AN ABSTRACT OF THE THESIS OF

Hyewon Lee for the degree of Master of Science in Microbiology presented on April 22, 1999. Title: Silencing of *Agrobacterium tumefaciens* T-DNA oncogenes by Cosuppression.

Abstract approved: Redacted for Privacy

Walter Ream

We have developed crown-gall resistant transgenic plants capable of suppressing *Agrobacterium tumefaciens* T-DNA oncogenes. Crown gall tumors result from overproduction of auxin and cytokinin in plant cells transformed by *A. tumefaciens*. High phytohormone levels result from expression of two auxin biosynthetic genes, tryptophan monooxygenase (*iaaM*) and indole acetamide hydrolase (*iaaH*), and isopentenyl transferase (*ipt*), which mediates cytokinin synthesis. Inactivation of *ipt* and either one of the two auxin biosynthesis genes prevents crown gall formation. To suppress T-DNA oncogene expression, we created transgenic tobacco that produce the corresponding untranslatable sense-strand RNAs. This phenomenon, called cosuppression, frequently blocks expression of transgenes in plants. Often, expression of an untranslatable sense-strand transgene elicits sequence-specific destruction of both the mutant mRNA and the corresponding wild-type mRNA.

Here we show that cosuppression can block expression of *A. tumefaciens* T-DNA oncogenes, resulting in plants that are resistant to gall induction by certain strains of *A. tumefaciens*.

Master of Science thesis of Hyewon Lee presented on April 22, 1999.

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Silencing of *Agrobacterium tumefaciens* T-DNA oncogenes by Cosuppression

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To my parents,
Man Hyung Lee and Jong Sook Bae

Silencing of *Agrobacterium tumefaciens* T-DNA oncogenes by Cosuppression

Chapter 1. LITERATURE REVIEW

Introduction

The genus *Agrobacterium* includes the species *A. tumefaciens*, which carries a Ti (tumor-inducing) plasmid and causes crown gall tumors, *A. rhizogenes*, which carries a Ri (root-inducing) plasmid and causes hairy root, and *A. radiobacter*, which is avirulent. *Agrobacterium tumefaciens* strains are commonly referred to by the type of modified amino acids, known as opines, that are produced by the infected plant tissue. The opines are a major carbon and nitrogen source for *A. tumefaciens*. The best studied are octopine- and nopaline-type Ti plasmids which share a highly homologous region of 9kb (called common DNA) covering all the oncogenes (Chilton et al., 1978; Depicker et al., 1978).

The *Agrobacterium*-plant interaction is the only known natural example of DNA transport between kingdoms (reviewed in Ream, 1989; Christie, 1997; Sheng and Citovsky, 1996). *A. tumefaciens* causes crown gall tumors in plants when a DNA segment called T-DNA (transferred DNA) is transferred from the extrachromosomal Ti plasmid in the bacterium to the plant genome (reviewed in Das, 1998; Kado, 1991; Zupan and Zambryski, 1995). Three genetic components -

T-DNA, virulence (*vir*) genes, and chromosomal virulence (*chv*) genes- are needed for tumor induction (Zambryski, 1992). The T-DNA, which contains oncogenes that encode enzymes for plant growth factor biosynthesis, becomes incorporated into the plant cell chromosomal DNA (Chilton et al., 1980; Willmitzer et al., 1980). The T-DNA does not encode proteins necessary for its own transfer but requires the T-DNA border repeats, which are imperfect repeats that delimit the T-DNA, for transmission (Leemans et al., 1982; Ream et al., 1983). The T-DNA and the *vir* region do not have to be on the same plasmid to cause tumor formation (Hoekema et al., 1983). This characteristic has allowed people to replace the oncogenes between the T-DNA borders with genes of interest, thus enabling people to introduce beneficial genes into the plant genome and facilitating the development of plant genetic engineering.

T-DNA Transfer Mediated by Agrobacterium

A. tumefaciens infection is a process in which the bacteria have to sense their surroundings and respond in an appropriate manner. Plant wounding is required for pathogenesis (Stachel et al., 1985; Stachel et al., 1986b). For transfer of the T-DNA to occur, the bacteria first have to attach to the plant cells at the wound site, which in nature is usually at the root-stem interface (crown of the plant). The bacteria first loosely bind to the plant cell surface, and then the bound bacteria synthesize cellulose filaments that stabilize the initial binding, resulting in a tight association between the bacteria and the plant cell (Binns and Thomashow, 1988; Hooykaas and Schilperoort, 1984; Matthysse, 1987).

Phenolic compounds secreted by the wounded plant stimulate the transcription of the virulence (*vir*) genes on the Ti plasmid (Winans, 1992). The *vir* region of the Ti plasmid encodes proteins that mediate processing and transfer of T-DNA (Stachel and Nester, 1986). The *vir* region is located adjacent to the left border repeat of the T-region (Hoekema et al., 1983). The *vir* genes are tightly regulated so that expression occurs only in the presence of wounded plant cells (Stachel et al., 1986b). Control of the *vir* gene expression is mediated by VirA and VirG proteins, which form a two-component regulatory system (Winans, 1992). VirA, the sensor protein, responds to signal molecules, such as acetosyringone and hydroxyacetosyringone (Stachel et al., 1985; Stachel et al., 1986b), released by wounded plant cells. VirA autophosphorylates and transmits the signal to the regulator protein, VirG (Miller et al., 1989; Winans et al., 1988; Winans, 1992). Phosporylated VirG functions as a transcriptional activator that induces the expression of all the *vir* genes (Miller et al., 1989; Winans et al., 1988; Winans, 1992). Also, the chromosomally encoded protein ChvE, a homolog of the *Escherichia coli* sugar-binding protein, interacts with VirA to activate transcription of the *vir* regulon (Huang et al., 1990; Kemner et al., 1997). Induction of the *vir* regulon initiates the T-DNA transport process.

Induction of *vir* gene expression results in the production of a single stranded T-DNA copy (Stachel et al., 1986a; Yusibov et al., 1994), called the T-strand, which is the intermediate in the transfer (Tinland et al., 1995; Yusibov et al., 1994). T-strand production occurs in a 5' to 3' direction, which is initiated at the

right border of the T-DNA and terminated at the left border (Shaw et al., 1984; Stachel et al., 1986a; Wang et al., 1984). Two proteins encoded by the *virD* operon, VirD1 and VirD2, are both required to produce an endonucleolytic cleavage between the third and fourth nucleotides in the bottom strand of the T-DNA borders (Albright et al., 1987; Jasper et al., 1994; Jayaswal et al., 1987; Stachel et al., 1986a; Wang et al., 1987). During cleavage, VirD2 covalently attaches to the 5' end of the T-strand at the right border nick (Herrera-Estrella et al., 1988; Howard et al., 1989; Pansegrau et al., 1993). The excised T-strand is displaced, perhaps by replacement strand synthesis (Albright et al., 1987; Stachel et al., 1986a).

The *virB* operon encodes 11 membrane-associated proteins that form a transport channel through which T-strand DNA is transferred into the cytoplasm of the plant cell (reviewed in Christie, 1997). Ten of the 11 VirB proteins are essential for tumor formation while VirB1 is not essential (Berger et al., 1994). VirB2 and VirB3 are involved in the assembly of a *vir*-dependent pilus (Jones et al., 1994; Jones et al., 1996; Lai and Kado, 1998). VirB2 is exported out of the *Agrobacterium* cell and forms the pilus (Lai and Kado, 1998). VirB4 and VirB11 are ATPases (Christie et al., 1989; Shirasu et al. 1994). VirB6, VirB7, VirB8, VirB9, and VirB10 form the transport pore (Finberg et al., 1995; Winans et al., 1996). VirD4, an essential virulence protein, also associates with the bacterial inner membrane and has significant homology with the TraG protein encoded by broad-host-range IncP plasmid RP4 (Farrand et al., 1996; Lin and Kado, 1993). TraG is thought to link the RP4 relaxosome (*oriT*-Mob complex) to the membrane-

associated transport channel (Lessl and Lanka, 1994). Similarly, VirD4 may form a link between the transported T-strand and the VirB channel.

T-DNA transfer to plants occurs by a mechanism analogous to bacterial conjugation (Lessl and Lanka, 1994; Stachel et al., 1986a; Stachel and Zambryski, 1986; Wang et al., 1984). The virulence system of *A. tumefaciens* appears related to the well-studied conjugative transport system of the plasmid RP4 (Lessl and Lanka, 1994). After the T-strand enters the plant cell, it integrates randomly (Thomashow et al., 1980; Yadav et al., 1980; Zambryski et al., 1982) in the plant nuclear genome (Chilton et al., 1980; Willmitzer et al., 1980), and expression of this DNA segment in the cell causes the transformed cells to grow as crown gall tumors.

The molecular mechanism by which the integration of the T-DNA occurs is still unknown. Recently VirD2 and a single-stranded (ss) DNA binding protein, VirE2, have been implicated in the integration process (Tinland et al., 1995; De Neve et al., 1997). VirD2 and VirE2 both have nuclear localization signals (NLS; reviewed in Silver, 1991). These proteins form the T-complex with the T-strand, which provides the T-strand with NLSs so that it is targeted into the plant nucleus (Citovsky et al., 1992; Howard et al., 1992; Koukolikova-Nicola et al., 1993; Shurvinton et al., 1992). A short amino acid sequence located downstream of the VirD2 NLS called the ω domain is thought to be required for efficient integration of T-DNA into the plant genome (Narasimhulu et al., 1996; Mysore et al., 1998; Shurvinton et al., 1992). VirE2 binds ssDNA without sequence specificity (Citovsky et al., 1989; Christie et al., 1988; Das, 1998). It is also transported into

the plant cell by *A. tumefaciens*. The single-stranded T-strand is protected against nucleases by the VirE2 protein (Citovsky et al., 1989, 1997; Sen et al., 1989). Sundberg et al. (1996) demonstrated that VirE1 is essential for the transfer of VirE2 into plant cells, but VirE1 is not required for transfer of T-strand DNA.

In addition to the gene products encoded by the Ti plasmid, chromosomally encoded gene products also participate in the infection of plants by *A. tumefaciens* strains. So far, 11 chromosomal genes necessary for tumorigenesis have been found. *chvA*, *chvB*, *pscA* (*exoC*) and *att* gene products are involved in attachment of *A. tumefaciens* to the plant cells at the wound site (Cangelosi et al., 1987; Douglas et al., 1982; Matthysse, 1987; Thomashow et al., 1987). *chvD*, *chvE*, *miaA*, and *ros* gene products provide regulation of *vir* expression in addition to the VirA/G system (Close et al., 1985; Gray et al., 1992; Huang et al., 1990; Kemner et al., 1997). *chvG* and *chvI* provide an additional two-component system that is required for virulence (Charles and Nester, 1993; Mantis and Winans, 1993). The inducing signal as well as the genes that are regulated by this system are not yet known (Charles and Nester, 1993). The *acvB* gene product is thought to bind to the T-complex in the periplasm to mediate its transfer into the cytoplasm of the plant cell (Wirawan et al., 1993; Wirawan and Kojima, 1996).

Transferred DNA (T-DNA)

T-DNA is flanked by two imperfect direct repeats designated T-DNA borders. The octopine Ti plasmid has two separated T regions, TL and TR, both of which are flanked by 24-base-pair (bp) imperfect repeats (Barker et al., 1983;

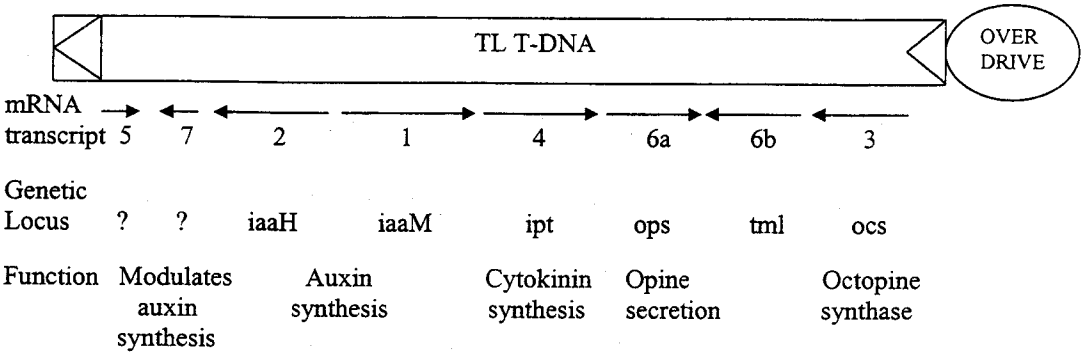


Figure 1.1. Genetic map of the TL T-DNA of an octopine type Ti plasmid. The arrows in the box represent the left and right borders of the T-DNA. *iaaH* (indoleacetamide hydrolase), *iaaM* (tryptophan monooxygenase), *ipt* (isopentenyl transferase), *ops* (opine secretion), *tml* (tumor morphology large), *ocs* (octopine synthase). (This picture was taken from Ream, 1989 and modified).

Gielen et al., 1984; Holster et al., 1983; Thomashow et al., 1980). The TL region harbors the oncogenes and the octopine synthase (*ocs*) gene (Figure 1.1) (Ooms et al., 1981). Deletion of this region results in the loss of tumorigenicity (Ooms et al., 1982). However, deletion of the TR region does not lead to avirulence, indicating that the TR region is not essential for tumor induction (Ooms et al., 1982; Thomashow et al., 1980). Studies on the border fragments show that deletion of the right border abolishes transfer, while manipulation of the left border has little effect (Jen and Chilton, 1986a; Miranda et al., 1992; Peralta et al., 1986; Peralta and Ream, 1985). Also reversing the orientation of the right border greatly reduces T-DNA transfer (Miranda et al., 1992). Thus, T-DNA transmission requires a right border, repeat which functions directionally.

T region genes have no effect on the efficiency of T-DNA transfer (Leemans et al., 1981). T-DNA transmission required only the border repeats (Caplan et al., 1985; Jen and Chilton, 1986b; Wang et al., 1984), but flanking sequences influenced transfer efficiency (Peralta and Ream, 1985). Peralta et al. (1986) found a specific 24 bp DNA sequence, designated *overdrive*, flanking the right border, which stimulated the activity of the right border repeat. *Overdrive* is essential for efficient octopine TL and TR T-DNA transfer (Peralta et al., 1986) and acts as an enhancer element because it stimulates transfer when placed upstream or downstream, and up to 6 kb from the border repeats (van Haaren et al., 1987). The VirC1 protein and *overdrive* likely interact, which may cause the *virC* operon to enhance tumorigenesis (Toro et al., 1989). *Overdrive* may also have a role in distinguishing the right and left T-DNA borders.

The T-DNA is directed out of *A. tumefaciens* through the VirB channel into the plant cell, where upon entry it becomes stably integrated into the plant DNA. T-DNA carries genes that direct the synthesis of unusual compounds called opines (Klapwijk et al., 1978). The infected plant cells are induced to synthesize opines even though the plant cannot utilize them. Instead, the opines are used as a nutrient source for *A. tumefaciens*, and specific opines induce conjugal transfer of the Ti plasmid (Petit et al., 1978; Veluthambi et al., 1989).

The T-DNA oncogenes cause neoplastic growth of plant cells due to overproduction of auxin and cytokinin in transformed cells (Budar et al., 1986; Meins, 1989; van Slogteren et al., 1984). Differential synthesis of auxin and/or

cytokinin determines tumor morphology. High levels of auxin cause rooty tumors, while high levels of cytokinin induce tumors with shoots (Akiyoshi et al., 1984; Ooms et al., 1981). Mutational analysis has shown that three oncogenes are of primary importance (Garfinkel and Nester, 1980; Garfinkel et al., 1981; Ooms et al., 1981). Two of these genes, *iaaM* (tryptophan monooxygenase) and *iaaH* (indoleacetamide hydrolase), code for enzymes that convert tryptophan to indoleacetic acid, an auxin (Inze et al., 1984; Schroder et al., 1984; Thomashow et al., 1984, 1986). The third gene, *ipt* (isopentenyl transferase), is involved in the biosynthesis of the cytokinin isopentenyl-AMP (Akiyoshi et al., 1984; Barry et al., 1984) (Figure 1.2).

Gene 5 modulates the activity of the growth factors produced by major oncogenes (Korber et al., 1991; Schell et al., 1993). It also catalyzes the synthesis of an auxin analogue, indole-3-lactate, which acts as an antagonist to auxin (Korber et al., 1991). Garfinkel et al. (1981) found that mutations in the region of T-DNA transcripts 6a or 6b led to the formation of tumors much larger than normal size on *Kalanchoe* stems. Hooykaas et al. (1988) have shown that the 6b gene causes the formation of small tumors on wild tobacco (*Nicotiana glauca*) and on *Kalanchoe tubiflora*. This gene is conserved among all T-DNAs examined. Its role is not well defined, but it is thought to modulate the activity of cytokinins and auxins in the tumors (Spanier et al., 1989; Tinland et al. 1990). The 6a (*ons*) gene is responsible for octopine and nopaline secretion (Messens et al., 1985).

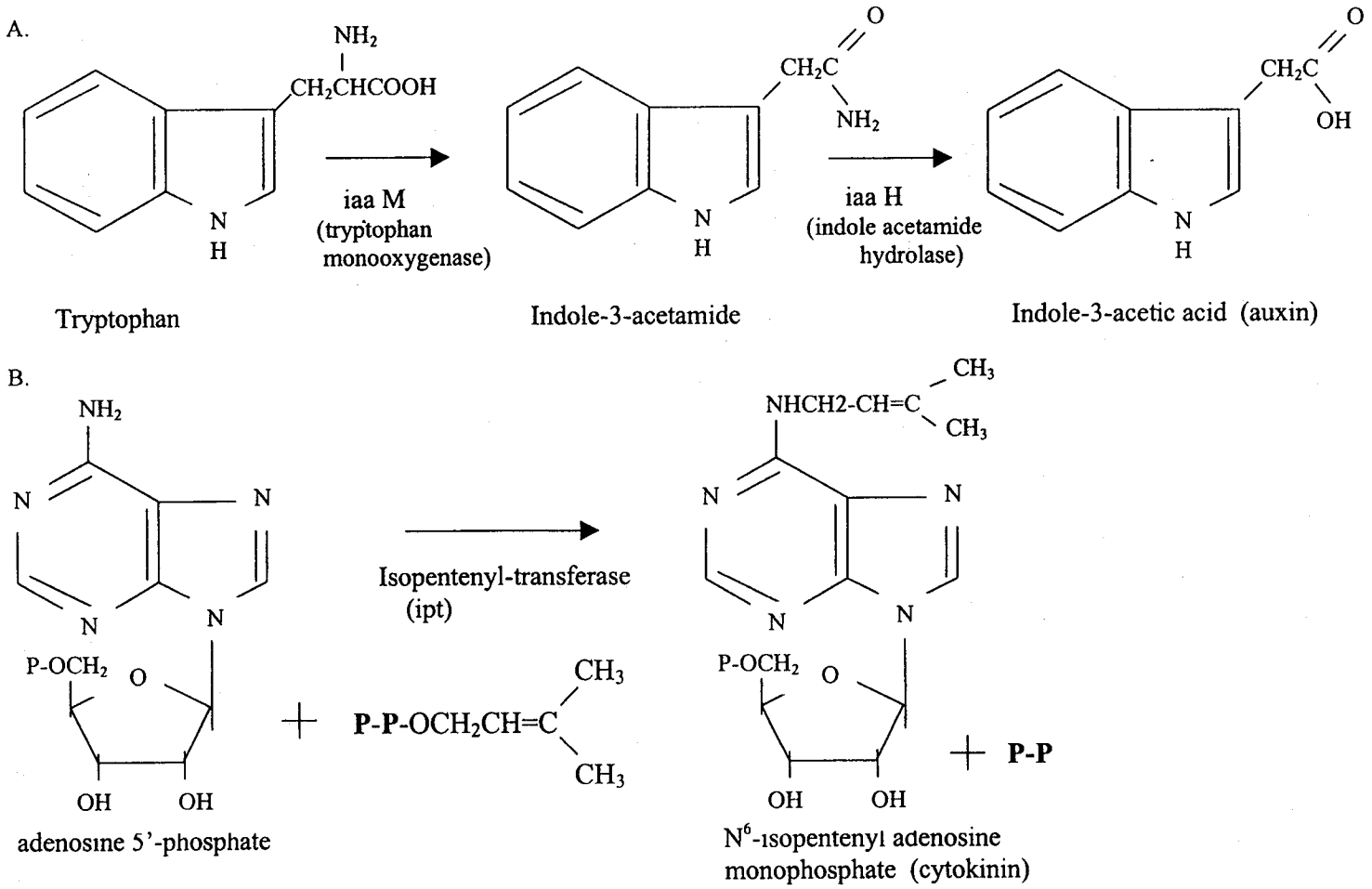


Figure 1.2. Plant hormone biosynthesis. A. Auxin pathway. B. Cytokinin pathway.

Homology-Dependent Gene Silencing

Transgenes introduced into plants can suppress the expression of homologous endogenous genes or transgenes already present in the plant genome, a phenomenon called gene silencing or co-suppression (Flavell, 1994; Matzke and Matzke, 1995; Meyer and Saedler, 1996; Stam et al., 1997). The discovery of co-suppression came about when transgenes downstream from strong promoters were not expressed uniformly in plants. Presently, gene silencing represents one of the most puzzling and intriguing phenomena in transgenic plants. Gene silencing was initially perceived as an unwanted response in plant genetic engineering and is a major impediment in the application of plant biotechnology. Understanding the mechanisms underlying this phenomenon is important because they could represent genetic controls involved in plant growth, developmental regulation and responses to environmental factors. Also, a deeper understanding of the ways that plants modify gene expression of homologous genes as a means to control excess production of RNA or proteins may help in eliminating unwanted gene expression and in improving plant biotechnology (reviewed in Senior, 1998).

Inhibition of gene expression is induced at two different levels, transcriptional and post-transcriptional. Transcriptional inactivation occurs when the transgene shares homology with the promoter of the silenced gene and has been associated with increased promoter methylation (Hobbs et al., 1990; Matzke and Matzke, 1995; Park et al., 1996). In contrast, silencing at the post-transcriptional level occurs when there is homology in the transcribed regions of the genes and

involves RNA turnover (Baulcombe, 1996; Depicker and Van Montagu, 1997). Gene silencing seems to be a general phenomenon in transgenic plants. It has been observed in several plant species, such as petunia, tomato, *Arabidopsis thaliana*, and tobacco that have been transformed with a variety of different chimeric genes (reviewed in Flavell, 1994). Gene silencing has also been observed in fungi, yeast, and animals (reviewed in Bingham, 1997; Cogoni et al., 1996; Garrick et al., 1998; Montgomery and Fire, 1998; Pal-Bhadra et al., 1997).

Various mechanisms have been suggested to explain the silencing phenomenon. However, the precise mechanism(s) of gene silencing is still not well understood. Some of the mechanisms thought to be involved are DNA methylation (Malagnac et al., 1997), the establishment of stably repressed chromatin (Pal-Bhadra et al., 1997), or RNA-mediated RNA degradation (Cogoni and Macino, 1997; Metzlaff et al., 1997). Methylation is known to regulate gene activity in prokaryotes and eukaryotes. In several studies, transgene silencing was associated with DNA methylation (Hobbs et al., 1993; Ingelbrecht et al., 1994; Matzke et al., 1994a). DNA methylation might silence genes by changing the structure of DNA, which influences the nucleic acid interactions, or it could interfere with the binding of transcription factors or enhance the binding of repressors (reviewed in Jost and Bruhat, 1997; Kass et al., 1997). Matzke and Matzke (1995) have proposed that a DNA-DNA interaction, such as pairing of homologous DNA sequences, can also function in plants as a signal for *de novo* methylation. RNA-directed methylation of transgene sequence has also been shown by Wassenegger et al. (1994). Jones et al.

(1998) looked at the alteration in the methylation status of a transgene following virus-induced post-transcriptional gene silencing. They found that *de novo* methylation occurred only in plants that silence the transgene and only after virus infection. Methylation was restricted to sequences homologous to the virus. So, methylation may contribute to both transcriptional and post-transcriptional silencing. However, gene silencing was induced in cytosine methylation defective *Neurospora crassa* strains (Cogoni et al., 1996). Therefore, methylation may not be essential for gene silencing.

Several examples of transgene silencing are considered to be similar to paramutation, which involves an allelic interaction that causes meiotically heritable alterations in gene expression (Hollick et al., 1997; Martienssen, 1996). Ectopic pairing (physical recognition) between the homologous loci has been suggested for silencing-related phenomena in other organisms, for example transvection in *Drosophila melangaster* (Pirrotta et al., 1990) or repeat-induced mutation in fungi (Selker, 1990; Rossignol and Faugeron, 1994). In plants, ectopic pairing of DNA sequences has been proposed as a basis for allelic interactions between endogenous genes (Coen and Carpenter, 1988), repeated transgenes (Assaad et al., 1993), allelic transgenes (Meyer et al., 1993), as well as non-allelic transgenes (Jorgensen, 1990). Van Blokland et al. (1994) reported that a promoterless transgene can cause co-suppression. This suggested DNA-DNA pairing between transgene and host genes or transgenes and transgenes. It was proposed that the level of the transgene expression may not be important for inducing co-suppression (Van Blokland et al.,

1994). It is likely that several parameters determine the probability of interaction between different loci and therefore influence the differences in the kinetics of inactivation and restoration of gene expression. One factor will be the accessibility of the loci for pairing, which is influenced by the steric organization of the chromosomal DNA. For example, an open chromatin structure may facilitate interaction between allelic or ectopic gene pairs that triggers silencing or provide access for cellular proteins involved in DNA methylation or heterochromatinization (Meyer, 1995). Also, the relative positions of the inserts in the genome may be important (Meyer, 1995). The structure of the transgenic loci appears to play a role in determining whether a given locus can interact with another (English and Jones, 1998). Transgene inactivation is favored by multiple inserts at a given locus (Linn et al. 1990; Scheid et al., 1991; Hobbs et al., 1993; Ingelbrecht et al., 1994; Matzke et al., 1994c) and by a repeated insert structure (Assaad et al., 1993; Meyer, 1996). However, such a structure is not an obligatory prerequisite for inactivation, since single copy transgene silencing has also been observed (Elmayan and Vaucheret, 1996; Que et al., 1997).

Antisense suppression is a natural system used in bacteria to control gene expression (reviewed in Wagner and Simons, 1994). Antisense RNAs are also known to downregulate the expression of eukaryotic genes (reviewed in Vanhee-Brossollet and Vaquero, 1998). Gene silencing may result from antisense suppression. Antisense RNA may hybridize with the target mRNA to form double-stranded (ds) RNA intermediates that are rapidly eliminated from the cell due to

degradation by RNAses specific for dsRNA, or dsRNA may inhibit RNA processing and transport (Nellen and Lichtenstein, 1993). Fire et al. (1998) have shown that dsRNA-mediated interference in *Caenorhabditis elegans* eliminates the endogenous mRNA transcript and is also sequence-specific. Alternatively, silencing could be due to the accumulation of RNA that may trigger an unknown process of RNA degradation that downregulates expression of specific genes (Dehio and Schell, 1994; Dougherty and Parks, 1995; Elmayan and Vaucheret, 1996). The RNA turnover threshold may be defined by both qualitative and quantitative aspects of the RNA transcripts (Dougherty et al., 1994). The autoregulation theory postulates that the inhibition of transcription and/or translation occurs by feedback from a RNA or protein that accumulates in aberrantly high concentrations in the transgenic plants (Meins and Kunz, 1995; Metzlaff et al., 1997). Silencing in transgenic plants may be mediated by a cellular pathway involved in the targeted elimination of aberrant RNAs (Dougherty and Parks, 1995; Metzlaff et al., 1997). Plant cells contain RNA-directed RNA polymerase (RdRp) activity (Schiebel et al., 1993a). This RdRp will randomly copy any RNA (Schiebel et al., 1993b; Schiebel et al., 1998) and make small complementary RNAs (10-75 nucleotides). These small RNAs may bind to a target and determine which RNA is eliminated.

However, none of these models have been able to explain the variety of examples where loss of gene expression has occurred. Thus, several different mechanisms are probably involved in gene silencing. Moreover, the different

mechanisms could be related at the most fundamental level. A recent review implies that transcriptional gene silencing and post-transcriptional gene silencing could be mediated by the same RNA-based mechanism (Wassenegger and Pelissier, 1998).

This suppression of gene activity is not homogenous throughout the plant. Palauqui et al. (1997) has shown by grafting experiments that silencing is transmitted throughout the plant. They suggest that a non-metabolic, transgene-specific, diffusable messenger mediates the propagation of *de novo* post-transcriptional silencing throughout the plant. Other studies of gene silencing point to the existence of a gene-specific, mobile signal molecule that transmits the co-suppressed state through the plant's transport system (Jorgensen et al., 1998; Smyth, 1997; Voinnet and Baulcombe, 1997). However, the identity of the transmitting signal is still unknown. A likely candidate for this mobile signal molecule is an RNA molecule derived from the suppressed gene. RNA molecules such as degraded transcripts (Metzlaff et al., 1997), malformed transcripts (Baulcombe and English, 1996), or complementary RNA molecules synthesized from sense transcripts by RNA-directed RNA polymerases (Lindbo et al., 1993; Dougherty and Parks, 1995) may be the signal molecules that transmit co-suppression. Identification of this signal will be crucial in deciphering the mechanism(s) underlying the gene silencing phenomenon.

Recent studies imply that this gene silencing phenomenon is a protection mechanism against viruses (Al-Kaff et al., 1998; Covey et al., 1997; English et al.,

1996; Lindbo et al., 1993; Mueller et al., 1995; Ratcliff et al. 1997; Sijen et al., 1996; Smith et al., 1994). Resistance is thought to occur through a sequence specific inactivation mechanism in the plant, which is similar to co-suppression that interferes with normal production of viral RNA. As a result, the plant overcomes the viral infection and recovers (Lindbo et al., 1993). Normal plants inoculated with DNA or RNA viruses were able to overcome virus infection by RNA turnover, demonstrating the similarity between this natural virus-mediated effect and transgene-induced gene silencing (Covey et al., 1997; Ratcliff et al., 1997). RNA viruses carrying sequences homologous to a transgene in the plant can be both targets and triggers of co-suppression. When plants contain a transgenic copy of a gene from the same virus, transcripts of the transgene can interfere with normal production of viral RNA as well as reduce the level of RNA produced from the transgene (Baulcombe, 1996; English et al., 1997). Plants seem to have a sequence-specific mechanism for recovery from viral infection that allows the plant to resist infection (Covey et al., 1997; Waterhouse et al., 1998). This systemic acquired silencing may allow the plant to identify, track, and destroy viral RNA in a sequence specific manner. The genes governing these silencing mechanisms in plants are still unknown. Recently, Elmayan et al. (1998) have isolated *Arabidopsis thaliana* mutants carrying a recessive monogenic mutation that define two genetic loci called *sgs* (suppressor of gene silencing). These *sgs* mutants are impaired in triggering post-transcriptional silencing. The characterization of these mutants may

provide further insight into the silencing mechanism, such as defining which and how many components are involved in silencing.

Chapter 2. GENE SILENCING OF T-DNA ONCOGENES

Introduction

Sanford and Johnston (1985) proposed a theory of pathogen-derived resistance which predicts that host resistance to a particular pathogen would be best derived from a pathogen's own genetic material. Gene products from the pathogen, if present in a dysfunctional form, in excess, or at the wrong developmental stage, might disrupt a certain pathogen-encoded cellular function essential to the pathogen but not to the host. Therefore, resistance to a particular pathogen could be achieved by introducing the appropriate pathogen gene into the host genome.

Virus resistance in transgenic plants was found to be mediated by an RNA-based mechanism that is related to post-transcriptional gene silencing in transgenic plants (English et al., 1996; Lindbo and Dougherty, 1992a, 1992b; Marano and Baulcombe, 1998; Mueller et al., 1995; Smith et al., 1994). Transgenic plants expressing viral genes display various levels of protection against virus infection. Lindbo and Dougherty (1992b) have shown that transgenic plants expressing untranslatable sense transcript of the tobacco etch virus (TEV) coat protein were highly resistant to TEV when the plants were infected with the virus. If indeed virus resistance mediated by RNA surveillance and destruction occurs in the plant, then crown gall disease might also be attenuated or prevented by a similar mechanism directed against T-DNA oncogenes. Here we have engineered

transgenic tobacco plants, similar to those used in viral studies (Lindbo and Dougherty, 1992a, 1992b; Lindbo et al., 1993), to investigate whether T-DNA oncogenes can be suppressed, in hopes of producing tumor-resistant plants.

Materials and Methods

For more details, look in Appendices.

Bacterial strains and plant lines

The plant lines used in this study are listed in Table 2.1 and the bacterial strains used in this study are listed in Table 2.2.

Construction of binary plant transformation vectors

The constructs in this study were based on the study done by Lindbo and Dougherty (1992a, 1992b). The T-DNA oncogenes were mutated using PCR primers (Figure 2.1.) so that the third codon of each gene was converted to a stop codon. These mutant oncogenes were first cloned into a pUC vector, sequenced, and then subcloned from pUC into pPEV6, a binary vector for *A. tumefaciens*-mediated plant cell transformation (Lindbo and Dougherty, 1992a). The *iaaM*-stop gene was made by replacing the third codon with a stop codon (TGA) and deleting TC from the fourth codon, which created a new *Bsp*HI site and also caused a frameshift creating two more stop codons (TGA, TAA) downstream (Figure 2.1A). This 1810-bp PCR product was ligated into pPEV6 at the *Bam*HI site. *ipt*-stop was constructed by deleting a cytosine from the third codon and replacing a cytosine with an adenine in the fourth codon to make a stop codon (TGA), which introduced

Table 2.1. Transgenic plant lines constructed in this study.

Plant line		Genotype	Co-suppression*
TDP1	B7	pPEV::*iaaM* stop transformed into *N. tabacum* cv. Burley	Worked
	B17	pPEV::*iaaM* stop transformed into *N. tabacum* cv. Burley	Worked
	B27	pPEV::*iaaM* stop transformed into *N. tabacum* cv. Burley	Worked
	B31	pPEV::*iaaM* stop transformed into *N. tabacum* cv. Burley	Failed
CW1	K27	pPEV::*ipt* stop transformed into *N. tabacum* cv. Kentucky	Worked
	K52	pPEV::*ipt* stop transformed into *N. tabacum* cv. Kentucky	Failed
CW4	B22	pPEV::*ipt* stop *iaaM* stop transformed into *N. tabacum* cv. Burley	Failed
	B30	pPEV::*ipt* stop *iaaM* stop transformed into *N. tabacum* cv. Burley	Partial
PEV6	B2	Vector only transformed into *N. tabacum* cv. Burley	Failed
	B14	Vector only transformed into *N. tabacum* cv. Burley	Failed

* :Inhibition of tumorigenesis when inoculated with the appropriate bacterial strain.

Table 2.2. *Agrobacterium tumefaciens* strains used in this study.

Strain	Genotype	Reference/source
A136	No Ti plasmid, C58 cured of pTiC58	Watson et al., 1975
A348	Octopine-type, A136(pTiA6NC)	Garfinkel et al., 1981
338	*ipt*::Tn*5* in pTiA6NC	Garfinkel et al., 1981
328	*iaaM*::Tn*5* in pTiA6NC	Garfinkel et al., 1981
C58	Nopaline-type, C58(pTiC58)	Holster et al., 1980
A208	Nopaline-type, A136(pTiT37)	Chilton et al., 1980
R1000	*A. rhizogenes*, A136(pRiA4)	Huffman et al., 1984

A. iaaM gene

Octopine wild type 5'end:

5'TCTTTTCTAACA*ATG*TCAGCTTCACCTCTCCTTGATAACC···3'

5'primer:

5'-CGGGATCC*ATG*TCA**TGA**ACCTCTCCT**TGATAA**C-3'

*Bsp*HI

Octopine wild type 3'end:

5'···ATTCGGATCCTGCGACTCATAGTCC···3'

3'primer:

5'-CGGGATCCTGCGACTCATAGT-3'

B. ipt gene

Octopine wild type 5'end:

5'TGCAAAAAACTT*ATG*GACCTGCATCTAATTTTCGGTCC···3'

5'primer:

5'-GAAGATCTGATC*ATG*GAC**TGA**ATC**TAA**TTTTCGGTCC-3'

*Hin*fI

Octopine wild type 3'end:

5'ACGCAGGGCTGGCGTAAC**CTA**ATACATTCCGAACGG···3'

3'primer:

5'-GAAGATCTGATCA**CTA**ATACATTCCGAACGG-3'

Figure 2.1. Sequences of octopine wild type T-DNA oncogenes and their primers used in this study. The start codon is shown in italics. The stop codon is shown in bold. Deleted nucleotides are underlined. Nucleotide changes are indicated with arrows.

a new *Hin*fI site and a second stop codon (TAA) downstream. The normal stop codon (TAG) was included at the 3' end (Figure 2.1B). The 747-bp PCR product was inserted into the *Bam*HI site of pPEV6 using *Bcl*I overhangs. The *iaaM*-stop gene was inserted into the *Bam*HI site that occurs 291-bp from the 5' end of the *ipt* stop gene. This gene fusion was then ligated into pPEV6 creating *ipt* stop-*iaaM* stop. The transgenes were expressed from the cauliflower mosaic virus (CaMV) 35S promoter and flanked by the CaMV 5' and 3' untranslated regions (UTS); the *nptII* gene expressed from the nopaline synthase promoter was also present

between the right and left T-DNA borders (Figure 2.2A). The *Bam*HI site, where all the mutated T-DNA oncogenes were ligated, is located between the 5' and 3' UTS. Thus, the oncogenes are transcribed from the CaMV 35S promoter. The binary vectors were transformed into haploid *Nicotiana tabacum* cultivar (cv) Burley (B) or cv. Kentucky (K).

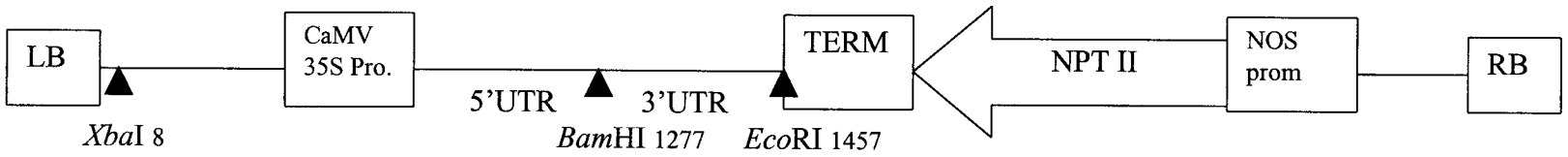
Inoculation of transgenic plants

A. tumefaciens was grown on AB minimal agar plates with 0.5% glucose as the carbon source or YEP agar plates (Chilton et al., 1974) at 30°C for 3 days. No antibiotics were added to the media. A sterile toothpick was used to wound the stem of the plant, and then a quantity of bacteria sufficient to fill the wound was inoculated into the wound site. Plants were grown in the greenhouse or the growth room (24°C, day length 16 hours). Initially, wounds were covered with parafilm to prevent bacteria from drying; wounds were uncovered 4 weeks post-inoculation. Tumor formation was scored visually 4 weeks, 6 weeks, and 10 weeks post-inoculation.

RNA isolation and analysis

Total RNA was isolated from the leaves of transgenic plants by LiCl precipitation (Verwoerd et al., 1989). The supernatant after the LiCl precipitation step was set aside for DNA extraction. All glassware was baked at 230°C prior to use, and other small apparatus was soaked for 30 minutes in 0.2% SDS/0.2% EDTA solution. The 0.2% SDS/0.2% EDTA solution was heated to a boil and poured onto the small

A. T-DNA of pPEV6 vector



B. TRANSGENE CONSTRUCT

CONSTRUCT SIZE

TRANSGENIC PLANT LINE

a. iaaM stop

*Bsp*HI

1800bp

5'-***ATG***TCA**TGA**ACCTCTCCT**TGATAA**C-3'

TDP1

b. ipt stop

*Hin*fI *Bam*HI

5'-CGTTCGGAATGTAT**TAG**TG -3'

752bp

5'-***ATG***GAC**TGA**ATC**TAA**TTT-3'

CW1

c. ipt stop-iaaM stop

2520bp

CW4

Figure 2.2. Binary vector and transgene constructs. ▨ :ATG start codon ⁘ :TGA, TAG, TAA stop codon.

apparatus, which was then rinsed with dH_2O. The gel apparatus was wiped with RNase-Off (CPG, Inc. Lincoln Park, NJ) or RNase Away (Molecular Bio-Products) before use. MilliQ water (ddH_2O) was used in all the solutions. 10μg of total RNA was subjected to electrophoresis on 1% agarose/formaldehyde denaturing gels and blotted onto Gene Screen Plus nylon membrane (NEN Life Science Products) overnight. RNA transferred to filters was stained with methylene blue and then prehybridized at 65°C or 42°C for at least 4 hours and hybridized at 65°C or 42°C overnight with ^{32}P-labeled probes. The blots were washed and then exposed to X-ray film for a week. 0.24-9.5 kb RNA ladder from Gibco BRL was used as the standard marker. Northern blots were hybridized with either a strand-specific ^{32}P-UTP labeled antisense RNA probe or a nick translated ^{32}P-dCTP labeled probe. RNA probes were created by cloning *iaaM* stop into pCR-TOPO vector (Invitrogen); *in vitro* transcription was done using the riboprobe transcription system from Promega. The 1.2 kb ^{32}P-dCTP labeled polyubiquitin (Burke et al., 1988) was from pTB112 (five repeats of ubiquitin inserted into pGEM-4) and used as a standard internal control probe to hybridize to northern blots.

DNA isolation and analysis

Plant genomic DNA was isolated from the supernatant of the LiCl RNA precipitation and ethanol precipitated twice and dissolved in TE(10/0.1). Plant DNA was further purified using CsCl density gradient centrifugation and digested with restriction enzymes, subjected to electrophoresis in a 0.8% agarose gel and blotted onto Gene Screen Plus nylon membrane (NEN Life Science Products)

overnight. Southern blots were performed according to procedures described by Ream and Field (1999, Academic Press). Blots were probed with either ^{32}P-dCTP labeled *iaaM* stop PCR product or ^{32}P-dCTP labeled *ipt* stop PCR product. The probes were synthesized by nick translation using the Nick Translation Kit from Gibco BRL. Prepacked NICK spin columns (Pharmacia) were used according to manufacturer's directions to separate the incorporated label from the unincorporated. Radioactivity of the purified probe was then measured in a scintillation counter (Beckman LS 6800). Depending on the probe, the number of counts per minute (cpm) ranged from 10^5 to 10^8 cpm/0.1μg of input DNA. 5μg or 10μg of TDP1 and CW4 plant genomic DNA was digested with either *Bam*HI or *Eco*RI restriction enzyme. 10μg of CW1 plant genomic DNA was digested with either *Hin*fI or *Eco*RI.

Results

Plant studies

Defective RNAs such as untranslatable sense RNA and antisense RNA were more efficient than translatable sense RNA in eliciting the silencing response in virus-resistant plants (Lindbo and Dougherty, 1992a, 1992b; Smith et al., 1994). Therefore, three of the T-DNA oncogenes that control the morphology of plant tumors were mutated to generate nonsense stop codons downstream of the AUG start codon, rendering the mRNAs untranslatable. The various mutant oncogenes were cloned into a plasmid vector (pPEV6) that allowed *Agrobacterium*-mediated

transformation of tobacco tissue. Because silencing occurs much more efficiently when the T-DNA is homozygous (de Carvalho Niebel et al., 1992; Dehio and Schell, 1994; Elmayan and Vaucheret, 1996; Hart et al., 1992), we transformed haploid tobacco and doubled the chromosome number to produce plants homozygous for the transgene. The resulting homozygous plant lines were TDP1 lines, which have the mutated monooxygenase (*iaaM* stop), TDP8 lines carrying the mutated indoleacetamide hydrolase (*iaaH* stop) (data not shown), and CW1 lines, which have the mutated isopentenyl transferase (*ipt* stop). The *ipt* stop-*iaaM* stop plant lines, CW4, were constructed in hopes of eliminating both phytohormone pathways with a single construct (Figure 2.2B), thus rendering the plant resistant to wild type *A. tumefaciens*. The PEV6 plants refer to the lines transformed with the empty vector plasmid pPEV6 (Figure 2.2A).

Plant lines that silenced T-DNA oncogenes were identified based on tumor formation and morphology at wounds inoculated with wild-type and T-DNA oncogene mutant *Agrobacterium tumefaciens*. Octopine-type *A. tumefaciens* induce unorganized tumors on tobacco (Ooms et al., 1981). Mutations in *iaaM* or *iaaH* result in shooty tumors that contain high levels of cytokinin, whereas mutations in *ipt* cause rooty tumors that contain high levels of auxin (Black et al., 1994; Garfinkel et al., 1981; Joos et al., 1983; Ooms et al., 1981). We should not see any tumor growth if the production of both auxin and cytokinin is blocked. The mutant bacterial strains that were used to inoculate the transgenic plants were selected to complement the mutated transgene in the plant so that expression of the genes for

both the auxin and cytokinin pathways should be suppressed. For example, the putative auxin pathway suppressing plant lines, TDP1 and TDP8, were inoculated with *A. tumefaciens* strain 338, an *ipt*::Tn5 mutant strain (Garfinkel et al., 1981). Plant lines designed to suppress the cytokinin pathway (CW1 lines, which contain *ipt* stop transgenes) were inoculated with *A. tumefaciens* strain 328, an *iaaM*::Tn5 mutant (Garfinkel et al., 1981). Both 338 and 328 were inoculated onto CW4 plant lines and PEV6 plant lines. All the transgenic plant lines were inoculated with *A. tumefaciens* strains A136 (no Ti plasmid) and A348 (octopine-type Ti plasmid) as negative and positive controls, respectively. The predicted results are shown in Table 2.3.

Table 2.3. Expected tumor phenotypes.

Bacterial strains / Transgenic plant line	328 (*iaaM*)	338 (*ipt*)	A348 (wild type)
TDP1 (*iaaM* stop)	ND*	No tumors	Shooty tumors
TDP8 (*iaaH* stop)	ND*	No tumors	Shooty tumors
CW1 (*ipt* stop)	No tumors	ND*	Unorganized tumors
CW4 (*ipt* stop-*iaaM* stop)	No tumors	No tumors	No tumors
PEV6 (vector only)	Shooty tumors	Unorganized tumors	Unorganized tumors

*ND : not done

Responses to inoculation with *A. tumefaciens* were first analyzed in tissue culture on 63 TDP1, 25 TDP8, 45 CW1, 30 CW4, and 11 PEV6 lines. Plant lines

that looked promising were selected and grown in the greenhouse and the growth room for further testing. All of the TDP8 lines analyzed in the tissue culture produced tumors when inoculated with strain 338, the *ipt*::Tn5 mutant (data not shown). Therefore, these lines were not further studied. Twelve TDP1 lines, 11 CW1 lines, 10 CW4 lines and 4 PEV6 lines were tested in the greenhouse. Tumor phenotypes displayed by the plant lines were recorded 5 week post-inoculation (Table 2.4 & Figure 2.3). Most TDP1 lines showed no signs of tumor growth when inoculated with 338, which was the result we expected in plants where the *iaaM* stop transgene elicited co-suppression of the wild-type *iaaM* gene on the incoming T-DNA. Several TDP1 lines, such as B7 and B17, produced shooty tumors when inoculated with the wild-type strain A348, confirming that suppression of the auxin pathway had occurred (Figure 2.3A & B). TDP1 B31, a line that failed to cosuppress *iaaM* in tissue culture plants, was used for comparison (Figure 2.3D).

The CW4 plant lines, which we constructed to suppress both phytohormone pathways, produced tumors when inoculated with *A. tumefaciens*. However, one line, CW4 B30, was nearly resistant to crown gall tumorigenesis (Table 2.4, Figure 2.3E). Inoculation of CW4 B30 with strain 338 produced no tumors with one exception, while strain 328 produced small tumors at 50% of the wounds inoculated (Table 2.4, Figure 2.3E), and wild-type *A. tumefaciens* (strain A348) induced tumors that were smaller than normal (Table 2.4, Figure 2.3E). On some of the CW4 and CW1 lines (e.g. CW4 B26, CW4 B42, CW4 B46, CW1 K19, CW1 K54), shooty tumors were observed on stems inoculated with 328 (Table 2.4,

Table 2.4. Tumor phenotypes- greenhouse trial[a].

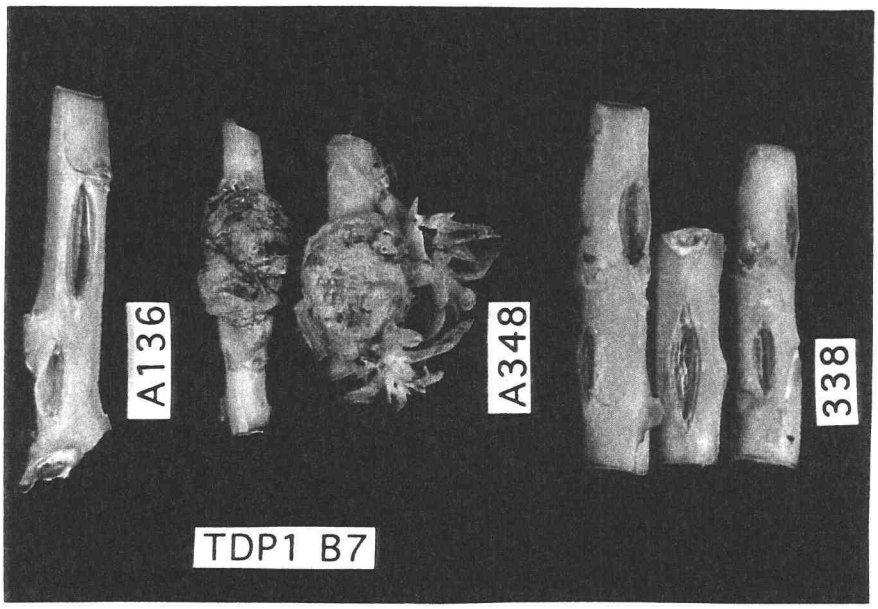
Plant line	Control		Bacterial strain inoculum	No. of plants inoculated	Results[b] (no. of plants)
	A136	A348			
TDP1 B3	- (1)	++ (2)	338	4	- (1) +/- (2) + (1)
TDP1 B7	- (1)	++ (2^{1S})[c]	338	3	- (3)
TDP1 B17	- (1)	+ (2^{1S})	338	6	- (6)
TDP1 B21	- (1)	+ (2)	338	3	- (3)
TDP1 B27	- (1)	+ (1)	338	3	- (2) ? (1)
TDP1 B30	- (1)	+ (2)	338	3	- (3)
TDP1 B31	- (1)	+ (1)	338	3	+ (3)
TDP1 B42	- (1)	+ (2)	338	2	+ (2)
TDP1 B55	- (1)	ND	338	1	- (1)
TDP1 B69	- (1)	+ (2)	338	3	- (3)
TDP1 B73	- (1)	++ (2^{2S})	338	2	- (2)
CW4 B1	- (1)	+++ (1)	338 328	1 2	+++ (1) - (1) + (1)
CW4 B4	- (1)	++ (1) +++ (1)	338 328	2 1	+ (2) + (1)
CW4 B11	- (1)	+++ (1)	338 328	1 1	- (1) +/- (1)
CW4 B22	- (1)	+++ (1)	338 328	2 2	- (1) + (1) - (1) + (1)
CW4 B26	- (1)	++ (1)	338 328	2 2	+ (2) + (2^{1S})
CW4 B30	- (1)	+ (1)	338 328	6 6	- (5) + (1) - (3) +/- (3)
CW4 B32	- (1)	+ (1) ++ (1)	338 328	2 2	+ (2) +/- (2)
CW4 B42	- (1)	+++ (1)	338 328	2 2	+ (2) + (2^{1S})
CW4 B46	- (1)	+ (1)	338 328	2 2	- (1) +/- (1) + (2^{2S})

Table 2.4 continued.

Plant line	Control		Bacterial strain inoculum	No. of plants inoculated	Results* (no. of plants)
	A136	A348			
CW1 K6	- (1)	++ (2)	328	3	+/- (1) + (2)
CW1 K17	- (1)	+ (1)	328	2	- (1) +/- (1)
CW1 K19	- (1)	+++ (5)	328	8	- (1) + (3) ++ (2) +++ (2^{1S})
CW1 K27	- (1)	++ (1)	328	2	- (1) + (1)
CW1 K32	- (1)	++ (1)	328	4	+/- (1) + (2) ++ (1)
CW1 K37	- (1)	+ (1)	328	3	- (1) + (2)
CW1 K51(A)	- (1)	+ (1)	328	2	- (2)
CW1 K52	- (1)	+++ (2)	328	1	- (1)
CW1 K53(B)	- (1)	+ (1)	328	2	+ (1) ++ (1)
CW1 K54	- (1)	+++ (1)	328	1	+ (1^{1S})
PEV6 B2	- (1)	++ (1)	338 328	2 1	+/- (1) + (1) + (1^{1S})
PEV6 B3	- (1)	+++ (1)	338 328	2 2	+ (2) + (1) ++ (1)
PEV6 B6	- (1)	++ (1)	338 328	1 2	+ (1) - (1) +/- (1)
PEV6 B14	- (1)	+++ (1)	338 328	2 1	+/- (1) ++ (1) +/- (1)

[a]All the tumors were observed 5 weeks post-inoculation.
[b]-: no tumors; ?: unsure about result; +/-: very slight response possible tumor; +: at least one small tumor; ++: moderate tumor; +++: large tumor.
[c]x^{1S}, x^{2S}, etc: the number of shooty tumors.

A.



B.

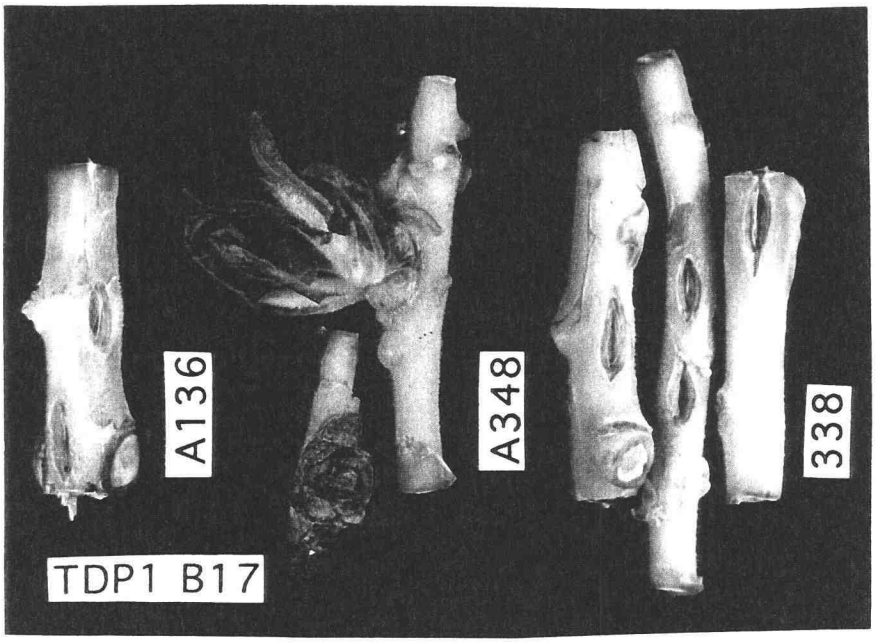
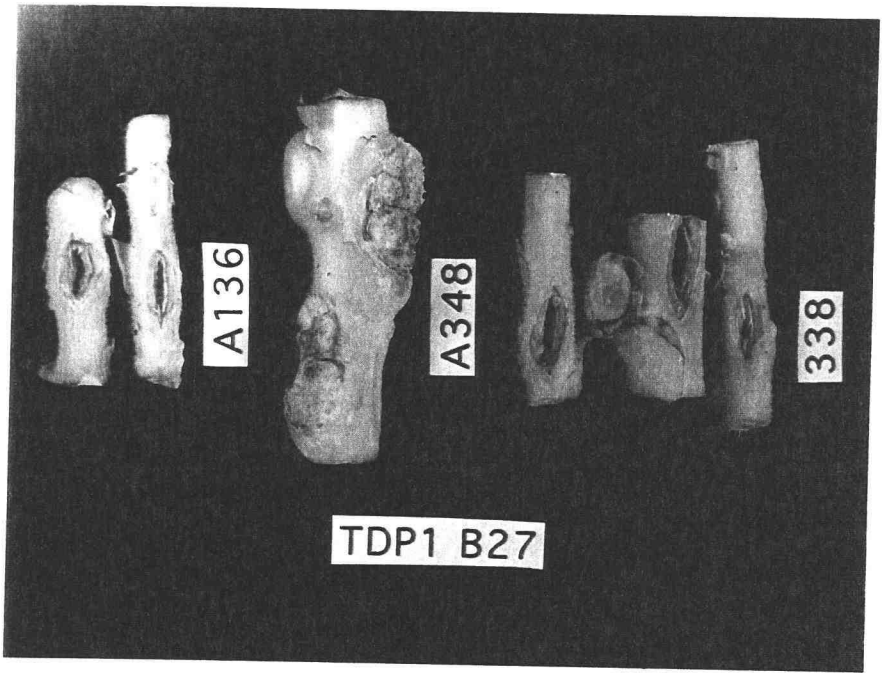


Figure 2.3. Suppression of T-DNA oncogenes in transgenic plants. The inoculated *A. tumefaciens* strain is indicated on the right side of the stems. A136 (no Ti plasmid), A348 (octopine-type Ti plasmid), 338 (*ipt*::Tn5 mutant), and 328 (*iaaM*::Tn5 mutant).
A.TDP1 B7 (*iaaM* stop) B. TDP1 B17 (*iaaM* stop) C. TDP1 B27 (*iaaM* stop) D. TDP1 B31 (*iaaM* stop) E. CW4 B30 (*iaaM* stop-*ipt* stop) F. PEV6 (vector only control)

C.



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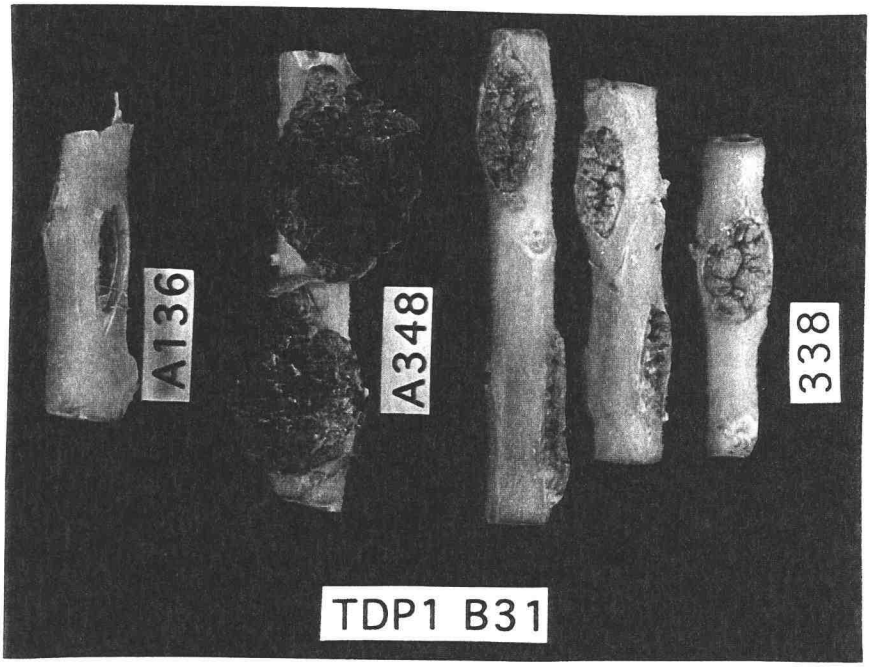
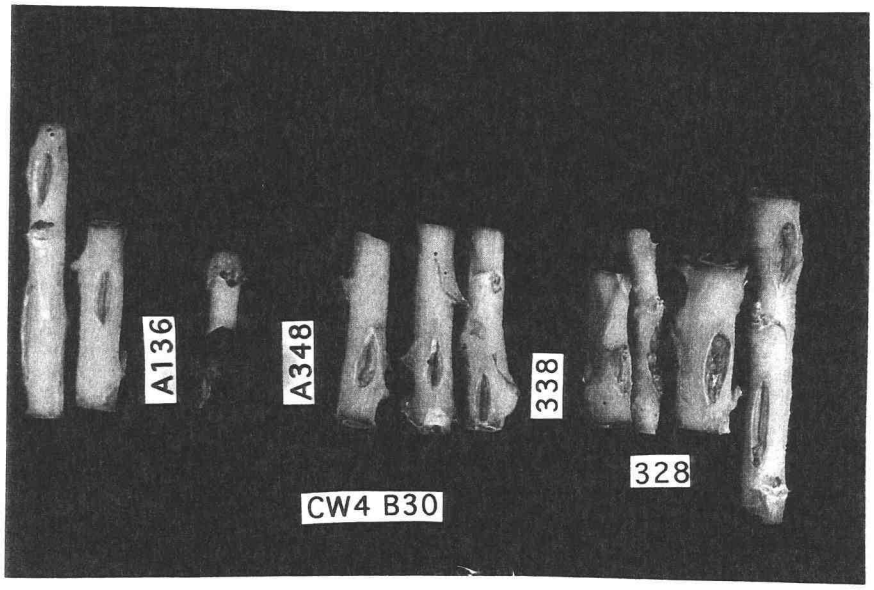


Figure 2.3., continued.

E.



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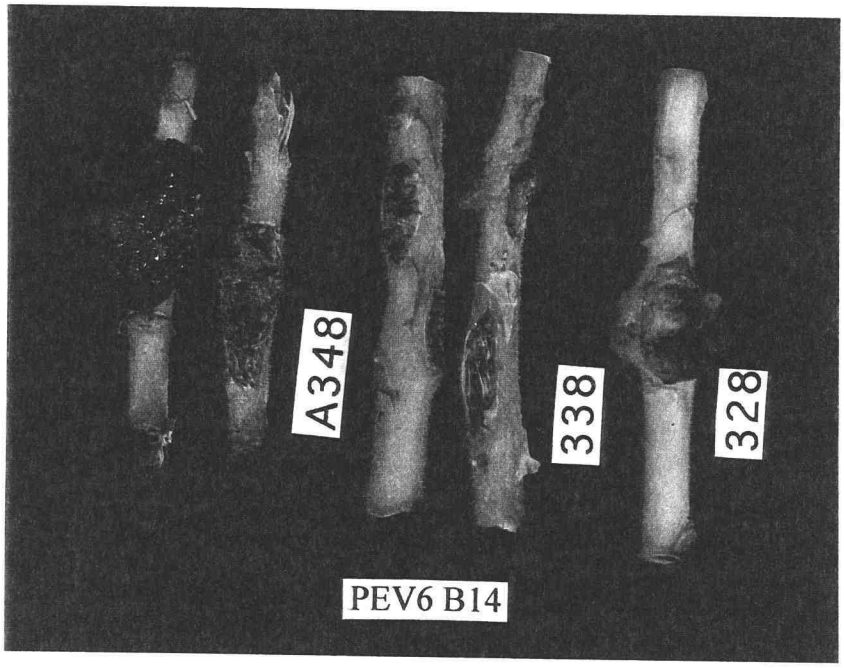


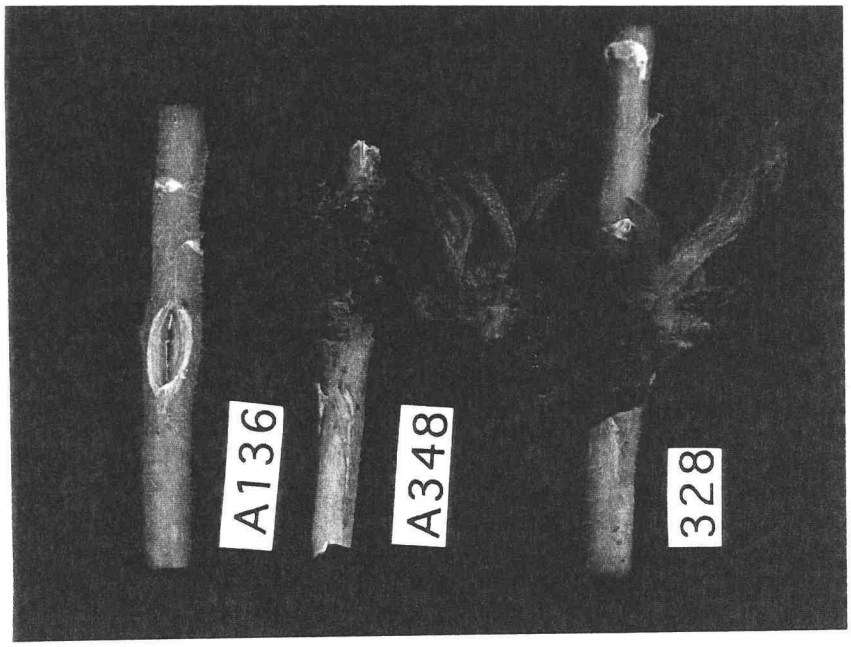
Figure 2.3., continued.

Figure 2.4). High levels of cytokinin cause shooty tumors (Ooms et al., 1981). Therefore, in these lines, the *ipt* gene is not suppressed. Three CW4 lines (e.g. B11, B30, and B46) gave little or no response when inoculated with strain 338, indicating that these lines suppressed the *iaaM* gene effectively, even though none of these lines abolished tumorigenesis by strains 328 and A348. Thus, the hybrid *ipt* stop-*iaaM* stop transgene elicited suppression of *iaaM* but not *ipt*.

Plants that looked promising in the greenhouse trial were re-tested in the growth room. Plants were inoculated on the stem as before, and the inoculation sites were covered with parafilm to ensure that the bacteria did not desiccate. The parafilm was removed 4 weeks post-inoculation. Tumor phenotypes were observed (Table 2.5) and plants were harvested and photographed after 10 weeks. The PEV6 plant lines had tumor growth on all of the wound sites except those inoculated with the negative control A136 (Figure 2.5). The TDP1 lines gave responses similar to those observed in the greenhouse: *iaaM* stop lines B7, B17, B21, B27, and B30 suppressed the *iaaM* oncogene (Table 2.5, Figure 2.6). Line CW4 B30, occasionally produced small tumors when inoculated with strain 338, whereas most inoculations with strain 328 yielded small tumors, indicating that this line effectively suppressed the *iaaM* oncogene but only attenuated the *ipt* oncogene (Table 2.5). These tumors were small, sometimes no more than one or two bumps (Figure 2.7).

Ten plant lines, including control plant lines, were tested further. These plants were grown from seeds in the growth chamber for 6 weeks, then transplanted

A.



B.

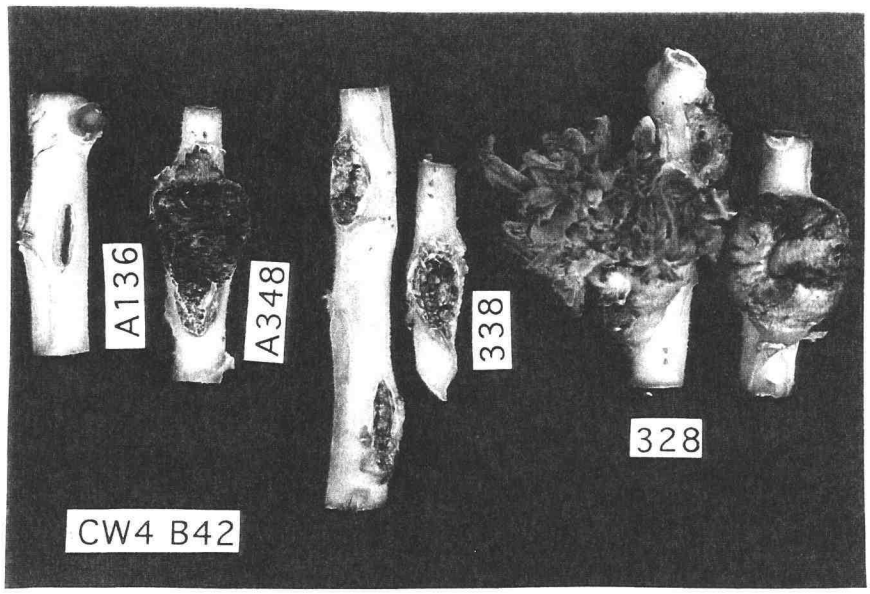


Figure 2.4. Shooty tumors on transgenic plants inoculated with 328 (*iaaM*::Tn5 mutant). The inoculated *A. tumefaciens* strain is indicated on the right side of the stems. A136 (no Ti plasmid), A348 (octopine-type Ti plasmid), 338 (*ipt*::Tn5 mutant), and 328 (*iaaM*::Tn5 mutant).
A. CW1 K54 (*ipt* stop) B. CW4 B42 (*iaaM* stop-*ipt* stop)

Table 2.5. Tumor phenotypes - growth room trial.

Plant line	Controls		Bacterial strain inoculum	No. of plants inoculated	Results[a] (no. of plants)
	A136	A348			
TDP1 B7	- (1)	+ (1)	338 C58 A208	3 1 1	- (3) + (1) +/- (1)
TDP1 B17	- (1)	+(1) ++(1^{1S})[b]	338 C58 A208	6 4 4	- (3) +/- (3) - (1) +/- (1) + (1^{1S}) ++ (1^{1S}) +/- (2) + (2^{2S})
TDP1 B21	- (1)	++ (1)	338 C58 A208	4 2 2	- (2) +/- (2) - (1) + (1) - (1) + (1)
TDP1 B27	- (1)	+++(2)	338 C58 A208	5 1 2	- (3) +/- (2) - (1) + (1) ++ (1^{1S})
TDP1 B30	- (1)	++ (1)	338 C58 A208	3 1 1	- (3) +/- (1) +/- (1)
TDP1 B31 (control)	- (1)	++ (1)	338 C58 A208	2 2 1	+ (2) + (2^{1S}) + (1)
TDP1 B53	- (1)	+ (1)	338	2	- (1) +/- (1)
TDP1 B55	- (1)	++ (1)	338	4	- (3) +/- (1)
TDP1 B69	- (1)	+++ (1)	338	4	- (4)
TDP1 B73	- (1)	+ (1)	338	4	- (2) +/- (2)
CW4 B1	- (1)	++ (1)	338 328	3 3	- (1) + (2) +/- (2) + (1)
CW4 B11	- (1)	++ (1)	338 328 C58 A208 R1000	5 4 1 1 1	+ (3) ++ (2) +/- (2) ++ (1) +++ (1^{1S}) + (1) +/- (1) - (1)
CW4 B22 (control)	- (1)	++ (1)	338 328 C58 A208 R1000	2 2 1 1 2	++ (1) + (1) ++ (2^{2S}) + (1) ++ (1) +/- (1) + (1)
CW4 B30	- (1)	++(1) +/-(1)	338 328 C58 A208 R1000	8 6 4 4 5	- (4) +/- (3) +(1) +/- (1) + (4) ++ (1) + (4) - (1) + (2) ++(1) - (4) +/- (1)

Table 2.5 continued

Plant line	Controls		Bacterial strain inoculum	No. of plants inoculated	Results* (no. of plants)
	A136	A348			
CW1 K6	- (1)	++ (1)	328	3	+ (1) ++ (2)
CW1 K17	- (1)	+(1) ++(1)	328	5	+ (3) ++ (2)
CW1 K27	- (1)	+ (1)	328	3	- (3)
CW1 K37	- (1)	++ (1)	328	4	+ (2) ++ (2)
CW1 K51(A)	- (1)	++ (1)	328	7	+ (7)
CW1 K52	- (1)	++ (1)	328	5	++ (5^{1S})
CW1 K53(B)	- (1)	++ (1)	328	4	+ (2) ++ (2)
CW1 K54	- (1)	++ (1)	328	4	+ (2) ++ (2)
PEV6 B2	ND	ND	338 328 C58 A208 R1000	1 1 1 1 1	+/- (1) ++ (1) +/- (1) + (1) - (1)
PEV6 B3	- (1)	++ (1)	338 328 C58 A208 R1000	1 1 1 1 1	+ (1) +++ (1) + (1) + (1) - (1)
PEV6 B6	- (1)	+ (1)	338 328	1 1	+/- (1) - (1)
PEV6 B14	ND	+ (1)	338 328 C58 A208 R1000	1 1 1 1 1	+ (1) ++ (1^{1S}) ++ (1) ++ (1) +/- (1)

[a] -: no tumors; +/-: very slight response possible tumor; +: at least one small tumor; ++: moderate tumor; +++: large tumor.

[b] x^{1S}, x^{2S}, etc: the number of shooty tumors.

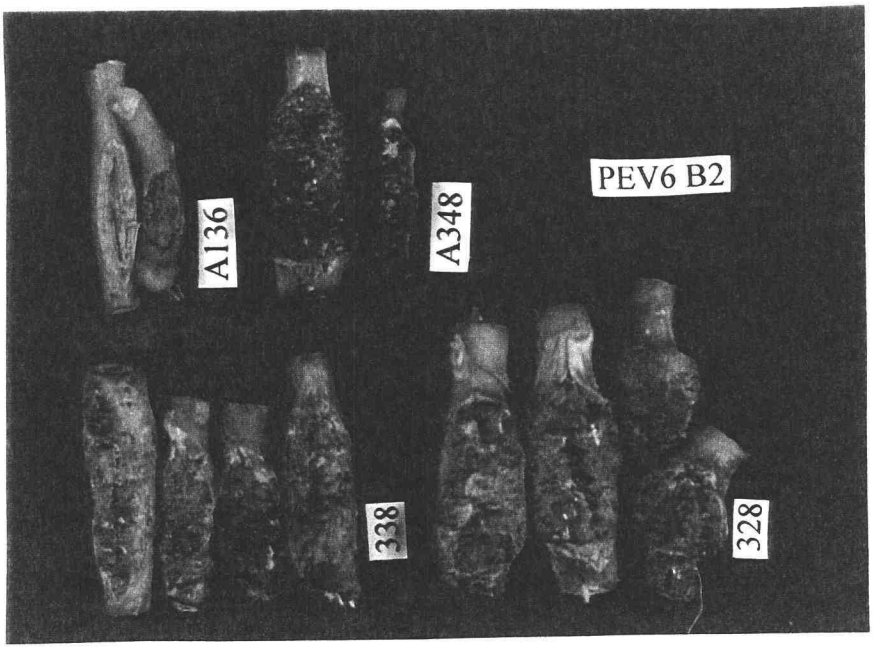
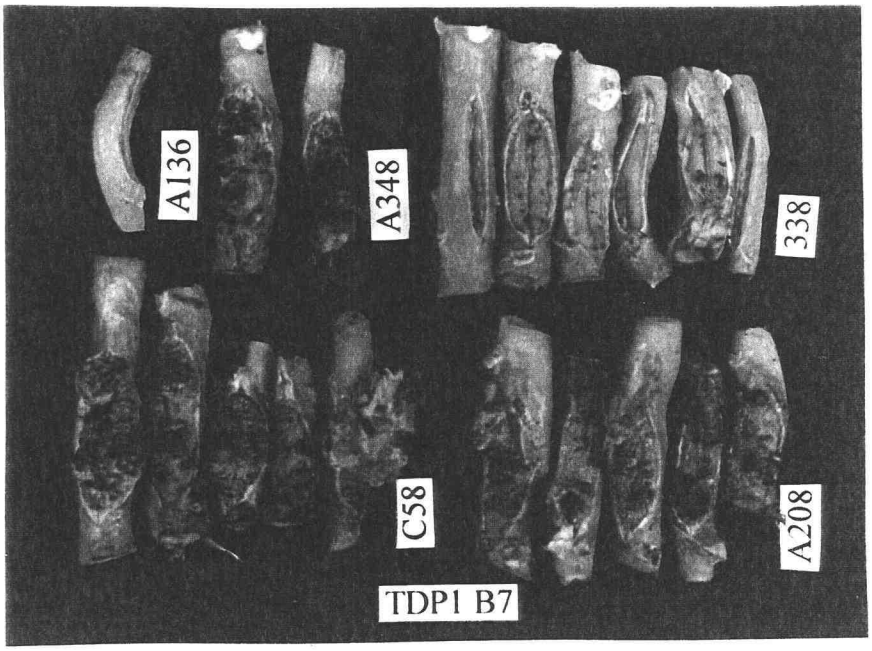


Figure 2.5. Tumors on PEV6 plant line. The inoculated *A. tumefaciens* strain is indicated on the right side of the stems. A136 (no Ti plasmid), A348 (octopine-type Ti plasmid), 338 (*ipt*::Tn5 mutant), and 328 (*iaaM*::Tn5 mutant).

A.



B.

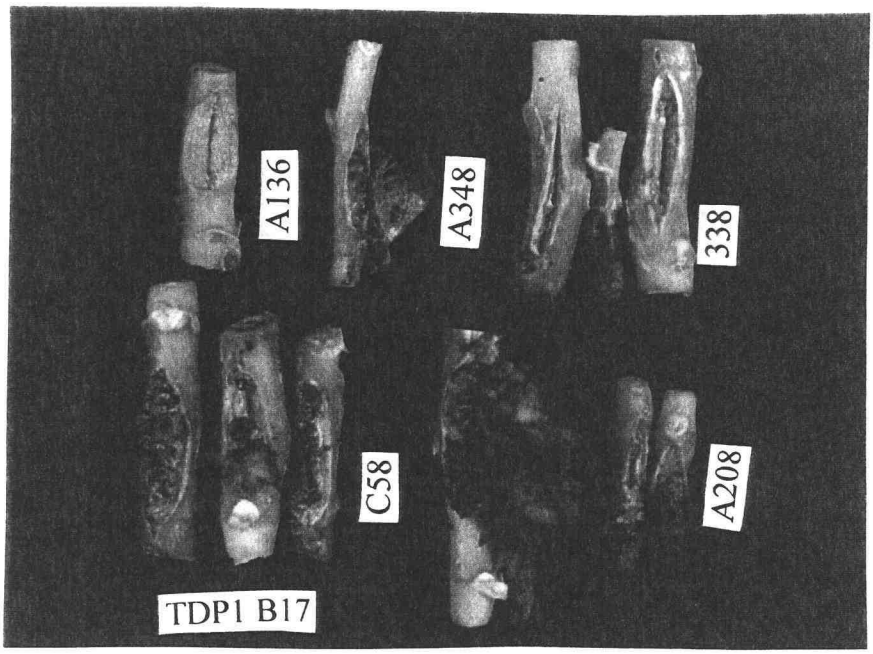


Figure 2.6.Suppression of *iaaM* oncogene by TDP1 (*iaaM* stop) plant lines. Inoculated bacterial strain is indicated on the right side of the stems. A136 (no Ti plasmid), A348 (octopine-type Ti plasmid), 338 (*ipt*::Tn5 mutant), 328 (*iaaM*::Tn5 mutant), C58 (nopaline-type Ti plasmid), and A208 (nopaline-type Ti plasmid).

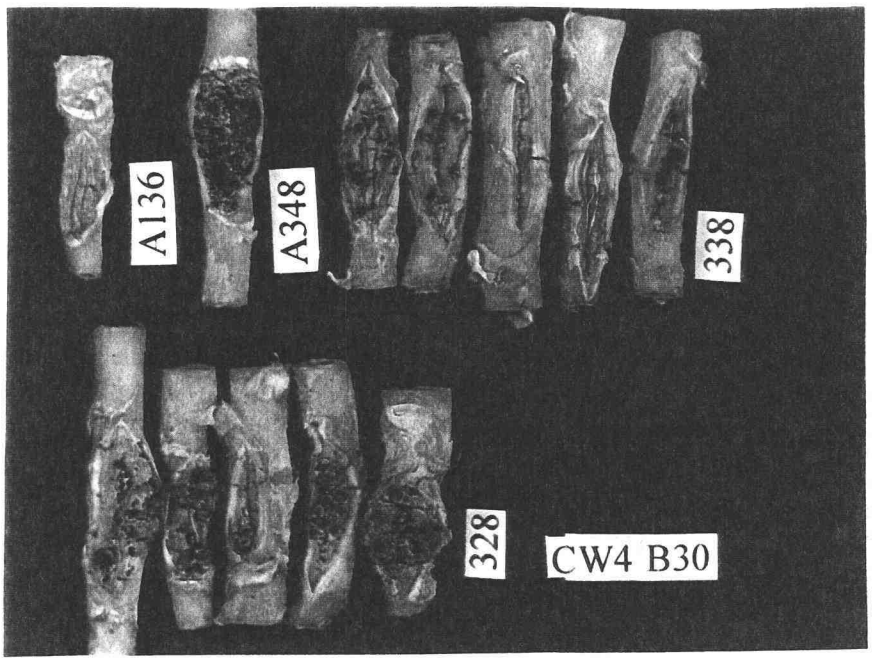


Figure 2.7. Suppression of *iaaM* and attenuation of *ipt* by *ipt* stop- *iaaM* stop line CW4 B30. Inoculated bacterial strain is indicated on the right side of the stems. A136 (no Ti plasmid), A348 (octopine-type Tį plasmid), 338 (*ipt*::Tn5 mutant), and

into separate pots and moved to the growth room. The plants were inoculated 16 days after the transplant. Tumor phenotype was scored and results similar to other trials were observed in these plants (Table 2.6). CW4 B1 and B11 produced tumors on all the stems except those that had been inoculated with A136 (Table 2.6). Further testing on 6 TDP1 lines, 1 CW1 line, and 1 CW4 line was done. These plants were grown 6 weeks in the growth chamber, then transplanted to bigger trays in the greenhouse. 7 weeks after the transplant, plants were inoculated with the appropriate *A. tumefaciens* strains and tumor phenotypes were observed. For each transgene construct, at least one line known to not elicit co-suppression was included as a positive control. The PEV6 B14 line was used as the vector-only control plant line. These inoculations confirmed the results observed in previous experiments (Table 2.7).

The growth room plants were also inoculated with nopaline-type *A. tumefaciens* strains (C58, A208), to see whether the transgenes would silence a slightly different oncogene sequence. Inoculation *A. rhizogenes* (strain R1000) with hairy root inducing *rol* genes in the T-DNA, were designed to test whether the transgenes disrupted a host gene necessary for infection. Because *rol* genes are not related to *iaaM* or *ipt*, transgenes that elicit suppression of these oncogenes should not affect *rol* expression, due to the sequence specific nature of co-suppression. Plants resistant to *A. tumefaciens* due to co-suppression of T-DNA oncogenes should remain sensitive to *A. rhizogenes* whereas plants in which transgenes have disrupted plant loci necessary for bacterial infection should not exhibit resistance to

Table 2.6. Tumor phenotype of selected plant lines- second growth room trial.

Plant line	Controls		Bacterial strain inoculum	No. of plants inoculated	Results[a] (no of plants)
	A136	A348			
TDP1 B7	- (1)	+(1) ++(1)	338 C58 A208	6 5 5	- (4) +/- (2) + (2) ++ (3^{1S})[b] - (1) + (4^{4S})
TDP1 B17	- (1)	+ (1^{1S})	338 C58 A208	3 3 3	- (3) + (3^{1S}) + (2) ++ (1^{1S})
TDP1 B27	- (1)	+++ (1)	338 C58 A208	6 4 4	- (2) +/- (1) + (3) + (2) ++ (2) + (2) ++ (2)
TDP1 B31 (control)	- (1)	++ (1)	338 C58 A208	4 2 2	+ (4) + (2) ++ (2)
CW4 B1	- (1)	++ (1)	338 328 C58 A208 R1000	5 4 3 3 3	+ (4) ++ (1) ++ (4) + (1) ++ (2) ++ (3) + (3)
CW4 B11	- (1)	++ (1)	338 328 C58 A208 R1000	5 4 3 3 2	+ (4) ++ (1) + (1) ++ (3) + (3) + (2) ++ (1) +/- (2)

Table 2.6 continued

CW4 B22 (control)	- (1)	+++ (1)	338 328 C58 A208 R1000	2 2 2 1 2	+ (2) + (1) ++ (1) + (1) ++ (1) + (1^{1S}) +/- (2)
CW4 B30	- (1)	++ (1)	338 328 C58 A208 R1000	4 3 2 1 2	- (2) +/- (1) + (1) + (3) - (1) +/- (1) +/- (1) - (2)
PEV6 B2	-(2)	++(1) +++ (1)	338 328 C58 A208 R1000	4 4 2 3 3	+ (1) ++ (2) +++(1) ++ (1) +++ (3) + (1) ++ (1) ++ (1) +++ (2) - (1) + (2)
PEV6 B14	- (1)	+++ (1)	338 328 C58 A208 R1000	2 2 2 2 2	+ (1) ++ (1) + (2^{1S}) ++ (2) ++ (1) +++ (1) +/- (2)

[a] -: no tumors; ?: unsure about result; +/-: very slight response possible tumor; +: at least one small tumor; ++: moderate tumor; +++: large tumor.
[b] x^{1S}, x^{2S}, etc: the number of shooty tumors.

Table 2.7. Tumor phenotype of tobacco plants- second greenhouse trial.

Plant lines	Controls		Bacterial strain inoculum	No. of plants inoculated	Results[a] (no. of plants)
	A136 (-)	A348 (+)			
TDP1 B7	- (1)	+ (1)	338	13	- (13)
TDP1 B17	- (1)	+++ (1^{1S})[b]	338	3	- (3)
TDP1 B27	- (1)	++ (2)	338	7	- (4) +/- (3)
TDP1 B30	- (1)	+++ (1)	338	4	- (3) +/- (1)
TDP1 B31	- (1)	++ (2)	338	8	+ (5) ++ (3)
TDP1 B46	- (1)	++++ (1)	338	3	+ (1) ++ (2)
TDP1 B69	- (1)	+++ (1^{1S})	338	5	- (3) +/- (2)
CW1 K27	- (1)	+++ (2)	328	9	- (5) +/- (2) + (2)
CW1 K52	- (1)	+++ (2)	328	8	+/- (1) ++ (6) +++ (1)
CW4 B22	- (1)	++ (2)	338 328	6 6	+ (6) + (1) ++ (5)
CW4 B30	- (1)	+ (2)	338 328	3 2	- (2) + (1) + (2)
PEV6 B14	- (1)	++ (1) +++ (1)	338 328	7 7	+ (7) ++ (3) +++ (4)

[a] -: no tumors; ?: unsure about result; +/-: very slight response possible tumor; +: at least one small tumor; ++: moderate tumor; +++: large tumor.
[b] x^{1S}, x^{2S}, etc: the number of shooty tumors.

both pathogen. Gene silencing is effective only if there is a high level of sequence homology (at least 70%) between the target RNA and the transgene (Lindbo et al., 1993; Marano and Baulcombe, 1998). The T-regions of wide-host-range Ti plasmids share a 9 kb homologous region that encompasses the oncogenes (Chilton et al., 1978; Depicker et al., 1978). The nopaline-type *A. tumefaciens* strains produced smaller tumors than those induced by the octopine-type strain A348 (Table 2.6 & 2.7). On plant lines inoculated with strain R1000, we expect to see very small galls and, in time, adventitious roots should appear. All the plant lines that were inoculated with R1000 produced small tumors, proving that these lines retained their ability to receive and inherit T-DNA (Figure 2.8). Some of the plant lines, such as TDP1 B7, B17, and CW4 B22, inoculated with the nopaline-type strains C58 and A208 had shooty tumors (Figure 2.6 & 2.8), indicating suppression of the nopaline-type *iaaM* gene. Nopaline-type strains inoculated on the CW4 B30 plants caused small bumps to form, indicating that the octopine-type *ipt* stop-*iaaM* stop hybrid transgene elicited partial suppression of nopaline-type T-DNA oncogenes(Figure 2.9). Plant lines that suppressed octopine-type T-DNA oncogenes did not suppress nopaline-type oncogenes. However, not enough number of plants were inoculated with nopaline-type strains to come to a definite conclusion.

Most CW1 lines did not suppress expression of the T-DNA *ipt* oncogene and produced either shooty or unorganized tumors when inoculated with strain 328 (Figure 2.10B). In contrast, one CW1 line, CW1 K27, did not respond to

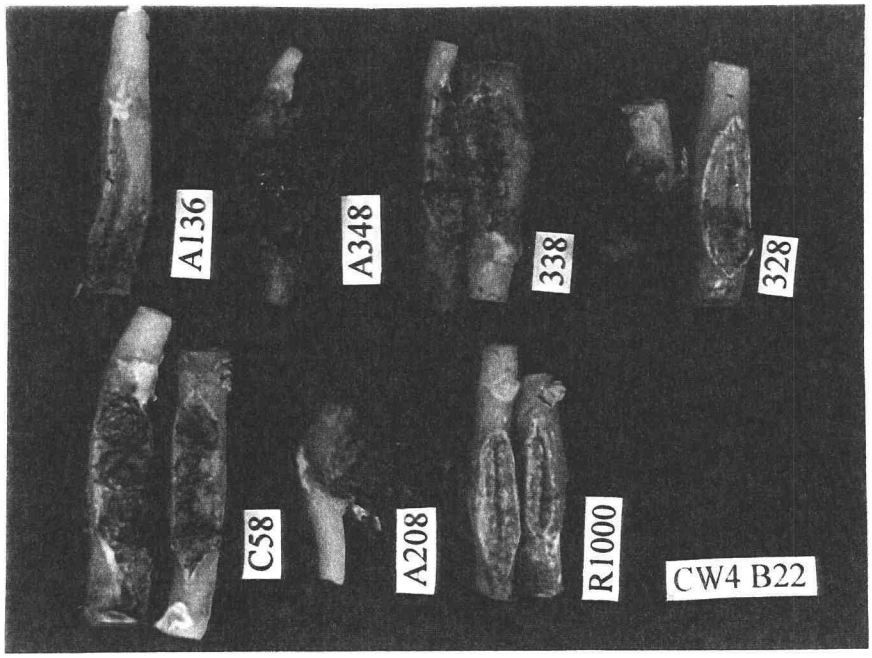


Figure 2.8. Ineffective *ipt* stop-*iaaM* stop plant line inoculated with nopaline-type *A. tumefaciens* (C58 or A208) and *A. rhizogenes* (R1000).

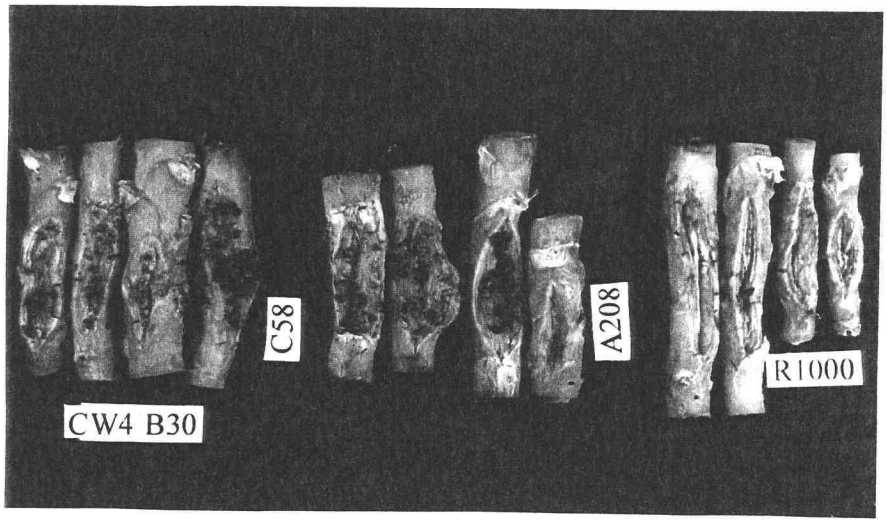
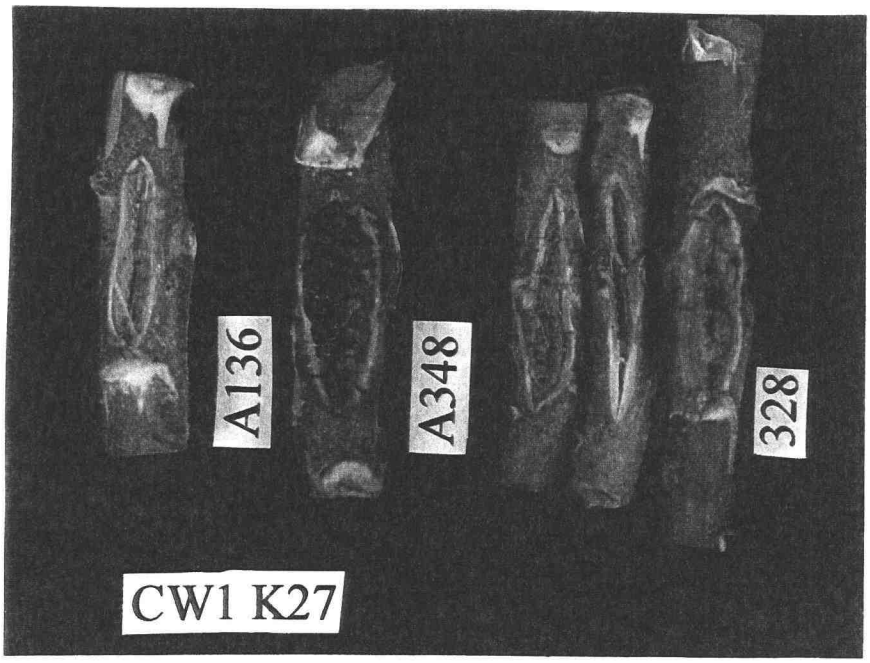


Figure 2.9. Partial suppression of nopaline-type T-DNA oncogenes by *ipt* stop-*iaaM* stop transgene. CW4 B30 line inoculated with nopaline-type *A. tumefaciens* (C58 or A208) and *A. rhizogenes* (R1000).

A.



B.

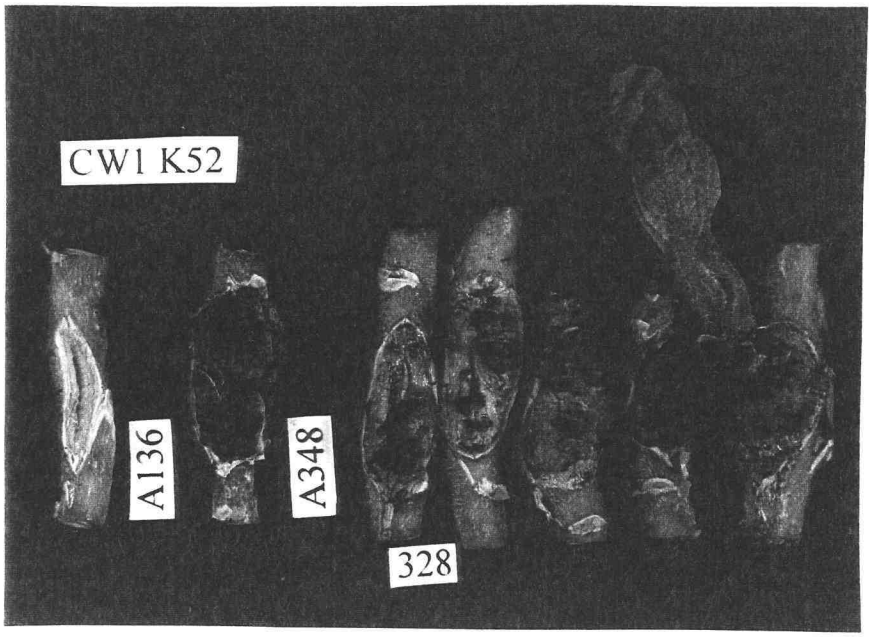
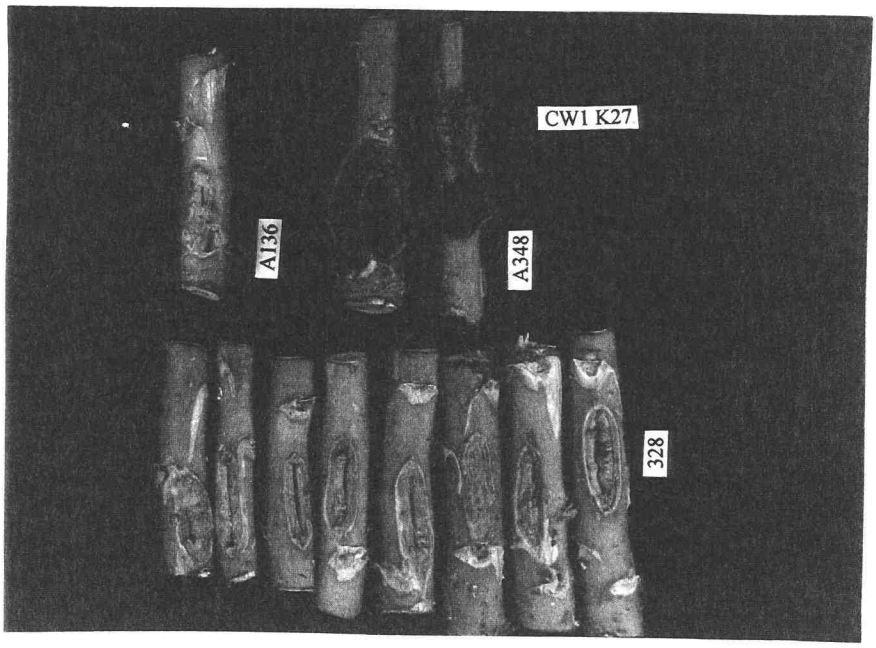


Figure 2.10. Tumor phenotypes on suppressing and non-suppressing *ipt* stop transgenic plant lines (CW1). Plants were inoculated with 328 (*iaaM*::Tn5 mutant). A136 (no Ti plasmid), A348 (octopine-type Ti plasmid).
A. CW1 K27 (suppressing line), growth room B. CW1 K52 (non-suppressing line), growth room C. CW1 K27, greenhouse D. CW1 K52, greenhouse

C.



D.

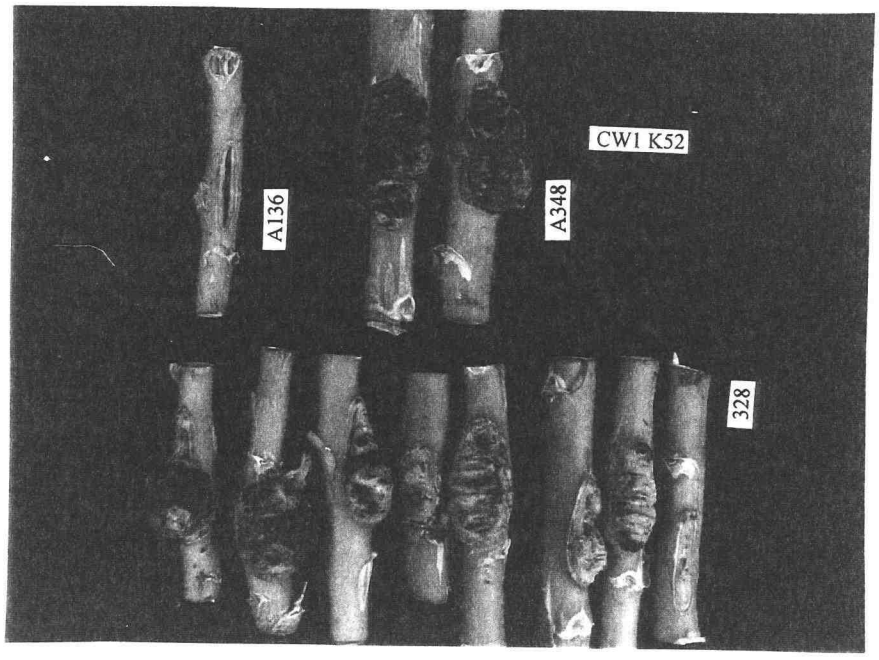


Figure 2.10, continued.

inoculation with strain 328 (Figure 2.10A). However, this line needed further investigation due to the insufficient number of plants studied. The CW1 K27 line was tested in a second greenhouse trial, which confirmed that it was generally resistant when inoculated with strain 328 (Figure 2.10C). Occasionally, small tumors formed (Figure 2.10C bottom right-hand corner stem), but when compared with the large tumors formed on the CW1 K52 line, which was did not suppress *ipt*, tumor incidence and size was quite reduced.

Table 2.8 summarizes the tumor phenotypes observed in the 4 sets of experiments. TDP1 B7 and B17 consistently showed no tumor formation when inoculated with strain 338. The CW1 and CW4 lines were susceptible to *A. tumefaciens* with two exceptions: tumor size and incidence was greatly reduced on CW1 K27 inoculated with strain 328 and on CW4 B30 inoculated with strain 328, 338, and A348. These results indicate that the expression of the incoming T-DNA can be suppressed by the mutant T-DNA oncogenes already present in the plant.

Transgene analysis

DNA and total RNA were extracted from the leaves harvested from selected transgenic plant lines grown in the greenhouse, and molecular analyses of the plants were done. Southern blot analysis was performed with the extracted plant genomic DNA to determine the structure and copy number of the transgene integrated into the plant genome. Genomic DNA from TDP1 and CW4 plant lines was digested with either *Bam*HI, which excises the 1800-bp *iaaM* stop fragment or *Eco*RI, which cuts only once in the T-DNA (Figure 2.2A). DNA from the CW1 *ipt*

Table 2.8. Summary of tumor phenotypes.

Bacterial strains / Transgenic plant line	328 (*iaaM*⁻)	338 (*ipt*⁻)	A348 (wild type)
TDP1 (*iaaM* stop)	ND*	Some resistant No tumors	Some shooty tumors Some unorganized tumors
CW1 (*ipt* stop)	One resistant Some shooty tumors Mostly unorganized	ND*	All unorganized tumors
CW4 (*ipt* stop-*iaaM* stop)	Some shooty tumors Some unorganized tumors, reduced tumor size	Mostly unorganized tumors, reduced tumor size	Some shooty tumors Some unorganized tumors, reduced tumor size
PEV6 (vector only)	All unorganized tumors	All unorganized tumors	All unorganized tumors

*ND : not done

stop transgenic plants was digested with either *Hin*fI, which releases the 682-bp *ipt* stop insert or *Eco*RI, which cuts the T-DNA once (Figure 2.1B & 2.2). The blots were probed with either nick translated ^{32}P-labeled *iaaM* stop PCR product or ^{32}P-labeled *ipt* stop PCR product. In most of the lines, the expected 1800-bp *iaaM* stop insert was detected in the *Bam*HI digest blot (Figure 2.11), so the *iaaM* stop transgene seems to be intact. PEV6 B2 and PEV6 B14 (lanes 9&10), which are vector-only controls, did not have a band, as expected. The upper bands in lanes 1&4-8, which are about 3.4 to 3.6 kb in size, may be rearranged *iaaM* stop insert.

In many cases, transgene silencing is associated with a locus containing multiple copies of the transgene (Assaad et al., 1993). Therefore, the number of

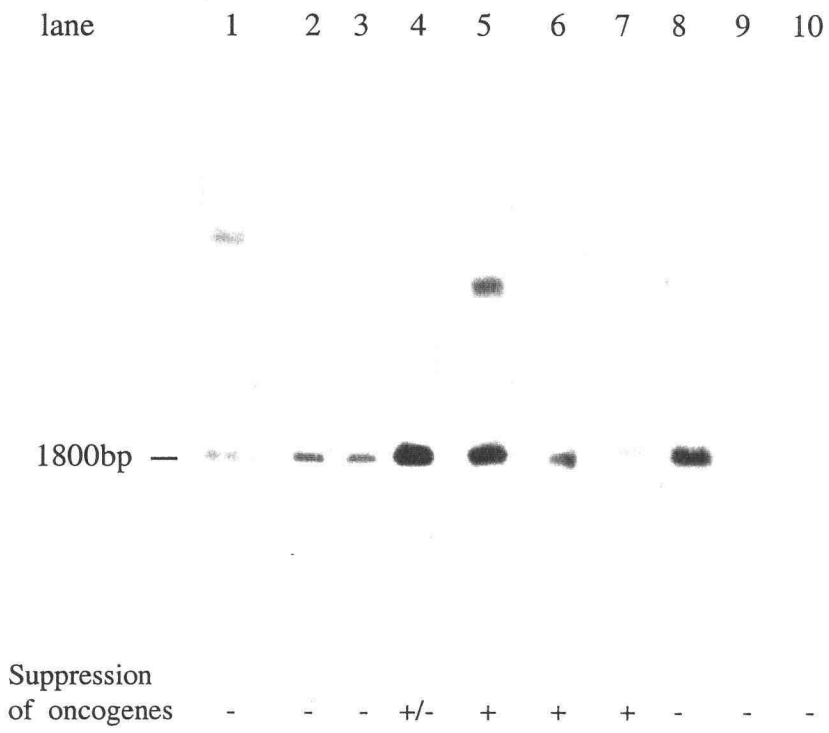


Figure 2.11. Southern blot analysis of transgenic plant genomic DNA digested with *Bam*HI. 5mg of DNA was used unless noted otherwise. Lane 1:CW4 B1(10mg), lane 2: CW4 B11, lane3: CW4 B22, lane 4: CW4 B30, lane 5: TDP1 B7, lane 6: TDP1 B17, lane 7: TDP1 B27(10 mg), lane 8: TDP1 B31(10mg), lane 9: PEV6 B2 (4 mg), lane 10: PEV6 B14

copies integrated into the plant genome was examined by digesting genomic DNA with *Eco*RI. Because, there is only one *Eco*RI restriction site in the T-DNA (Figure 2.2A), *Eco*RI digestion produced T-DNA/ plant DNA junction fragments, which enabled us to estimate the copy number by counting the number of bands produced. The left-hand junction fragments in CW4 plant lines should be larger than 4 kb and those for the TDP1 plant lines should be larger than 3.3 kb, if the T-DNA remained intact from the *Eco*RI site to the left border sequence. Except for CW4 B1 (Figure 2.12; lane 2), all left-hand junction fragments were bigger than the estimated minimum size (Figure 2.12). The smallest junction fragments in CW4 B1 were about 2.3 kb and 2.9 kb, which suggests that a truncated T-DNA was integrated into the plant. Most likely the left border of the T-DNA was truncated, because T-DNA transfer occurs in a 5' to 3' polar manner from right to left (Shaw et al., 1984; Stachel et al., 1986a; Wang et al., 1984). In the pPEV6 vector, the CaMV promoter lies 235-bp to the left of the *Eco*RI site, whereas insertion of *ipt* stop and *iaaM* stop in pCW4 increased this distance to 2.75 kb (Figure 2.2). Thus, these truncated transgenes may not be transcribed unless a host promoter occurs nearby. Weak signals around 6.5 kb and 9.4 kb were also detected in CW4 B1. CW4 B30 contained at least six copies of the transgene (Figure 2.12, lane 5). Although only four bands (12, 6.2, 5.7, and 5.1 kb) were detected, the bands at 5.7 kb and 6.2 kb had stronger signals compared to the other two bands in the lane. Thus, comigration of similar size fragments may have occurred. Two copies of T-DNA were detected in both CW4 B11 (17.5 and 6 kb) and CW4 B22 (9.6 and 4.6 kb) (Figure 2.12,

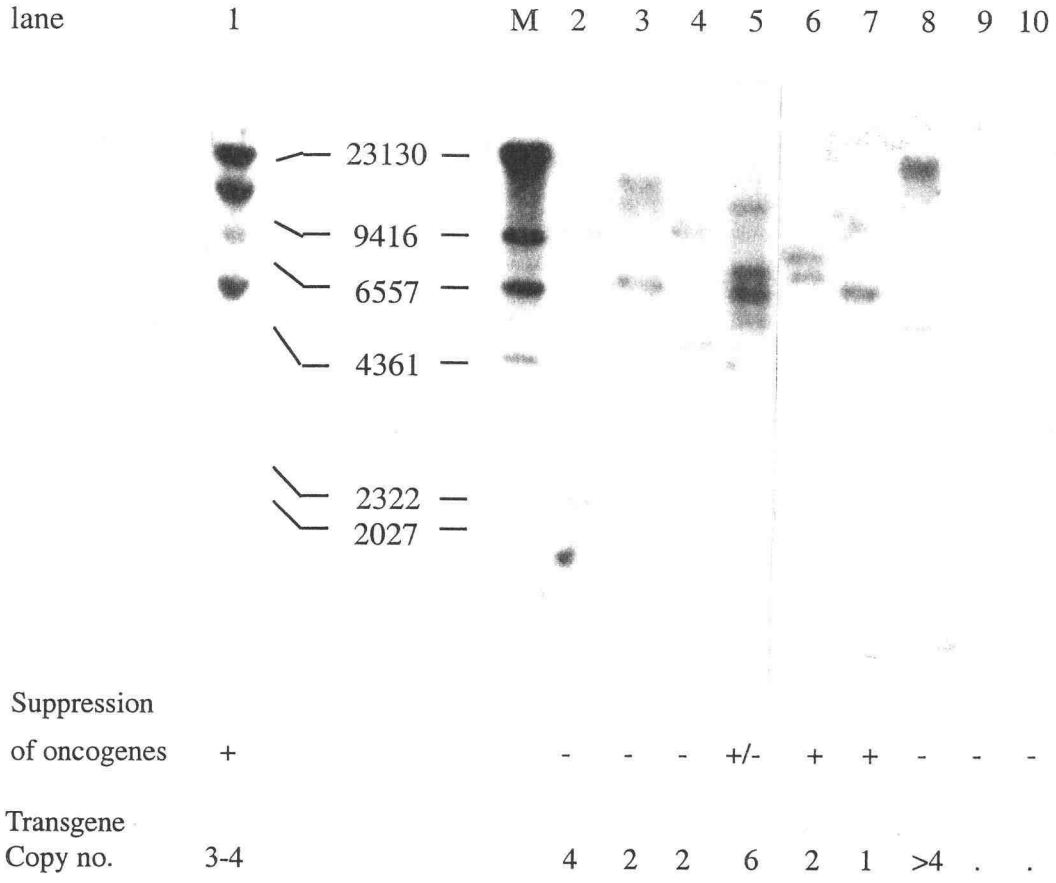


Figure 2.12. Southern blot analysis of transgenic plant genomic DNA (5 mg) digested with *Eco*RI. M: *Hin*dIII lambda DNA marker, lane 1: TDP1 B7, lane 2: CW4 B1, lane 3: CW4 B11, lane 4: CW4 B22, lane 5: CW4 B30, lane 6: TDP1 B17, lane 7: TDP1 B27, lane 8: TDP1 B31, lane 9: PEV6 B2, lane 10: PEV6 B14

lanes 3&4). Three strong bands (18.5, 11, and 5.1 kb) and a weak fourth band (7.4 kb) were observed in TDP1 B7 (Figure 2.12, lane 1), whereas two and one copies were detected in TDP1 B17 (7.1 and 6.2 kb) and TDP1 B27 (5.8 kb), respectively (Figure 2.12, lanes 6&7). In TDP1 B31, clear bands occurred at approximately 20 kb and 5 kb (Figure 2.12, lane 8). However, two other faint bands were observed at 7.4 kb and 10 kb. No bands were detected in the vector-only control lines, PEV6 B2 and PEV6 B14 (Figure 2.12, lanes 9&10).

In the CW1 lines, a 682-bp fragment is expected when genomic DNA is digested with *Hin*fI, and a left-hand junction fragment larger than 2.2 kb is anticipated when genomic DNA is digested with *Eco*RI. The 682-bp *Hin*fI fragment was detected in both CW1 K27 and CW1 K52 (Figure 2.13). CW1 K27 had two other *Hin*fI fragments that were larger than the 682-bp fragment. These may result from T-DNA rearrangements. The *ipt* stop transgene (contained in pUC119) was also digested with *Hin*fI to estimate the copy number of each T-DNA. When compared with the 5 copy reconstruction, CW1 K27 had at least two copies while CW1 K52 had one copy (Figure 2.13, lanes 1-3). The *Eco*RI-digested DNAs confirmed this result (Figure 2.13, lanes 4 & 5).

Post-transcriptional gene silencing is characterized by reduced levels of mRNA accumulation while transcription is not affected (Baulcombe, 1996). Northern blot analysis of total RNA from various transgenic plants was done to determine the levels of transcript accumulation. Methylene blue staining of the RNA blot revealed the rRNAs, which indicates the relative amounts of RNA loaded

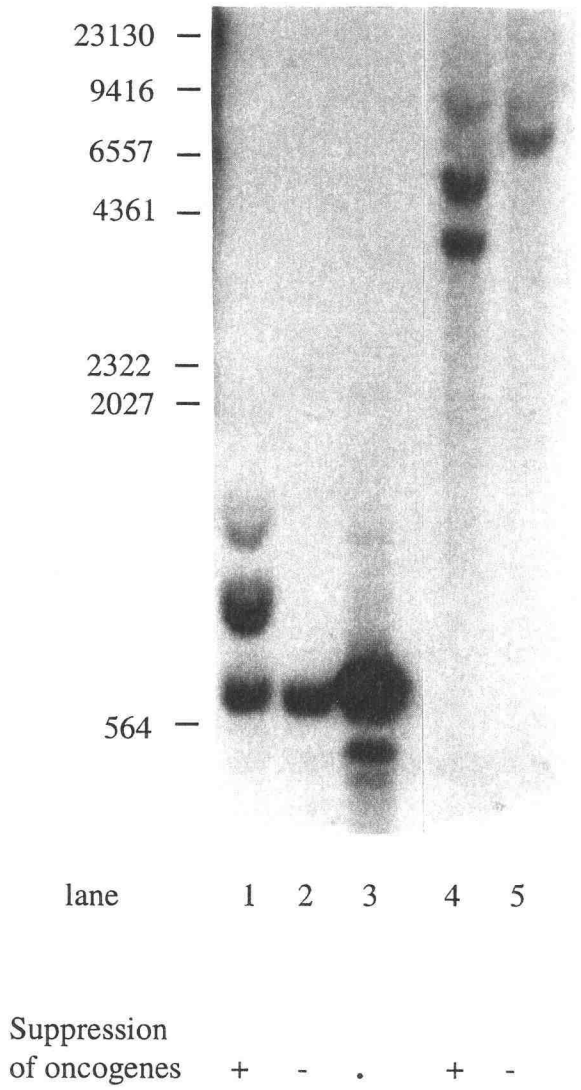


Figure 2.13. Southern blot analysis of CW1 plant lines. 10 mg of plant genomic DNA. Lane 1: CW1 K27 (*Hin*fI digest), lane 2: CW1 K52 (*Hin*fI digest), lane 3: pUC119 + *ipt* stop (*Hin*fI digest, 5 copy reconstruction, 100ng), lane 4:CW1 K27 (*Eco*RI digest), lane 5: CW1 K52 (*Eco*RI digest).

in each lane. To quantify accumulated levels of a constitutively expressed mRNA in the different transgenic plant lines, the blot was probed with a ^{32}P-labeled ubiquitin DNA as an internal control. *iaaM* stop transcripts are expected to be about 2.1 kb. *ipt* stop and *ipt* stop-*iaaM* stop transcripts should be 1 kb and 2.8 kb, respectively.

We detected transcripts of the appropriate size in the CW4 and CW1 lines (Figure 2.14). Different amounts of *ipt* stop transgene mRNA accumulated in two CW1 lines, which indicates that the transgene mRNA of tumor suppressing line, CW1 K27, may be degraded while *ipt* stop mRNA accumulated to a high level in the CW1 K52 line. The northern blot of TDP1 and CW4 hybridized with ^{32}P-labeled *iaaM* antisense RNA showed no *iaaM* transgene mRNA in the TDP1 lanes (Figure 2.15, lanes 1-4), while transcripts in CW4 lines were detected at the appropriate size (Figure 2.15, lanes 5-8). There are two ways to explain undetectable mRNA in this northern blot (Figure 2.15): 1) the mRNA is transcribed but degraded to undetectable levels due to PTGS, or 2) the promoter in this particular transgene is inactive, so mRNA is not made. For lines TDP1 B7, B17, and B27, because these lines co-suppress *iaaM* of the incoming T-DNA, the first explanation is likely to have occurred (Figure 2.15, lanes 1-3). While in the case of the non-suppressing TDP1 B31 line, the promoter may be inactive and so the transgene was not be transcribed (Figure 2.15, lane 4). The light bands detected in all the lanes are a background of rRNAs as shown in the methylene blue staining of the blot. When this northern blot was probed with a nick translated ^{32}P-labeled

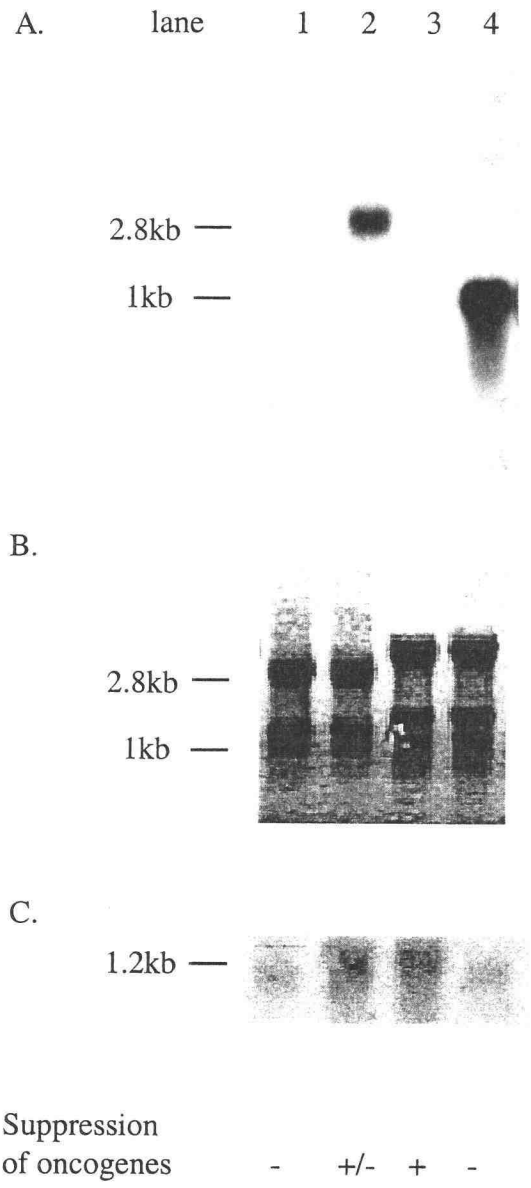


Figure 2.14. Northern blot analysis of CW4 and CW1 plant lines (10 mg of total RNA).
Lane 1: CW4 B22, lane 2: CW4 B30, lane 3: CW1 K27, lane 4: CW1 K52.
A. RNA blot probed with ^{32}P-labeled *ipt* stop. B. Methylene blue stained RNA blot.
C. RNA blot probed with ^{32}P-labeled TB112 (ubiquitin).

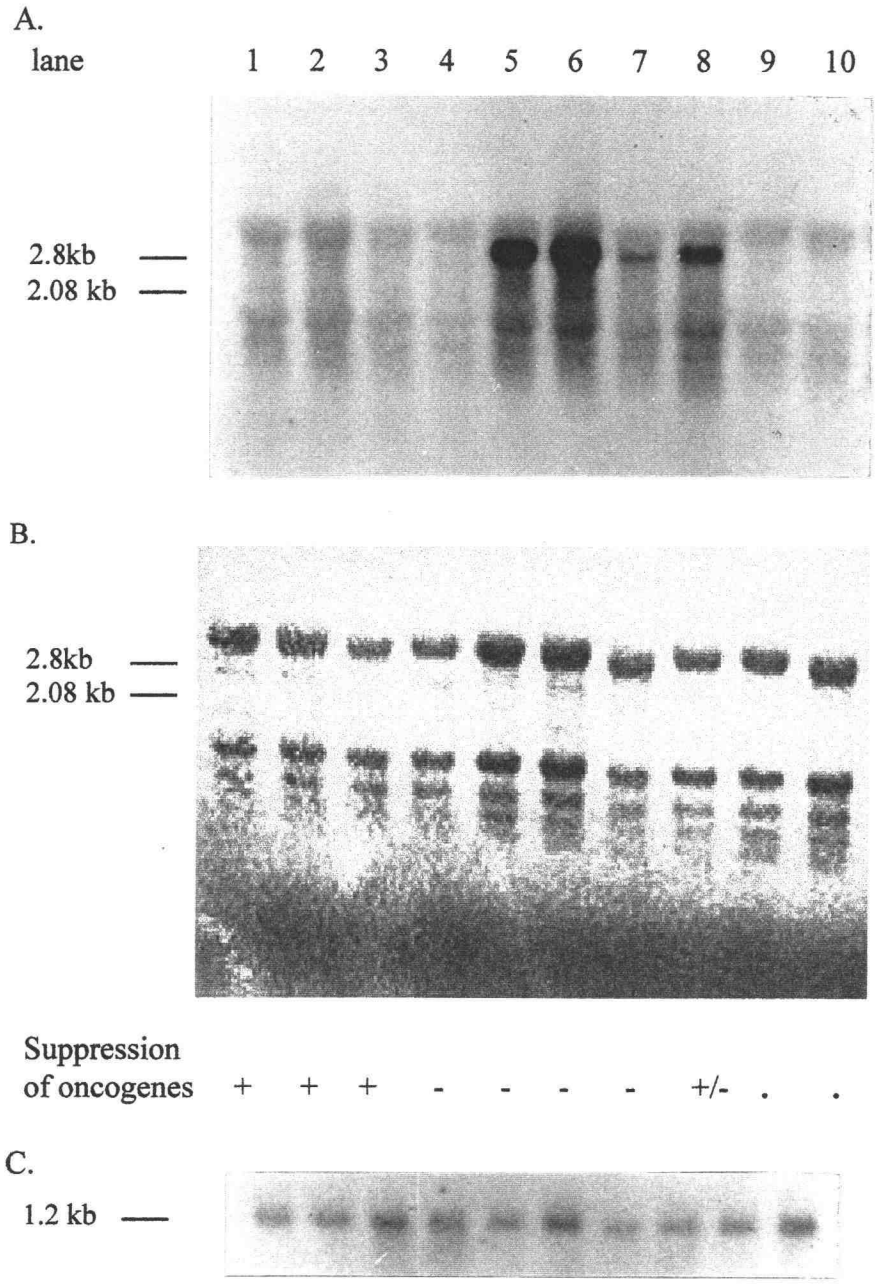


Figure 2.15. Northern blot analysis of total RNA (10 μg) from transgenic plant lines. Lane 1: TDP1 B7, lane 2: TDP1 B17, lane 3: TDP1 B27, lane 4: TDP1 B31, lane 5: CW4 B1, lane 6: CW4 B11, lane 7: CW4 B22, lane 8: CW4 B30, lane 9: PEV6 B2, lane 10: PEV6 B14.

A. RNA blot hybridized with ^{32}P-labeled *iaaM* antisense riboprobe.

B. Methylene blue stained RNA blot.

C. RNA blot probed with ^{32}P-labeled TB112 (ubiquitin).

iaaM PCR product, we detected an additional band of approximately 3.4 kb in all the TDP1 lanes (Figure 2.16, lanes 1-4). Because this band was not detected with the strand-specific antisense RNA probe (Figure 2.15, lanes 1-4), it apparently results from antisense transcription of the transgene, perhaps from the adjacent nopaline synthase *npt*II gene. However, the nick-translated probe did not allow us to detect the 2.1 kb sense-strand *iaaM* stop transcript, while those of CW4 were detected.

Discussion

We generated transgenic plant lines expressing nonsense mutated T-DNA oncogenes driven by an enhanced CaMV 35S promoter to determine whether these transgenic plants were resistant to incoming T-DNA oncogenes. The transgenic plants were inoculated with *A. tumefaciens* strains selected to complement suppression of the mutated transgene(s) in the plant. We found that the level of suppression in our transgenic plants varied among the individual plant lines. This variation has been observed in other gene silencing studies as well (Matzke et al., 1994b; Van der Krol et al., 1990). The variability may be due to features of the transgene themselves, such as integration sites, structures of the integrated T-DNAs, expression level, and copy number.

35 out of 63 *iaaM* stop transgenic plant line (TDP1) suppressed the target oncogenes. In contrast, only one line expressing *ipt* stop (CW1) out of 45 exhibited co-suppression of T-DNA *ipt* genes. Our *ipt* stop-*iaaM* stop plant line (CW4), when inoculated with the wild-type *A. tumefaciens*, did not prevent tumor growth.

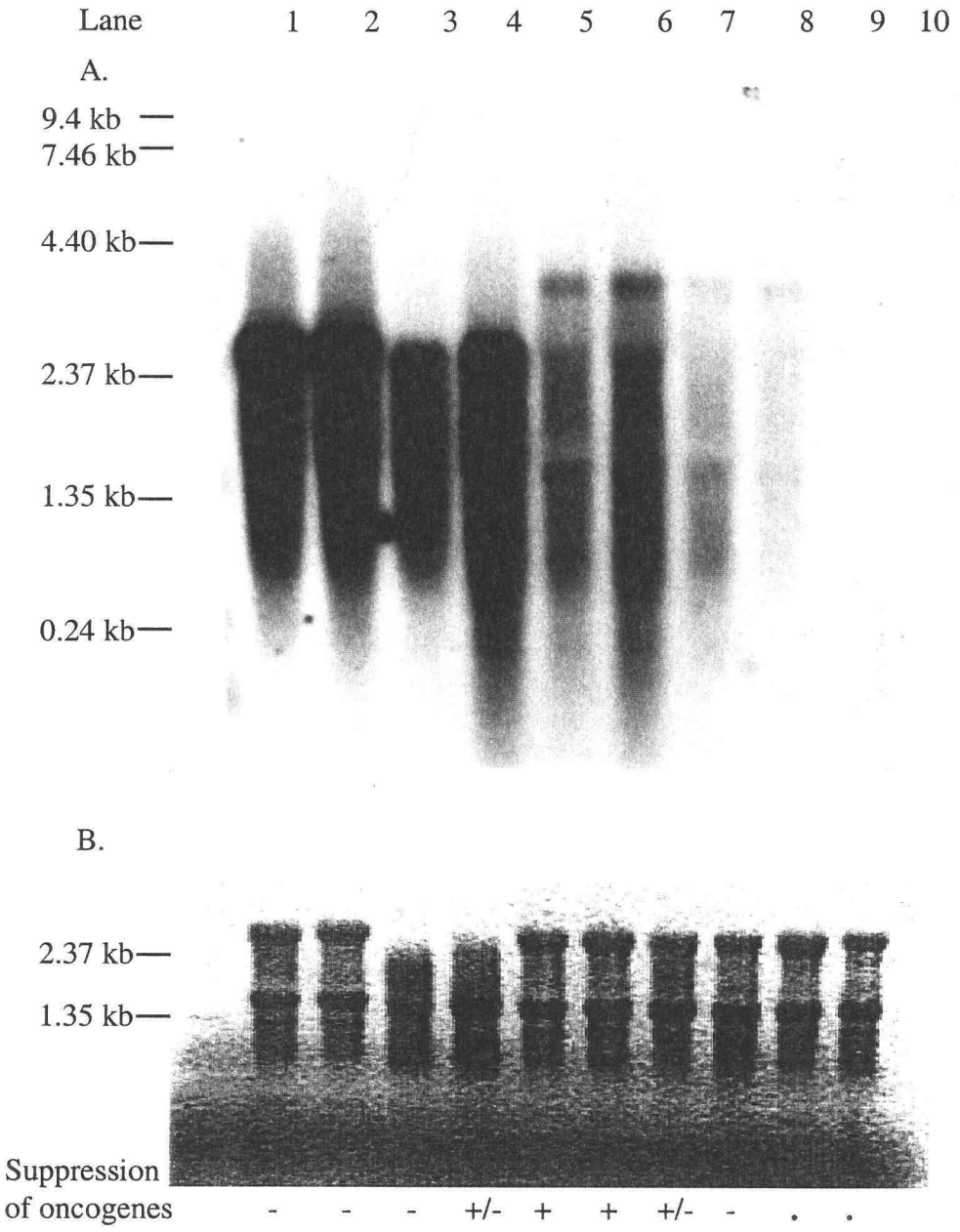


Figure 2.16. Northern blot analysis of RNA blot hybridized with nick translated ^{32}P-labeled double stranded *iaaM* probe. Lane 1: CW4 B1, lane 2: CW4 B11, lane 3: CW4 B22, lane 4: CW4 B30, lane 5: TDP1 B7, lane 6: TDP1 B17, lane 7: TDP1 B27, lane 8: TDP1 B31, lane 9: PEV6 B2, lane 10: PEV6 B14.
A. Film exposed for 2 weeks.
B. Methylene blue stain of RNA blot.
C. Film exposed for 1 week.

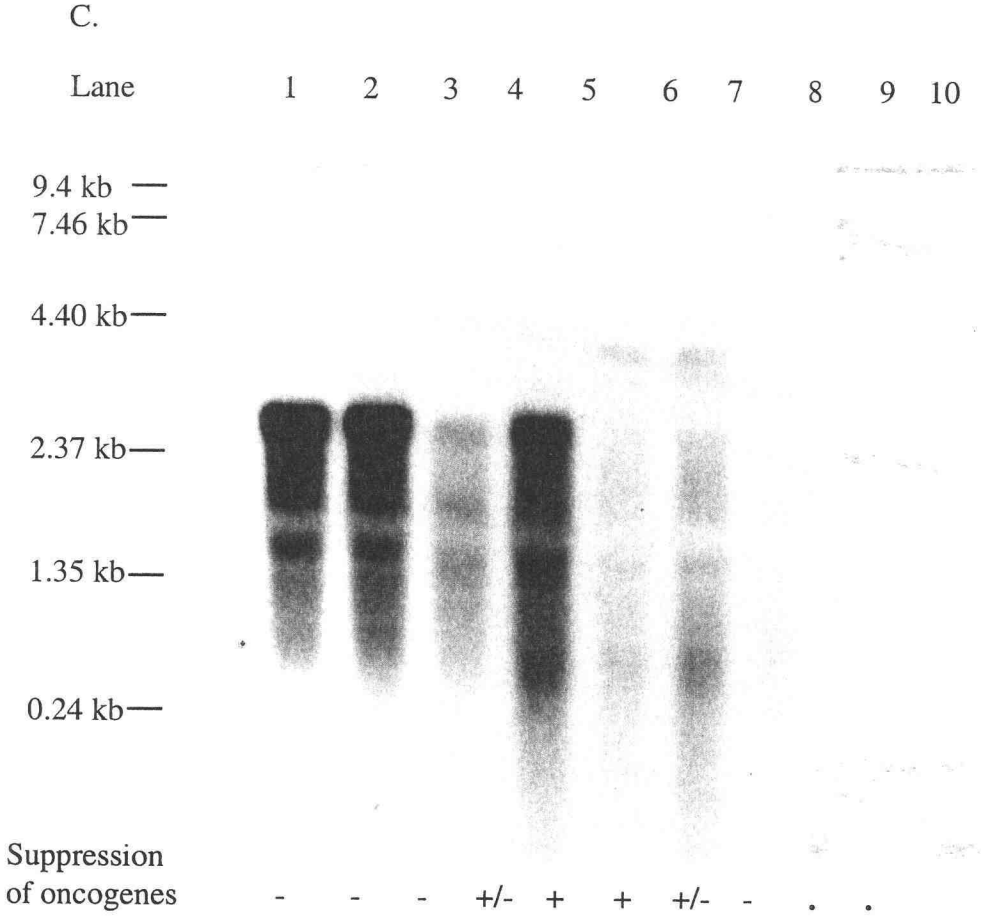


Figure 2.16, continued.

However, one of these lines exhibited partial suppression when inoculated with other *A. tumefaciens* strains. Since the flanking sequences are identical for all the transgenes in the plants and because of the number of lines tested for each transgene, the transgene sequence affected its efficiency at eliciting co-suppression. Specifically *iaaM* stop was much more effective than the other constructs, and embedding *iaaM* stop within *ipt* stop diminished the effectiveness of *iaaM* stop. Some studies indicate there might be consensus sequence regions in the RNA target where the silencing signal identifies the target thereby triggering the silencing mechanism (English et al., 1996; Goodwin et al., 1996; Marano and Baulcombe, 1998; Sijen et al., 1996). However, a recent study done by Palauqui et al., (1999) suggests that it is the length of the target rather than the sequence that triggers silencing. They show that bombarding a 247-bp *Nia*2 gene caused localized silencing but not systemic acquired silencing (Palauqui et al., 1999). This may be the reason our *ipt* stop-*iaaM* stop construct does not work, since we reduce the length of the *ipt* gene by inserting *iaaM* stop into it.

High copy number has been shown to be more effective silencers (Hobbs et al., 1993; Matzke et al., 1993). In our studies, high transgene copy number did not always correlate with strong suppression. Line CW4 B30 (*ipt* stop-*iaaM* stop) contains six copies (Figure 2.12, lane5) but did not completely suppress the T-DNA oncogenes as well as line TDP1 B31 (*iaaM* stop), which has at least four copies (Figure 2.12, lane 8) and it does not suppress at all. Line TDP1 B27 (*iaaM* stop) has one copy but suppresses *iaaM* gene (Figure 2.12, lane7).

The degree of co-suppression is indicated by mRNA accumulation (Baulcombe, 1996; Depicker and Van Montagu, 1997). Generally, transgene mRNA accumulation was high in non-suppressing lines and undetected or low in suppressing lines, such as the *ipt* stop (CW1) lines where the non-suppressing line accumulated to high levels while the suppressing line showed no accumulation (Figure 2.14, lane 3-4). The one *ipt* stop-*iaaM* stop line (CW4 B30) that exhibited partial suppression of target genes accumulated reduced levels of transgene mRNA relative to lines that did not suppress (e.g. CW4 B1 and B11) (Figure 2.15, lane 5-8). An *iaaM* stop (TDP1 B31) line was an exception, this line did not suppress *A. tumefaciens iaaM* expression contained undetectable transgene mRNA which may be due to promoter inactivation (Figure 2.15, lane 4).

Putative antisense transgene transcript was detected only in *iaaM* stop (TDP1) lines, which were the only effective inducers of co-suppression. The strength of the signal was strongest in the two lines (TDP1 B7 and B17) that most effectively suppressed tumor growth (Figure 2.16, lane 5-6). This suggests that dsRNA may elicit co-suppression; the putative antisense transcript of *iaaM* stop hybridizes with transgene mRNA to form dsRNA which may be degraded by ds-specific RNAses. Recent studies done with the nematode *C. elegans* have shown that microinjection of dsRNA caused more effective silencing than either sense or antisense strands individually (Fire et al., 1998; Montgomery et al., 1998; Montgomery and Fire, 1998). dsRNA-induced gene silencing has also been observed in transgenic plants (Waterhouse et al., 1998). However, the one

suppressing *ipt* stop plant line did not produce antisense transcript. Therefore, it seems that several means rather than one exclusive process may initiate PTGS.

At present, we are unable to prove the mechanism by which T-DNA oncogene silencing occurs. However, we think T-DNA oncogene silencing is due to co-suppression. No single mechanism proposed so far can explain all the examples where loss of gene expression has occurred. However, in spite of the complexity of gene silencing in transgenic plants, the different mechanisms may be related at the most fundamental level. Gene silencing-derived resistance may be an effective way for developing pathogen-resistant transgenic plants. Better understanding of the gene silencing mechanism will enable us to not only decipher how the plant regulates unwanted gene expression but also improve our plant biotechnology. The production of *Agrobacterium*-resistant transgenic plants will play a significant role in agricultural improvement.

Chapter 3. Summary and Future Plans

In summary, we have shown that nine *iaaM* stop (TDP1) lines and one *ipt* stop (CW1) line effectively suppressed the target T-DNA oncogenes when inoculated with the appropriate mutant bacteria. We also have one *ipt* stop-*iaaM* stop (CW4) line that partially suppressed both oncogenes. The present study demonstrates that T-DNA oncogenes can be suppressed. The resistance status seems to be associated with low levels of mRNA accumulation. Our study indicates, with the correct type of transgene expression, T-DNA oncogenes can be suppressed completely. Because broad-host-range *A. tumefaciens* share homology in their oncogenes, plants resistant to one strain should be resistant to other strains.

Further studies will focus on improving the constructs used in generating resistant transgenic plants and trying to determine the mechanism(s) involved in silencing. One of the *iaaM* stop suppressing lines was crossed with *ipt* stop suppressing line to produce plants containing both *ipt* stop and *iaaM* stop transgenes on separate T-DNA to investigate the resistance of these plants with wild-type *Agrobacterium*. Constructs designed to produce dsRNA will also be tested. Virus resistance induced by dsRNA has been shown in plants (Waterhouse et al., 1998). A hairpin mRNA and a dsRNA formed from independent molecules were both effective triggers for silencing (Waterhouse et al., 1998). Other studies show that microinjection of dsRNA induced a homology-dependent and highly

effective decrease in the activity of the corresponding homologous gene in nematodes (Fire et al., 1998; Montgomery et al., 1998; Montgomery and Fire, 1998). Fire et al. (1998) found that only a few molecules of dsRNA were required to achieve effective silencing, while a high dose ($3.6x10^6$ molecules per gonad) was required to observe similar silencing using either single sense or antisense RNA strands. Fire et al. (1998) also show that even though annealing of sense and antisense strand RNA before injection is not a prerequisite, more than an hour interval between the sequential injection of sense and antisense RNA resulted in a dramatic decrease of silencing. Because we believe dsRNA may play a critical role in eliciting PTGS, our constructs will generate both sense and antisense transcripts.

dsRNA-mediated interference is extremely target specific and is able to cross cellular boundaries (Fire et al., 1998). Palauqui et al. (1997) have demonstrated that silencing could be transmitted from silenced stocks to nonsilenced scions expressing the corresponding transgene but silenced scions grafted onto wild-type stocks or nonsilenced stocks did not cause silencing to occur in the stocks. The transmission was systemic, unidirectional, and transgene specific. Systemic acquired silencing seems to propagate upward (Palauqui et al., 1997; Voinnet and Baulcombe, 1997). Infiltration studies and bombardment studies show the systemic silencing signal through the vascular tissues (Palauqui et al., 1999; Voinnet and Baulcombe, 1997). This systemic induction of PTGS will help us in transmitting *Agrobacterium*-resistance to plants by grafting from silenced stocks to non-silenced scions and/or vice versa. In plants (e.g. walnut and grape)

where *Agrobacteria*-infection is a problem, resistant rootstocks will be an advantage. Because relatively few rootstocks are used compared to the large variety of different scions in use. And since only the rootstocks are genetically altered and not the scions, the fruits of the plant will not be genetically engineered.

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APPENDICES

Appendix 1: Inoculating Tobacco plants with Agrobacteria

Materials;

- 20x AB salt (1 liter), autoclaved
  - NH_4Cl 20g
  - $MgSO_4 \cdot 7H_2O$ 6g
  - KCl 3g
  - $CaCl_2$ 0.2g (or 1.36ml/l of 1M $CaCl_2$)
  - $FeSO_4 \cdot 7H_2O$ 50mg (or 18ml/l of 10mM $FeSO_4$)
- 20x AB buffer (1 liter), autoclaved
  - K_2HPO_4 60g
  - NaH_2PO_4 20g
- YEP broth (1 liter), autoclaved
  - Peptone 10g
  - Yeast extract 10g
  - NaCl 5g
- YEP plates, autoclaved: YEP broth + 1.5% Bacto agar
- AB minimal/ Glu plates
  - 0.5% Glucose + 1.5% Bacto agar + dH_2O, autoclave
  - Add sterile 20x AB salts and sterile 20x AB buffer
- Sterile Petri-plates
- Sterile toothpicks, autoclaved

Method;

1. Grow Agrobacteria on AB min/Glu plates or YEP plates at 29°C for 3 days.

2. Using a sterile toothpick wound the stem of the plant (about 1cm).

3. Then with a new sterile toothpick, scrape off a sufficient amount of bacteria from the plate to fill the inoculation site.

4. Parafilm the wound site to prevent the bacteria from drying up.

5. Take off the parafilm after 4weeks postinoculation. Observe for tumor growth.

Appendix 2: Extracting DNA from Tobacco Leaves

Material;

- 1.7 ml epphendorf tubes
- 100% ethanol
- TE (10/0.1):10mM Tris pH 7.5, 0.1mM EDTA, autoclaved
- 7.5M ammonium acetate, autoclaved
- Microcentrifuge

Methods;

1. Aliquot 500µl of supernatant saved from RNA extraction per epphendorf tubes.

2. Add 1ml of 100% ethanol. Mix well. Incubate at 4°C for at least 15 minutes.

3. Centrifuge at top speed in microcentrifuge for 15 minutes.

4. Resuspend the pellet in 200µl of TE(10/0.1).

5. Add 100µl of 7.5 M ammonium acetate and 600µl of 100% ethanol. Mix well. Incubate at 4°C for 15 minutes.

6. Centrifuge at top speed for 15 minutes.

7. Resuspend pellet in 50µl of TE(10/0.1).

8. Quantitate DNA. Store at –20°C.

A260 x dilution factor x 50 = µg/ml

Appendix 3: Purifying Plant Genomic DNA using CsCl Density Gradient Centrifugation

Materials;

- RNase A (100mg/ml)
  10mg/ml of RNase A dissolved in ddH_2O. heat to 100°C for 15 minutes. Allow to cool slowly to room temperature. Store at –20°C.
- Cesium Chloride (CsCl)
- TES buffer: 50mM Tris pH 8, 5mM EDTA, 50ml NaCl, autoclaved
- Ethidium bromide (EtBr) – always wear gloves when handling EtBr
- H_2O-saturated n-butanol: add equal volumes of H_2O and n-butanol, shake well
- TE(10/0.1):10mM Tris pH 7.5, 0.1mM EDTA, autoclaved
- 100% EtOH
- dH_2O
- 15ml Falcon tubes
- Pasteur pipets
- Polyallomer 13x51mm quick-seal tubes no. 342412
- Beckman sealer
- Beckman vTi80 rotor
- Beckman L8-70M ultracentrifuge
- microcentrifuge
- 18ga syringe needle
- 1cc syringe

Methods;

1. Add 2.5µl of RNase A (100mg/ml) to DNA extractions. Leave at room temperature.

2. Pre-weigh 4.5g of CsCl into 15ml Falcon tube.

3. Add TES buffer and DNA extraction into a new pre-weighed Falcon tube and bring to 4g.

4. Add 4.5g of CsCl.

5. Using a Pasteur pipet as a funnel, load the solution (DNA/TES/CsCl solution) in Beckman polyallomer quick-seal tube.

6. Layer 30µl of 10mg/ml EtBr on top of the CsCl solution.

7. Fill to the base of the neck of the quick-seal tube with dH_2O. Avoid getting bubbles in.

8. Weigh the tubes. (Tubes should weigh about 9.6g.) Balance tubes within 50 to 100mg.

9. Heat seal the tube.

Beckman sealer should be warmed up beforehand.
Place metal cap on the tube. Then place under the heat sealer. Tap on the metal cap every few seconds. When it starts to feel a little mushy after you press down, hold for 3 seconds. Then move the tube over to the switch at the right hand side and press down for 20 seconds or so until the metal cap is slightly touching the shoulders of the tube.
It is important that you do not press down too hard.
Let the cap cool for a couple of minutes. Then take the cap off by twisting it with your fingers or by pulling up with pliers.

10. Check the seal by squeezing on the tube. Weigh the tube again.

11. Centrifuge at 65000rpm for 4 hours at 15°C, accel at 1, decel at 9, in Beckman vTi80 rotor.

12. Gently remove the tubes from the rotor so as not to disturb the band(s).

13. Punch a hole in the top of the tube to provide an air inlet. Recover the DNA band by inserting needle through tube wall directly below the band.

14. Extract EtBr by adding 1 volume of H_2O-saturated n-butanol. Mix by inverting. Spin for 2 seconds in microcentrifuge. Remove the butanol (top) layer and repeat extraction (4 to 5 times).

15. Dilute with 2 volumes of TE(10/0.1).

16. If sample volume is greater than 500µl, divide it between two tubes.

17. Add 2 volumes of 100% EtOH based on the volume of sample+TE.

18. Incubate at 4°C for at least 1 hour.

19. Centrifuge at top speed for 5 minutes in microcentrifuge.

20. Dry pellet. Resuspend pellet in TE(10/0.1).

21. Measure OD at 260nm.

Appendix 4: Extracting RNA from Tobacco Leaves

Material;

- Mortar and pestle
- 50ml disposable Falcon tubes
- 1.7ml epphendorf tubes
- Microcentrifuge
- Tobacco leaf
- Phenol
- Chloroform
- Cold 100% (or 95%) ethanol
- Cold 70% ethanol
- 4M LiCl, autoclaved*
- 5M NH_4OAc, autoclaved*
- Extraction buffer:100mM LiCl, 1% SDS, 100mM Tris pH 9, 10mM EDTA, dH_2O, autoclaved*
- ddH_2O, autoclaved*

* Solutions were put into bottles that had been baked beforehand to get rid of RNase.

Method;

1. Prechill mortar and pestle in the -20°C freezer.

2. Grind the tobacco leaf to fine powder in the presence of liquid nitrogen.
*Be careful not to let the leaf thaw. The tobacco leaf had been pick beforehand and keep in the -80 °C freezer so it was difficult to weigh out the amount of leaf used. About half of a moderate size tobacco leaf was used.

3. Carefully pour the ground sample into a sterile, disposable polypropylene tube (50ml Falcon tube) so that sample does not spatter.

4. Allow the liquid nitrogen to boil off completely.

5. Immediately add 2.5ml of RNA extraction buffer. Immediately add 2.5ml of phenol:chloroform. Vortex for 1 minute.

6. While the solution is still homogeneous, divide the solution into three 1.7ml epphendorf tubes. Discard any that remains.
* It is easier to pipet the solution if you cut off the front part of the tip a little before pipetting.

7. Centrifuge the epphendorf tubes in a microcentrifuge at top speed for 10 minutes.

8. Remove the aqueous (upper) phase from each of the three tubes and combine into a tube or tubes.

9. Add an equal volume of 4M LiCl to each tube.

10. Precipitate RNA overnight at -20°C.

11. Centrifuge at top speed for 10 minutes.

12. Remove the supernatant (save for DNA extraction). Resuspend pellet in 100 to 200 µl ddH_2O. Combine.

13. Add 0.4 volumes of 5M NH_4OAc. Add 2.5 volumes of cold 100% (or 95%) ethanol.

14. Precipitate RNA at -20 °C for 2 hours. Centrifuge at top speed for 10 minutes.

15. Decant the supernatant. Wash the pellet with cold 70% ethanol. Air-dry pellet. Resuspend in 25 µl of ddH_2O.

16. Quantitate RNA. Store at –20°C.

A_{260} x dilution factor x 40 = µg/ml

Appendix 5: Southern Blot

Restriction Digest of DNA

Materials;

- 37°C waterbath
- 1.7ml epphendorf tubes, sterile
- *Eco*RI restriction enzyme (NE Biolabs) & *Eco*RI buffer
- *Bam*HI restriction enzyme (NE Biolabs) & *Bam*HI buffer
- *Hin*fI restriction enzyme (NE Biolabs) & NE buffer 2

Method;

1. Combine:

5 to 10µg of plant genomic DNA	__µl
enzyme	0.5 to 1µl
10x enzyme buffer	3µl
dH_2O	__ µl
	Total 29µl

Incubate at 37°C for 4 hours.

Add 1 µl of enzyme. Incubate at 37°C overnight to ensure complete digestion.

Agarose Gel Electrophoresis

Materials;

- Ultra pure agarose (Gibco BRL)
- Ethidium bromide (EtBr) – always wear gloves when handling EtBr
- TAE: 0.04M Tris-acetate, 2mM EDTA

  1 liter of 50x:

Tris base	242g
Glacial acetic acid	57.1ml
0.5M EDTA pH 8	100ml

- *Hin*dIII-cut lambda DNA ladder
- Loading buffer: 0.25% bromphenol blue, 50% glycerol
- Gel apparatus: comb, gel tray, gel chamber, EC600 power supply (E-C Apparatus Corporation, St. Petersburg, Florida)
- UV light
- Camera
- Microwave

Method;

1. Make 0.8% agarose gel (150ml of TAE+ 1.2g of agarose). Melt in microwave. Add 15µl of EtBr. Cool to about 70°C.

2. Pour gel into bed with comb in place. Allow to solidify (30 minutes). Pour a little of running buffer (TAE) and remove the comb.

3. Add 5µl of loading buffer to each sample.

4. Place gel in the buffer chamber. Fill the chamber with TAE.

5. Load entire sample into the individual wells of the gel.

6. Apply 100 volts. DNA will migrate to the positive electrode.

7. When the bromphenol blue dye nears the bottom of the gel, observe the gel under UV light and photograph. To protect the gel from UV light, layer a plastic wrap between the UV and gel surface.

Denaturation and Blotting of DNA Gel

Materials;

- Container or tray
- Gloves
- Gene Screen Plus
- Whatman 3mm filter paper
- Paper towels
- Plastic wrap
- Heat block (used as weight)
- Stratagene 1800 Stratalinker
- 0.25M HCl
- NaOH/NaCl solution: 0.5M NaOH, 1.5M NaCl
- Tris/NaCl neutralization buffer: 1M Tris pH 7.5, 1.5M NaCl

Tris base	157.6g
NaCl	87.7g
concentrated HCl	67.7ml
dH_2O	810ml

- 20x SSC: 3M NaCl, 0.3M sodium citrate pH 7

NaCl	350.4g
sodium citrate $2H_2O$	176.5g
concentrated HCl	7.2ml

Add dH_2O to final volume of 2 liters

- 0.2M Tris, pH 7.5 + 2x SSC

Tris base	24.2g
Concentrated HCl	14ml
dH_2O	880ml
20x SSC	100ml

Method;

1. Wash the gel twice for 8 minutes each in 0.25M HCl (100-200ml/wash). Rinse the gel with dH_2O after second wash.

2. Wash the gel twice for 15 minutes each in NaOH/NaCl solution. Rinse the gel with dH_2O after second wash.

3. Wash the gel twice for 15 minutes each in Tris/NaCl neutralization buffer.

4. Cut membrane (Gene Screen Plus) to exact size of gel. Wear gloves. Mark one corner of the membrane with soft pencil.

5. Float the membrane on dH_2O in a tray to wet it by capillary action.

6. Soak membrane in 10x SSC for 15 minutes.

7. Cut 8 sheets of Whatman 3mm filter paper to the exact size of the gel. Saturate the filters with 10x SSC and set 7 sheets on a large piece of plastic wrap.

8. Place agarose gel on the SSC-saturated Whatman 3mm paper. Invert the gel so the bottom face will contact the membrane. Remove air bubbles trapped between the gel and the filters.

9. Lay the membrane on top of the gel with the pencil mark down. Do not move the membrane once it has contacted the gel even if the gel and the filter are not properly aligned. Remove air bubbles.

10. Place 1 sheet of SSC-saturated Whatman 3mm paper on top of the membrane. Remove air bubbles. Put 3 inch stack of dry paper towels. Wrap the entire stack in plastic wrap. Set a modest weight on top.

11. Allow DNA transfer to continue for 2 to 16 hours. Transfer is complete when the gel becomes 1mm thick.

12. Wash membrane for 20 minutes at room temperature with 0.2M Tris, pH 7.5 + 2x SSC.

13. Place membrane (pencil marked side up) on dry Whatman 3mm paper. Just as the membrane begins to dry, irradiate it with 1200μJoules of UV light using Stratagene 1800 Stratalinker or bake at 80°C in a vacuum oven for 2 hours.

*Store membrane dry between sheets of Whatman 3mm paper for several months at room temperature. For long term storage, place membranes in a desiccator at room temperature or 4°C.

Probe preparation

Materials;

- 1.7ml epphendorf tubes, sterile
- Nick translation-Gibco BRL kit
- Riboprobe *in vitro* transcription -Promega kit
- 16°C waterbath
- Scintillation counter
- Microcentrifuge
- Beckman GP centrifuge (swinging-bucket rotor)
- NICK spin column (Pharmacia)
- Geiger counter
- Area to handle radioactive isotopes and prepare radioactive reactions
- ^{32}P-labeled dCTP (3000Ci/mmole)
- ^{32}P-labeled rUTP (3000Ci/mmole)
- TE-saturated phenol:$CHCl_3$
- $CHCl_3$:isoamyl alcohol (24:1)
- 7.5M ammonium acetate
- 100% EtOH
- 70% EtOH
- ddH_2O
- ice

Method;

A. Nick translation-Gibco BRL kit

1. In 1.7 ml epphendorf tube (mix on ice)
   1µg of plasmid DNA
   5µl of dNTP minus dCTP
   add dH2O to 35µl total
2. Add 10µl of ^{32}P-labeled dCTP (3000Ci/mmole).
3. Add 5µl of DNase I/ DNA polymerase I.
4. Incubate at 16°C for 30 minutes.
5. Separate unincorporated label from the incorporated label with NICK spin column (Pharmacia).
6. Determine the number of counts per minute (cpm) in a scintillation counter. A good probe should have 10 to 100 million cpm/µg of input DNA.

B. Riboprobe *in vitro* transcription -Promega kit

1. Mixture should be kept at room temperature during addition of each successive component, since DNA can precipitate in the presence of spermidine if kept at 4°C.

5x transcription buffer	5 µl

100mM DTT	2.5 µl
Recombinant RNasin Ribonuclease inhibitor(20U)	0.5 µl
2.5mM rNTP minus rUTP	4 µl
linearized template DNA	2 µl
^{32}P-rUTP (3000Ci/mmole)	10 µl
T7 RNA polymerase(17U)	1 µl

Add dH_2O to total 25µl
2. Incubate at 37°C for 1 hour.
3. Add 1 µl of RQ1 RNase-free DNase to a concentration of 1U/µg of template DNA.
4. Incubate at 37°C for 15 minutes.
5. Extract with 1 volume of TE-saturated phenol:$CHCl_3$. Vortex for 1 minute.
6. Centrifuge (12000xg) for 2 minutes.
7. Transfer the upper, aqueous phase to a fresh tube.
8. Add 1 volume of $CHCl_3$:isoamyl alcohol (24:1). Vortex for 1 minute. Centrifuge for 2 minutes.
9. Transfer the upper, aqueous phase to a fresh tube. Add 0.5 volumes of 7.5M ammonium acetate and 2.5 volumes of 100% EtOH.
10. Mix and place at –70°C for 30 minutes. Centrifuge for 20 minutes.
11. Remove the supernatant and wash pellet with 1ml of 70% EtOH. Resuspend pellet with ddH_2O.
12. Determine the number of counts per minute (cpm) in a scintillation counter.

Hybridization & Membrane Wash

Materials;

- Plastic container or bag
- Kodak XAR X-ray film
- Film cassette
- Prehybridization/hybridization solution: 50% Formamide,1% SDS, 1M NaCl, 10% dextran sulfate, 1mg/ml of denatured salmon sperm DNA
- Salmon sperm DNA (10mg/ml), sonicated for 2 minutes, boiled for 3 minutes and chilled on ice
- 2x SSC
- 2x SSC+ 1% SDS
- 0.1x SSC + 1% SDS
- Denatured probe

Method;

1. Place membrane in a plastic container or bag.

2. Prehybridize membrane in 45ml of prehybridization solution. Agitate gently for at least 2 to 4 hours.

3. Add 10^6 to 10^7 cpm of denatured probe (Boil the probe for 5 minutes. Chill on ice.).

4. Incubate at 42°C overnight, gently agitating.

5. Remove hybridization solution.

6. Wash the membrane twice for 5 minutes with 100ml of 2x SSC at room temperature.

7. Wash membrane twice for 30 minutes each with 200ml of 2x SSC+ 1% SDS at 65°C.

8. Wash membrane twice for 30 minutes each with 100ml of 0.1x SSC + 1% SDS at 42°C.

9. Seal the membrane in plastic wrap will it is still damp. This will allow you to later strip the probe from the membrane and re-hybridize with different probe.

10. Under a safelight, load the membrane into a film cassette. Set a sheet of Kodak XAR X-ray film on top, then place an intensifying screen (DuPont Cronex) over both. Expose film for 2 weeks at –80°C. exposure time will vary with the specific activity of the probe and how much probe is bound to the target.

11. Develop film with X-OMat (Kodak RP X-OMat Processor, Model M6B).

Stripping Southern blot- to re-probe the membrane

Materials;

- Glass beaker
- Glass container or tray
- Whatman 3mm paper
- 0.1% of SDS

Method;

Boil 200ml of 0.1% of SDS. Place membrane in glass tray. Pour the boiling solution onto the membrane. Cool to room temperature. Place membrane onto dry Whatman 3mm paper and let membrane dry. Store membrane between Whatman 3mm paper at room temperature for later use.

Appendix 6: Northern blot

All solutions and apparatus used with RNA must be RNases-free.
All glassware and bakable apparatus were baked at 230°C. Big plastic apparatus were wiped with RNase Away (Molecular Bio-Products, available through Fisher) or RNase-OFF (CPG, Inc. Lincoln Park, NJ) while small ones were soaked in boiled 0.2% SDS/0.2% EDTA solution.
All the solutions were made with ddH_2O (MilliQ dH_2O).
Important to always **wear gloves** when working with RNA to prevent ribonuclease contamination.

Getting Rid of RNase

Material;

- 0.2% SDS + 0.2% EDTA solution
- Big glass container

Method;

1. 0.2% SDS + 0.2% EDTA solution was heated to a boil.

2. Pour the heated solution into a big container.

3. Soak the small glassware, gel apparatus (comb, spacer, etc) for 30 minutes.

4. Rinse with dH_2O.

Agarose/formaldehyde Gel Electrophoresis

Material;

- 0.5M EDTA (EDTA has to be dissolved in pH 8) FW=372.24
  7.44g EDTA + 30ml ddH_2O
  pH to pH 8 to dissolve EDTA. Bring up to 40ml.
- 3M sodium acetate
- 3M NaOH
- 10x MOPS buffer (1 liter)
  41.8g MOPS [3-(N-morpholino)-propanesulfonic acid] FW=209.3
  800ml ddH_2O
  Adjust to pH 7.
  Add 16.6ml of 3M sodium acetate and 20ml of 0.5M EDTA.
  Bring to 1 liter final volume. Filter sterilize. Store at 4°C.
- 50mM NaOH, autoclaved
- 6x formaldehyde loading buffer: 1mM EDTA pH 8, 0.25% bromphenol blue, 50% glycerol
- 37% formaldehyde
- formamide
- 0.24-9.5 Kb RNA Ladder (Gibco BRL)

- Ultra pure agarose (Gibco BRL)
- Gel apparatus: comb, gel tray, gel chamber, EC600 power supply (E-C Apparatus Corporation, St. Petersburg, Florida)
- 1.7ml epphendorf tubes, sterile
- 55°C waterbath

Method;
1. Heat 1% agarose/ddH_2O suspension (in microwave) to dissolve. 2g of agarose + 174ml ddH_2O. Cool down to 60°C.

2. Add 20ml of 10x MOPS buffer and 6ml of 37% formaldehyde. (200ml total.) Pour into gel tray and let it set.

3. Combine:

5 to 10µg of RNA sample	
10x MOPS	6µl
37% formaldehyde	10.7µl
formamide	25µl
ddH_2O	
total	60µl

Incubate at 55°C for 15 minutes.
Add 10µl of formaldehyde loading buffer.

4. Pour some 1x MOPS buffer on the gel and remove the comb.

5. Place gel in the gel chamber and fill the chamber with 1x MOPS buffer as the running buffer.

6. Load the samples into the individual wells of the gel.

7. Apply 100 volts until the bromphenol blue dye migrates half way down the gel.

Denaturation and Blotting of RNA Gel
Material;
- 50mM NaOH
- Tris/NaCl neutralization buffer
- 10x SSC
- Container or tray
- Gloves
- Gene Screen Plus nylon membrane (DuPont)
- Whatman 3mm filter paper
- Paper towels
- Plastic wrap

- Heat block (used as weight)
- Stratagene 1800 Stratalinker

Method;
1. Rinse gel 3 times with 200ml dH_2O to remove fomaldehyde; 6 minutes total.

2. Soak the gel with agitation in 250ml of 50mM NaOH for 30 minutes at room temperature.

3. Neutralize for 30 minutes in Tris/NaCl neutralization buffer, then place the gel in 10x SSC.

4. Cut Gene Screen Plus to exact size of gel. Mark one corner of the membrane with a soft pencil.
Wear gloves and use the liner sheet to keep the nylon membrane clean.

5. Float the membrane on distilled water in a tray to wet the membrane by capillary action.

6. Soak the membrane in 10x SSC for 15 minutes.

7. Cut 8 sheets of Whatman 3mm filter paper to exact size of the gel and saturate in 10x SSC.

8. Place 7 sheets on a large piece of plastic wrap.

9. Place the agarose gel on the SSC-saturated Whatman 3mm paper.

10. Invert the gel so the bottom face will contact the membrane. Use finger pressure to remove air bubbles.

11. Lay membrane with the pencil mark down on top of the gel.

12. Once the membrane contacts the gel, do not move it. Use finger pressure to remove the air bubbles.

13. Place 1 sheet of SSC-saturated Whatman 3mm paper on top of the membrane and remove air bubbles.

14. Cover this with a 3 inch stack of dry paper towels cut to the same size as the gel.

15. Wrap the entire stack in the plastic wrap and set a modest weight on top of the paper towels.

16. Allow RNA transfer to continue for 2 to 16 hours. Transfer is complete when the gel becomes 1mm thick.

17. Rinse membrane in 2x SSC for 5 minutes at room temperature with agitation. Place membrane pencil-marked side up on dry Whatman 3mm paper. Just as the membrane begins to dry, irradiate it with 1200μJoules of UV light. This links the RNA permanently to the membrane.

Methylene Blue Staining of RNA blot

Materials;

- 5% CH_3COOH
- 0.5M NaOAc/0.04% methylene blue
- Container

Method;

1. Soak membrane in 5% CH_3COOH for 15 minutes.

2. Then soak the membrane in0.5M NaOAc/0.04% methylene blue for 5 to 10 minutes.

3. Rinse with ddH_2O until bands appear.

4. Take picture by placing the membrane on top of a light box. Make sure that the membrane does not dry out.

Probe preparation

Same as the procedures in Southern blot.

Hybridization and Membrane Wash

Materials;

- Prehybridization/hybridization solution: 50% Formamide,1% SDS, 1M NaCl, 10% dextran sulfate, 1mg/ml of denatured salmon sperm DNA
- 2x SSC
- 2x SSC+ 1% SDS
- 0.1x SSC + 1% SDS
- Denatured probe
- Plastic container or bag
- Kodak XAR X-ray film
- Film cassette

Method;

1. Place membrane in a plastic container or bag.

2. Prehybridize membrane in 45ml of prehybridization solution. Agitate gently for at least 2 to 4 hours.

3. Add 10^6 to 10^7 cpm of denatured probe (Boil the probe for 5 minutes. Chill on ice.).

4. Incubate at 42°C overnight, gently agitating.

5. Remove hybridization solution and wash the membrane twice for 5 minutes with 100ml of 2x SSC at 42°C.

6. Wash membrane 3 times for 20 minutes (each) with 100ml of 2x SSC + 1% SDS at 65°C.

7. Wash membrane 3 times for 20 minutes (each) with 100ml of 0.1x SSC + 1% SDS at 42°C.

8. Seal the membrane in plastic wrap while it is still damp. This will allow you to later strip the probe from the membrane and re-hybridize with different probe.

9. Under a safelight, load the membrane into a film cassette. Set a sheet of Kodak XAR X-
ray film on top, then place an intensifying screen (DuPont Cronex) over both. Expose film for 2 weeks at –80°C. exposure time will vary with the specific activity of the probe and how much probe is bound to the target.

10. Develop film.

Stripping RNA blot
Same as that of Southern blot.