 26 nucleotides, which afford sequence complementarity to

either DNA or RNA targets that are transcriptionally or posttranscriptionally silenced, respectively (Figure 1.3). RNA silencing is divided into different pathways based on the origin of dsRNA, the silenced targets, and the genes involved (Baulcombe, 2004; Matzke et al., 2004; Matzke and Birchler, 2005; Xie et al., 2004). The micro-RNA (miRNA) and *trans*-acting-siRNA (ta-siRNA) pathways are involved in endogenous gene regulation (Allen et al., 2005; Dugas and Bartel, 2004; Peragine et al., 2004; Vazquez et al., 2004b) (Figures 1.4 and 1.5). miRNA biogenesis and function require the Dicer- like protein, DCL1 (Schauer et al., 2002), ARGONAUTE1 (AGO1, a RISC

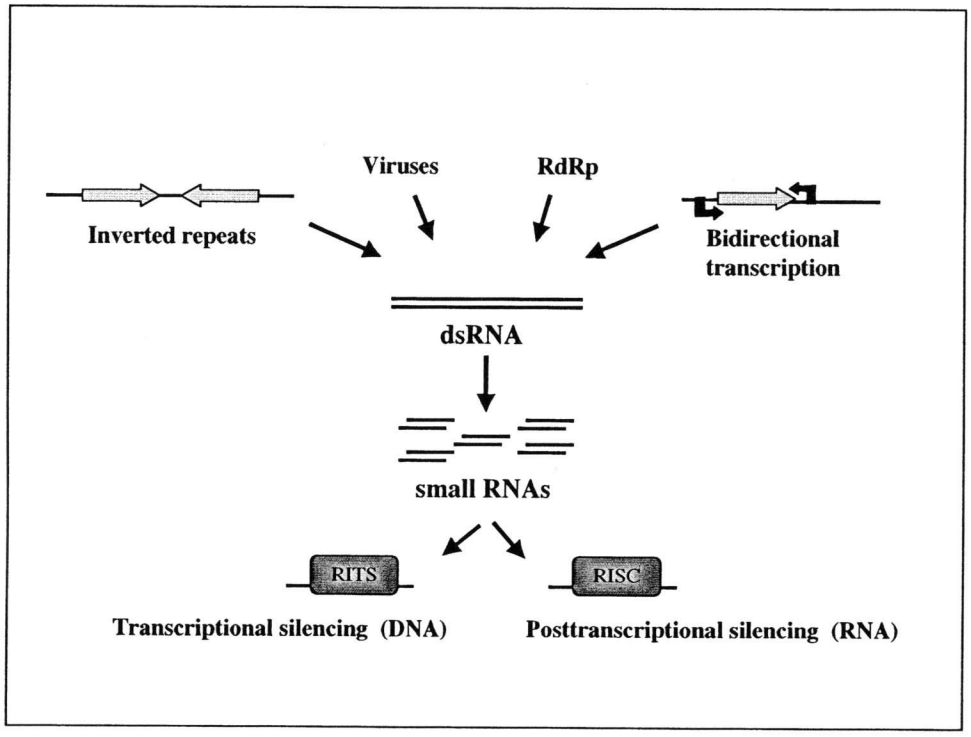

Figure 1.3 Summary of RNA silencing in plants. dsRNA arising from sources such as inverted-repeat DNA sequences, bidirectional transcription, viruses, or RNA-dependent RNA polymerases (RdRP) are processed to small RNAs which afford sequence specificity to the RNA-induced transcriptional silencing complex (RITS) or the RNA-induced silencing complex (RISC) to silence gene expression from DNA or RNA, respectively.

component (Vaucheret et al., 2004), the RNA methyltransferase HEN1 (Yu et al., 2005), and two other nuclear-localized proteins, HYL1 and HST, for miRNA maturation and nuclear export, respectively (Han et al., 2004; Hiraguri et al., 2005; Park et al., 2005; Vazquez et al., 2004a) (Figure 1.4). miRNAs arise from protein-noncoding transcripts encoded by *MIR* genes (Reinhart et al., 2002). RNA polymerase II-derived *MIR* transcripts form stem-loop secondary structures called primary-miRNAs (pri-miRNAs) that are processed by DCL1 and the dsRNA-binding protein HYL1 to small RNAs (Han et al., 2004; Hiraguri et al., 2005; Park et al., 2002; Reinhart et al., 2002; Schauer et al., 2002; Vazquez et al., 2004a). HEN1 methylates the 3'-end of each strand of the small RNAs to yield mature miRNAs (Yu et al., 2005). Mature miRNAs are exported from the nucleus via the Exportin-5-like protein, HST (Lund et al., 2004; Park et al., 2005) (Figure 1.4). These small RNAs then target mRNAs containing complementary sequences for RISC-mediated cleavage and subsequent destruction or further processing in the case of ta-siRNAs (see below) (Allen et al., 2005; Tang et al., 2003) (Figures 1.4 and 1.5). miRNAs can also function as translational repressors (Aukerman and Sakai, 2003; Chen, 2004). Many miRNA-regulated genes are involved in plant developmental processes and many encode transcription factors (Table 1.1) (Allen et al., 2005; Dugas and Bartel, 2004; Kasschau et al., 2003; Mallory et al., 2005; Vazquez et al., 2004a). Whereas protein-coding targets are downregulated by miRNAs, some targets require miRNAs for functional maturation. These target RNAs are protein-noncoding transcripts that generate ta-siRNAs with sequence complementarity to yet other RNAs. In the ta-siRNA pathway, miRNA-cleaved transcripts are templates for dsRNA synthesis by the RNA-dependent RNA polymerase (RdRp), RDR6, and a protein of unknown function, SGS3, which is required for RDR6-dependent RNA silencing (Mourrain et al., 2000; Peragine et al., 2004; Vazquez et al., 2004b) (Figure 1.5). Another Dicer-like protein, DCL4, likely processes this dsRNA to ta-siRNAs in a 21-nucleotide phase set by the original miRNA cleavage (Allen et al., 2005) (Xie and Carrington, personal communication). ta-siRNAs target protein-coding transcripts and may target the RNA

from which they originate (Allen et al., 2005; Peragine et al., 2004; Vazquez et al., 2004b).

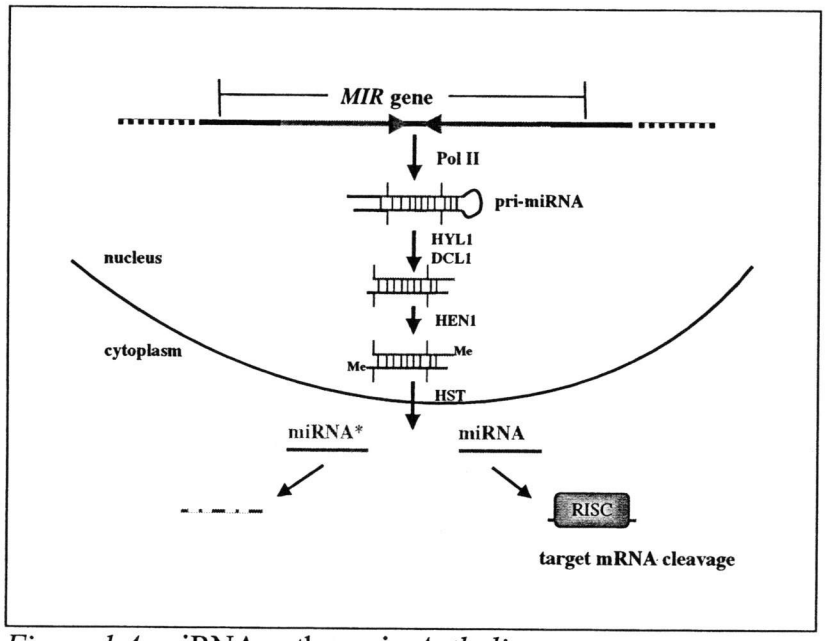

Figure 1.4 miRNA pathway in *A. thaliana.*

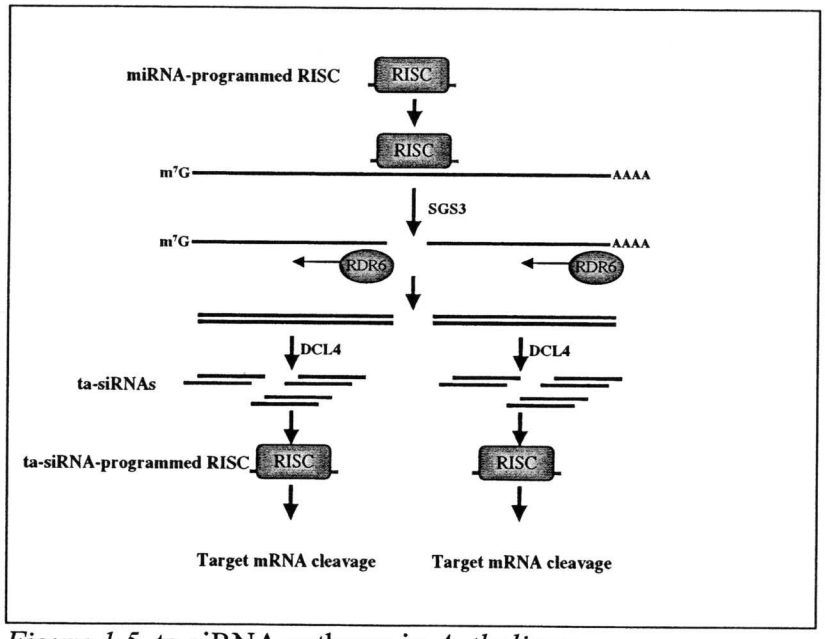

Figure 1.5 ta-siRNA pathway in *A. thaliana.*

Table 1.1 Arabidopsis miRNA and ta-siRNA Targets
[Adapted from (Allen et al., 2005; Jones-Rhoades and Bartel, 2004).]

miRNA or ta-siRNA Family	Target Family	Target Family Function	References
miR156	Squamosa promoter-binding protein (SBP)-like	Transcription factor	(Kasschau et al., 2003; Vazquez et al., 2004a)
miR159	MYB	Transcription factor	(Achard et al., 2004; Palatnik et al., 2003; Vazquez et al., 2004a)
miR160	Auxin response factor (ARF)	Transcription factor	(Kasschau et al., 2003; Vazquez et al., 2004a)
miR161	Pentatricopeptide repeat-containing protein (PPR)	Unknown	(Allen et al., 2004; Vazquez et al., 2004a)
miR162	Dicer-like (DCL)	miRNA metabolism	(Xie et al., 2004)
miR163	S-adenosyl-L-methionine: carboxyl methyltransferase (SAMT)	Transcription factor	(Allen et al., 2004)
miR164	NAC	Transcription factor	(Kasschau et al., 2003; Laufs et al., 2004; Mallory et al., 2004; Vazquez et al., 2004a)
miR166	homeobox domain-leucine zipper protein (HD-ZIPIII)	Transcription factor	(Emery et al., 2003; Tang et al., 2003)
miR167	ARF	Transcription factor	(Kasschau et al., 2003; Vazquez et al., 2004a)
miR168	Argonaute 1 (AGO1)	miRNA metabolism	(Vaucheret et al., 2004; Vazquez et al., 2004a)
miR169	CCAAT-binding factor (HAP2)	Transcription factor	(Jones-Rhoades and Bartel, 2004)
miR171	Scarecrow (SCR)	Transcription factor	(Llave et al., 2002; Vazquez et al., 2004a)

(continued)

Table 1.1 (continued)

miRNA or ta-siRNA Family	Target Family	Target Family Function	References
miR172	APETALA 2 (AP2) floral homeotic protein	Transcription factor	(Aukerman and Sakai, 2003; Chen, 2004; Kasschau et al., 2003)
miR173	TAS1, TAS2	ta-siRNA biogenesis	(Allen et al., 2005)
miR319	TCP	Transcription factor	(Palatnik et al., 2003)
miR390	TAS3	ta-siRNA biogenesis	(Allen et al., 2005)
miR393	Transport inhibitor response protein (TIR1)/F-box; bHLH	Hormone signaling	(Jones-Rhoades and Bartel, 2004)
miR394	F-box	Transcription factor	(Jones-Rhoades and Bartel, 2004)
miR395	ATP-sulfurylase (ATPS); sulfate adenylyltransferase (AST)	Metabolism	(Allen et al., 2005; Jones-Rhoades and Bartel, 2004)
miR396	GRF	Transcription factor	(Jones-Rhoades and Bartel, 2004)
miR397	laccase/copper oxidase	Metabolism	(Jones-Rhoades and Bartel, 2004)
miR398	copper/zinc/superoxide dismutase (CSD); Cytochrome C oxidase	Stress response; metabolism	(Jones-Rhoades and Bartel, 2004)
miR399	E2-UBC	Ubiquitin conjugation	(Allen et al., 2005)
miR447	2-phosphoglycerate kinase (PGK) – related protein	Metabolism	(Allen et al., 2005)
miR403	Argonaute 2 (AGO2)	miRNA metabolism	(Allen et al., 2005)
TAS1	Unclassified	Unknown	(Peragine et al., 2004; Vazquez et al., 2004b)
TAS2	PPR	Unknown	(Allen et al., 2005)
TAS3	ARF	Transcription factor	(Allen et al., 2005)

Another endogenous RNA silencing pathway in *Arabidopsis* silences transcription of target genes via heterochromatinization (chromatin condensation by DNA and histone methylation). In this chromatin-associated RNA silencing pathway, highly repeated DNA sequences (such as ribosomal RNA sequences, transposons, retroelements, and centromeric repeats) are sources and targets of siRNAs that mediate transcriptional gene silencing (Chan et al., 2004; Hamilton et al., 2002; Lippman et al., 2004; Lippman and Martienssen, 2004; Onodera et al., 2005; Xie et al., 2004; Zilberman et al., 2003; Zilberman et al., 2004). This chromatin RNAi pathway involves the Dicer-like enzyme DCL3, (the RdRp) RDR2, and RNA polymerase IV for siRNA production; an Argonaute protein, AGO4, is probably part of the transcriptional silencing complex (Table 1.2) (Figure 1.6) (Chan et al., 2004; Herr et al., 2005; Onodera et al., 2005; Xie et al., 2004; Zilberman et al., 2003; Zilberman et al., 2004). Other proteins in the chromatin RNAi pathway are involved in methylation of histones and DNA, including the CpNpG methyltransferase CHROMOMETHYLASE 3 (CMT3) (Table 1.2) (Cao and Jacobsen, 2002a; Lindroth et al., 2001). Two other RNA silencing pathways target foreign nucleic acid species, namely viruses and transgenes. RDR1 and DCL2 function in antiviral defense (Xie et al., 2004; Yu et al., 2003). RDR6 and SGS3 contribute to silencing sense transgenes and some viruses (Butaye et al., 2004; Dalmay et al., 2000; Mourrain et al., 2000). There is genetic overlap among the different RNA silencing pathways (Figure 1.6) (Table 1.2).

Table 1.2 *Arabidopsis thaliana* RNA silencing pathways, associated proteins, and their functions.

PROTEIN	DESCRIPTION & FUNCTION	PATHWAY					REFERENCES
		miRNA	trans-acting-siRNA	Anti-viral	Chromatin RNAi	Transgene silencing	
AGO1	Binds and/or cleaves target RNA (RISC component)	X	X				(Vaucheret et al., 2004)
AGO4	Directs chromatin modifications	-			X		(Chan et al., 2004; Zilberman et al., 2003; Zilberman et al., 2004)
AGO7	unknown		X				(Fagard et al., 2000; Hunter et al., 2003; Peragine et al., 2004)
CMT3	DNA (CpNpG) methylation				X		(Cao and Jacobsen, 2002a; Lindroth et al., 2001)
DCL1	RNAseIII-like; Cleaves pre-miRNA to miRNA	X	X				(Park et al., 2002; Reinhart et al., 2002; Schauer et al., 2002)
DCL2	RNAseIII-like; Cleaves dsRNA to siRNAs			X			(Xie et al., 2004)
DCL3	RNAseIII-like; Cleaves dsRNA to siRNAs				X	X	(Chan et al., 2004; Xie et al., 2004)
DCL4	RNAseIII-like; Cleaves dsRNA to siRNAs		X				(Xie and Carrington, personal communication)
DRM1	de novo DNA methylation				X		(Cao and Jacobsen, 2002a, 2002b)
DRM2	de novo DNA methylation				X		(Cao and Jacobsen, 2002a, 2002b)
HEN1	miRNA methylation/maturation	X	X		X		(Boutet et al., 2003; Chen et al., 2002; Park et al., 2002; Yu et al., 2005)
HST	Homologous to mammalian Exportin 5; miRNA nuclear export	X	X				(Lund et al., 2004; Park et al., 2005)
HYL1	dsRNA-binding protein; miRNA stabilization and/or maturation; modulation of DCL1 function	X	X				(Han et al., 2004; Hiraguri et al., 2005; Vazquez et al., 2004a)

(continued on next page)

Table 1.2, continued.

PROTEIN	DESCRIPTION & FUNCTION	PATHWAY					REFERENCES
		miRNA	trans-acting-siRNA	Anti-viral	Chromatin RNAi	Transgene silencing	
KYP	Histone methyltransferase				X		(Jackson et al., 2002)
MET1	Cytosine methyltransferase; DNA (CpG) methylation				X		(Finnegan et al., 1996, 2000; Kishimoto et al., 2001; Ronemus et al., 1996; Vongs et al., 1993)
RDR1	RNA-dependent RNA polymerase (RdRP); viral RNA turnover			X			(Yu et al., 2003)
RDR2	RdRP; copies sense RNA or stabilizes DCL3				X	X	(Chan et al., 2004; Xie et al., 2004)
RDR6 (SDE1/SGS2)	RdRP; amplification of silencing signal, spreading of silencing; viral RNA turnover		X	X		X	(Allen et al., 2005; Butaye et al., 2004; Dalmay et al., 2000; Mourrain et al., 2000; Parizotto et al., 2004; Peragine et al., 2004; Vazquez et al., 2004b; Yu et al., 2003)
SDE3	unknown					X	(Dalmay et al., 2000)
SGS3	spreading of silencing		X	X		X	(Butaye et al., 2004; Mourrain et al., 2000; Peragine et al., 2004)
NRPD1a (previously SDE4)	Subunit of RNA polymerase IV (produces chromatin-associated siRNAs)				X		(Chan et al., 2004; Hamilton et al., 2002; Herr et al., 2005; Onodera et al., 2005)
NRPD2a	Subunit of RNA polymerase IV (produces chromatin-associated siRNAs)				X		(Onodera et al., 2005)
SUVH2	histone methylation				X		(Naumann et al., 2005)

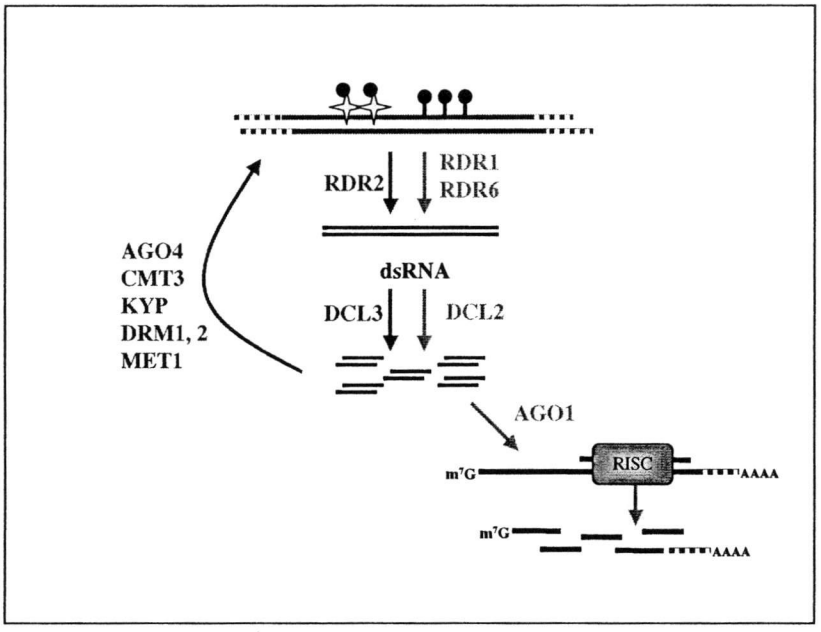

Figure 1.6 Transcriptional and posttranscriptional silencing pathways in *Arabidopsis thaliana*. Chromatin-associated RNA silencing pathway is on the left-hand side of the diagram; stars and lollipops represent RNA-directed heterochromatinization of DNA. Viral and transgene silencing pathways are on the right-hand side of the diagram. See Table 1.2 for a description of the proteins.

RNA silencing and crown gall disease

Crown gall disease (tumorigenesis) results from T-DNA gene expression in plant cells. RNA silencing reduces expression of genes with sequences matching the initiating dsRNA molecule (Tomari and Zamore, 2005). Therefore, RNA silencing can inhibit T-DNA gene expression if the host expresses dsRNA of T-DNA-derived gene sequences. This strategy produced crown gall disease-resistant plants in two cases (Escobar et al., 2001; Escobar et al., 2002; Viss et al., 2003). Interestingly, silencing required that the initiating RNA contain translatable sequences, as

untranslatable transgenes did not initiate silencing (Lee et al., 2003). Thus, RNA silencing can be exploited for generating crown gall disease resistance.

A. tumefaciens-induced tumorigenesis also requires plant cell functions to facilitate transformation, T-DNA gene expression, and response to hormones (Gelvin et al., 2002; Zhu et al., 2003). Plant cell transformation involves several steps including bacteria-plant cell binding, emission of signals for *vir* gene induction, T-DNA transfer, nuclear import, and integration. T-DNA genes must be expressed and plant cells must respond to auxin and cytokinin produced by these gene products. In roots, plant cells need only respond to auxin overproduction as root tissue is insensitive to cytokinin (Ream et al., 1983). Disruption of any of these functions can alter tumorigenesis. As protection against foreign nucleic acids, RNA silencing may target T-DNA or T-DNA-derived mRNAs. Disruption of the protective RNA silencing pathways may increase T-DNA integration, expression, and ultimately increase the frequency of tumorigenic transformation events. Also, the miRNA pathway regulates several genes including those encoding transcription factors and hormone response factors, such as auxin response factors (ARFs) (Table 1.1). Therefore, disruption of the miRNA pathway may disrupt regulation of genes required for transformation and tumor development.

Crown gall disease resistance by RNA silencing

Two cases of crown-gall-resistant plants have been reported (Escobar et al., 2001; Escobar et al., 2002; Viss et al., 2003), but this technology has yet to become commercially available. In both cases, disease resistance was based on RNA silencing (Baulcombe, 2000; Lindbo and Dougherty, 1992a, 1992b; Napoli et al., 1990; Tijsterman et al., 2002; van der Krol et al., 1990). Crown gall disease was prevented in transgenic *Arabidopsis thaliana* and tomato (*Lycopersicon esculentum*) (Escobar et al., 2001), walnut (Escobar et al., 2002), and apple trees (Viss et al., 2003) expressing double-stranded RNA (dsRNA) of the *A. tumefaciens* oncogenes, *iaaM* and *ipt*.

Chapter 2 of this document describes the disease resistance in apple trees mediated by RNAi. Transgene constructions were designed to generate both sense and antisense transcripts of *iaaM* and *ipt*. The transgene sequences, *iaaM*-stop and *ipt*-stop, contained premature stop codons and frameshift mutations two codons downstream of the first AUG to ensure that no functional oncogenic proteins would be made *in planta* (see Figures 2.1 and 3.1). These constructions successfully silenced the wild-type *iaaM* gene and prevented tumorigenesis on apple roots (Figures 2.3 and 2.4). Interestingly, despite both *iaaM*-stop and *ipt*-stop being fused and in the same T-DNA, only the *iaaM* oncogene was silenced; *ipt* was not. Fortunately, roots do not respond to cytokinin (Ream et al., 1983), so *iaaM*-silencing is sufficient for disease resistance. Thus, RNA silencing can be exploited to prevent T-DNA gene expression and crown gall disease.

In the study reported here, *iaaM* silencing may require translatable RNA to initiate silencing. Despite the intentional design of our transgenes to be untranslatable, it is likely that the *iaaM*-stop transgene is translatable from alternative AUG codons downstream from the premature stop and frameshift mutations. Two reports demonstrate that translatable transgene sequences induce RNA silencing better than nontranslatable sequences (Que et al., 1997; Tanzer et al., 1997). In petunia, premature termination codons in transgene mRNAs reduced silencing of the cognate endogenous gene (Que et al., 1997). Ribosomes associate with low-molecular-weight silencing-derived RNAs (Tanzer et al., 1997). Also, studies in other organisms demonstrate that RNAi and mRNA translation are linked. In *Drosophila melanogaster*, oocyte mRNAs are subject to RNAi only during oocyte maturation when mRNAs are actively translated but not during the oocyte's arrested stage when mRNAs are present but not translated (Kennerdell et al., 2002). Also, in *Drosophila* and *Trypanosoma bucei*, siRNAs and RISC component proteins associate with translating ribosomes (Djikeng et al., 2003; Ishizuka et al., 2002; Pham et al., 2004). Finally, two studies demonstrate that RNAi and mRNA turnover are linked (Gazzani

et al., 2004; Souret et al., 2004). The *Arabidopsis* AtXRN4 5'-3' exoribonuclease degrades micro-RNA (miRNA)-cleaved mRNAs (Souret et al., 2004) and can eliminate mRNA substrates for small-interfering RNA (siRNA) generation (Gazzani et al., 2004). Chapter 3 shows that translatable *iaaM* sequences initiate silencing. Untranslatable *iaaM* or *ipt* sequences do not trigger silencing.

RNA silencing may affect T-DNA-directed tumorigenesis

T-DNA-directed tumorigenesis requires T-DNA integration into the plant genome and subsequent expression of the oncogenes, *iaaM*, *iaaH*, and *ipt*. T-DNAs can also be engineered to contain transgenes (Bevan, 1984). Wild-type or transgene-containing T-DNAs are foreign genetic elements in plant cells and they frequently form inverted repeats (Jorgensen et al., 1987). Therefore, they are likely targets and initiators of RNA silencing. Several reports indicate that T-DNA is subject to silencing (Amasino et al., 1984; Butaye et al., 2004; Chan et al., 2004; Dalmay et al., 2000; Francis and Spiker, 2005; Mourrain et al., 2000; Schubert et al., 2004; Xie et al., 2004). In transformed plant tissues, silenced T-DNA genes are hypermethylated. After treating these tissues with the demethylating agent, 5-azacytidine, T-DNA genes are less methylated and their expression is reactivated (Amasino et al., 1984). *A. thaliana* plants with mutations in silencing-associated genes, *RDR6* and *SGS3*, exhibited 7- and 2.5-fold increased expression of a 35S cauliflower mosaic virus promoter (p*35S*)-driven ß-glucuronidase (GUS) *uidA* transgene, respectively, compared to wild-type plants (Butaye et al., 2004). Integration patterns of the transgene T-DNA included single and multiple copies and inverted and tandem repeats (Butaye et al., 2004). An *Agrobacterium*-delivered *FWA* transgene in *A. thaliana* required the chromatin-RNA silencing genes *DRM2, RDR2, DCL3, SDE4,* and *AGO4* for methylation and silencing (Chan et al., 2004). The *Arabidopsis FWA* gene (encoding a homeodomain-containing transcription factor and involved in timing of flowering) is normally methylated within direct repeats in its promoter, silencing *FWA* expression (Soppe et al., 2000). Epigenetic *fwa* mutants have lost this methylation and ectopic *FWA* expression causes

a dominant late flowering phenotype. The methylation and silencing of *FWA* can be restored by introduction of an extra copy of *FWA* via *Agrobacterium tumefaciens* transformation (Soppe et al., 2000). However, this silencing does not occur in *Arabidopsis* lines with mutations in the chromatin RNA-silencing genes *DRM2, RDR2, DCL3, SDE4,* and *AGO4* (Cao and Jacobsen, 2002b; Chan et al., 2004; Soppe et al., 2000). Also, PCR-based identification of T-DNA-transformed *A. thaliana* lines revealed silenced T-DNA (Francis and Spiker, 2005). Thus, disruption of the protective roles of RNA silencing may increase T-DNA-directed tumorigenesis. This hypothesis was tested in Chapter 4.

Disruption of the miRNA (or ta-siRNA) pathway may cause a different response to *A. tumefaciens* infection. Tumorigenesis requires that plant cells respond to the hormones produced as a result of T-DNA gene expression. miRNAs are predicted to regulate at least 109 genes, including at least seven auxin-response factors (ARF) (Table 1.1) (Allen et al., 2005; Jones-Rhoades and Bartel, 2004). An *A. thaliana* line with a mutation in *HYL1*, a gene involved in miRNA maturation, demonstrated reduced sensitivity to exogenous auxin and cytokinin (Lu and Fedoroff, 2000). Altered miRNA metabolism may affect ARF gene regulation and ultimately how plant cells respond to *Agrobacterium* infection.

Chapter 4 of this document examines tumorigenesis and T-DNA transfer in *A. thaliana* lines with single mutations in RNA silencing genes. Only one line, *hen1-1*, showed a significant (10-fold) reduction in tumorigenesis compared to wild-type. The same line showed no significant reduction in T-DNA transfer and nuclear uptake. If T-DNA integration and gene expression are not altered in this line, the reduction in tumorigenesis may be due to altered hormone response.

Chapter 2

Crown-Gall-Resistant Transgenic Apple Trees that Silence *Agrobacterium tumefaciens* Oncogenes

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Abstract

Crown gall disease is an economically significant problem in fruit and nut orchards, vineyards, and nurseries worldwide. Tumors on stems and leaves result from excessive production of the phytohormones auxin and cytokinin in plant cells genetically transformed by *Agrobacterium tumefaciens*. High phytohormone levels result from expression of three oncogenes transferred stably into the plant genome from *A. tumefaciens*: *iaaM, iaaH*, and *ipt*. The *iaaM* and *iaaH* oncogenes direct auxin biosynthesis, and the *ipt* oncogene causes cytokinin production. In contrast to other tissues, roots do not respond to high cytokinin levels, and auxin overproduction is sufficient to cause tumor growth on roots. Inactivation of *iaaM* abolished gall formation on apple tree roots. Transgenes designed to express double-stranded RNA from *iaaM* and *ipt* sequences prevented crown gall disease on roots of transgenic apple trees.

Introduction

Crown gall is a significant agricultural problem worldwide. *Agrobacterium tumefaciens*, a ubiquitous soil bacterium (Bouzar and Moore, 1987), causes this disease in a wide variety of plants including fruit and nut trees, grapevines, cane berries, chrysanthemum, rose, and other nursery crops (DeCleene and DeLey, 1976; Pinkerton et al., 1996). *A. tumefaciens* genetically transforms plant cells to grow as tumors; therefore, after a few hours of infection the disease will progress even if the tumor-inducing bacteria are killed with antibiotics. Thus, prevention is the only effective way to control crown gall.

Crown gall tumors result from overproduction of two plant growth hormones, auxin and cytokinin, in plant cells transformed by *A. tumefaciens* (Winans, 1992; Zambryski, 1992). These abnormally high phytohormone levels result from expression of three genes transferred stably into plant cells from the *A. tumefaciens*

tumor-inducing (Ti) plasmid: *iaaM* (tryptophan monooxygenase), *iaaH* (indole-3-acetamide hydrolase), and *ipt* (AMP isopentenyl transferase) (Garfinkel et al., 1981; Ream et al., 1983). IaaM converts tryptophan into indole-3-acetamide, which IaaH converts into indole-3-acetic acid (auxin) (Inze et al., 1984; Schroeder et al., 1984; Thomashow et al., 1984; Thomashow et al., 1986; Van Onckelen et al., 1986). Loss of either enzyme prevents auxin production and tumorigenesis on plant roots (Ream et al., 1983). Ipt converts adenosine monophosphate into isopentenyl adenosine monophosphate, a cytokinin (Akiyoshi et al., 1984; Barry et al., 1984; Buchmann et al., 1985). Inactivation of *ipt* and either one of the two auxin biosynthesis genes will abolish crown gall formation on any plant tissue (Ream et al., 1983).

Adequate means do not exist to prevent crown gall disease in non-transgenic plants. Inoculation of plants with *Agrobacterium radiobacter* K84 affords some protection against specific strains of *A. tumefaciens* (Jones et al., 1988; Kerr and Tate, 1984; Moore, 1979, 1988; Shim et al., 1987); however, crown gall disease remains a serious problem (Pinkerton et al., 1996). *A. radiobacter* K84 produces a toxin called agrocin 84 (Hayman and Farrand, 1988; Murphy and Roberts, 1979; Ryder et al., 1987), which inhibits growth of bacterial cells (Murphy and Roberts, 1979; Ryder et al., 1987). Agrocin 84 enters *A. tumefaciens* via the active transport system that mediates uptake of the agrocinopine class of opines (Murphy and Roberts, 1979). Because only agrocinopine-type *A. tumefaciens* have the permease that confers sensitivity to agrocin 84, *A. radiobacter* K84 offers limited protection against crown gall.

Arabidopsis thaliana plants resistant to crown gall were produced by screening for mutations that affect host genes necessary for *A. tumefaciens* infection or transfer and integration of bacterial oncogenes (Nam et al., 1997; Nam et al., 1999). Testing tens of thousands of potential mutants is feasible in *A. thaliana*. However, this strategy is not applicable to woody plants in which crown gall is a commercial problem because generating and testing large numbers of mutants is impractical in woody species.

Our strategy to produce crown gall resistant plants was based on the phenomenon called post-transcriptional gene silencing (PTGS) or RNA interference (RNAi) (Lindbo and Dougherty, 1992a, 1992b; Napoli et al., 1990; van der Krol et al., 1990) for reviews see (Baulcombe, 2000; Sharp and Zamore, 2000; Tijsterman et al., 2002b; Voinnet, 2001). Transgenes that elicit PTGS trigger sequence-specific destruction of transgene-encoded messenger RNA (mRNA) and other mRNAs that have sufficient sequence identity (Dougherty and Parks, 1995; Jorgensen et al., 1998). A number of transgene constructions can trigger PTGS. Early observations implicated high levels of sense-strand transcription across an untranslatable (or wild-type) transgene as an elicitor of PTGS; silencing often correlated with multiple-copy transgenes (Dougherty et al., 1994; Napoli et al., 1990; van der Krol et al., 1990). Transgenes in an inverted repeat configuration also elicit PTGS (Cluster et al., 1996; Stam et al., 1997; Wang and Waterhouse, 2000). Recent work in plants (Angell and Baulcombe, 1997; Chuang and Meyerowitz, 2000; Escobar et al., 2001; Hamilton and Baulcombe, 1999; Waterhouse et al., 1998; Wesley et al., 2001), nematodes (Fire et al., 1998; Montgomery and Fire, 1998; Montgomery et al., 1998; Timmons and Fire, 1998), neurospora (Cogoni and Macino, 1999a; Cogoni and Macino, 1999b), insects (Kennerdell and Carthew, 1998), and trypanosomes (Ngo et al., 1998) demonstrated that double-stranded RNA (dsRNA) molecules induce systemic, sequence-specific PTGS.

We have produced *Nicotiana tabacum* (tobacco) plants resistant to crown gall by introducing transgenes that silenced the *iaaM* and *ipt* oncogenes. Escobar et al. used this approach to produce transgenic *A. thaliana, Lycopersicon esculentum* (tomato), and *Juglans regia* (walnut) that are resistant to crown gall (Escobar et al., 2001; Escobar et al., 2002). The goal of our present research was to provide a new, effective method to produce fruit and nut trees, grapevines, and other crop plants resistant to crown gall disease. Using a gene silencing strategy we produced six transgenic apple lines that were completely resistant to crown gall.

Materials and methods

Oncogene silencing constructions

Figure 2.1 shows the T-DNA regions of two plant transformation plasmids used to generate transgenic apple lines that target the *A. tumefaciens iaaM* and *ipt* oncogenes for silencing. Although blocking *iaaM* expression prevented gall formation on apple roots (Figure 2.2), we also hoped to silence *ipt* in order to prevent tumor growth on stems(Ream et al., 1983). Both plasmids (pJP17 and pJP20) are based on pCGN5927, which contains pRi and ColE1 origins of replication, a bacterially expressed gentamicin resistance gene, and T-DNA border sequences flanking multiple cloning sites and a neomycin phosphotransferase gene (*nptII*) fused to the nopaline synthase (*nos*) promoter (McBride and Summerfelt, 1990). The transgenes in pJP17 and pJP20 were designed to produce complementary sense and antisense RNAs corresponding to the first 1797 base pairs (bp) of *iaaM* and the entire *ipt* coding sequence. The third codon of each gene was converted to a stop codon by polymerase chain reaction (PCR) using primers containing the desired mutations. In addition to the nonsense codon, a frameshift mutation was introduced into each transgene. Sense-strand RNAs that contain premature stop codons elicit gene silencing if they can initiate translation from a start codon downstream of the premature stop (D. C. Baulcombe, personal communication) (Que et al., 1997; Tanzer et al., 1997). The *ipt*-stop construction lacked alternative translation start sequences, but *iaaM*-stop contained an obvious in-frame start at codon 18. This codon is surrounded by excellent sequence context for efficient translation, including A at -3 and G at +4: CCA ACC **AAA** **AUG** **G**. Nucleotides that match the consensus sequence for "ideal" translational context (GCC GCC **A**/GCC **AUG G**) (Kozak, 1989, 1991) are underlined; the start codon and critical nucleotides at -3 and +4 are bold. In pJP17, *iaaM* and *ipt* were fused and situated between the cauliflower mosaic virus (CaMV) 35S and figwort mosaic virus (FMV) promoters. Plasmid pJP20 contained separate copies of *iaaM* and *ipt*, each

flanked by opposing CaMV and FMV promoters (Figure 2.1). The following paragraphs describe the construction of pJP17 and pJP20.

To build a plasmid with two strong plant promoters, an enhanced FMV promoter was inserted into pCGN8059, which contains the CaMV promoter (McBride and Summerfelt, 1990). An amplicon that contained the FMV promoter was produced using pCGN8063 (McBride and Summerfelt, 1990) as template. The primer oligonucleotides (shown 5' to 3') (GCCGAGATCTAGCTTCTGCAGGTCCTGCTC and AATAATTCTAGAAGGCCTGAATTCGAGCTCGGTACCGGATCCGTGTCCTTC AAATGGGAATG) generated a 1067-bp amplicon with a *Bgl*II site upstream and *Bam*HI, *Kpn*I, *Sac*I, *Eco*RI, *Stu*I, and *Xba*I sites downstream of the promoter. This PCR product was digested with *Bgl*II and *Xba*I and inserted into the *Bam*HI and *Xba*I sites of pCGN8059. In the resulting plasmid, pJP8, the CaMV and FMV promoters are positioned such that they drive transcription in opposite directions through a region with multiple cloning sites.

We amplified *iaaM* and *ipt* from template sequences derived from an octopine-type Ti plasmid (pTiA6NC) (Garfinkel et al., 1981); these templates were harbored in pUC119-based plasmids. Reaction mixtures (50 µl) contained 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.001% gelatin, deoxynucleoside triphosphates at 0.25 mM each, 2 units of *Tfl* DNA polymerase (Epicenter), primer oligonucleotides at 1 µM each, and 400 ng of template DNA. Reactions were incubated in a Perkin Elmer 480 thermal cycler at 94°C for 1 min followed by 30 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 2 min. Upon conclusion of thermal cycling, reactions were cooled to 15°C. The upstream primer used to amplify *iaaM*-stop (CGGGATCC***ATG***TCA**TGA**ACCTCTCCTTGATAAC) contained a *Bam*HI site (GGATCC) upstream of the first *iaaM* codon (bold italicized *ATG*). This primer contained a stop codon (bold TGA) in place of the third codon (GCT) of *iaaM*, and it

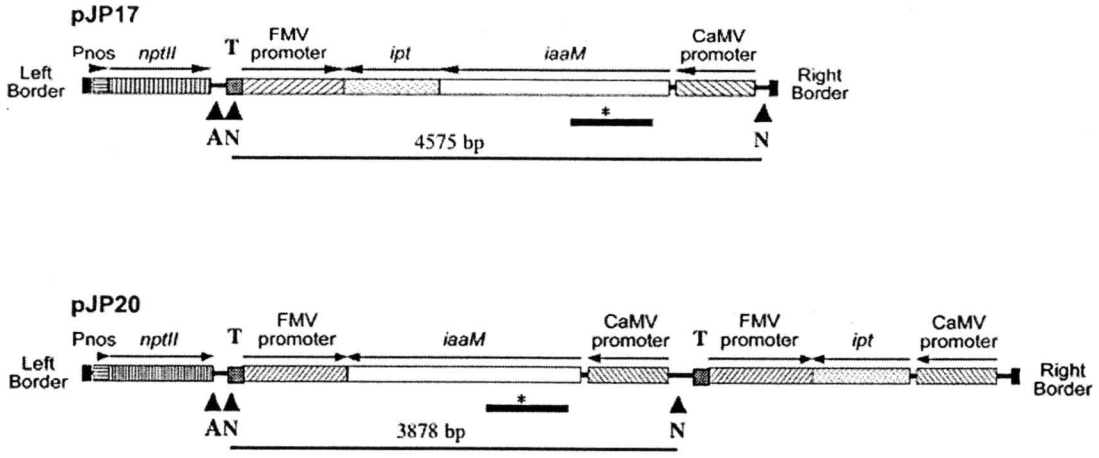

Figure 2.1. Oncogene-silencing constructions. The T-DNA (transferred DNA) portions of plasmids pJP17 and pJP20 are shown. Black boxes at the ends of each map represent the right and left T-DNA border sequences. Arrows above promoter sequences indicate the direction of transcription, and those above oncogenes show the orientation of the coding sequences. "Pnos," "FMV," and "CaMV" are the nopaline synthase, figwort mosaic virus, and cauliflower mosaic virus promoters, respectively. Shaded boxes labeled "T" indicate the nopaline synthase 3' untranslated region (3'-UTR) and polyadenylation signal from pCGN8059 (McBride and Summerfelt 1990); these 3'-UTR sequences are oriented so that they can terminate sense-strand oncogene RNAs expressed from the CaMV promoter. "*iaaM*" and "*ipt*" indicate sequences from the tryptophan monooxygenase and AMP-isopentenyl transferase genes. "*nptII*" is the neomycin phosphotransferase II gene, which was used to select apple cells containing the transgenes; a nopaline synthase 3'-UTR (not shown) is located at the 3' end of the *nptII* gene. Black triangles labeled "N" show the location of *Not*I sites, and those labeled "A" mark the position of the *Avr*II site. Lines labeled "4575 bp" and "3878 bp" show the size and location of the *Not*I restriction fragments. Black bars labeled "*" indicate the *iaaM* sequences used as a radiolabeled probe for Southern and northern blots.

lacked the first two bases (TC) of the fourth codon. The downstream primer used to produce *iaaM*-stop (CGGGATCCTGCGACTCATAGT) included the *Bam*HI site within the *iaaM* gene (Barker et al. 1983) and an *Xba*I site (TCTAGA) at the 5' end. The upstream primer used to amplify *ipt*-stop (GAAGATCTGATC*ATG*GAC**TGA**ATCTAATTTTCGGTCC) contained an *Xba*I site

upstream of the *ipt* start codon (italicized). Two mutations in this primer, deletion of the first base (C) of the third codon and conversion of the first base (C) of the fourth codon to A, created a TGA nonsense codon (bold type) and a frameshift mutation. The downstream primer (GAAGATCTGATCA**CTA**ATACATTCCGAACGG) contained the complement of the *ipt* termination codon (bold CTA) and a *Bcl*I site (TGATCA) at the 5' end. All PCR products were inserted into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) via topoisomerase-mediated recombination and sequenced.

To place *ipt*-stop between opposing CaMV and FMV promoters, this amplicon was digested with *Xba*I and *Bcl*I and inserted into pJP8 cleaved with *Xba*I and *Bam*HI to produce pJP12. Next the *iaaM*-stop amplicon was fused to *ipt*-stop in pJP12; the *iaaM*-stop amplicon was digested with *Xba*I and *Bam*HI and ligated to pJP12 cleaved with *Xba*I and *Bgl*II to form pJP15, which contains the fused *iaaM*-stop and *ipt*-stop sequences flanked by opposing CaMV and FMV promoters. This region of pJP15 was excised as a 4585-bp *Not*I fragment and inserted into the *Not*I site of the plant transformation vector pCGN5927 to form pJP17 (Figure 2.1).

Plasmid pJP20 was constructed so that separate pairs of CaMV and FMV promoters flank the *iaaM* and *ipt* sequences. To place *iaaM*-stop between the CaMV and FMV promoters, the *iaaM*-stop amplicon was cleaved with *Xba*I and *Bam*HI and inserted into pJP8 digested with *Xba*I and *Bgl*II to form pJP14. In separate steps, the *ipt*-stop and *iaaM*-stop sequences and the flanking CaMV and FMV promoters were excised from pJP12 and pJP14 and inserted into the pCGN5927 plant transformation plasmid (McBride and Summerfelt, 1990). First, *ipt*-stop was excised from pJP12 by cleavage with *Not*I and *Hin*dIII and ligated into pCGN5927 cut with the same restriction enzymes; the resulting plasmid was called pJP18. Next, *iaaM*-stop was removed from pJP14 as a 3883-bp *Not*I fragment and inserted into the *Not*I site of pJP18 to form pJP20 (Figure 2.1).

Apple transformation and regeneration

Apple tissues were cocultivated with disarmed *A. tumefaciens* EHA101 (Hood et al., 1986) harboring either pJP17 or pJP20. Prior to inoculation, the bacteria were cultured overnight at 25°C with aeration in 523 medium (James et al., 1993) containing 20 μg ml^{-1} of gentamicin. The bacteria were harvested by centrifugation, suspended in 4 volumes of MS 20 medium (James et al., 1993), and incubated at 25°C with aeration for 4 to 5 h until the optical density at 600 nm reached 0.5.

Jonagold bud cultures were grown at 25°C for three weeks on propagation medium (4.33 g l^{-1} Murashige-Skoog [MS] salts, pH 5.6, 30 g l^{-1} sucrose, 6.8 g l^{-1} agar, 1 mg l^{-1} 6-benzylaminopurine [BAP], 0.1 mg l^{-1} indole-3-butyric acid [IBA]) with 16 h of daily illumination. Elongated shoots were cut and placed on root induction medium (4.33 g l^{-1} MS salts, pH 5.5, 30 g l^{-1} sucrose, 6.8 g l^{-1} agar, 3 mg l^{-1} IBA) and incubated in the dark at 25°C for three days. Shoots were then transferred to root expression medium (2.15 g l^{-1} MS salts, pH 5.6, 30 g l^{-1} sucrose, 7 g l^{-1} agar) and incubated at 25°C under 16 h daily illumination. Newly expanded leaves 0.5-1 cm in length were used for transformation experiments.

Leaf tissue was cut into 3-4 mm strips (perpendicular to the midvein) and incubated in the bacterial suspension for 10-15 min. Explants were blotted on sterile filter paper, placed abaxial side up on cocultivation medium, and incubated in the dark 24°C for 72 h. Explants were transferred to stage 1 selection medium (4.33 g l^{-1} MS salts, pH 5.6, 40 g l^{-1} sorbitol, 2.5 g l^{-1} Gelrite, 3 mg l^{-1} BAP, 0.5 mg l^{-1} α-napthalenenacetic acid, 2 mg l^{-1} zeatin, 50 mg l^{-1} kanamycin, 300 mg l^{-1} cefotaxime) and incubated in the dark. Explants were transferred to fresh stage 1 selection medium after 25 days. Fifty days after cocultivation the explants were moved to stage 2 selection medium (4.33 g l^{-1} MS salts, pH 5.6, 30 g l^{-1} sucrose, 6.8 g l^{-1} agar, 5 mg l^{-1} BAP, 0.5 mg l^{-1} IBA, 50 mg l^{-1} kanamycin, 300 mg l^{-1} cefotaxime) and incubated in the light. Green shoots were excised from leaf explants and placed on propagation medium containing 25 mg l^{-1} kanamycin and 100 mg l^{-1} cefotaxime. Elongated shoots were rooted as described

previously (James et al., 1993), and root explants were challenged with wild-type *A. tumefaciens* A348.

Tumorigenesis assays

Axenic apple root explants were infected with wild-type *A. tumefaciens* A348, the octopine-type strain (Garfinkel et al., 1981) that was the source of the oncogene sequences in pJP17 and pJP20.

The nucleotide sequence of *iaaM* from an octopine-type Ti plasmid (Barker et al., 1983) (accession AF242881) is 95% identical to *iaaM* from nopaline-type pTiC58 (Otten et al., 1999) (accession AF126446) and 90% identical to *iaaM* from pTiTm4, which resides in biovar III *Agrobacterium vitis* (Bonnard et al., 1991) (accession X56185). Because PTGS requires at least 23 consecutive nucleotides of sequence identity between the target gene and the transgene used to elicit gene silencing (Thomas et al., 2001), we expected the constructions in pJP17 and pJP20 to silence *iaaM* genes from many strains of *A. tumefaciens*. The bacteria were cultured at 23°C in 523 medium (James et al., 1993) to a titer of 3 x 10^8 cells ml^{-1} and diluted 5 fold in Murashige-Skoog (MS) medium (pH 5.8) supplemented with 0.8% sucrose, 1 mM proline, and 0.1 mM acetosyringone. Root explants were immersed in 25 ml of diluted *A. tumefaciens* culture for 15 min and transferred to MS agar (pH 5.8) containing 3% sucrose, 1 mM proline, 0.1 mM acetosyringone, and 2.7 g l^{-1} Phytagel agar. Inoculated roots were incubated at 23°C with a 16-hour photoperiod. Root explants were transferred to maintenance medium (MS, pH 5.6, containing 7 g l^{-1} Phytagel agar and 300 mg l^{-1} cefotaxime) two days post inoculation and monthly for three months until roots were scored for tumor formation.

RNA extraction and real-time reverse transcriptase-PCR (RT-PCR)

RNA was extracted from 0.2 g of shoot or root tissue using a Qiagen RNeasy mini kit (Qiagen, Valencia, CA) as modified by Shevchenko and Brunner

(www.fsl.orst.edu/tgerc/pubs/PoplarRNAextraction.pdf). Shoot tips were harvested from trees growing in soil, whereas root tissue was taken from axenic trees propagated on Gamborg's B-5 medium (Sigma, St. Louis, MO) in Magenta boxes. Plant tissue was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. One ml of RLT buffer (Qiagen, Valencia, CA) containing 0.01 g of polyvinylpyrrolidone (PVP-40) (Sigma, St. Louis, MO) was added to the ground tissue and shaken for one min. The tissue was then homogenized for 30 s at maximum speed with a Virtis tissue homogenizer. After homogenization, the sample was mixed with 0.4 volumes of 5 M potassium acetate, incubated on ice for 15 min, and then centrifuged for 15 min at 12,000 rpm in a microcentrifuge. The supernatant solution was mixed with 0.5 volume of 100% ethanol and transferred to an RNeasy spin column (Qiagen, Valencia, CA). RNA was purified according to the manufacturer's instructions.

Real-time RT-PCR was performed using an ABI Prism 7700 Sequence Detector with version 1.7 software (Applied Biosystems, Foster City, CA) and a Quantitect SYBR Green RT-PCR kit (Qiagen, Valencia CA). Three RNAs were examined: *ipt*, *iaaM*, and 5.8S rRNA. PCR primers were designed to amplify products of 100, 150, and 165 base pairs, respectively. PCR primers for *iaaM* and *ipt* amplified regions near the 3' ends of RNAs encoded by these transgenes. The primers used to amplify *iaaM* sequences were: GAACCAAGCGGTTGATAACAGCC (sense strand) and CTGCGACTCATAGTCCAGGAATAC (antisense strand). The primers used to amplify *ipt* RNAs were: CCATGCGCGCCAACAGGA (sense strand) and GAAGATCTGATCACTAATACATTCCGAAC (antisense strand). The entire 5.8S rRNA was amplified using primers corresponding to the 5' (GACTCTCGGCAACGGATATCTC) and 3' (GGGGCAACGGCGTGTG) ends. A standard curve was constructed for each primer set with template RNA transcribed *in vitro* from plasmid templates using a Riboprobe kit (Promega, Madison, WI). Eight reactions used to generate each standard curve contained template RNA ranging from 10-1280 pg in twofold increments. To produce *iaaM* and *ipt* RNA templates for

standard curves, plasmids that contained *iaaM* (pHL183) or *ipt* (pJH3) inserted in pCR2.1 (Invitrogen) were linearized with *Sac*I and transcribed *in vitro*. Apple 5.8S rRNA was amplified by standard RT-PCR and inserted into pCR4-TOPO (Invitrogen) to form pJH13, which was linearized with *Pst*I and transcribed *in vitro* to generate template RNA for real-time RT-PCR standard curves. RNA levels for *iaaM*, and *ipt* were normalized to 5.8S rRNA levels. Real-time RT-PCR products were examined by electrophoresis through 2% agarose gels.

DNA and RNA extraction

DNA and RNA was extracted from leaves of transgenic apple using SDS/phenol followed by LiCl precipitation as described previously (Ream and Field, 1999) or by using a Qiagen DNeasy plant maxi kit (Qiagen, Valencia, CA) . Leaves (1.0 g) were frozen and ground to a fine powder in liquid nitrogen. Nucleic acids were extracted by mixing the ground tissue vigorously with 10 ml extraction buffer (100 mM LiCl, 1% SDS [w/v], 10 mM EDTA, 100 mM Tris, pH 9) and 10 ml phenol:chloroform (24:1). After centrifugation, the aqueous phase was recovered, mixed with an equal volume of 4 M LiCl, and incubated overnight at -20°C to precipitate total RNA, which was collected by centrifugation. The supernatant solution, which contained genomic DNA, was mixed with 2 volumes of ethanol and incubated at 4°C to precipitate the DNA. The RNA and DNA pellets were dissolved in 200 µl of water or DNA buffer (10 mM Tris, pH 7.5, 0.1 mM EDTA), precipitated again with 3 volumes of ethanol and 0.5 volume of ammonium acetate, and washed with 70% ethanol. The pellets were dissolved in 1.0 ml of water, and the absorbance at 260 nm was measured. DNA samples prepared by SDS/phenol extraction and by the Qiagen method were pooled and further purified by CsCl-ethidium bromide density gradient centrifugation as described (Ream and Field, 1999).

Southern and northern blot analysis

Samples containing 2.5 to 10 μg of genomic DNA were digested with either *Not*I or *Avr*II. The restriction fragments were separated by agarose gel electrophoresis and transferred to Gene Screen Plus nylon membranes (Perkin Elmer, Boston, MA). Procedures for capillary blotting and hybridization were as described (Ream and Field, 1999). DNA probes were generated using a random primer DNA labeling system (Invitrogen, Carlsbad, CA) to incorporate α-^{32}P-labeled dCTP (3000 Ci/mmole; ICN Pharmaceuticals, Irvine CA). Samples containing 12 μg of total RNA were subjected to formaldehyde-agarose gel electrophoresis and transferred to Hybond XL nylon membranes (Amersham Pharmacia, Little Chalfont, UK). Strand-specific RNA probes were created by inserting an *iaaM*-stop PCR amplicon into pCR-TOPO topoisomerase-bound plasmid DNA (Invitrogen, Carlsbad, CA) as directed by the manufacturer. An *in vitro* transcription system (Promega, Madison, WI) was used to produce *iaaM*-stop RNA containing α-^{32}P-labeled UTP (3000 Ci/mmole). Specific activity of the probes varied from 10^7 to 10^8 cpm per μg. Signals were detected on Kodak XAR film and on a Molecular Dynamics storage phosphor screen. We used a Molecular Dynamics phosphorimager with ImageQuant software (version 5.2) to measure the intensity of each 3,878-bp *Not*I band. Uniform rectangles were placed around each band in the image, and the integrated intensity of all pixels in each enclosed area was reported in arbitrary units. A rectangle located in an empty lane was used to measure background signal, which was subtracted from the signal in each band.

Results and Discussion

Blocking auxin overproduction conferred crown gall resistance to apple roots

Mutations in the *A. tumefaciens iaaM* oncogene abolish gall formation on roots and tubers of several non-woody plant species (Ream et al., 1983). Non-transgenic apple roots were challenged with *iaaM*- and *ipt*-mutant *A. tumefaciens* strains to see whether

this was also true on apple roots. Non-transgenic roots readily formed unorganized galls when inoculated with wild-type *A. tumefaciens* A348 (an octopine-type strain) or an *ipt*-mutant (A338) (Garfinkel et al., 1981), whereas an *iaaM*-mutant (A328) and a Ti-plasmidless strain did not produce a significant response (Figure 2.2). Thus, blocking *iaaM* expression prevented gall formation on apple roots, the area most affected by crown gall disease in the field.

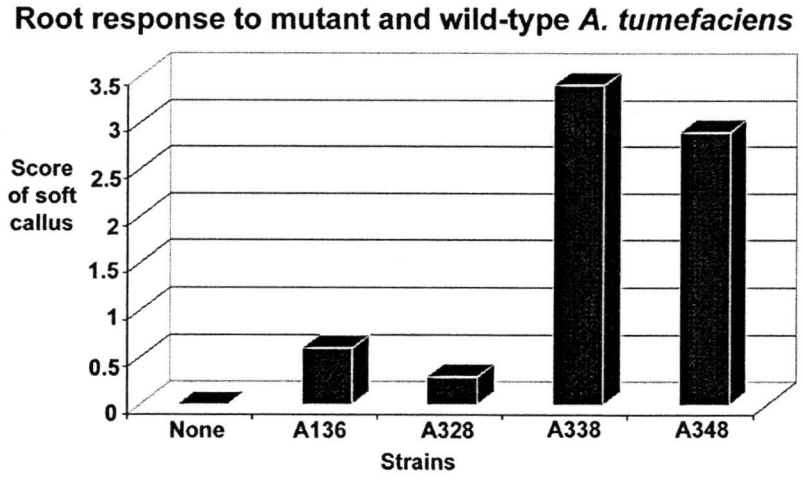

Figure 2.2. Apple root response to mutant and wild-type *A. tumefaciens*. Non-transgenic apple roots were inoculated with wild-type (A348), *iaaM*-mutant (A328), *ipt*-mutant (A338), and Ti-plasmidless (A136) strains. The amount of soft callus formed on inoculated roots was scored on a scale from 0 (no response) to 3.5 (extensive callus growth).

Roots of transgenic apple lines that silenced iaaM were resistant to crown gall

Fifty transgenic apple lines were generated that contained the oncogene-silencing constructions from pJP17 and pJP20 shown in Figure 2.1. After initial tests for gall resistance, fourteen clones were selected for further testing, and parental nontransgenic lines were used as controls. For each line, at least 180 root explants

were inoculated with wild-type *A. tumefaciens* A348. Root explants from six of the fourteen lines were completely resistant to crown gall (Figures 2.3 & 2.4). Three of the resistant lines (330-1, 426-14, 426-15) contained T-DNA from pJP17, in which *iaaM*-stop and *ipt*-stop are fused and situated between opposing CaMV and FMV promoters. The other three gall-resistant lines (705-3, 705-4, 705-5) were transformed with pJP20, in which *iaaM*-stop and *ipt*-stop are separate, and each is flanked by opposing CaMV and FMV promoters (Figure 2.1). Thus, both constructions were capable of silencing the wild-type *iaaM* oncogene.

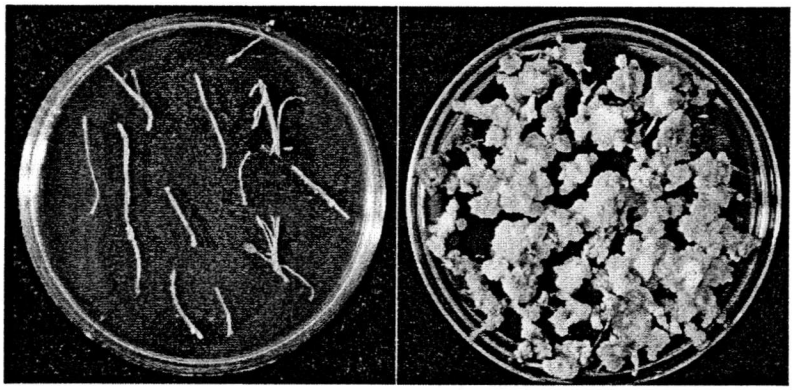

Figure 2.3. Crown-gall-resistant apple roots. Transgenic (left) and non-transgenic (right) apple roots infected with wild-type *A. tumefaciens* A348. Tumorigenesis was scored three months after inoculation. Large tumors were present on all non-transgenic roots, but no galls formed on transgenic roots.

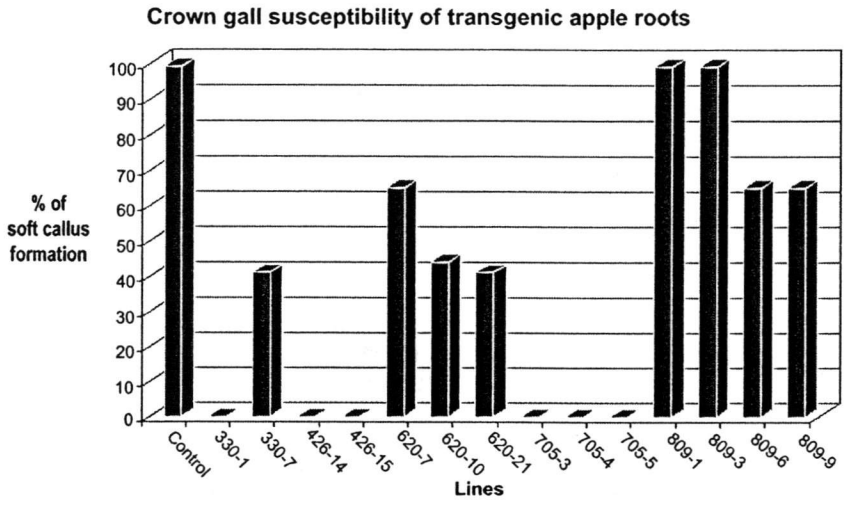

Figure 2.4. Crown gall susceptibility of roots from 14 transgenic apple lines infected with wild-type *A. tumefaciens* A348. Four kanamycin-resistant lines (330-1, 330-7, 426-14, and 426-15) were transformed with the T-DNA from pJP17; the remaining ten kanamycin-resistant lines contained the pJP20 T-DNA. "% soft callus formation" indicates the fraction of root explants that developed visible tumors within 3 months after inoculation with *A. tumefaciens* A348. A score of 100% indicates that all inoculated root explants developed at least one tumor; a score of 0% means that none of the explants developed a tumor.

Transgene copy number and structure

We performed Southern blot analyses on three gall-resistant apple lines, two sensitive lines, and one line transformed with vector sequences only. Each DNA sample was digested with *Not*I, which produced a 3,878-bp fragment from intact copies of the pJP20 T-DNA; the pJP17 T-DNA contained a 4,575-bp *Not*I fragment (Figure 2.1). The blot was probed with radiolabeled *iaaM* sequences (Figure 2.1). All gall-resistant lines examined (705-3, 705-4, 705-5) contained intact copies of the pJP20 T-DNA, as did one sensitive line (620-10) (Figure 2.5A). Another sensitive line (330-7) did not contain an intact copy of the pJP17 T-DNA (Figure 2.5A).

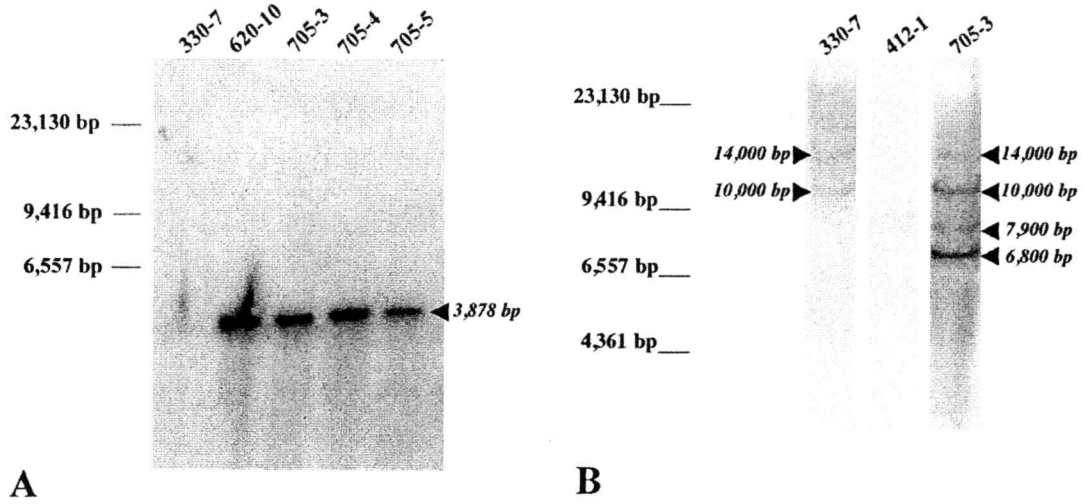

Figure 2.5. Southern blot analysis of transgene structure and copy number. The transgenic apple lines from which genomic DNA was isolated are indicated above each lane. Numbers and lines on the left edge of each panel indicate the positions and sizes (in base pairs) of restriction fragments produced by digestion of phage lambda DNA with *Hin*dIII. Arrowheads and italicized numbers indicate the positions and estimated sizes (in base pairs) of *Not*I (panel A) or *Avr*II (panel B) restriction fragments that hybridized to radiolabeled *iaaM* sequences. Each lane contained 2.5 to 10 μg of genomic DNA: 330-7 (10 μg), 412-1 (10 μg), 620-10 (10 μg), 705-3 (2.5 μg), 705-4 (6 μg), 705-5 (3 μg). The blot of *Not*I-digested DNA (panel A) was exposed to a Molecular Dynamics phorphorimager screen. Band intensities (for each 3,878-bp *Not*I fragment) were expressed in arbitrary units using ImageQuant software: 620-10 (99 units), 705-3 (47 units), 705-4 (55 units), 705-5 (27 units).

The T-DNAs each contained a single *Avr*II site located near the left border, between the *nptII* gene and the FMV promoter (Figure 2.1). Each integrated copy of the T-DNA, when digested with *Avr*II, produced a restriction fragment extending from this *Avr*II site through the right T-DNA border to the nearest *Avr*II site in the plant genome. The resulting fragments contained unknown lengths of plant DNA. Thus, the number of *Avr*II fragments indicated the minimum transgene copy number. Gall-resistant line 705-3 yielded four *Avr*II fragments: 6.8 kb, 7.9 kb, 10 kb, and 14 kb (Figure 2.5B). The dark bands at 6.8 kb and 10 kb presumably represent the intact T-DNA copies detected by *Not*I digestion. Restriction fragments (in a particular lane) that contained all of the sequences in the probe should produce bands with the same signal intensity. The light bands at 7.9 kb and 14 kb may represent partially deleted T-DNAs that lack some of the *iaaM* sequences present in the radiolabeled probe. Gall-sensitive line 330-7 contained two partially deleted T-DNAs represented by light *Avr*II fragments of 10 kb and 14 kb (Figure 2.5B). The weak signal, despite the large amount of DNA (10 μg) loaded in this lane, indicates that these fragments had very little overlap with the probe. Although lane 330-7 contained fourfold more DNA, lane 705-3 had much stronger bands (Figure 2.5B). Line 412-1 was transformed with vector sequences only, and its DNA did not hybridize with the *iaaM* probe (Figure 2.5B). This line was fully sensitive to crown gall tumorigenesis, as expected (data not shown).

Analysis with *Not*I showed that four lines (620-10,705-3, 705-4, and 705-5) contained at least one intact copy of the pJP20 T-DNA (Figure 2.5A). Fragments produced by *Avr*II indicated that line 705-3 contained two intact copies of the pJP20 T-DNA (Figure 2.5B). We measured the signal intensity in each *Not*I band in Figure 2.5A. These signals were normalized to the amount of DNA loaded in each lane. The signal per μg of DNA in sample 705-3 was approximately twice that detected in the other three samples. Thus, line 705-3 had two intact copies of the T-DNA whereas lines 620-10, 705-4, and 705-5 each contained only one intact T-DNA.

Gall-resistant apple lines accumulated more ipt transgene RNA than iaaM or iaaM-ipt RNA

In general, post-transcriptional gene silencing significantly reduces, but does not eliminate, accumulation of the target and transgene RNAs (Chuang and Meyerowitz, 2000; Wesley et al., 2001). Therefore, we expected apple lines with gall-resistant roots (due to silencing of the wild-type *iaaM* oncogene) to accumulate low levels of *iaaM*-stop transgene RNA in uninfected tissues. None of the transgenic apple lines silenced the wild-type *ipt* oncogene in shoots or leaves (data not shown), and so we expected *ipt*-stop transgene RNA to accumulate to higher levels than *iaaM*-stop transgene RNA in these uninfected plants. To avoid the problem of distinguishing transgene RNA from wild-type oncogene messenger RNA (mRNA), we measured transgene RNA levels in uninfected tissue.

We used the presence or absence of tumorous growth to assess levels of wild-type *iaaM* oncogene mRNA in infected root explants. This assay is extremely sensitive, and it clearly indicates whether a useful level of silencing occurred. In cultured crown gall tumor cells, mRNA encoded by the wild-type *iaaM* oncogene is present in very low concentrations, and it is difficult to detect by northern blot analysis (Gelvin et al., 1982; Willmitzer and Wagner, 1981). Therefore, we expected that full-length *iaaM* oncogene mRNA would be impossible to detect by northern blot analysis of small amounts of infected root tissue. In gall-resistant roots that silence the *iaaM* oncogene, most (or all) of the oncogene-encoded *iaaM* RNA will be partially or fully degraded and unable to form a discrete band during gel electrophoresis. Thus, degraded RNAs are difficult to detect by northern blot analysis. Although RT-PCR is extremely sensitive, this method cannot distinguish full-length *iaaM* mRNA from degraded mRNA, transgene-encoded RNA, or mRNA expressed by bacteria present in infected tissue. For these reasons, we used auxin-promoted tumor growth to assess *iaaM* oncogene silencing.

Transgene RNA accumulation was measured by RT-PCR in uninfected roots (and shoots) of three gall-resistant apple lines, two gall-sensitive transgenic lines, and one sensitive line transformed with vector sequences only. The apple lines examined contained very little *iaaM*-stop or *iaaM-ipt*-stop transgene-encoded RNA. These RNAs were not detectable by northern blot analysis, despite several attempts; however, ribosomal RNAs in these preparations formed discrete bands indicating that they were not degraded (data not shown). Therefore, we used a more sensitive method, real-time RT-PCR, to measure small quantities of transgene-encoded RNA. Transgene RNA levels were normalized to the levels of 5.8S ribosomal RNA, which were also determined by real-time RT-PCR. Gall-resistant lines derived from pJP20 (705-3, 705-4, and 705-5) contained *iaaM* and *ipt* sequences as separate transgenes, each transcribed in both directions from the same strong viral promoters (Figure 2.1). Although RNA levels produced by each transgene could vary independently because they were not fused into a single transcript, high RNA levels would be expected from

both transgenes unless transcriptional or post-transcriptional gene silencing affected one (or both) transgenes. As predicted (based on their ability to silence the wild-type *iaaM* oncogene), transgene-encoded *iaaM*-stop RNA accumulated to very low levels (10-30 fg of *iaaM* RNA per pg of 5.8S rRNA) in these three gall-resistant lines (Figure 2.6). In contrast, *ipt*-stop RNA was tenfold more abundant (100-250 fg/pg 5.8S rRNA; Figure 2.6), suggesting that the *ipt*-stop transgene was not subject to gene silencing in these apple lines. Nevertheless, the roots of these plants were resistant to gall formation because *iaaM*, which (together with *iaaH*) causes tumors on apple roots, was silenced enough to prevent tumorigenesis.

A fourth apple line (620-10) contained similar levels of transgene RNA (Figure 2.6) and exhibited reduced susceptibility to gall formation on root explants (Figure 2.4). In this line, *iaaM*-stop RNA levels were only fivefold lower than *ipt*-stop RNA levels, suggesting that silencing of *iaaM* may have occurred to a lesser extent in line 620-10 than in the lines (705-3, 705-4, and 705-5) in which tumorigenesis was blocked completely.

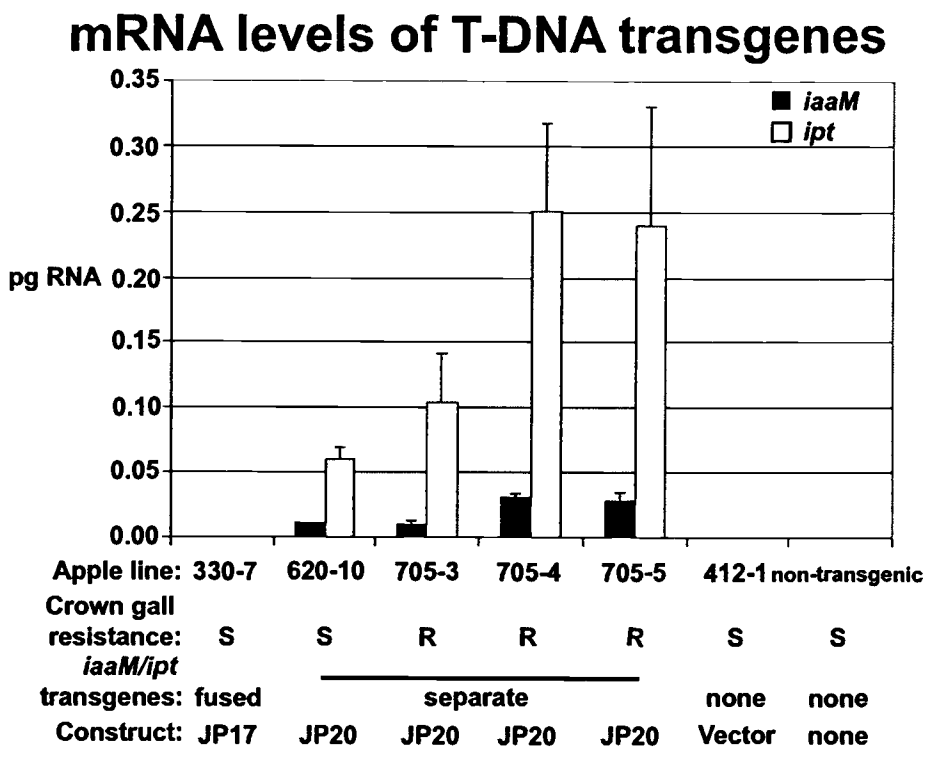

Figure 2.6. T-DNA-encoded RNA accumulation in gall-resistant and gall-sensitive transgenic apple lines. Transgene-encoded RNA in apple shoots was measured by real-time RT-PCR. RNA levels were normalized to the amount of 5.8S ribosomal RNA present and expressed as pg of transgene RNA/pg of 5.8S rRNA. Black and white bars depict the amount of RNA detected with PCR primers specific for *iaaM* and *ipt*, respectively. "R" indicates apple lines that were completely resistant to gall formation; "S" indicates lines that were partially (620-10) or fully (330-7 and 412-1) sensitive to tumorigenesis.

RNAs produced by the fused *iaaM-ipt*-stop transgene were present at low levels in roots excised from two gall-resistant lines derived from pJP17. Similar amounts of *iaaM-ipt*-stop RNA were detected with PCR primers specific for *iaaM* or *ipt* sequences: line 330-1 contained 0.25-0.46 fg *iaaM-ipt* RNA/pg 5.8S rRNA and line 426-14 contained 0.04-0.09 fg *iaaM-ipt* RNA/pg 5.8S rRNA. Root explants from these lines were resistant to gall formation (Figure 2.4), indicating that the *iaaM-ipt*-stop transgene triggered silencing of the wild-type *iaaM* oncogene.

Gall-sensitive apple lines contained trace amounts of transgene RNA

Transgene-encoded RNA levels were lowest in the five gall-sensitive lines we examined. Line 330-7 (transformed with pJP17) accumulated only 0.013-0.03 fg *iaaM-ipt* RNA/pg 5.8S rRNA (Figure 2.6). The other lines (620-7, 809-1, 809-3, and 809-9) were transformed with pJP20. In roots from these lines, transgene-encoded *iaaM*-stop RNA levels ranged from <0.005 to 0.1 fg/pg 5.8S rRNA, and *ipt*-stop transgene-encoded RNA accumulation ranged from <0.0001 to 0.3 fg/pg 5.8S rRNA (data not shown). These gall-sensitive lines did not accumulate appreciable levels of *iaaM*-stop, *ipt*-stop, or *iaaM-ipt*-stop RNA, and they failed to silence the wild-type *iaaM* oncogene.

Several circumstances may lead to extremely low transgene RNA accumulation in the absence of PTGS. The transgenes in these gall-sensitive apple lines may have been weakly transcribed due to chromosomal location, or the presence of multiple viral promoters may have triggered transcriptional gene silencing (Neuhuber et al., 1994). Alternatively, gene rearrangements may have removed promoters or transgene sequences from the integrated T-DNAs. This was the case for line 330-7, which did not contain an intact copy of the pJP17 T-DNA (Figure 2.5A). Instead, this line contained two partially deleted T-DNA copies that lacked some of the *iaaM*-stop sequences (Figure 2.5B). Thus, insufficient *iaaM*-stop transgene RNA correlated with failure to silence the wild-type *iaaM* oncogene in all gall-sensitive lines examined.

Translatable RNA elicits gene silencing better than untranslatable RNA

Sense-strand RNAs that contain premature stop codons elicit gene silencing if they can initiate translation from a start codon downstream of the premature stop (D. C. Baulcombe, personal communication) (Que et al., 1997; Tanzer et al., 1997). In such cases, low molecular weight transgene RNA from silenced plant tissue is bound to polyribosomes in the cytoplasm, where transgene RNA degradation appears to occur

(Tanzer et al., 1997). At the suggestion of Dr. Baulcombe, we searched the sequences of the *iaaM*-stop and *ipt*-stop constructions for start codons downstream of the stop/frameshift mutations we had introduced at the third codon. We sought AUG codons surrounded by sequences that promote efficient translation. We also considered the length of the coding sequence between an AUG and the closest in-frame stop codon downstream. The *ipt*-stop transgene lacked alternative translation start sequences, but *iaaM*-stop contained an obvious in-frame start at codon 18 surrounded by excellent sequence context for efficient translation (CCA ACC **A**AA **AUG G**). Nucleotides that match the consensus sequence for "ideal" translational context (GCC GCC **A/**GCC **AUG G**) (Kozak, 1989, 1991) are underlined; the start codon and critical nucleotides at -3 and +4 are bold. Codon 18 may be the true initiation codon of the *iaaM* gene. The presumed initiation codon is the first AUG in the open reading frame. However, it lies in a less favorable context for translation initiation: UUU CUA ACA AUG U. Predicted IaaM protein sequences from *A. tumefaciens, A. rhizogenes*, and *A. vitis* are closely related (58-64% identical), except for the first 17 amino acids, which are not conserved; compare accession numbers NP_536129, AAD30493, AAC77909, and Q09109. Both wild-type and *iaaM*-stop mRNAs may initiate translation at codon 18 (in the customary predicted coding sequence).

High levels of *ipt*-stop transgene RNA in *iaaM*-silencing lines, which contained little *iaaM*-stop transgene RNA, suggest that both transgenes were expressed strongly. In these lines, the *iaaM*-stop RNA apparently triggered gene silencing (and its own degradation) whereas the *ipt*-stop RNA did not. In an earlier study (manuscript submitted), we showed that *iaaM*-stop RNA triggers gene silencing efficiently because this RNA is translatable, due to an alternative translation start downstream of the nonsense mutation we introduced at the third codon. In contrast, *ipt*-stop RNA is untranslatable and elicits gene silencing poorly. To examine sequence requirements for silencing *iaaM*, we deleted 189 bp from the 5' end of *iaaM*-stop in pJP17, removing the alternative translation initiation site. This untranslatable RNA contained

90% of the *iaaM* sequences present in the translatable RNA encoded by pJP17. The translatable RNA encoded by pJP17 was highly effective at silencing the *iaaM* oncogene (Figure 2.4), but the untranslatable RNA was unable to silence *iaaM* (manuscript submitted).

Levels of transgene RNA in *iaaM*-silencing and nonsilencing lines support our conclusion that translatable *iaaM*-stop sense-strand RNA elicited gene silencing very effectively whereas untranslatable *ipt*-stop RNA did not. If a transgene-encoded RNA was a potent elicitor of gene silencing, we expected it to silence the target gene in any transgenic line that strongly expressed this RNA. In addition, we predicted weak (or no) transgene expression in lines that failed to silence the target gene. This is exactly what we observed with the translatable *iaaM*-stop construction: all nonsilencing lines expressed very little or no transgene RNA, but all *iaaM*-silencing lines contained low but detectable amounts of *iaaM*-stop RNA. Conversely, we reasoned that the untranslatable *ipt*-stop RNA, which was unable to silence *ipt*, might be present at high levels in many lines. This was the case in all lines we examined, except one (330-7) in which the integrated T-DNAs had suffered deletions. This supports our conclusion that the untranslatable *ipt*-stop RNA was unable to trigger PTGS, in contrast to translatable *iaaM*-stop RNA, which was highly effective in transgenic lines that expressed this RNA.

Gall formation on apple root explants was prevented by blocking overproduction of auxin that normally results from infection by *A. tumefaciens*. Six apple lines that contained a transgene designed to produce double-stranded RNA from a portion of the *iaaM* oncogene triggered effective silencing of this target gene. Root explants from these apple lines exhibited complete resistance to *A. tumefaciens*-induced gall development, presumably due to post-transcriptional silencing of the *iaaM* oncogene. As expected, tissues from these apple lines accumulated *iaaM* transgene RNA at low levels. Thus, silencing the *A. tumefaciens iaaM* oncogene provides a simple and effective means to prevent crown gall disease in crop plants such as apple trees.

Acknowledgements

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Chapter 3

RNA Translatability Affects RNA Silencing

Jennifer Pitrak

Abstract

Agrobacterium tumefaciens oncogenes cause transformed plant cells to overproduce auxin and cytokinin. Two oncogenes encode enzymes that convert tryptophan to indole-3-acetic acid (auxin): *iaaM* (tryptophan monooxygenase) and *iaaH* (indole-3-acetamide hydrolase). A third oncogene (*ipt*) encodes AMP isopentenyl transferase, which produces cytokinin (isopentenyl-AMP). Inactivation of *ipt* and *iaaM* (or *iaaH*) abolishes tumorigenesis. Previously, crown gall disease-resistant apple roots were created by introducing transgenes designed to elicit post-transcriptional gene silencing (PTGS) or RNA interference (RNAi) of *iaaM* and *ipt*. Transgenes that elicit silencing trigger sequence-specific destruction of the inducing RNA and messenger RNAs with related sequences. Although PTGS has proven effective against a variety of target genes, we found that crown-gall-resistant transgenic lines silenced *iaaM*, but none silenced *ipt*, suggesting that transgene sequences influenced the effectiveness of PTGS. Sequences required for oncogene silencing included a translation start site. The ineffective *ipt* transgene contained a premature stop codon and a –1 frameshift at the third codon. In contrast, a transgene encoding both translatable sense-strand RNA and antisense RNA from the 5' end of *iaaM* silenced the *iaaM* oncogene, but deletion of the translation start site abolished the ability of the transgene to silence *iaaM*. Likewise, single nucleotide changes in potential mRNA translation initiation codons also abolished *iaaM* silencing. These results suggest that mRNA translatability plays a role in PTGS.

Introduction

Crown gall tumors result from overproduction of auxin and cytokinin in plant cells transformed by *Agrobacterium tumefaciens* (Winans, 1992). These abnormally high phytohormone levels result from expression of three genes transferred stably into the plant genome from the *A. tumefaciens* tumor-inducing (Ti) plasmid: *iaaM* (tryptophan monooxygenase), *iaaH* (indole-3-acetamide hydrolase), and *ipt* (AMP isopentenyl

transferase) (Garfinkel et al., 1981; Ream et al., 1983). IaaM converts tryptophan into indole-3-acetamide, which IaaH converts into indole-3-acetic acid (auxin) (Inze et al., 1984; Schroeder et al., 1984; Thomashow et al., 1984; Thomashow et al., 1986; Van Onckelen et al., 1986). Loss of either enzyme prevents auxin production. Ipt converts adenosine monophosphate (AMP) into isopentenyl-AMP, a cytokinin (Akiyoshi et al., 1984; Barry et al., 1984; Buchmann et al., 1985). Inactivation of *ipt* and either one of the two auxin biosynthesis genes abolishes crown gall formation on all plant tissues (Ream et al., 1983). However, tumor formation on roots requires only auxin overproduction.

Adequate means do not exist to control crown gall disease on grapes, fruit and nut trees, cane berries, chrysanthemum, rose, and other nursery crops. Inoculation of plants with *Agrobacterium radiobacter* K84 affords some protection against specific strains of *A. tumefaciens* (Moore, 1988), however, crown gall disease remains a significant problem. *Arabidopsis thaliana* plants resistant to crown gall disease have been produced by traditional genetic approaches (Nam et al., 1997; Nam et al., 1999), but this strategy is not applicable to plants in which crown gall is a problem.

Two cases of genetically engineered crown-gall-resistant plants have been reported (Escobar et al., 2001; Escobar et al., 2002; Viss et al., 2003). In both cases, disease resistance was based on the phenomenon called post-transcriptional gene silencing (PTGS) or RNA interference (RNAi) (Baulcombe, 2000; Lindbo and Dougherty, 1992a, 1992b; Napoli et al., 1990; Tijsterman et al., 2002; van der Krol et al., 1990). Transgenes that elicit PTGS trigger sequence-specific destruction of transgene-encoded messenger RNA (mRNA) and other mRNAs that have sufficient sequence identity (Jorgensen, 2003; Tomari and Zamore, 2005). PTGS has effectively silenced a number of genes in plants, including endogenous genes, transgenes, and viral RNAs (Angell and Baulcombe, 1997; Conner et al., 1997; Escobar et al., 2001; Marano and Baulcombe, 1998; Ruiz et al., 1998). Transgenes that elicit PTGS need not reside in all cells of the plant to induce systemic silencing of target genes. Leaves infiltrated

with a PTGS-eliciting transgene can trigger silencing in untreated leaves (Voinnet and Baulcombe, 1997; Voinnet et al., 1998), and transgenic rootstock can silence homologous genes in grafted non-transgenic tissue (Palauqui et al., 1997).

A number of transgene constructions can trigger PTGS. Early observations implicated high levels of transcription across a transgene as an elicitor of PTGS (Que et al., 1997; Tanzer et al., 1997); silencing often correlated with multiple-copy transgenes (Dougherty et al., 1994; Napoli et al., 1990). It is now well established that double-stranded RNA (dsRNA) is the trigger for PTGS/RNAi (Tomari and Zamore, 2005). dsRNA can arise from viral genome replication intermediates, transcription of inverted-repeat sequences that generate "hairpin RNAs," sense and antisense transcription of the same gene sequence (bidirectional transcription), or from RNA-dependent RNA-polymerase (RdRp) activity (Baulcombe, 2004; Dalmay et al., 2000; Montgomery and Fire, 1998; Mourrain et al., 2000; Waterhouse et al., 1998; Xie et al., 2004). In plants, dsRNA is processed by a dsRNA-specific RNAseIII-type nuclease, Dicer-like (DCL) (Schauer et al., 2002), into 21-24-nucleotide short interfering RNAs (siRNAs) (Hamilton et al., 2002). These siRNAs afford sequence specificity to the RNA-induced silencing complex (RISC) to direct cleavage of cognate mRNAs (Tang et al., 2003).

Previously crown gall disease resistance was demonstrated on roots of apple trees engineered to silence the *A. tumefaciens* oncogenes, *iaaM* and *ipt* (Viss et al., 2003). Although transgenic lines were designed to target both oncogenes, *iaaM* was silenced whereas *ipt* was not, suggesting that either transgene or target gene sequences influenced the effectiveness of gene silencing. In another study, both *iaaM* and *ipt* genes were silenced (Escobar et al., 2001). Contrary to the transgene sequences used in apple trees, the transgenes used by Escobar and colleagues were not designed to be untranslatable (Escobar et al., 2001). This suggests that translatability of the *ipt* sequence influences silencing. To examine the effects of transgene sequence on silencing, the *iaaM* transgene sequence was altered by deletion and single nucleotide

changes of potential mRNA translation initiation sites. In another construction, the promoter driving antisense transcription from the *iaaM* transgene was deleted. These altered sequences were tested in transient gene silencing assays and revealed that translation initiation sites and antisense RNA were both necessary for induction of PTGS.

Materials and methods

Transgene construction

To produce both sense and antisense RNAs from *iaaM* and *ipt* sequences, I fused modified *iaaM* and *ipt* sequences (Lee et al., 2003) in tandem and situated them between opposing promoters (Figure 3.1). First, I constructed a plasmid (pJP8) that contained opposing Cauliflower Mosaic Virus (CaMV) and Figwort Mosaic Virus (FMV) promoters. I amplified the enhanced FMV promoter from pCGN8063 (McBride and Summerfelt, 1990) using: GCCG**AGATCT**AGCTTCTGCAGGTCCTGCTC (forward primer) and AATAAT**TCTAGA**AGGCCT**GAATTC**GAGCTCGGTACC**GGATCC**GTGTCCTT CAAATGGGAATG (reverse primer). (Restriction sites used for cloning are in bold type; sequences complementary to the template are underlined.) This amplicon was inserted into pCR2.1-TOPO (Invitrogen, Carlsbad, CA), excised with *Bgl*II and *Xba*I, and ligated to pCGN8059 (McBride and Summerfelt, 1990) digested with *Bam*HI and *Xba*I to yield pJP8. The *ipt* sequences were amplified from pUC119::*ipt*-stop (Lee et al., 2003) using: GA**AGATCT**AGAATGGACTGAATCTAATTTTCGGTCC (forward primer) and GA**AGATCT**GATCACTAATACATTCCGAACGG (reverse primer). The "*ipt*-stop" transgene contains nonsense (TGA) and –1 frameshift mutations at the third codon. The amplicon was inserted into pCR2.1-TOPO. Next, *ipt*-stop was excised with *Xba*I and *Bcl*I and ligated to pJP8 digested with *Bam*HI and *Xba*I to yield pJP11. Truncated *iaaM* sequences were amplified from pUC119::*iaaM*-stop (Lee et al., 2003) using: CG**GGATCC**ATGTCATGAACCTCTCCTTGATAAC

(forward primer) and GGAATTCTAGATCCTGCGACTCATAGTCCAGGC (reverse primer). The "*iaaM*-stop" transgene contains the first 1813 base pairs (bp) of *iaaM*, with a stop (TGA) codon at ORF position 3 and a –2 bp deletion in codon 4. The PCR product was inserted into pCR2.1-TOPO, excised with *Bam*HI and *Xba*I, and ligated to pJP11 digested with *Bgl*II and *Xba*I to yield pJP15. A *Not*I fragment containing the *iaaM-ipt*-stop transgene flanked by the CaMV and FMV promoters was excised from pJP15 and inserted into the *Not*I site of pCGN5927 (McBride and Summerfelt, 1990) to form pJP17 (Figure 3.1). pJC14 was constructed in the same manner as pJP17, except that the *iaaM*-stop forward primer was: TTGCTGATCACTGGCCGATGGTCGCTTCCCC and *Bcl*I was used instead of *Bgl*II. The vector-only control plasmid (pJP21) was constructed by inserting a *Not*I fragment that contains the CaMV and FMV promoters from pJP8 into the *Not*I site of pCGN5927.

Plasmid pJP27 was constructed by digestion of pJP17 with *Bam*HI and subsequent ligation to remove the peFMV promoter (Figure 3.1). Plasmids pJP25, pJP30, and pJP33 (Figure 3.5) were constructed by replacing the *Sbf*I/*Stu*I fragment of pJP17 with *Sbf*I/*Stu*I fragments containing G-to-T nucleotide changes at *iaaM* positions 69 (codon 23), 54 (codon 18), and both 69 and 54, respectively. First, pJP17 sequences were amplified in three separate PCR reactions using forward primer, SBFI (sequence 5' to 3', AGTGCCAAGCTTTCCTGCAGGGCGGCCGCG), and a reverse primer containing the C-to-A mutation at the appropriate position(s) (underlined nucleotides indicate C-to-A mutation): GACCATTGTCAGATCCACAATTTTGGTTGGG (position 54), CGACAATTGTCAGATCCACCATTTTGGTTGGG (position 69), CGACAATTGTCAGATCCACAATTTTGGTTGGG (positions 54 and 69). Next, pJP17 sequences were amplified in three separate PCR reactions using reverse primer, STUI (TGTCCTGATTTCAAGGCCTGAGTAATAGCG), and a forward primer containing the G-to-T mutation at the appropriate position(s) (underlined nucleotides indicate G-to-T mutation): CCCAACCAAAATTGTGGATCTGACAATGGTCG (position 54), CCCAACCAAAATGGTGGATCTGACAATTGTCG (position 69),

CCCAACCAAAATTGTGGATCTGACAATTGTCG (positions 54 and 69). Finally, pJP17 sequences containing the G-to-T mutations were amplified by overlap extension PCR. Reactions contained amplicons with the G-to-T mutation and the SBFI and STUI primers. Overlap extension products were inserted into pCR4-TOPO (Invitrogen), sequenced, excised with *Sbf*I and *Stu*I, and ligated to pJP17 digested with *Sbf*I and *Stu*I.

Mixed infection assays

A. tumefaciens cultures were grown overnight in YEP broth (Garfinkel et al., 1981) at 28°C with aeration. *K. daigremontiana* stems were wounded with a sterile toothpick, and each wound was inoculated with 5 μL of overnight broth culture containing EHA101(pJP17) (*iaaM*-silencing strain) and A348 (wild-type *A. tumefaciens*) mixed in a 4:1 ratio. Plants were maintained in a greenhouse or growth room and scored 6-12 weeks after inoculation. Potato disks were inoculated in the same manner except cultures were washed once in PBS buffer and suspended in 1 volume of PBS prior to inoculation (Pueppke and Benny, 1981). Potato disks (Red Norland or Pontiac cultivars) 3 mm thick were cut from 7-mm-diameter cores, placed on water agar, and inoculated with 5 μL of mixed culture within 10 minutes of cutting. Disks from 3-6 potatoes were inoculated with each culture mixture such that each mixture was used to inoculate disks from each potato. Culture mixtures represent experimental treatment groups, and each potato was one experimental replication. Disks were incubated at room temperature and the number of tumor foci per disk was scored after 3 weeks. Data from 2-6 replications were compiled and a Student's T-test (for comparisons of 2 treatment groups) or Analysis of Variance (ANOVA) (for 3 or more treatment groups) was used to assess statistical significance of differences between or among treatments (Turner and Thayer, 2001). If ANOVA indicated a significant difference among treatments, then paired comparisons (Z-tests) were done with each treatment group to the control group(s) (Turner and Thayer, 2001).

Results

Construction of genes encoding sense and antisense RNA

Double-stranded RNAs are potent inducers of gene silencing (Angell and Baulcombe, 1997; Chuang and Meyerowitz, 2000; Fire et al., 1998; Montgomery and Fire, 1998; Montgomery et al., 1998; Timmons and Fire, 1998; Waterhouse et al., 1998; Wesley et al., 2001). Transcription of sense and antisense RNAs from a target gene can trigger PTGS in plants (Chuang and Meyerowitz, 2000; Waterhouse et al., 1998). In an attempt to silence both *ipt* and *iaaM*, a transgene was designed to express both sense and antisense RNA from fused *iaaM* and *ipt* sequences. *iaaM*-stop and *ipt*-stop (Lee et al., 2003) were joined in tandem and inserted into a plant transformation vector between opposing CaMV and FMV promoters (Figure 3.1). The sequence of this construction (pJP17) was verified and tested for its ability to silence the *iaaM* and *ipt* oncogenes.

Gene silencing during mixed infections

Two mixed-infection assays were developed to evaluate the efficacy of oncogene-silencing transgenes. In these assays, *Kalanchoe daigremontiana* stems or potato tuber disks were infected with a mixture of two bacterial strains: a) wild-type *A. tumefaciens* (A348) and b) disarmed *A. tumefaciens* (EHA101) containing the oncogene-silencing plasmid, pJP17. Disarmed strains lack oncogenes, but they retain virulence (*vir*) genes required to move transgenes from a binary plasmid vector (e.g. pJP17) into plants. Mixed infection assays obviated the need to create transgenic plants. In mixed infections most plant cells are transformed with both the transgenes and tumorigenic T-DNA. Transgene-derived RNA should initiate silencing against the oncogenic T-DNA genes. This assay involves multiple transformation events, thereby

masking chromosomal position effects that can influence the effectiveness of a particular integrated transgene.

K. daigremontiana stems develop pronounced tumors in response to *A. tumefaciens*. These tumors have distinct morphologies corresponding to overproduction of cytokinin, auxin, or both (Garfinkel et al., 1981; Miranda et al., 1992; Ream et al., 1983). If auxin overproduction is blocked, tumors are smooth and lack adventitious roots due to high cytokinin levels, whereas wild-type tumors are rough and produce adventitious roots. Co-inoculation of *K. daigremontiana* with wild-type *A. tumefaciens* (A348) and EHA101 carrying pJP17 (Figure 3.1) resulted in cytokinin-driven tumors (Figure 3.2A), indicating that *iaaM*, but not *ipt*, was silenced. Co-inoculation with wild-type *A. tumefaciens* and EHA101 containing the empty vector (pJP21) did not disrupt normal tumorigenesis by A348 (Figure 3.2B).

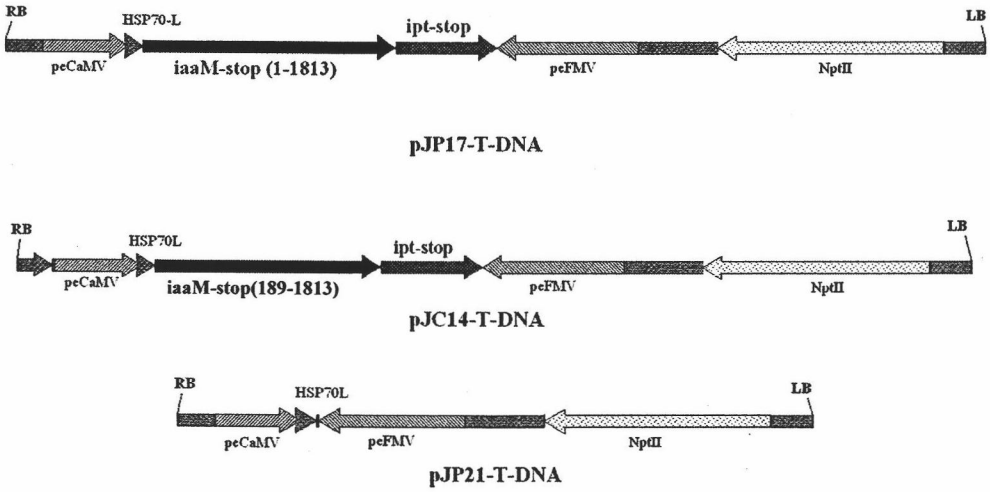
To quantify the degree of *iaaM* silencing, potato disks were inoculated with the same *A. tumefaciens* mixtures used in the assays on *K. daigremontiana*. Potato tubers respond to auxin but not to cytokinin (Ream et al., 1983). Individual tumor foci develop on potato disks when they are inoculated with wild-type (or *ipt*-mutant) *A. tumefaciens*, but potatoes do not respond when inoculated with an *iaaM* mutant (Ream et al., 1983). This system has been used to quantitatively compare the virulence of *A. tumefaciens* strains (Dombek and Ream, 1997; Pueppke and Benny, 1981; Shurvinton et al., 1992; Shurvinton and Ream, 1991; Sundberg et al., 1996). The *iaaM*-silencing plasmid (pJP17) significantly reduced tumorigenesis on potato compared to the empty vector (pJP21) (Figures 3.2C,D and 3.3A). In three tests, three- to five-fold fewer tumor foci formed on disks co-inoculated with wild-type and *iaaM*-silencing strains, compared to disks co-inoculated with wild-type and vector-only strains. The decrease in tumor formation caused by the silencing strain was statistically significant: $p<0.001$ in a Student's T-test. In this assay, the *iaaM*-silencing transgene did not prevent tumorigenesis completely. Perhaps some potato cells received only an oncogenic T-DNA. Alternatively, the *iaaM*-silencing transgene may have inserted

ipt

wt *ipt*	(1) **ATG**GACCTGCATCTAATTTTCG....GAATGTATTAG **(723)**
ipt-stop	(1) **ATG**GAC_*TGA*ATC*TAA*TTTTCG....GAATGTATTAG **(723)**

iaaM

wt *iaaM*	(1) **ATG**TCAGCTTCACCTCTCCTTGATAACCA...GCAGGATCC...GAAATTAG **(2268)**
iaaM-stop	(1) **ATG**TCA*TGA*__ACCTCTCCT*TGATAA*CCA...GCAGGATCC **(1813)**



B

Figure 3.1. Transgene sequences and constructions. **A**, Wild-type gene and transgene sequences showing premature stop codons and frameshift mutations in transgene sequences. Numbers in parentheses indicate nucleotide positions in the wild-type sequence. **B**, Transgene constructions encoding sense and antisense RNA. RB and LB indicate right and left T-DNA borders, respectively. Arrows labeled peCaMV and peFMV indicate the location and orientation of enhanced CaMV 35S and FMV promoters. The arrowhead labeled HSP70L represents the 5'-untranslated leader sequence from the petunia (*Petunia hybrida*) heat shock protein 70 gene. Arrows labeled iaaM-stop and ipt-stop show the location, orientation, and length of oncogene-silencing transgenes containing *iaaM* and *ipt* sequences. Numbers in parentheses indicate the portions of the *iaaM*-stop transgene sequence in pJP17 and pJC14. The arrow labeled NptII shows the position and orientation of the neomycin phosphotransferase gene, which is flanked by the nopaline synthase promoter and 3'-UTR and polyadenylation signal. Sb, St, and B indicate approximate positions of *Sbf*I, *Stu*I, and *Bam*HI sites, respectively.

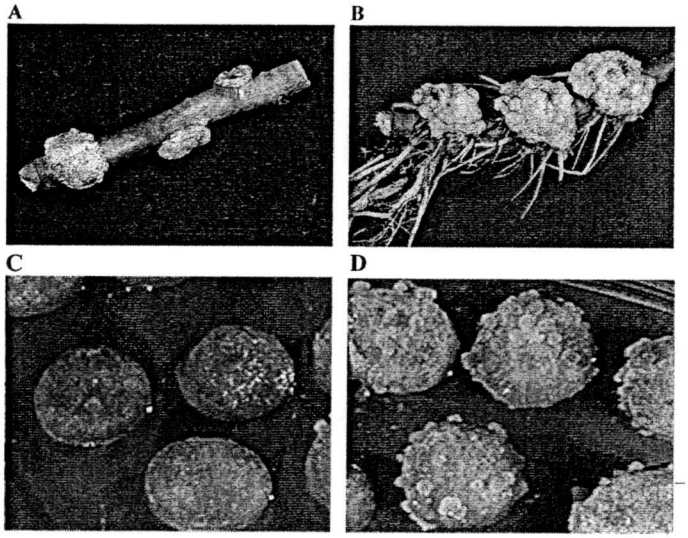
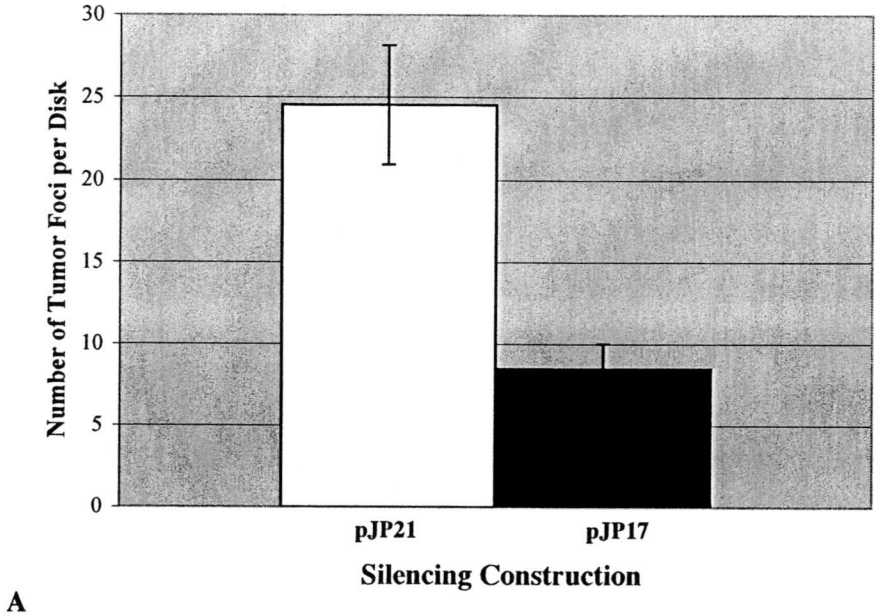


Figure 3.2 IaaM gene silencing during mixed infections. *K. daigremontiana* stems (**A** and **B**) and potato disks (**C** and **D**) co-inoculated with wild-type *A. tumefaciens* (A348) and disarmed strain EHA101 containing oncogene-silencing plasmid pJP17 (**A** and **C**) or an empty vector pJP21 (**B** and **D**).

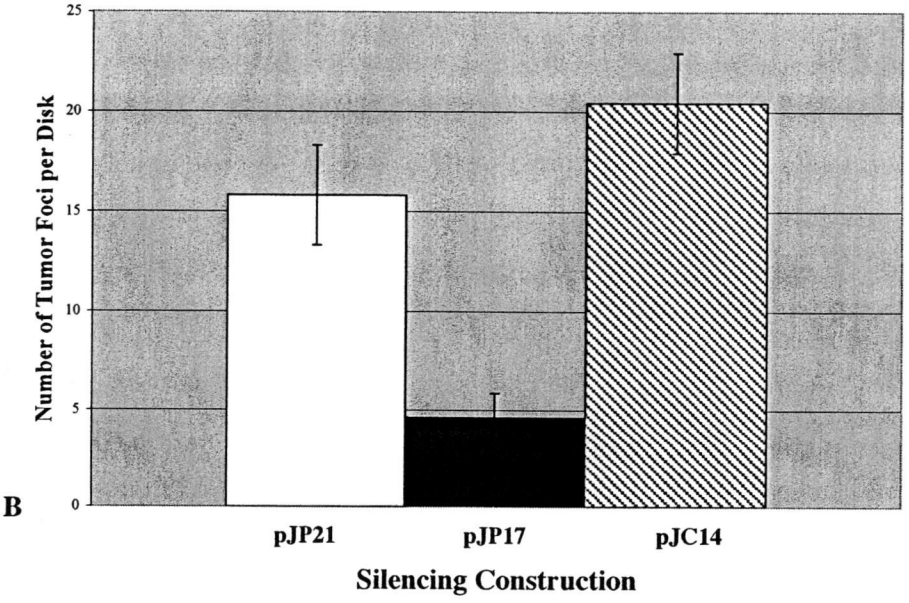
into transcriptionally silent chromatin in some potato cells. Nevertheless, this assay is a rapid and quantitative means to evaluate the efficacy of *iaaM*-silencing transgenes; constructions that reduced tumorigenesis in this assay prevented tumorigenesis on roots of transgenic apple trees containing these transgenes (Viss et al., 2003).

Effects of RNA sequence on gene silencing

Sense-strand RNAs that contain premature stop codons elicit gene silencing if they can initiate translation from a start codon downstream of the premature stop (D. C. Baulcombe, personal communication) (Que et al., 1997; Tanzer et al., 1997). In such cases, low-molecular-weight transgene RNA from silenced plant tissue is bound to polyribosomes in the cytoplasm, where transgene RNA degradation appears to occur (Tanzer et al., 1997). I searched *iaaM*- and *ipt*-stop for start codons downstream of



A



B

Figure 3.3 *IaaM* gene silencing on potato by the translatable transgene construction pJP17. **A.** Tumor formation on potato co-inoculated with wild-type *A. tumefaciens* A348 and EHA101(pJP17), containing the *iaaM*-stop sequence, or vector-only control, EHA101(pJP21). ($p<0.001$, t-test) **B.** Tumor formation on potato co-inoculated with A348 and EHA101(pJC14), EHA101(pJP17), or EHA101(pJP21). Error bars indicate the 95% confidence intervals. (See Tables A.1 and A.2 for counts of tumor foci on each disk.)

the nonsense mutations at codon 3. The *ipt* transgene lacked an alternative translation start, but *iaaM* contained two obvious in-frame starts at codons 18 and 23. These codons are surrounded by appropriate sequence context for translation initiation: CCAAAAUGGU (codon 18) and CUGACAAUGGU (codon 23) (nucleotides that match the consensus sequence are underlined) (Figure 3.4). The consensus sequence for translation initiation in dicotyledonous plants is: AAAA/CAAUGGCU; the start codon is underlined and most common nucleotides are bold (Joshi et al., 1997). Codon 1 of *iaaM*-stop lies in slightly less favorable context for translation initiation than codons 18 and 23 (Figure 3.4). Due to termination codons immediately downstream of codon 1 in the *iaaM*-stop transgene (Figure 3.5), translation is likely to reinitiate at one or both of the downstream AUGs (Kozak, 1987).

To examine sequence requirements for silencing *iaaM*, 188 bp was deleted from the 5' end of *iaaM*-stop to make an untranslatable construction (pJC14) (Figures 3.1 and 3.5). *K. daigremontiana* developed wild-type tumors when co-inoculated with A348 and EHA101 containing this untranslatable *iaaM* transgene, indicating that the region containing translation initiation sequences was necessary for silencing *iaaM*. Likewise, potato disks co-inoculated with A348 and EHA101(pJC14) developed as many tumor foci as those co-inoculated with A348 and vector-only control strain, EHA101(pJP21) (Figure 3.3B). In side-by-side comparisons, the translatable *iaaM* construct (pJP17) reduced tumor foci fivefold relative to a vector-only control ($p<0.001$, z-test), whereas the untranslatable derivative (pJC14) had no significant effect: tumor foci were 1.3-fold higher than the vector-only control. Although the untranslatable transgene contained 90% of the *iaaM* sequences present in the successful *iaaM*-silencing transgene, the deletion mutant did not silence *iaaM*.

CONSENSUS (position)	A (-5)	A (-4)	* A (-3)	* A/C (-2)	A (-1)	A (+1)	U (+2)	G (+3)	* G (+4)	* C (+5)
iaaM-stop putative start site 1 (codon 1)	G	A	U	C	C	A	U	G	U	C
iaaM putative start site 2 (codon 18)	C	C	A	A	A	A	U	G	G	U
iaaM putative start site 3 (codon 23)	U	G	A	C	A	A	U	G	G	U

Figure 3.4 Comparison of potential translation initiation sequences within the *iaaM*-stop transgene mRNA. Asterisks indicate the most common nucleotides of translation initiation sequences (Joshi et al., 1997). Codons 18 and 23 and their context sequences are the same in *iaaM*-stop and the wild-type *iaaM*. Numbers in parentheses indicate relative positions of the consensus sequence.

To determine whether translation initiation sequences affect PTGS, single nucleotide changes were made to pJP17 in *iaaM* codons 18 and 23. Potential start (AUG) codons were changed to isoleucine (AUU) codons, resulting in plant transformation vectors pJP30 (codon 18 changed to AUU), pJP25 (codon 23 changed to AUU), and pJP33 (codons 18 and 23 changed to AUU) (Figure 3.5). When these new constructions were tested in mixed infections on potato disks, none silenced *iaaM* (Figure 3.6). In paired comparisons (z-tests) to the vector control, $p>0.4$ for all strains except EHA101(pJP17) for which $p<0.01$.

To determine whether antisense transcription was necessary for the silencing elicited by pJP17, the peFMV promoter was removed from pJP17 (Figure 3.1) and this new construction, pJP27 (see Materials and Methods), was tested. In mixed infections on potato disks, pJP27 did not reduce tumorigenesis (Figure 3.6).

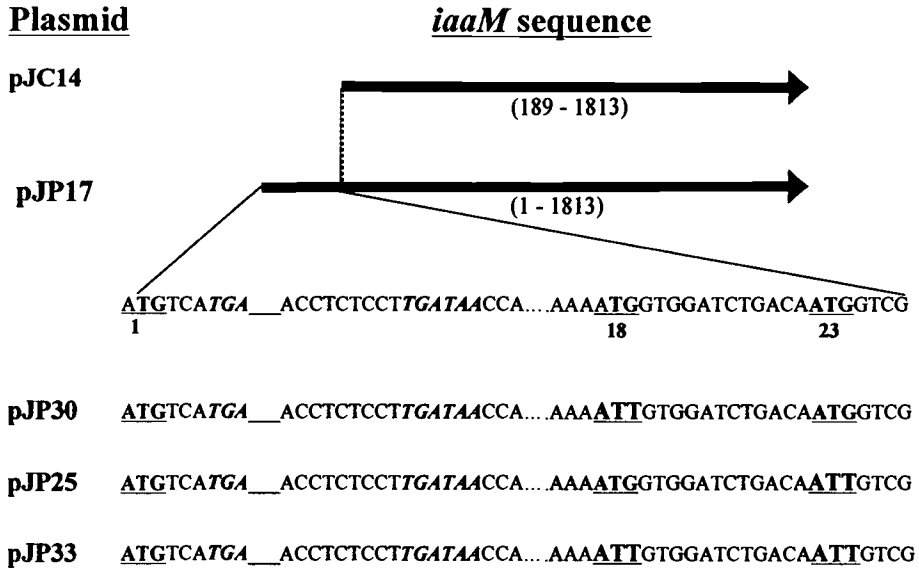


Figure 3.5 Transgene constructions containing altered translation initiation sequences. Schematic diagram of the sequence changes made to *iaaM*-stop in pJP17. pJC14 contains a 188-nucleotide deletion of the 5' end of *iaaM*-stop. pJP30, pJP25, and pJP33 have single nucleotide changes (G-to-T) at wild-type *iaaM* codons 18, 23, and both 18 and 23, respectively. These mutations change potential start codons (AUG) to isoleucine codons (AUU) in the mRNA transcribed from these genes.

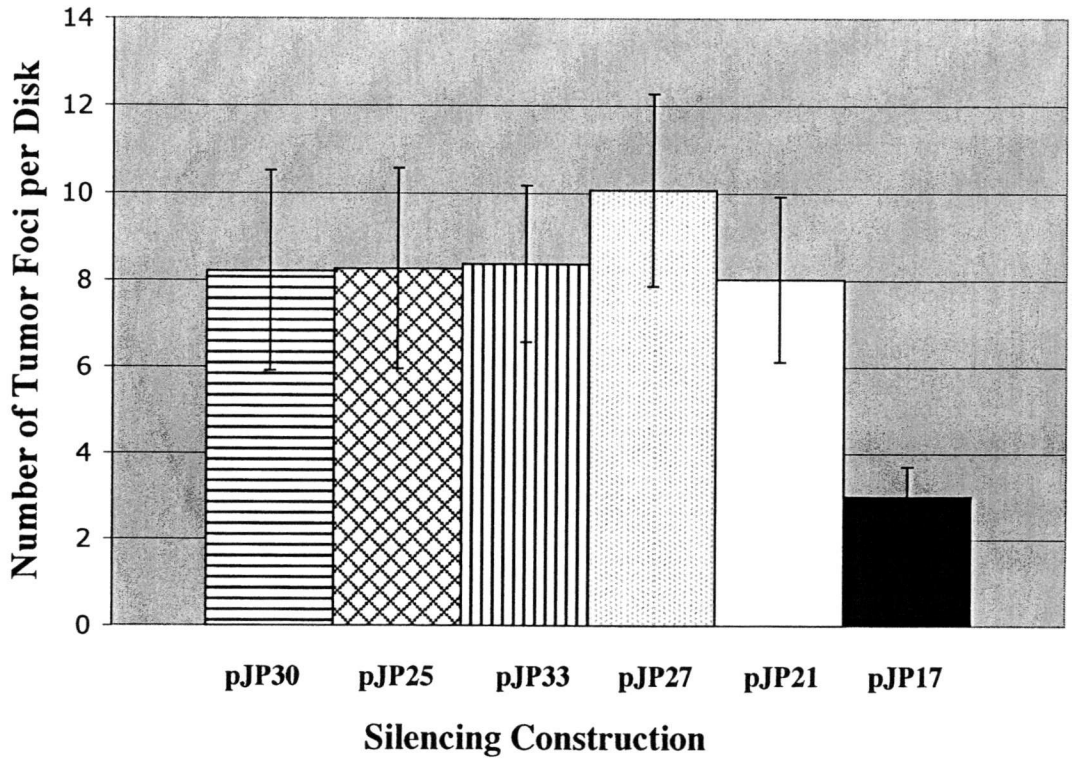


Figure 3.6 AUG codon changes eliminate *iaaM* gene silencing on potato. The graph shows tumor formation on potato co-inoculated with wild-type *A. tumefaciens* A348 and EHA101 carrying transformation construction pJP30, pJP25, or pJP33 (with single nucleotide changes at pJP17 *iaaM*-stop codons 18, 23, and both 18 and 23, respectively) or pJP27 (lacking the FMV promoter, see Materials and Methods). Error bars indicate the 95% confidence intervals. (See Table A.3 for counts of tumor foci on each disk.)

Discussion

Transgene constructions designed to posttranscriptionally silence both *A. tumefaciens* oncogenes, *iaaM* and *ipt*, decreased gene expression for *iaaM* but not *ipt*. This was the case in previous work with transgenic plants (Lee et al., 2003; Viss et al., 2003) and in transient assays reported here (Figure 3.2). Gene expression was assessed by tumorigenesis and mRNA levels in transgenic tissues (Viss et al., 2003) and by tumorigenesis in transient assays. Although both transgenes were designed to be untranslatable [they contained premature stop codons and frameshift mutations

immediately downstream of the first AUG (Figure 3.1)], further inspection of the *iaaM*-stop sequence revealed two in-frame start codons in good sequence context for efficient translation initiation downstream from these mutations (Figures 3.4 and 3.5). Despite both transgenes being fused, *iaaM* gene expression was consistently reduced, but *ipt* gene expression was not. This distinct difference in PTGS between the two transgenes suggests that transgene sequence affects PTGS. I believe the difference in silencing is due to a difference in translatability of the *iaaM*-stop and *ipt*-stop mRNA sequences.

One line of support for this hypothesis comes from a study done by Escobar and colleagues. In their study, transgenes designed to generate self-complementary *iaaM* and *ipt* transcripts silenced the oncogenes in *Arabidopsis thaliana* and *Lycopersicon esculentum* (tomato) (Escobar et al., 2001). Both the *iaaM* and *ipt* transgene sequences were amplified by PCR from *A. tumefaciens* strain 20W-5A. Although these transgene sequences were not provided by the authors, homology of *A. tumefaciens* oncogene sequences is approximately 90% (Depicker et al., 1978; Drummond and Chilton, 1978). Therefore, these sequences are probably highly similar to those used in this study (derived from *A. tumefaciens* A348). Escobar and colleagues created self-complementary *ipt* and *iaaM* sequences driven by 35S cauliflower mosaic virus promoters (Escobar et al., 2001). A sense copy of *ipt* (from 20W-5A) bp 45-627 was fused to an identical fragment in the antisense orientation, but separated by a 1000-bp region of noncoding linker DNA. The self-complementary *iaaM* construction was made by fusing an antisense copy of *iaaM* (from 20W-5A) bp 1274-2224 to the 5' end of a full-length sense *iaaM* fragment (Escobar et al., 2001). The authors do not indicate that any alterations were made in the coding sequence of these transgenes, such as frameshift mutations or premature stop codons. Therefore, these transgenes likely produced translatable RNA, unlike those reported in the current study. Although Escobar and colleagues used self-complementary sequences, similar "hairpin" sequences of the pJP17 transgenes did not silence *ipt*. Thus, it is likely that silencing depends on the translatability of the transgene RNA.

RNAi is induced by dsRNA, sources of which include viral genomes or replication intermediates, transcription of inverted-repeats, bidirectional transcription, or products of RNA-dependent RNA-polymerases (RdRps) (Baulcombe, 2004; Montgomery and Fire, 1998; Waterhouse et al., 1998) which can generate dsRNA from single-stranded templates. RdRps are required for silencing initiated by single-stranded RNAs (Dalmay et al., 2000; Tang et al., 2003). In plants, dsRNA is processed by Dicer-like enzymes into siRNAs, which are then incorporated into the RNAi effector complex, RISC, to direct cleavage of cognate mRNAs (Tang et al., 2003). Spreading of silencing into gene sequences adjacent to the RNAi-trigger sequences depends on the RNA-dependent RNA polymerase (RdRp), RDR6 (also called SGS2/SDE1) (Vaistij et al., 2002). Cleaved mRNA molecules may serve as templates for RDR6 (Allen et al., 2005; Gazzani et al., 2004). The resulting dsRNA molecules provide a secondary source of siRNAs, thus amplifying the original dsRNA trigger. In *Arabidopsis*, cleaved mRNAs are also targets of the 5'-3' exoribonuclease, AtXRN4, which can destroy RdRp templates (Gazzani et al., 2004; Souret et al., 2004).

Translatability affects the capacity of an RNA molecule to elicit PTGS (D.C. Baulcombe, personal communication) (Que et al., 1997; Tanzer et al., 1997). Transgene mRNA molecules containing premature nonsense codons reduced the degree and frequency of PTGS of an endogenous chalcone synthase gene (*Chs)* in petunia, compared to translatable mRNAs (Que et al., 1997). Tanzer and colleagues (1997) found silencing-specific low-molecular-weight RNAs associated with ribosomes in transgenic tobacco plants. In *Drosophila melanogaster*, oocyte mRNAs are subject to RNAi only during oocyte maturation when mRNAs are actively translated, not during the oocyte's arrested stage when mRNAs are present but not translated (Kennerdell et al., 2002). Likewise, in *Drosophila* and *Trypanosoma brucei*, siRNAs and RISC component proteins associate with translating ribosomes (Djikeng et al., 2003; Ishizuka et al., 2002; Pham et al., 2004). Thus, it is clear that RNAi and mRNA translation are linked.

The pJP17 transgene construction reported here silenced wild-type *iaaM* gene expression and reduced tumorigenesis driven by this oncogene (Figures 3.2 and 3.3) (Lee et al., 2003; Viss et al., 2003). The effectiveness of pJP17 required promoters driving transcription in both the sense and antisense directions (relative to *iaaM*-stop and *ipt*-stop sequences). The two start codons (AUG) downstream of premature termination codons were also required (Figures 3.5 and 3.6). Silencing by the pJP17 construction may be explained as follows. First, sense and antisense transcription of the transgene sequences provide complementary RNAs containing *iaaM* and *ipt* sequences. These RNA molecules inefficiently anneal to form dsRNA that initiates the RNAi process starting with Dicer-like cleavage of the dsRNA. Subsequent incorporation of the resulting siRNAs into RISC then directs cleavage of the sense-strand transgene mRNA while it is associated with translating ribosomes. Each AUG codon downstream of the premature stop codons in *iaaM*-stop may serve equally well to initiate translation. Ribosomes terminate translation when they encounter the premature stop codon located in the 5'-end of *ipt*-stop, thus preventing mRNA cleavage by RISC within the *ipt* sequence. The resulting RNA molecules can serve as templates for dsRNA synthesis by RdRps and thus propagate RNAi. However, the 3' RISC cleavage products (containing *ipt*-stop sequences) are also subject to degradation by an AtXRN4-like 5'-3' exoribonuclease. RdRp-dependent amplification of *iaaM*-stop-derived siRNAs, and therefore *iaaM* gene silencing, is more likely to occur than RdRp-dependent amplification of *ipt* RNA, which is probably not associated with ribosomes.

Without antisense transcription of the transgenes, as in the case of pJP27, which did not silence *iaaM*, dsRNA formation is less likely than it is with both sense and antisense transcription; thus RNAi initiation is, at best, very inefficient. Constructions containing single nucleotide changes (AUG to AUU) within one or both of two potential translation start codons in the pJP17 sequence also failed to silence *iaaM* (Figures 3.5 and 3.6). Both codons are in similar sequence context and may each

serve equally well as alternative translation initiation sites for *iaaM*-stop codon 1, which is immediately followed by premature stop codons and frameshift mutations. Together, both alternative AUGs may contribute ribosome-associated mRNAs that can be used for siRNA biosynthesis. Apparently, neither AUG by itself provides sufficient mRNA for RNAi amplification, perhaps due to competition between translation and nonsense-mediated decay of the mRNAs (Maquat, 2004).

If the silencing construction pJP17 silences *iaaM* as described, a construction designed to generate transgene dsRNA that is independent of RdRp activity may silence both target oncogenes very efficiently. Constructions designed to generate "hairpin" RNAs from the transgene sequences prevented tumorigenesis on potato disks as well as pJP17, but did not inhibit *ipt* expression in *Kalanchoe* mixed infections (data not shown). Perhaps, in this case nonsense-mediated decay destroys the transgene-derived mRNA prior to complete transcription of the antisense portion of the "hairpin" RNA. One would also predict that *iaaM* silencing by pJP17 could be disrupted by introduction of a translation termination codon directly downstream of *iaaM* codon 23, which is the last in-frame AUG in good translation initiation context in the gene sequence. This experiment, as well as examination of the stability and translatability of pJP17-derived mRNAs (such as analysis of mRNA transcription, accumulation, and ribosome loading), could provide further support for how pJP17 silences *iaaM*.

Chapter 4

A Mutation in the *Arabidopsis thaliana HEN1* Gene Reduces *Agrobacterium*-Induced Tumorigenesis

Jennifer Pitrak

Abstract

RNA silencing is an evolutionarily conserved mechanism of gene regulation and genome protection. In plants, RNA silencing regulates gene expression, defends against viruses and transposable elements, and inhibits expression from transgenes. *Agrobacterium tumefaciens* is the causative agent of crown gall disease and is widely used to deliver transgenes to plants. Wild-type *A. tumefaciens* transfers DNA from its tumor-inducing plasmid (Ti plasmid) to the plant cell where the transferred DNA (T-DNA) integrates into the plant genome. T-DNA-encoded genes direct auxin and cytokinin biosynthesis and opine production. Auxin and cytokinin cause unorganized proliferation of T-DNA-transformed plant cells, and opines provide nutrition to *A. tumefaciens*. Manifestation of crown gall disease requires several steps: bacterial binding to the plant cell, *A. tumefaciens* virulence gene induction, T-DNA transfer, nuclear import, and integration into the plant genome, T-DNA gene expression, and, in roots, response to auxin. Mutations in host genes can affect any of these steps. T-DNA molecules, like viruses and transposons, are foreign invasive nucleic acids and are, therefore, likely targets for protective RNAi. Likewise, RNAi naturally regulates many plant genes that may be involved in *Agrobacterium*-mediated tumorigenesis. To assess whether RNAi influences crown gall disease, eleven RNAi-mutant *Arabidopsis thaliana* lines were tested for susceptibility to crown gall disease. An *A. thaliana* line with a mutation in *HEN1*, a gene involved in methylation and maturation of micro-RNAs (miRNAs), showed reduced *A. tumefaciens*-induced tumorigenesis compared to wild-type *A. thaliana* (11% of wild type). None of the other lines demonstrated a significant difference. The same line showed no significant difference in transient expression of a non-tumorigenic reporter T-DNA.

Introduction

RNA silencing was first identified in plants as "co-suppression" of an *Agrobacterium tumefaciens*-introduced transgene (Napoli et al., 1990). Now RNA silencing is well established as an evolutionarily conserved mechanism of gene regulation and genome protection that relies on double-stranded RNA (dsRNA) (Tomari and Zamore, 2005). In plants, dsRNA originates from various sources and is processed by an RNAseIII-like enzyme, Dicer-like (DCL), into small-interfering RNAs (siRNAs). Comprised of approximately 22 nucleotides, siRNAs contribute sequence specificity to the RNA-induced silencing complex (RISC) or to cellular machinery that targets the genome for chromatin modification (Baulcombe, 2004; Matzke et al., 2004; Matzke and Birchler, 2005; Tang et al., 2003; Tomari and Zamore, 2005).

RNA silencing in *Arabidopsis* regulates endogenous gene expression and protects the genome. RNA silencing can be divided into several pathways based on silencing targets and the genes involved (Baulcombe, 2004; Matzke et al., 2004; Matzke and Birchler, 2005; Xie et al., 2004) (Table 4.1). The micro-RNA (miRNA) and *trans*-acting-siRNA (ta-siRNA) pathways are involved in endogenous gene regulation (Allen et al., 2005; Dugas and Bartel, 2004; Peragine et al., 2004; Vazquez et al., 2004b). miRNA biogenesis and function require the Dicer-like protein, DCL1 (Schauer et al., 2002), ARGONAUTE1 (AGO1, a RISC component) (Vaucheret et al., 2004), the RNA methyltransferase HEN1 (Yu et al., 2005), and two other nuclear-localized proteins, HYL1 and HST, for miRNA maturation and nuclear export, respectively (Han et al., 2004; Hiraguri et al., 2005; Park et al., 2005; Vazquez et al., 2004a). miRNAs arise from protein-noncoding transcripts encoded by *MIR* genes (Reinhart et al., 2002). *MIR* transcripts form stem-loop ("hairpin") secondary structures that are processed by DCL1 to miRNAs. These small RNAs then target mRNAs containing complementary sequences for RISC-mediated cleavage and subsequent destruction or further processing in the case of ta-siRNAs (see below) (Allen et al., 2005; Tang et al., 2003). miRNAs can also function as translational repressors by binding to partially complementary sequences within the 3' UTR of their

target RNAs (Aukerman and Sakai, 2003; Chen, 2004). Many miRNA-regulated genes are involved in plant developmental processes and many encode transcription factors (Allen et al., 2005; Dugas and Bartel, 2004; Kasschau et al., 2003; Mallory et al., 2005; Vazquez et al., 2004a). Whereas protein-coding targets are downregulated by miRNAs, some targets require miRNAs for functional maturation. These target RNAs are protein-noncoding transcripts that generate ta-siRNAs with sequence complementarity to yet other RNAs. In the ta-siRNA pathway, miRNA-cleaved transcripts are templates for dsRNA synthesis by the RNA-dependent RNA polymerase (RdRp), RDR6, and a protein of unknown function, SGS3, which is required for RDR6-dependent RNA silencing (Mourrain et al., 2000; Peragine et al., 2004; Vazquez et al., 2004b). Another Dicer-like protein, DCL4, likely processes this dsRNA to ta-siRNAs in a 21-nucleotide phase set by the original miRNA cleavage (Allen et al., 2005)(Xie and Carrington, personal communication). ta-siRNAs target protein-coding transcripts and may target the RNA from which they originate (Allen et al., 2005; Peragine et al., 2004; Vazquez et al., 2004b).

Another endogenous RNA silencing pathway in *Arabidopsis* silences transcription of target genes via heterochromatinization (chromatin condensation by DNA and histone methylation). In this chromatin-associated RNA silencing pathway, highly repeated DNA sequences (such as ribosomal RNA sequences, transposons, retroelements, and centromeric repeats) are sources and targets of siRNAs that mediate transcriptional gene silencing (Chan et al., 2004; Hamilton et al., 2002; Lippman et al., 2004; Lippman and Martienssen, 2004; Onodera et al., 2005; Xie et al., 2004; Zilberman et al., 2003; Zilberman et al., 2004). This chromatin RNAi pathway involves the Dicer-like enzyme DCL3, (the RdRp) RDR2, and RNA polymerase IV for siRNA production; an Argonaute protein, AGO4, is probably part of the transcriptional silencing complex (Chan et al., 2004; Herr et al., 2005; Onodera et al., 2005; Xie et al., 2004; Zilberman et al., 2003; Zilberman et al., 2004) (Table 4.1). Other proteins in the chromatin RNAi pathway are involved in methylation of

histones and DNA, including the CpNpG methyltransferase CHROMOMETHYLASE 3 (CMT3) (Cao and Jacobsen, 2002a; Lindroth et al., 2001) (see Table 4.1). Two other RNA silencing pathways target foreign nucleic acid species, namely viruses and transgenes. RDR1 and DCL2 function in antiviral defense (Xie et al., 2004; Yu et al., 2003). RDR6 and SGS3 contribute to silencing sense transgenes and some viruses (Butaye et al., 2004; Dalmay et al., 2000; Mourrain et al., 2000). There is genetic overlap among the different RNA silencing pathways (Table 4.1).

Agrobacterium tumefaciens, the causative agent of crown gall disease, genetically transforms plant cells to grow rapidly and produce opines, a bacterial-specific nutrition source. *A. tumefaciens* transfers DNA (T-DNA) from its tumor-inducing plasmid to plant cells where the T-DNA integrates into the plant genome, often in more than one location and oriented in direct or inverted repeat configurations (Grevelding et al., 1993; Jones et al., 1987; Jorgensen et al., 1987; Jorgensen et al., 1996; Spielmann and Simpson, 1986). After integration, T-DNA genes for phytohormone and opine biosynthesis are expressed by the host (Winans, 1992; Zhu et al., 2000). Crown gall tumors result from overproduction of auxin and cytokinin in transformed plant cells (Winans, 1992). The T-DNA-encoded genes, *iaaM* (tryptophan monooxygenase) and *iaaH* (indole-3-acetamide hydrolase) are responsible for auxin overproduction; *ipt* (AMP isopentenyl transferase) is required for cytokinin overproduction (Garfinkel et al., 1981; Ream et al., 1983). These genes are required for tumorigenesis, but not for T-DNA transmission (transfer, nuclear import, and integration) (Sheng and Citovsky, 1996). Therefore, *A. tumefaciens* can transform plant cells with any genes in place of T-DNA genes (Bevan, 1984). Wild-type or engineered T-DNAs (transgenes) are foreign genetic elements in recipient plant cells, and T-DNAs often form direct and inverted repeats. Therefore, it is not surprising that they are subject to RNA silencing (Butaye et al., 2004; Dalmay et al., 2000; Francis and Spiker, 2005; Mourrain et al., 2000; Schubert et al., 2004; Xie et al., 2004). Greater T-DNA gene expression would be expected in plants with mutations in genes

of the chromatin-associated or transgene silencing pathways than in wild-type plants. Indeed, *A. thaliana* mutated in *RDR6* or *SGS3* showed increased *uidA* (beta-glucuronidase) transgene expression (7- and 2.5-fold, respectively) compared to wild-type plants (Butaye et al., 2004). Likewise, an *Agrobacterium*-delivered *FWA* transgene in *A. thaliana* required the chromatin-RNA silencing genes *DRM2, RDR2, DCL3, SDE4,* and *AGO4* for methylation and silencing (Chan et al., 2004). The *Arabidopsis FWA* gene (encoding a homeodomain-containing transcription factor and involved in timing of flowering) is normally methylated within direct repeats in its promoter, silencing *FWA* expression (Soppe et al., 2000). Epigenetic *fwa* mutants have lost this methylation and ectopic *FWA* expression causes a dominant late flowering phenotype. The methylation and silencing of *FWA* can be restored by introduction of an extra copy of *FWA* via *Agrobacterium tumefaciens* transformation (Soppe et al., 2000). However, this silencing does not occur in *Arabidopsis* lines with mutations in the chromatin RNA-silencing genes *DRM2, RDR2, DCL3, SDE4,* and *AGO4* (Cao and Jacobsen, 2002b; Chan et al., 2004; Soppe et al., 2000).

Successful tumorigenesis from wild-type *Agrobacterium* transformation requires plant cells to respond to auxin and cytokinin produced by the T-DNA-encoded proteins, IaaM, IaaH, and Ipt. miRNAs regulate at least seven auxin response factor (ARF) genes (Allen et al., 2005; Dugas and Bartel, 2004). Thus, mutations in the miRNA pathway may affect the response of *Arabidopsis* to *Agrobacterium* transformation.

To determine whether RNAi affects *Agrobacterium*-induced tumorigenesis, eleven *Arabidopsis* lines containing mutations in RNA silencing pathways were tested for *Agrobacterium*-mediated tumorigenesis on root explants. Most lines showed no significant difference in tumorigenesis compared to wild-type controls. However, one line (*hen1-1)* with a mutation in *HEN1* demonstrated a tenfold reduction in tumorigenesis. The same line demonstrated no significant reduction in transient

expression of a reporter transgene, indicating that reduced tumorigenesis in *hen1-1* is not due to reduced T-DNA transfer or nuclear import.

Table 4.1 *Arabidopsis thaliana* RNA silencing pathways, associated proteins, and their functions.

PROTEIN	DESCRIPTION & FUNCTION	PATHWAY					REFERENCES
		miRNA	trans-acting-siRNA	Anti-viral	Chromatin RNAi	Transgene silencing	
AGO1	Binds and/or cleaves target RNA (RISC component)	X	X				(Vaucheret et al., 2004)
AGO4	Directs chromatin modifications				X		(Chan et al., 2004; Zilberman et al., 2003; Zilberman et al., 2004)
AGO7	unknown		X				(Fagard et al., 2000; Hunter et al., 2003; Peragine et al., 2004)
CMT3	DNA (CpNpG) methylation				X		(Cao and Jacobsen, 2002a; Lindroth et al., 2001)
DCL1	RNAseIII-like; Cleaves pre-miRNA to miRNA	X	X				(Park et al., 2002; Reinhart et al., 2002; Schauer et al., 2002)
DCL2	RNAseIII-like; Cleaves dsRNA to siRNAs			X			(Xie et al., 2004)
DCL3	RNAseIII-like; Cleaves dsRNA to siRNAs				X	X	(Chan et al., 2004; Xie et al., 2004)
DCL4	RNAseIII-like; Cleaves dsRNA to siRNAs		X				(Xie and Carrington, personal communication)
DRM1	de novo DNA methylation				X		(Cao and Jacobsen, 2002a, 2002b)
DRM2	de novo DNA methylation				X		(Cao and Jacobsen, 2002a, 2002b)
HEN1	miRNA methylation/maturation	X	X		X		(Boutet et al., 2003; Chen et al., 2002; Park et al., 2002; Yu et al., 2005)
HST	Homologous to mammalian Exportin 5; miRNA nuclear export	X	X				(Lund et al., 2004; Park et al., 2005)
HYL1	dsRNA-binding protein; miRNA stabilization and/or maturation; modulation of DCL1 function	X	X				(Han et al., 2004; Hiraguri et al., 2005; Vazquez et al., 2004a)

(continued on next page)

Table 4.1, continued.

PROTEIN	DESCRIPTION & FUNCTION	PATHWAY					REFERENCES
		miRNA	trans-acting-siRNA	Anti-viral	Chromatin RNAi	Transgene silencing	
KYP	Histone methyltransferase				X		(Jackson et al., 2002)
MET1	Cytosine methyltransferase; DNA (CpG) methylation				X		(Finnegan et al., 1996, 2000; Kishimoto et al., 2001; Ronemus et al., 1996; Vongs et al., 1993)
RDR1	RNA-dependent RNA polymerase (RdRP); viral RNA turnover			X			(Yu et al., 2003)
RDR2	RdRP; copies sense RNA or stabilizes DCL3				X	X	(Chan et al., 2004; Xie et al., 2004)
RDR6 (SDE1/SGS2)	RdRP; amplification of silencing signal, spreading of silencing; viral RNA turnover		X	X		X	(Allen et al., 2005; Butaye et al., 2004; Dalmay et al., 2000; Mourrain et al., 2000; Parizotto et al., 2004; Peragine et al., 2004; Vazquez et al., 2004b; Yu et al., 2003)
SDE3	unknown					X	(Dalmay et al., 2000)
SGS3	spreading of silencing		X	X		X	(Butaye et al., 2004; Mourrain et al., 2000; Peragine et al., 2004)
NRPD1a (previously SDE4)	Subunit of RNA polymerase IV (produces chromatin-associated siRNAs)				X		(Chan et al., 2004; Hamilton et al., 2002; Herr et al., 2005; Onodera et al., 2005)
NRPD2a	Subunit of RNA polymerase IV (produces chromatin-associated siRNAs)				X		(Onodera et al., 2005)
SUVH2	histone methylation				X		(Naumann et al., 2005)

Materials and methods

Plant materials and bacterial strains

All plants were grown in sterile culture as described (Nam et al., 1997) except that Phytatrays (Sigma-Aldrich) were used in lieu of baby food jars, and each Phytatray contained four plants. Dr. James Carrington provided all *Arabidopsis thaliana* lines. The *hen1-1*, *cmt3-7*, and *ago4-1* mutant lines were previously described; they are ethylmethane sulfonate (EMS)-induced mutants in the Landsberg *erecta* (L*er*) ecotype (Cao and Jacobsen, 2002a; Chen et al., 2002; Lindroth et al., 2001; Park et al., 2002; Zilberman et al., 2003). The *hen1-1* allele has a G-to-A mutation that introduces a stop codon in the HEN1 C-terminal domain coding sequence (Chen et al., 2002). The *cmt3-7* allele has a stop codon after 27 amino acids of *CMT3* (Lindroth et al., 2001). The *dcl2-1*, *dcl3-1*, *rdr1-1,* and *rdr2-1* mutant lines have been described (Xie et al., 2004); all are T-DNA insertion mutants in the Columbia (Col) ecotype. *dcl2-1* has a T-DNA insertion in predicted exon 9 (after nucleotide 2,842 relative to the first ATG in the genomic sequence) of *DCL2* (At3g03300) (Xie et al., 2004). *dcl3-1* has a T-DNA insertion within predicted exon 7 of *DCL3* (At3g43920) 2,136 nucleotides after the first ATG in *DCL3*, introducing a premature stop codon just after codon 288 (Xie et al., 2004). *rdr1-1* has a T-DNA insertion within predicted exon 1 after nucleotide 2,366 relative to the first ATG of *RDR1* (At1g14790) (Xie et al., 2004). *rdr2-1* has a T-DNA insertion within predicted exon 1 (before nucleotide 316 from ATG) of *RDR2* (At4g11130) (Xie et al., 2004). *hyl1*-2, and *hen1-5* are T-DNA insertion mutants in the Columbia background and were identified in the SALK mutant collection and confirmed by Vazquez et al. (2004a). *hyl1-2* (SALK_064863) contains a T-DNA insertion within a predicted exon of *HYL1* (At1g09700) (Alonso et al., 2003). *hen1-5* (SALK_049197) contains a T-DNA insertion within the fifth intron of *HEN1* (At4g20910) (Alonso et al., 2003). The *hst-15* mutant (SALK_079290) contains a T-DNA insertion at position 1584 relative to the start codon of wild-type *HST* (Alonso et

al., 2003). The *rdr6-15* mutant (SAIL_617) contains a T-DNA insertion at position 312 relative to the start codon of wild-type gene At3g49500 (Allen et al., 2005).

Agrobacterium tumefaciens A208 (Sciaky et al., 1978) or A281 (Hood et al., 1986) was used for tumorigenesis assays. *A. tumefaciens* EHA105 is a kanamycin-sensitive isolate of EHA101 (Hood et al., 1986) and was kindly provided by Dr. Stanton Gelvin. EHA105 was transformed with the intron-containing *uidA* (beta-glucuronidase) expression construction, pBISN1 (Narasimhulu et al., 1996a), and the resulting kanamycin-resistant strain EHA105(pBISN1) was used for GUS expression assays.

Tumorigenesis and GUS assays

Roots were inoculated and assessed for tumorigenesis and GUS expression as described (Nam et al., 1997), except that bacterial suspensions were diluted 1:1000 in 0.9% NaCl prior to inoculation. Briefly, *Arabidopsis* roots grown in sterile culture were excised, cut into 3-5 mm pieces in a small amount of sterile water, blotted onto sterile filter paper to remove excess water, then placed onto MS basal medium (4.32 g/L MS [Murashige and Skoog, Sigma-Aldrich] minimal salts, 0.5 g/L Mes, pH 5.7, 1 mL/L vitamin stock solution [0.5 mg/mL nicotinic acid, 0.5 mg/mL pyridoxine, and 0.5 mg/mL thyamine-HCl], 100 mg/L myoinositol, 10 g/L sucrose, and 0.75% bactoagar [Difco]). Bundles of root pieces were covered with bacterial suspension and after 10 minutes, most of the bacterial solution was removed. Roots and bacteria were cocultivated at 24°C for 2 days, after which the roots were rinsed in 100 mg/L timentin. 100-300 root pieces were separated onto MS plates containing 100 mg/L timentin and incubated at 24°C for 4 weeks (for tumorigenesis assays) at which time tumorigenesis was scored as the percentage of root pieces with tumors. Alternatively, (for GUS assays) roots and bacteria were cocultivated at 24°C for 2 days, after which the roots were rinsed in 100 mg/L timentin. Rinsed root bundles were blotted on sterile filter paper and then incubated for 4 days on callus-inducing medium (CIM) for

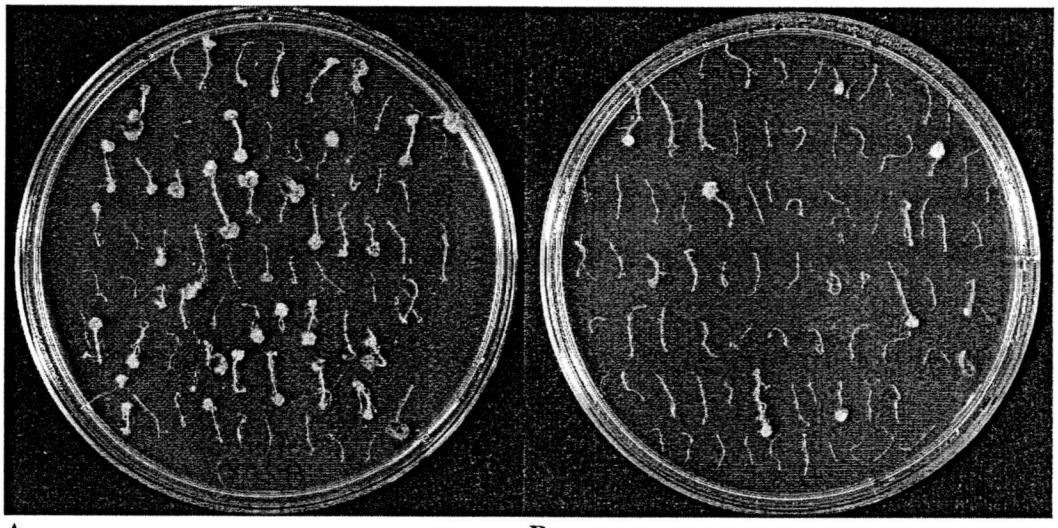
4-6 days, after which they were stained with 2 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) (Jefferson et al., 1987). Root bundles were incubated in approximately 1 mL 2mM X-gluc in 1.6 mL microfuge tubes overnight at 37°C. Root bundles were rinsed in 70% ethanol for 5 minutes. Then roots were placed in a small amount of water and separated onto water agar plates (0.75% bactoagar [Difco]). 100-300 root pieces were examined under a dissecting scope and GUS expression was scored as percentage of root pieces that stained blue. For both tumorigenesis and GUS assays, 2-4 replications were performed for each line. In each replication, roots segments from 8-16 plants per line were pooled, inoculated, and 100-300 root pieces were arbitrarily chosen for subsequent incubation (tumorigenesis) or staining (GUS). Analysis of Variance (ANOVA) was used to assess statistical significance of any differences in average tumorigenesis among the lines tested (Turner and Thayer, 2001). If ANOVA indicated a significant difference, then paired comparisons (Z-tests) were performed with each line and wild-type (Turner and Thayer, 2001). GUS expression of wild-type (L*er*) and *hen1-1* was compared with the Student's t-test.

Results

Arabidopsis hen1-1 demonstrates reduced tumorigenesis

To examine whether RNA silencing affects *Agrobacterium*-induced tumorigenesis, RNAi-mutant *Arabidopsis thaliana* lines *dcl2-1*, *dcl3-1*, *rdr1-1*, *rdr2-1*, *rdr6-15*, *ago4-1*, *cmt3-7*, *hst-15*, *hyl1-2*, *hen1-5*, and *hen1-1* were challenged with wild-type *A. tumefaciens*. Roots from these lines and from wild-type *A. thaliana* control lines were cut into 3-5 mm segments and inoculated with *A. tumefaciens*. After removal of the bacteria, the segments were grown on hormone-free medium for 4 weeks. Tumorigenesis was scored as the percentage of root segments forming tumors (Figures 4.1 and 4.2). Compared to wild-type *A. thaliana*, none of the lines with mutations in the antiviral and chromatin-associated RNAi pathways (Table 4.1; *dcl2, dcl3, rdr1,*

rdr2, rdr6, ago4, cmt3) showed a significant difference in rate of tumor formation (Figure 4.2B,C). Likewise, the miRNA and ta-siRNA pathway mutants *hyl1-2*, *hst-15*, and *hen1-5* did not show a significant difference in tumorigenesis (Figure 4.2A). In contrast, an *A. thaliana* line with a mutation in a miRNA pathway gene (*hen1-1*) developed significantly fewer tumors compared to wild-type *A. thaliana* (Figures 4.1 and 4.2C). The *hen1-1* line developed tumors at 11% the rate of wild-type *A.thaliana* (Figure 4.2C).



A **B**

Figure 4.1 Tumorigenesis on *A. thaliana* roots. *A. thaliana* roots were inoculated with *A. tumefaciens* A208, incubated on hormone-free medium for 4 weeks, then scored for tumorigenesis. **A.** Wild-type L*er* roots. **B.** *hen1-1* roots.

A. thaliana HEN1 mutant is not altered in T-DNA transfer and nuclear import

To assess whether the lower rate of tumorigenesis in the *hen1-1* line was due to a deficiency in T-DNA transfer and nuclear import, I inoculated roots with *A. tumefaciens* carrying a reporter T-DNA and examined the transient expression of the

reporter gene. *A. tumefaciens* EHA105(pBISN1) is a non-tumorigenic strain carrying a *uidA* reporter gene construction (Narasimhulu et al., 1996a). This *uidA* gene contains an intron to prevent functional GUS expression in bacterial cells (Narasimhulu et al., 1996a). *Arabidopsis* roots were inoculated with EHA105(pBISN1), rinsed in 1x timentin after 2 days, and grown on CIM for 4-6 days. Beta-glucuronidase (GUS) expression was assessed by staining with 5-bromo-4-chloro-3-indolyl glucuronide (Jefferson et al., 1987), and the rate of T-DNA expression was calculated as the percentage of root pieces staining blue (Figures 4.3 and 4.4). Compared to wild-type *Arabidopsis*, the *hen1-1* line showed no difference in GUS expression indicating that T-DNA transfer and nuclear import are not affected in the mutant. This test reflects *uidA* expression from nuclear-localized T-DNAs that are not necessarily stably integrated into the plant genome (Janssen and Gardner, 1990; Nam et al., 1997; Narasimhulu et al., 1996a; Narasimhulu et al., 1996b).

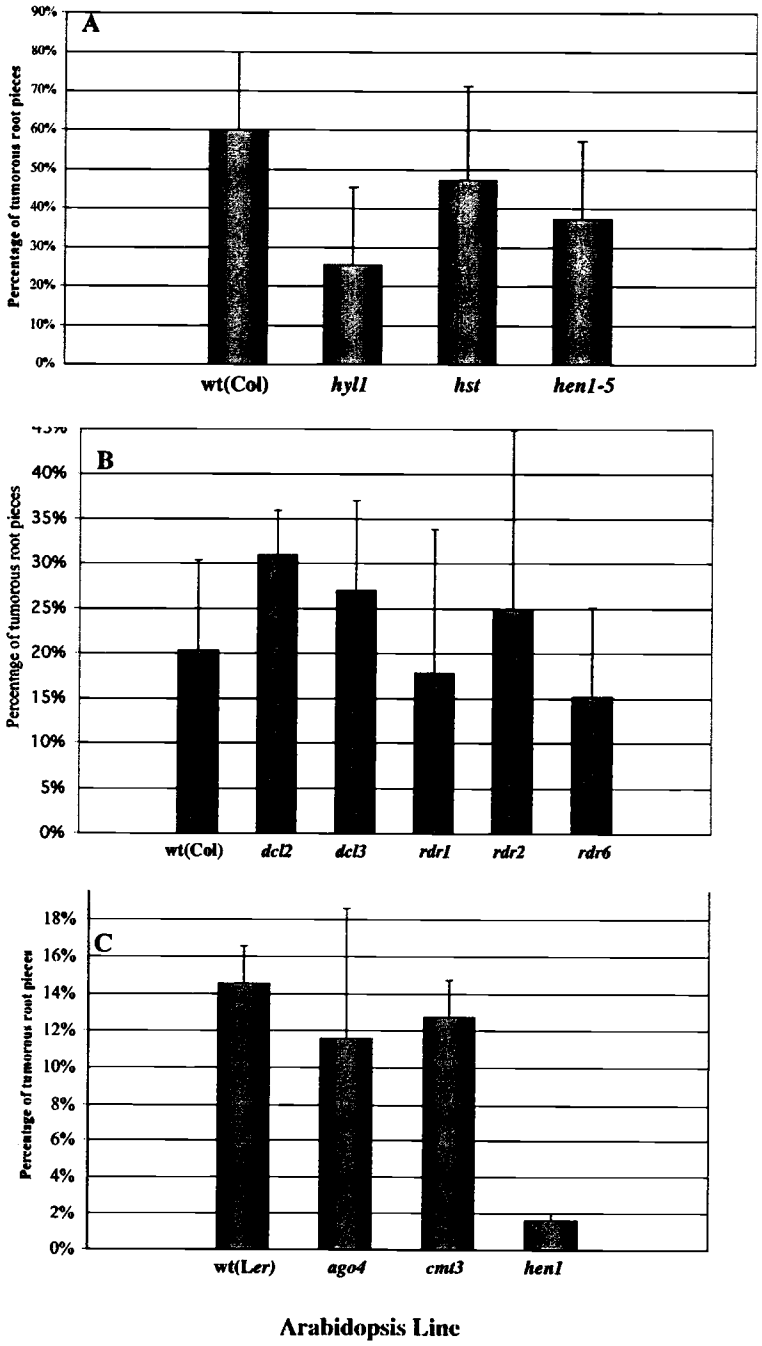


Figure 4.2 Root tumorigenesis on *A. thaliana* mutants. Analysis of variance (ANOVA) yielded p-value=0.2 for wt(Col), *hyl*, *hst*, and *hen1-5* (panel **A**); p-value=0.6 for wt(Col), *dcl2, dcl3, rdr1, rdr2,* and *rdr6* (panel **B**); ANOVA and subsequent paired comparisons to wt(L*er*) yielded the following p-values: 0.3(*ago4)*, 0.5(*cmt3*), <0.01(*hen1-1*) (panel **C**). Error bars indicate 95% confidence intervals.

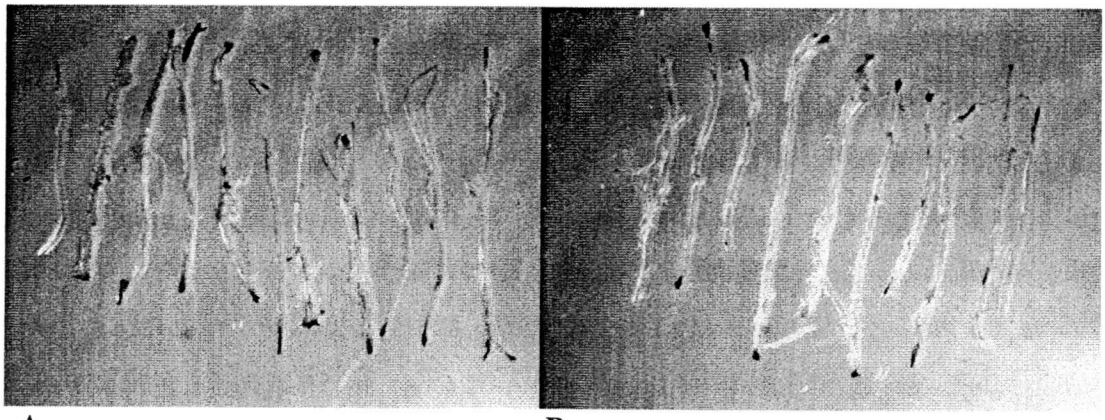


Figure 4.3 Transient GUS expression in *A. thaliana* roots. *A. thaliana* roots were inoculated with *A. tumefaciens* strain, EHA105(pBISN1), incubated on hormone-free medium for 4-6 days, then scored for GUS expression (percentage of pieces staining blue). Dark spots at cut ends of root pieces are blue. **A.** Wild-type L*er* roots. **B.** *hen1-1* roots.

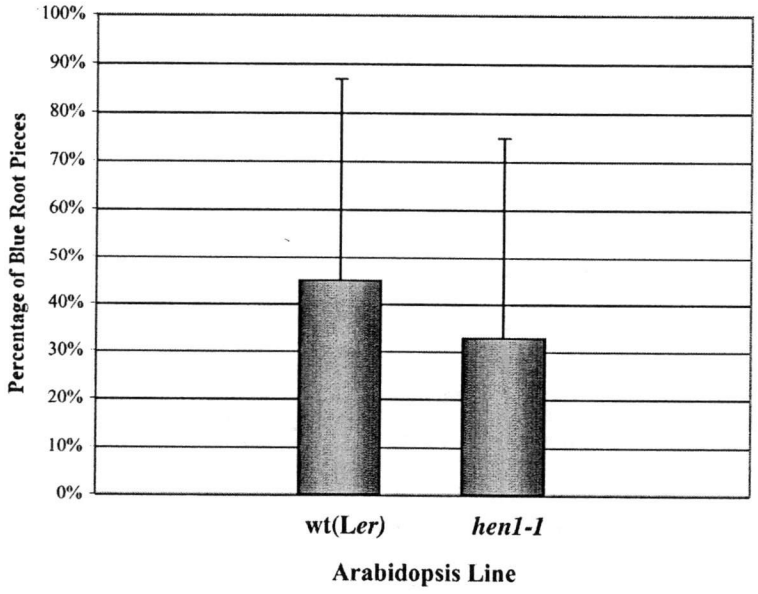


Figure 4.4 Transient GUS expression in *A. thaliana* wild-type and *hen1-1*. Graph shows the percentage of root pieces staining blue. A Student's T-test for no difference yielded p-value=0.73.

RNA silencing pathways in plants regulate endogenous gene expression and protect against expression of potentially harmful genetic elements such as transposons, viruses, and transgenes (Baulcombe, 2004; Lippman and Martienssen, 2004; Tomari and Zamore, 2005). *Agrobacterium* T-DNA is a foreign and harmful genetic element in plant cells, so it is reasonable that T-DNA sequences would be RNAi targets. Thus, an absence of the "protective" RNAi components may increase T-DNA-induced tumorigenesis. To test this, I challenged roots of seven RNAi-mutant *A. thaliana* with tumorigenic *A. tumefaciens* and compared their rates of tumorigenesis to that of wild-type *A. thaliana* roots. Tumorigenesis requires T -DNA transmission and expression as well as host response to auxin. None of these lines (*dcl2-1, dcl3-1, rdr1-1, rdr2-1, rdr6-15, cmt3-7, ago4-1*) demonstrated a significant increase in tumorigenesis compared to wild-type (Figure 4.2B,C). Instead, the *rdr6-15, ago4-1,* and *cmt3-7* lines developed slightly fewer (79-90% of wild-type) tumors than wild-type *Arabidopsis*. These results may be due to redundancy of the RNAi genes. *Arabidopsis* contains three RDR proteins (RDR1, RDR2, and RDR6), four DCL proteins (DCL1, DCL2, DCL3, DCL4), at least three AGO proteins (AGO1, AGO4, AGO7), and several histone and DNA methylase proteins (see Table 4.1 and references therein). Perhaps T-DNA silencing in wild-type plants occurs by a combination of RNAi pathways, and a difference in tumorigenesis would be seen in *Arabidopsis* lines with multiple RNAi mutations. However, this is unlikely because single mutations in RNA silencing genes have relieved silencing of transgene-containing T-DNAs. Mutations in *Arabidopsis RD6* and *SGS2* reduced silencing and methylation of a *uidA* transgene (Butaye et al., 2004) and *Arabidopsis rdr2, dcl3, sde4,* and *ago4* lines reduced silencing and de novo methylation of the *FWA* transgene (Chan et al., 2004). Alternatively, RNAi may not target wild-type T-DNA sequences. However, this is unlikely due to evidence that wild-type T-DNAs are silenced (Amasino et al., 1984; Escobar et al., 2001; John and Amasino, 1989; Lee et al., 2003; Viss et al., 2003). Finally, the similar tumorigenesis rates of the RNAi mutant lines compared to wild-type may be due to the presence of a silencing suppressor encoded

in the T-DNA. Many plant viruses encode silencing suppressors that inhibit RNAi at specific steps (Voinnet, 2005). Although the functions of most T-DNA genes have been determined (Zhu et al., 2000), they have not been tested specifically for suppression of silencing.

To assess whether the regulatory RNAi (miRNA and ta-siRNA) pathways play a role in *Agrobacterium*-induced tumorigenesis, I challenged three *Arabidopsis* lines (*hst-15, hyl1-2,* and *hen1-1*) with virulent *A. tumefaciens*. The *Arabidopsis* line *hen1-1* demonstrated a significant reduction in tumorigenesis compared to wild type (11% of wild type) (Figures 4.1 and 4.2C). Tumorigenesis requires T-DNA transfer, nuclear import, integration, stable oncogene expression, and host response to oncogene expression. The reduced tumorigenesis rate of *hen1-1* could be due to an alteration of any of these processes. However, non-stable (transient) T-DNA gene expression can occur from nuclear-localized T-DNA prior to integration into the plant genome (Janssen and Gardner, 1990; Nam et al., 1997; Narasimhulu et al., 1996a; Narasimhulu et al., 1996b). To assess T-DNA transfer and nuclear import in wild-type and *hen1-1 Arabidopsis*, I infected roots with avirulent *A. tumefaciens* EHA105(pBISN1) (containing the *uidA*-reporter T-DNA) and measured transient GUS expression 4-6 days post-inoculation (Janssen and Gardner, 1990; Narasimhulu et al., 1996a). There was no difference in GUS expression between the two lines, indicating that T-DNA transfer and nuclear uptake are not affected in *hen1-1* (Figures 4.3 and 4.4).

The different rate of tumorigenesis of the *hen1-1* mutant compared to *hen1-5* may be due to several factors. First, *hen1-1* is in the L*er* ecotype, which is less susceptible to A. *tumefaciens*, compared to the Col ecotype (Nam et al., 1997). Perhaps the 37% reduction in tumorigenesis of the *hen1-5* line compared to wild-type (Figure 4.2) is exacerbated in the L*er* background. This may also explain why the Columbia ecotype *A. thaliana* lines with mutations in *HYL1* and *HST*, genes which are also involved in miRNA biogenesis, do not demonstrate as pronounced a reduction in tumorigenesis

compared to the *hen1-1* line. Alternatively, the two *hen1* alleles may exhibit different residual or altered HEN1 function. The *hen1-1* allele contains a premature stop codon in the C-terminal domain coding sequence of *HEN1* (Chen et al., 2002). This domain is conserved among bacterial, fungal, and metazoan proteins and contains an *S*-adenosyl methionine (SAM)-binding motif (Park et al., 2002; Yu et al., 2005). Purified HEN1 protein demonstrated methylation of miRNA duplexes *in vitro*, whereas HEN1 containing an engineered mutation in the SAM-binding motif did not demonstrate methylase activity (Yu et al., 2005). Likewise, miRNAs isolated from wild-type *A. thaliana* were methylated, but those from *hen1-1* lines were not methylated (Yu et al., 2005). miRNAs from *hen1-1* and *hen1-5* lines accumulate to lower levels or demonstrate shifted electrophoretic mobility, but whether methylation of miRNAs is altered in the *hen1-5* line is not known (Chen et al., 2002; Park et al., 2002; Xie et al., 2004; Yu et al., 2005). The *hen1-5* allele contains a T-DNA insertion in the fifth intron of *HEN1* (SALK_049197) (Alonso et al., 2003). Perhaps this mutation is less disruptive to HEN1 function than is the *hen1-1* mutation. Both *A. thaliana* mutants, *hen1-1* and *hen1-5*, exhibit developmental abnormalities, such as narrowing or curling leaves, late flowering, short stature, and reduced fertility (Park et al., 2002; Vazquez et al., 2004a). In this work, the *hen1-1* line exhibited a much more compact stature and thicker less-pliable roots when grown in tissue culture for approximately 21 days compared to the *hen1-5* line. Although these alleles are in different genetic backgrounds (L*er* and Col, respectively), the *hen1-1* line was readily distinguishable from wild-type L*er*, Col, and the *hen1-5* plants. L*er* and Col wild-type plants were not readily distinguishable from each other. This indicates that the *hen1-1* allele may be more defective than the *hen1-5* allele.

It remains to be determined whether T-DNA integration and subsequent expression are altered in *hen1-1*. This can be accomplished by examining *uidA* expression or kanamycin resistance in roots 4 weeks post-inoculation with EHA105(pBISN1) (Nam et al., 1997; Narasimhulu et al., 1996a). If *hen1-1* exhibits reduced stable transgene

expression in these assays, then it is likely that reduced *hen1-1* tumorigenesis is due to a lack of stable T-DNA integration or expression. If, on the other hand, *hen1-1* exhibits stable transgene expression similar to that in wild-type *Arabidopsis*, then the difference in tumorigenesis reported here is probably due to differential response to the T-DNA oncogene products, IaaM and IaaH, which produce auxin. This seems plausible because several mi-RNAs and ta-siRNAs are complementary to *ARF* genes (Allen et al., 2005; Dugas and Bartel, 2004; Kasschau et al., 2003; Vazquez et al., 2004a) and a mutation in the miRNA-pathway gene *HYL1* alters *Arabidopsis* response to auxin (Lu and Fedoroff, 2000). MiRNA160 regulates *ARF10*, *ARF16*, and *ARF17* (Mallory et al., 2005; Rhoades et al., 2002). MiRNA167 is complementary to *ARF6* and *ARF8* (Bartel and Bartel, 2003; Rhoades et al., 2002) and TAS3 is complementary to *ARF1, ARF2, ARF3,* and *ARF4* (Allen et al., 2005). There are 23 *ARF* genes in *Arabidopsis*. ARF proteins either activate or repress transcription of target genes (Hagen and Guilfoyle, 2002) and mutations or overexpression of *ARF* genes in *Arabidopsis* results in patterning and developmental abnormalities similar to those of miRNA pathway mutants (Boutet et al., 2003; Han et al., 2004; Hardtke et al., 2004; Kasschau et al., 2003; Mallory et al., 2005; Park et al., 2002; Reinhart et al., 2002; Tian et al., 2004; Vaucheret et al., 2004; Vazquez et al., 2004a). ARFs can interact and modulate each other and some cooperate with transcriptional repressors, Aux/IAA proteins (Kim et al., 1997; Liscum and Reed, 2002; Rouse et al., 1998; Tiwari et al., 2004). Many Aux/IAA proteins are primary response genes of auxin (Theologis et al., 1985). The interaction between ARFs and Aux/IAA can result in negative feedback regulation of auxin. *A.thaliana* plants overexpressing *ARF8* had lower levels of free indole-acetic acid (IAA) in their hypocotyls compared to wild-type plants and exhibited phenotypes consistent with low auxin contents, such as suppression of lateral root formation and lower apical dominance (Tian et al., 2004). These observations are consistent with overexpression of several auxin-inducible genes that can adenylate IAA *in vitro* (Staswick et al., 2002), including GH3 proteins (Nakazawa et al., 2001; Takase et al., 2004; Takase et al., 2003), some of which are inducible by

ARF8 (Tian et al., 2004). Thus, overexpression of *ARF8* increases *GH3* expression leading to increased conjugation (and inactivation) of IAA. Likewise, *A. thaliana* containing a miRNA-resistant mutant *ARF17* gene demonstrated increased *GH3* family gene expression (Mallory et al., 2005). Perhaps the decreased *A. tumefaciens*-induced tumorigenesis observed in *Arabidopsis hen1-1* is due to similar negative feedback regulation induced by auxin overproduction. Depleted miRNA accumulation in *hen1-1* may increase mRNA levels and resultant expression of *ARF*s leading to depleted levels of auxin compared to wild-type plants in which miRNA accumulation is unaltered. To examine this hypothesis further, *Agrobacterium*-induced tumorigenesis should be assessed in plants with miRNA-resistant (or ta-siRNA-resistant) *ARFs* or those overexpressing miRNA/ta-siRNA-regulated *ARFs*.

Chapter 5

Conclusion

This work describes a practical application of RNAi to the problem of crown gall disease in apple trees (Chapter 2), the discovery that mRNA translatability affects its capacity to trigger RNAi (Chapter 3), and finally, that disruption of the miRNA-pathway gene, *HEN1*, reduces *Agrobacterium*-induced root tumorigenesis in *Arabidopsis* (Chapter 4). These findings have implications for efficient engineering of disease resistance via RNAi and may lead to better understanding of host factors involved in crown gall disease.

Crown gall disease is a persistent agronomic problem with losses of approximately $400,000 annually just in the Pacific Northwest (Pinkerton et al., 1996). Affected plants include fruit and nut trees, grapevines, chrysanthemum, rose, and other nursery crops (DeCleene and DeLey, 1976; Pinkerton et al., 1996). Control of this disease is particularly difficult due to the unique biology of the inciting organism, *Agrobacterium tumefaciens*. This ubiquitous soil-borne bacterium (Bouzar and Moore, 1987) genetically transforms wounded plant cells to rapidly grow in an unorganized fashion resulting in a gall, which reduces yield and compromises structural integrity of infected plants (Escobar and Dandekar, 2003; Winans, 1992; Zambryski, 1992). T-DNA transferred from the *Agrobacterium* Ti-plasmid integrates into the plant genome where genes for auxin and cytokinin overproduction are expressed (Escobar and Dandekar, 2003; Winans, 1992; Zambryski, 1992). The T-DNA-encoded genes *iaaM* (tryptophan monooxygenase) and *iaaH* (indole-3-acetamide hydrolase) are required for auxin production; *ipt* (AMP isopentenyl transferase) is required for cytokinin production (Garfinkel et al., 1981; Ream et al., 1983). Once plant cells are transformed, the bacterium need not be present for the disease to progress. Current control of crown gall disease includes identification and containment of *A. tumefaciens* infection, eradication of the bacterium by soil fumigation, control of plant-wounding insects and worms, and sterilization of horticulture equipment (Mullen and Hagan, 2005). These methods are not convenient or fail-safe. *Agrobacterium radiobacter* produces agrocin 84, a toxin that affects some strains of *A. tumefaciens* (Murphy and Roberts, 1979) and is used to control crown

gall disease (Jones et al., 1988; Kerr and Panagopoulos, 1977; Kerr and Tate, 1984; Moore, 1979, 1988; Shim et al., 1987). However, many *A. tumefaciens* strains are not susceptible to agrocin 84, so this control method is not adequate. Some disease-resistant crop varieties and cultivars have been identified (Beneddra et al., 1996; Bliss et al., 1999; Reynders-Aloisi and Pelloli, 1998; Sandor and Burr, 1995). But, plant resistance varies with the infecting strain of *Agrobacterium*, so crown gall remains a problem (Pinkerton et al., 1996).

Chapter 2 describes a strategy for control of crown gall disease based on RNAi or PTGS. Apple trees engineered to express double-stranded RNA (dsRNA) of two *Agrobacterium tumefaciens* oncogenes, *iaaM* and *ipt*, successfully prevented gall formation on *Agrobacterium*-infected roots. Transgene constructions were designed to produce sense and antisense sequences of *iaaM* and *ipt* to initiate RNA silencing of incoming wild-type *Agrobacterium* oncogenes. The transgene sequences, *iaaM*-stop and *ipt*-stop, contained premature stop codons relative to the first AUG and frameshift mutations to ensure that no functional oncogenic proteins would be made. These constructions successfully silenced the wild-type *iaaM* gene and prevented tumorigenesis on apple roots. However, they did not silence *ipt* even when both *iaaM*-stop and *ipt*-stop sequences were fused and under control of the same 5'- and 3'-elements (Figure 2.1). Fortunately, roots do not respond to cytokinin (the product of Ipt) (Ream et al., 1983), so *iaaM*-silencing is sufficient for disease resistance. But, the difference between *iaaM* and *ipt* silencing raised the question of why one sequence initiates RNA silencing, and the other does not.

When this question was presented to Dr. David Baulcombe. He suggested that the *iaaM*-stop transgene sequence might actually be translatable, perhaps from an AUG codon downstream from the early stop codons (Figure 3.1). Two studies demonstrated that translatable transgene sequences induce RNA silencing better than nontranslatable sequences (Que et al., 1997; Tanzer et al., 1997). Upon closer inspection of the *iaaM*-stop transgene sequence, I found two "start" codons (AUG) in good sequence context

for translation initiation (Joshi et al., 1997) and both were in-frame with the wild-type *iaaM* gene sequence (Figures 3.4 and 3.5). *Ipt*-stop did not contain alternative start codons. Deletion of 188 bp in the 5'-region of *iaaM*-stop removed the alternative start codons and abolished *iaaM* RNA silencing in transient assays (Figures. 3.1B and 3.3B). Likewise, single nucleotide changes of the two in-frame AUG codons (AUG to AUU) also abolished *iaaM* silencing (Figures 3.5 and 3.6). Therefore, untranslatable *iaaM* transgene sequences did not trigger RNAi. Likewise, the failure of *ipt*-stop to initiate RNAi is probably due to its untranslatability. In fact, Escobar et al. silenced the *ipt* oncogene with a translatable *ipt* sequence {Escobar, 2001 #232}. Also, studies in other organisms demonstrated that RNAi and mRNA translation are linked. In *Drosophila melanogaster*, oocyte mRNAs are subject to RNAi only during oocyte maturation when mRNAs are actively translated but not during the oocyte's arrested stage when mRNAs are present but not translated (Kennerdell et al., 2002). Also, in *Drosophila* and *Trypanosoma bucei*, siRNAs and RISC component proteins associate with translating ribosomes (Djikeng et al., 2003; Ishizuka et al., 2002; Pham et al., 2004). Finally, two studies demonstrate that RNAi and mRNA turnover are linked (Gazzani et al., 2004; Souret et al., 2004). The *Arabidopsis* AtXRN4 5'-3' exoribonuclease degrades micro-RNA-cleaved mRNAs (Souret et al., 2004) and can eliminate mRNA substrates for siRNA generation (Gazzani et al., 2004). As described in Chapter 3, the differences in *iaaM* silencing are likely due to differences in translatability of the *iaaM*-stop-derived sequences.

Chapter 4 addresses whether RNAi influences crown gall disease development. Eleven RNAi-mutant *A. thaliana* lines for root tumorigenesis in response to *A. tumefaciens*. Of these, only one (*hen1-1*) showed a significant difference (tenfold reduction) in tumorigenesis compared to wild-type *A. thaliana*. However, this same line demonstrated no difference in transient expression of a *gusA* reporter gene, indicating that the reduced tumorigenesis in *hen1-1* is not due to a deficiency in T-DNA transfer or nuclear import. This line contains a mutation in *HEN1*, the micro-RNA (miRNA) methylase gene involved in micro-RNA (miRNA) maturation (Park et

al., 2002; Yu et al., 2005). miRNAs are siRNAs that regulate endogenous genes, many of which are involved in plant development and encode transcription factors and hormone response factors (Allen et al., 2005; Dugas and Bartel, 2004). As described in Chapter 4, the difference in tumorigenesis between *hen1-1* and wild-type *Arabidopsis* may be due to differential response to T-DNA-encoded genes. However, first T-DNA integration and stable expression should be assessed in *hen1-1*. If T-DNA integration and stable expression are altered in *hen1-1*, then it is likely that reduced *Agrobacterium*-induced tumorigenesis is due to these factors. However, if integration and expression of T-DNA is not altered in *hen1-1*, then the altered tumorigenesis in this line may be due to altered response to T-DNA-directed auxin production, as tumorigenesis is assessed on cytokinin-resistant root tissue. Several miRNAs and ta-siRNAs are complementary to *ARF* genes (Allen et al., 2005; Dugas and Bartel, 2004; Kasschau et al., 2003; Vazquez et al., 2004a). *Arabidopsis* containing a mutation in the miRNA-pathway gene *HYL1* exhibits reduced sensitivity to auxin (Lu and Fedoroff, 2000). Inhibition of root growth in *hyl1* plants required exogenous indoleacetic acid (IAA) at concentrations fivefold higher than those required for wild-type root inhibition (Lu and Fedoroff, 2000). Likewise, in response to exogenous 2,4-dichlorophenoxyacetic acid (2,4-D), root elongation was reduced by 90% in wild-type plants compared to 50% for *hyl1* plants (Lu and Fedoroff, 2000). *ARF10*, *ARF16*, and *ARF17* are regulated by miRNA160 (Mallory et al., 2005; Rhoades et al., 2002). MiRNA167 is complementary to and likely regulates *ARF6* and *ARF8* (Bartel and Bartel, 2003; Rhoades et al., 2002) and TAS3 is complementary to *ARF1, ARF2, ARF3,* and *ARF4* (Allen et al., 2005). The 23 ARF proteins encoded in the *Arabidopsis* genome either activate or repress transcription of their target genes (Hagen and Guilfoyle, 2002). MiRNA pathway mutants exhibit patterning and developmental abnormalities similar to those of *Arabidopsis* lines with non-functional or overexpressing *ARF* genes, such as delayed flowering, curled leaves, and short stature (Boutet et al., 2003; Han et al., 2004; Hardtke et al., 2004; Kasschau et al., 2003; Mallory et al., 2005; Park et al., 2002; Reinhart et al., 2002; Tian et al., 2004; Vaucheret et al., 2004; Vazquez et al., 2004a). ARFs can interact and modulate each

other and some heterodimerize with Aux/IAA proteins which repress ARF transcriptional activity (Kim et al., 1997; Liscum and Reed, 2002; Rouse et al., 1998; Tiwari et al., 2004). Many Aux/IAA proteins are primary response genes of auxin (Theologis et al., 1985). ARF-Aux/IAA interaction can result in negative feedback regulation of plant auxin levels. Tian and colleagues showed that *A.thaliana* plants overexpressing *ARF8* had lower levels of free indole-acetic acid (IAA) in their hypocotyls compared to wild-type plants and exhibited phenotypes consistent with low auxin contents, such as suppression of lateral root formation and lower apical dominance (Tian et al., 2004). These observations are consistent with overexpression of auxin-inducible *GH3* family genes, *DFL1, DFL2,* and *YDK1* (Nakazawa et al., 2001; Takase et al., 2004; Takase et al., 2003). Most *GH3* family genes can adenylate IAA *in vitro* (Staswick et al., 2002). ARF8 induces expression of *GH3* family genes *DFL1, AtGH3a,* and At1g28130 (Tian et al., 2004). Thus, overexpression of *ARF8* increases *GH3* expression leading to increased conjugation (and inactivation) of IAA. Also, *A. thaliana* containing a miRNA-resistant mutant *ARF17* gene demonstrated increased expression of *GH3* family genes (Mallory et al., 2005). Perhaps the decreased *A. tumefaciens*-induced tumorigenesis observed in *Arabidopsis hen1-1* is due to similar negative feedback regulation induced by auxin overproduction. Depleted miRNA accumulation in *hen1-1* may increase mRNA levels and resultant expression of *ARF*s leading to depleted levels of auxin compared to wild-type plants in which miRNA accumulation is unaltered. To examine this hypothesis further, *Agrobacterium*-induced tumorigenesis should be assessed in plants with miRNA-resistant (or ta-siRNA-resistant) *ARFs* or those overexpressing miRNA/ta-siRNA-regulated *ARFs*.

In summary, this work explains a practical application of RNAi to the problem of crown gall disease in apple, the discovery that mRNA translatability affects its capacity to trigger RNAi, and finally, that disruption of the miRNA-pathway gene, *HEN1*, reduces *Agrobacterium*-induced root tumorigenesis in *Arabidopsis*. These findings can direct efficient engineering of crown gall disease resistance via RNAi in

susceptible crop species. The reduced tumorigenesis in *hen1-1* raises the possibility that crown gall disease resistance can be achieved by alteration of host genes.

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Table A.1 Tumor foci per disk (summarized in Figure 3.3A)

Replicate	1		2		3	
Strain tested*	EHA101 (pJP17)	EHA101 (pJP21)	EHA101 (pJP17)	EHA101 (pJP21)	EHA101 (pJP17)	EHA101 (pJP21)
Number of tumor foci per potato disk	1	1	4	10	0	0
	4	6	4	11	0	2
	4	7	4	12	1	2
	5	12	5	16	1	3
	5	15	6	16	1	4
	5	15	6	16	2	4
	6	20	7	17	2	11
	6	20	7	18	3	11
	7	22	9	18	3	14
	8	22	9	18	4	25
	8	22	10	24	4	26
	10	23	10	27	5	27
	11	25	11	29	5	29
	11	27	11	30	5	31
	11	27	11	31	7	35
	11	28	12	32	7	49
	11	28	16	32	8	50
	11	28	16	35	12	56
	12	30	16	36	28	80
	12	30	21	36		
	15	33	21	46		
	15	35				
	16	36				
	27	41				
		45				
		52				

* Strain indicated was mixed in a 4:1 ratio with wild-type *A. tumefaciens* A348.

Table A.2 Tumor foci per disk (summarized in Figure 3.3B)

Replicate	1			2		
Strain tested*	EHA101 (pJP17)	EHA101 (pJP21)	EHA101 (pJC14)	EHA101 (pJP17)	EHA101 (pJP21)	EHA101 (pJC14)
Number of tumor foci per potato disk	0	4	4	1	3	7
	0	4	5	1	4	9
	0	7	5	1	6	10
	0	10	6	3	7	10
	1	10	6	3	9	11
	1	11	7	3	11	13
	1	12	12	4	12	18
	2	13	13	4	13	18
	3	16	14	5	13	19
	3	16	14	5	14	22
	3	16	14	6	14	25
	3	17	15	6	14	26
	4	17	15	6	16	30
	4	18	15	8	16	30
	5	23	15	11	17	32
	5	23	16	11	17	35
	5	24	17	12	20	37
	5	29	20	15	22	42
	5	37	20	16	24	
	7		21		25	
	8		21		28	
			22		37	
			22			
			23			
			23			
			25			
			26			
			26			
			26			
			29			
			30			
			30			
			30			
			31			
			31			
			32			
			32			
			38			

* Strain indicated was mixed in a 4:1 ratio with wild-type *A. tumefaciens* A348.

Table A.3 Tumor foci per disk (summarized in Figure 3.6)

Replicate	1						2						3					
Strain tested*	17	21	27	25	30	33	17	21	27	25	30	33	17	21	27	25	30	33
Number of tumor foci per potato disk	0	1	1	0	1	1	0	0	5	0	0	0	0	3	1	3	0	1
	1	2	1	0	2	2	0	1	7	1	4	3	0	3	1	3	1	4
	1	3	2	1	2	2	1	6	9	6	4	3	1	4	2	4	1	4
	3	3	3	1	3	4	1	6	9	6	6	3	1	4	3	4	2	5
	3	3	3	2	3	4	1	6	10	6	6	4	1	4	4	4	3	5
	3	4	5	4	3	5	3	6	13	6	8	4	1	6	4	6	4	6
	3	4	5	4	3	8	4	7	13	7	12	5	1	6	4	6	4	6
	4	6	5	6	3	8	4	7	14	7	15	6	1	6	6	6	5	6
	4	6	6	7	6	9	5	8	15	8	15	7	1	8	7	8	5	6
	4	7	7	8	6	9	5	9	15	9	18	9	2	8	8	8	5	6
	4	11	8	8	7	9	6	18	17	18	19	10	2	10	10	10	7	7
	4	11	10	9	11	11	6	18	22	18	21	10	2	11	10	11	9	13
	4	11	15	11	13	11	6	21	26	21	21	18	2	11	12	11	13	18
	6	11	16	14	16	13	7	23	26	23	28	23	4		22			21
	6	19	18	14		13	13	23	27	23	30	30	4		26			25

* Strains are as follows: 17 - EHA101(pJP17); 21 - EHA101(pJP21); 27 - EHA101(pJP27); 25 – EHA101(pJP25); 30 – EHA101(pJP30); 33 – EHA101(pJP33). Each strain was mixed in a 4:1 ratio with *A. tumefaciens* A348.