A PRELIMINARY STUDY OF COTTON (+)-DELTA-CADINENE SYNTHASE WITH TRANSGENIC PLANTS

By

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A PRELIMINARY STUDY OF COTTON (+)-DELTA-CADINENE SYNTHASE WITH TRANSGENIC PLANTS

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NOMENCLATURE

bp	basepair
CaMV	cauliflower mosaic virus
CAT	chloramphenicol acetyltransferase
CDN1	(+)-delta-cadinene synthase
cdn1	(+)-delta-cadinene synthase gene
cfu	colony forming unit
CHS	chalcone synthase
cpm	count per minute
Dig	digoxigenin
FPP	farnesyl pyrophosphate
HR	hypersensitive reaction
min	minute
OMT	O-methyltransferase
PCR	polymerase chain reaction
PTGS	post-transcriptional gene silencing
rpm	rotation per minute
term	termination signal
Xcm	*Xanthomonas campestris* pv. *malvacearum*
Xcv	*Xanthomonas campestris* pv. *vesicatoria*

Chapter 1. Introduction

Phytoalexins and CDN1 Enzyme

Previous related work in our laboratory

Xanthomonas campestris pv. *malvacearum (Xcm)* is the agent of bacterial blight in cotton. When challenged with *Xcm*, bacterial-blight-resistant cotton lines respond by a hypersensitive reaction as well as by accumulating sesquiterpenoid phytoalexins, including 2,7-dihydroxycadalene (DHC), lacinilene C (LC) and lacinilene C 7-methyl ether (LCME) (Essenberg et al., 1982; Essenberg et al., 1990). In bacterial-blight-susceptible lines of cotton, the phytoalexin level remains low. A farnesol compound [presumably farnesyl pyrophosphate (FPP)] has been proven to be a precursor of those phytoalexins (Essenberg et al., 1985). In the proposed pathway leading from FPP to phytoalexins, the first intermediate is (+)-δ-cadinene, which has been identified as the product of cotton sesquiterpene cyclase activity, *i.e.* the activity of (+)-δ-cadinene synthase (CDN1) (Davis & Essenberg, 1995). The phytoalexins have been localized mainly in the hypersensitively necrotic cells around bacterial colonies in the inter-cellular spaces (Pierce & Essenberg, 1987; Essenberg et al., 1992a). A method has been developed to determine the concentration of phytoalexins in necrotic cells (Essenberg et al., 1992b). The concentrations were calculated to be enough to inhibit bacterial growth when observed tissue amounts of phytoalexins were assigned to hypersensitively responding cells (Pierce et al., 1996).

Related work by other groups

The role of phytoalexins in plant disease resistance has been studied in other groups too. Among these groups, some of them tried to change the sensitivity of pathogens to phytoalexins. For example, fungus *Nectria haematococca* possesses an enzyme, pisatin demethylase, which can detoxify the phytoalexin pisatin produced by its host pea (pisatin demethylation ability, pda). The pisatin demethylase gene was transformed into a pda$^-$, non-pathogenic *N. haematococca* by Vanetten's group (Ciuffetti et al, 1988), and changed the transformants to a pathogen to pea. This result was strong evidence that phytoalexin plays a very important role in pea's resistance to fungus *N. haematococca*. Schafer et al. also performed a similar experiment in which they expressed pisatin demethylase in *Cochliobolus heterostrophus*, a pathogen of maize but not of pea, and converted transformants to weak pathogens of pea. There are also other groups who studied the importance of phytoalexins by enhancing or interfering with phytoalexin production by genetic manipulations or chemical treatments. For example, a gene for biosynthesis of stilbene-type phytoalexin was transformed into tobacco, resulting in an enhancement of resistance of tobacco to the fungal pathogen *Botrytis cinerea* (Hain et al., 1993). Eldon and Hillocks (Eldon & Hillock, 1996) reduced the production of phytoalexin hemigossypol by treating cotton (*Gossypium hirsutum*) with an inhibitor of hydroxy-methylglutaryl (HMG) CoA reductase, which catalyzes a committed step on the terpenoid pathway. This resulted in a breakdown of resistance of cotton to verticillium wilt but not to fusarium wilt. Eldon and Hillocks concluded from their experiment that hemigossypol production in cotton is responsible for resistance to verticillium wilt, but not to fusarium wilt.

Reason to do the experiment

Although there is much evidence that phytoalexins play a role in resistance to bacterial blight and other diseases in cotton, there is no proof that phytoalexins are necessary for resistance. Are phytoalexins necessary for bacterial blight resistance? We undertook to

answer this question by interfering with the normal expression of CDN1 with antisense and sense constructs of *cdn1* cDNA via *Agrobacterium*-mediated transformation and subsequently obtaining transgenic plants.

The enzyme, CDN1, has been isolated, and the reaction it catalyzes may be a rate-controlling step of the pathway of phytoalexin production (Davis et al., 1996, Davis & Essenberg, 1995). cDNA of *cdn1* has been obtained by E. M. Davis in our laboratory (Davis et al., 1998). This enabled the construction of antisense and sense cDNA of *cdn1* for the purpose of transgenic plant analysis.

Transgenic Plants

Antisense RNA in transgenic plants

Antisense RNA has been used to inhibit specific gene expression in many organisms including various kinds of plants. Yet, the mechanism of antisense RNA function is not completely understood. The early studies in plants addressed the expression of antisense RNA and its inhibition of sense RNA expression. Ecker and his coworkers developed a transient assay system in which a mixture of sense and antisense constructs of chloramphenicol acetyltransferase (CAT) was electroporated into carrot protoplasts (Ecker & Davis, 1986). In this system, a maximum of 95% inhibition was achieved when the antisense to sense ratio was 50:1. Later, Delauney's group transformed tobacco plants with a sense construct of a CAT gene, which was not in tobacco originally, and obtained CAT activities in different levels (Delauney et al., 1988). They subsequently retransformed these transgenic tobacco plants with an antisense CAT construct and found the reduction or loss of CAT activity in some doubly transgenic plants. They also found the transgenes were intact in the cell lines they examined [transgene rearrangement could happen in either *Agrobacterium*-mediated system or direct gene transfer systems such as particle bombardment and microinjection (Negrutiu, 1995)], and there was a strong correlation between the degree of CAT gene inactivation and the level of antisense transcripts

accumulated. van der Krol's group showed that inhibition by antisense RNA is sequence specific. They found that antisense RNA of the chalcone synthase (CHS) gene from petunia could down-regulate the endogenous CHS gene in transgenic petunia and in tobacco, which shares 80% homology to the CHS gene of petunia, but other enzymes involved in the same flavonoid biosynthetic pathway as CHS in petunia were not altered by this antisense construct (van der Krol et al., 1990). These and other studies strongly suggest that there is a potential to use antisense RNA to inhibit specific gene activity.

The generally accepted mechanism of antisense RNA inhibition involves the formation of an antisense-sense RNA duplex followed by the rapid degradation of this duplex (Delauney et al., 1988; Crowley et al., 1985). This hypothesis was supported by *in vitro* run-on experiments on isolated nuclei in which RNA was transcribed *in vitro* with ^{32}P-labeled NTP, followed by RNA isolation and northern analysis. The results indicated that the transcription rates of sense genes were unaffected by the expression of antisense RNA (Sheehy et al., 1988; Crowley et al., 1985). The fact that no direct evidence of duplex formation has been found in plants was hypothesized to be due to the unstable nature and rapid degradation of this duplex. Whether the duplex formation and degradation happen in nuclei or cytoplasm is not clear.

In the hypothetical mechanism above, more active transcription of antisense RNA than that of sense RNA should be required in order to effectively inhibit the sense endogenous gene or transgene. But there is evidence in the transgenic petunia system that effective inhibition of flower pigmentation by antisense CHS gene does not require excess antisense RNA (van der Krol et al., 1990b). This experiment led van der Krol's group to propose an RNA-DNA interaction in this particular system. This suggests that there may be more than one mechanism of antisense RNA action.

In spite of the fact that the mechanism of antisense RNA action is not completely understood, there are many applications with antisense RNA to investigate the basic biochemical functions of enzymes and to improve the characters of crops and other

economically important plants. Typically, a full range of inhibition of sense gene expression by antisense RNA can be achieved in 20 independent primary transformants (Negrutiu, 1995; van der Krol et al., 1990b; Atanassova et al., 1995). In the published reports about antisense RNA application, high efficiencies of inhibition were achieved (those who achieved only low efficiency may not have published their results). For example, Smith and his coworkers reported that insertion of a single copy of an antisense gene produced 50% to 95% inhibition of polygalacturonase enzyme activity in transgenic tomatoes, while insertion of two copies can result in 99% inhibition (Smith, et al., 1990). In experiments on lignin modification (Atanassova et al., 1995), 15 out of 20 antisense transgenic tobacco plants had less than 50% of control plants' activity of O-methyltransferase (OMT), which is the key enzyme for lignin synthesis, with a minimum of 3% using a full-length antisense construct of the OMT gene. Using an antisense construct of OMT lacking the 5' non-translated region, a higher frequency of inhibition was achieved, in which all 20 transgenic plants showed less than 50% OMT activity, with 4 of them showing only a trace of activity.

Over-expression and cosuppression

When researchers introduced sense gene constructs into plant genomes with the purpose of over-expressing some genes, they encountered a great variability in transgene expression. There is a surprising phenomenon, called cosuppression, in which some transgenic lines completely shut down the expression of the homologous gene in plants, as well as the expression of the transgene (Atanassova et al., 1995; Jorgensen et al., 1995). Typically, 5 to 20 percent of sense transgenic plants lines exhibit cosuppression (Baulcombe et al., 1996). Cosuppression is also known as post-transcriptional gene silencing (PTGS) because it is believed that the suppression happens at the post-transcriptional RNA level (Jorgensen et al., 1995). The possibility that the over-expressed enzyme or its products feed back to induce cosuppression has been precluded by the

finding that even truncated, nonfunctional genes can induce cosuppression (Smith et al., 1990; van Blockand et al., 1994).

Currently, there are three models to account for PTGS (Baulcombe et al., 1996), all of them involving the action of antisense RNA. The first model deals with antisense RNA transcribed directly from an adjacent downstream promoter, which may be an endogeneous promoter or a promoter of the reporter gene or selectable gene (Baulcombe et al., 1996). The second model is also known as the "threshold model". In this model, it is proposed that there is a threshold for the transcription of a gene; once transcription exceeds this threshold, antisense RNA is induced and blocks further expression of this gene (Lindbo, 1993; Meins, 1994). Cosuppression has been studied extensively in transgenic petunia containing sense CHS genes due to the reason that any change in the floral pigmentation is easily identified (Jorgensen et al., 1995). It has been found that cosuppression is very sensitive to increases in gene expression or dosage. This strongly supports the threshold theory. The third proposed model is that the transgene in a plant genome is highly methylated and produces aberrant RNA which induces the transcription of antisense RNA (English et al., 1996; Ingelbrecht et al., 1994). This model is based on the finding that there is a correlation between the level of PTGS and the degree of transgene methylation in some transgenic plants, primarily transgenic tobacco. Any of these three models is capable of explaining part of the cosuppression phenomenon but not any of them can explain all. Are there multiple mechanisms responsible for cosuppression or are there any new mechanisms to be proposed? The answer is not clear.

Considerations for our constructs for transformation

Promoters

In plant genes, the class II promoter (promoter recognized by RNA polymerase II) normally contains two core elements, a conserved TATA sequence and a less conserved transcription start site, which is located 25 to 30 nucleotides downstream of the TATA

sequence. Lacking either of these two elements, transcription can start but less actively, but lacking both of them results in non-specific start sites of transcription (Sawadogo & Szentirmay, 1997).

To express a transgene in plants, one is required to construct a promoter upstream of a coding sequence. In earlier studies, because researchers were interested in showing that transgene integration from the *Agrobacterium* Ti plasmid and expression is a reality in plants, they simply used the promoters of the T-DNA genes, such as the nopaline synthase promoter (Galun & Breiman, 1997). Subsequently, Chua and collaborators isolated the CaMV 35S promoter which was found to be much stronger than T-DNA promoters (Odell et al., 1985). In later work, the 35S promoter, which is generally expressed constitutively in plant tissues, has been used extensively in petunia, tomato, *Nicotiana*, *Arabidopsis*, potato, and other plants. Currently, other promoters are also used, such as CaMV 19S promoter (Delauney et al., 1988), nopaline synthase promoter (Kumria et al., 1998), and chlorophyll a/b-binding protein promoter (Sandler et al., 1988). Although there are no reports comparing the activities of these promoters, the 35S promoter is used the most, and high expression of transgenes has been achieved with it (Atanassova et al., 1995; Jorgensen et al., 1995). Previous work on transgenic cotton has taken advantage of the 35S promoter to express 2,4-D monooxygenase, a bacterial enzyme that degrades the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) and achieved a 50-100 fold increase of resistance to 2,4-D in transgenic plants (Lyon et al., 1993). McCabe and Martinell also used the 35S promoter to express GUS in transgenic cotton when they were developing transformation protocols suitable for cotton (McCabe & Martinell, 1993).

Terminators

A termination signal is necessary for mRNA transcription, maturation and stability (Galun & Breiman, 1997). Not much attention has been paid to comparison of different terminators or to development of new terminators for plant transformation. But since a

terminator is necessary, either CaMV or nopaline synthase terminator has been used (Futterer et al., 1995).

Selectable genes

A selectable gene should also be included in the same construct as the desired transgene in order to distinguish transformed plants from non-transformed plants. There are several groups of selectable genes (Schrott, 1995): One group encodes products which confer resistance to antibiotics, such as kanamycin, hygromycin, or streptomycin. The second group of selectable genes confers resistance to herbicides and is widely used in transgenic crops. Other selectable genes are in the third group, which includes resistance to high nitrate or high amino acid levels.

The most commonly used selectable gene in transgenic plants, including transgenic cotton (Lyon et al., 1993), is neomycin phosphotransferase (*nptII*) which confers resistance to kanamycin, geneticin, paromomycin and neomycin (Galun & Breiman, 1997).

Full-length vs. truncated antisense RNA

Some groups reported that even a truncated antisense transgene can down-regulate sense gene expression. For example, Atanassova *et al.* reported that an antisense construct of the OMT gene containing the coding sequence and the 3' non-translated region, but lacking the 5' non-translated region, achieved a better inhibition of OMT activities than a full-length antisense construct (Atanassova et al., 1995). van der Krol's group also reported that an antisense construct containing the 3' half of the CHS cDNA sequences had an inhibitory effect on flower pigmentation resembling that of their full-length antisense construct (van der Krol et al., 1990).

Despite the reports on using truncated cDNA sequences, there are more successful examples of using full-length antisense cDNA sequences (van der Krol et al., 1990; Atanassova et al., 1995; Ecker & Davis, 1986). By using the term "full-length", however, they did not define how far they achieved to the 5' end and 3' end of the cDNA sequences.

Transformation strategy

In order to integrate a transgene into a plant genome, two methods have been widely used, which are *Agrobacterium*-mediated transformation and particle bombardment. Transformation of cotton that expressed GUS activity has been successful via particle bombardment (McCabe & Martinell, 1993). *Agrobacterium*-mediated transformation of cotton had been hindered because most cotton lines were found to be difficult to regenerate from explants (McCabe & Martinell, 1993). But by the time we were ready to transform cotton, Norma Trolinder (BioTex, Inc., Lubbock, TX) and her coworkers had developed a successful method for cotton regeneration (Trolinder, 1996, Personal communication). So, either of the two methods of transformation seems to be suitable for us.

Chapter 2. Construction of Intermediate Plasmids pIA, pIS and pIE

Introduction

The work described in this chapter is the construction of an expression cassette containing the *cdn1* cDNA which can subsequently be inserted into a binary vector.

The expression cassette comes from pRTL2 (Figure 1) (Topfer et al.; 1987; Carrington, unpublished) which contains a CaMV 35S promoter with a duplicated enhancer sequence, a CaMV 35S termination signal for transcription and an ampicillin resistance gene for selection. Kay et al. reported that duplication of the enhancer sequence in various 35S promoters increased the activities to more than 10-fold that of the original promoters (Kay et al., 1987). pRTL2 also contains a TL sequence which is from the tobacco etch virus leader sequence. We did not use this sequence in our constructs. pRTL2 was used previously to study tobacco etch virus (TEV) protein by expressing TEV protein fused to GUS in transgenic tobacco, and GUS activity was achieved in the transgenic plants (Carrington et al., 1990).

We decided to use full length *cdn1* cDNA in our constructs.

Materials and Methods

Source of materials

pRTL2 was given to us by Dr. J. Sherwood (Oklahoma State University) with permission from J. Carrington. *cdn1-C* cDNA is from E. Davis (Davis et al., 1998). After inoculation of cotton (*Gossypium hirsutum* L.) cotyledons with *Xcm*, cotyledons were collected at different times which ranged from 30 hours to 60 hours after inoculation, and

total RNA was extracted from the pooled cotyledons. cDNA was obtained by reverse transcription (Davis, 1998). *cdn1* cDNA was amplified by PCR with synthesized primers.

PCR primers used to amplify cdn1 cDNA

Davis and I designed two primers based on the partial sequence of Davis' earlier clone of *cdn1* (Davis, 1998), plus the restriction enzyme recognition sequences which would help later in cloning. These two primers, synthesized by the OSU Recombinant DNA/Protein Resource Facility, are 2033 and 2034 (Figure 2). Primer 2033 has 31 nucleotides: it is complementary to the nucleotides 1823 to 1840 of *cdn1* cDNA sense strand (The coding sequence of *cdn1* is nucleotides 1 to 1665), with an extension that contains the recognition sites for *Xho*I and *Xba*I restriction enzymes. It is the "antisense" primer. Primer 2034 is a 5' primer corresponding to nucleotides -47 to -30 of *cdn1* (*i.e.* the "sense" primer) with a 5' extension containing the restriction recognition sites for *Bam*HI and *Eco*RI restriction enzymes. S stands for G or C in primer 2034; the nucleotide of the native gene there was uncertain at the time primer 2034 was designed.

There was a restriction enzyme digestion test to confirm the absence in *cdn1* cDNA of those restriction enzyme recognition sites that would be incorporated into the primers 2033 and 2034. The plasmid containing *cdn1* in vector PCR2.1 (Davis, 1998) was incubated with *Xho*I, *Xba*I, *Bam*HI, or *Eco*RI at 37°C overnight and then loaded onto an agarose gel. The plasmid was resistant to *Bam*HI, *Xba*I, and *Xho*I digestion and only had one cut for *Eco*RI, which is from PCR2.1. The results indicated *cdn1* cDNA does not have any recognition site for *Xho*I, *Xba*I, *Bam*HI, or *Eco*RI.

Preparation of cdn1 DNA

cdn1 cDNA was amplified by PCR with primers 2033 and 2034 by E. Davis (Davis, 1998). I withdrew 0.5 µl of his PCR product mixture (DNA not quantitated) and re-amplified *cdn1* with primers 2033 and 2034 using the following program:

1. 95°C 3'
2. 85°C 20" add Taq DNA polymerase (Promega)
3. 94°C 45"
4. 45°C 45"
5. 72°C 45"
6. repeat step 3 to step 5, 35 times
7. 72°C 10'
8. 4°C 0 00 (infinite)

Besides the templates indicated above, the PCR mixture consisted of: 2 mM $MgCl_2$, 0.2 mM dNTP, 1 μM each of primers, 4 units of Taq DNA Polymerase (Promega) in 50 μl reaction.

cdn1 PCR product was extracted with phenol-chloroform (1:1) followed by ethanol precipitation. The pellet was dried and re-dissolved in H_2O.

Antisense construct

Insert

Purified *cdn1* PCR product, estimated to be 0.8 μg, was double digested with *Bam*HI and *Xho*I in buffer E (Promega). The desired fragment (1.9 kb) was separated by agarose gel electrophoresis and was purified with a QIAgen gel extraction kit. The resulting *cdn1* DNA was used as the insert in the antisense construct. The exact length of the insert based on sequence analysis by E. Davis of *cdn1* cDNA plus the primers was calculated to be 1914 bp (Davis, et al., 1997).

Vector

E. coli XL-1 blue said to contain pRTL2 (J. Sherwood) was inoculated into 30 ml terrific broth, cultured overnight at 37°C at 225 rpm. pRTL2 was prepared from the cells using an alkaline lysis method (Perkin Elmer). It was digested with *Hin*dIII and viewed on an agarose gel to confirm purification of a plasmid with the correct digestion pattern and we

obtained, as expected, two bands, one about 1.3 kb, and the other 2.6 kb. Intact plasmid was double digested with *Bam*HI and *Xho*I in buffer E. In this step, TL sequence (Figure 1) was deleted. About 2 μg of the desired fragment, (about 3.7 kb), was purified by gel electrophoresis followed by extraction with a QIAgen gel extraction kit. The resulting fragment of pRTL2 containing 35S promoter and termination signals was used for an intermediate plasmid in the antisense construct.

Ligation and transformation

The concentrations of insert and vector were estimated based on the brightness of ethidium bromide staining on the agarose gel compared to the 1.6 kb fragment in the 1 kb ladder. About 50-75 ng of insert and 25-75 ng of vector were used in each ligation reaction. Ligation was performed at 14°C overnight using T4 DNA ligase (Invitrogen). Two different vector : insert ratios were tried, 1:1 and 1:3. A control tube with only vector but no insert was also included. Two-μl ligation reactions were used to transform 20 μl INVαF' competent cells, and the transformation reactions were plated on LB agar with 50 μg/ml ampicillin. Transformants were screened by PCR using primers 919 and 918, which are internal to the *cdn1* cDNA. Both negative (no template) and positive (*cdn1* in PCR2.1) controls were included and both gave expected results.

Transformants were then confirmed by sequencing. Two sequence analyses, using primer T1600 or 2375 separately, were done to confirm the junction at either end of the *cdn1* cDNA. T1600 is an internal antisense primer, which is from Dr. Heinstein's lab (Chen et al., 1995), annealing to nucleotides 225 to 243 of *cdn1* cDNA, and pointing to the 5' end. Primer 2375 is another gene specific primer (designed by E. Davis), but is a sense primer corresponding to nucleotides 1508 to 1525 of *cdn1* cDNA.

This plasmid, named pIA, was used as intermediate antisense construct.

"Empty" construct

During the antisense construction, a control ligation with vector but no insert was done. After transformation, some transformants grew on amp$^+$ plates. One such clone was increased and was confirmed by sequence using an antisense "M13 forward" sequencing primer, which is complementary to sequence located directly downstream of the transcription termination signal in pRTL2, that the plasmid from this clone was derived from pRTL2 but contained neither *cdn1* nor TEV sequence. This plasmid, named pIE, was then used as an "empty" intermediate vector.

Sense construct

The construction of *cdn1* into pRTL2 in sense orientation used a similar procedure as the construction of the antisense construct. Both vector DNA (pRTL2) and insert DNA (*cdn1*) were double digested by *Eco*RI and *Xba*I. After gel electrophoresis, a 3.85 kb band as the vector fragment and a 1.9 kb band as the insert fragment were cut out and extracted with QIAgen gel extraction kit. These 2 fragments were ligated at 14°C and the ligation product was then transformed into INVαF' competent cells. Transformants were identified by restriction enzyme digestion pattern, and the 5' junction was confirmed by sequencing using primer T1600. The 3' junction was confirmed after the final binary vector was cloned.

This plasmid, named pIS, was used as intermediate sense construct.

Glycerol stocks

Eight hundred and fifty µl of fresh LB bacterial culture liquid containing ampicillin was added to 150 µl sterile glycerol; frozen in dry ice quickly and moved to -80°C.

Results

Three intermediate constructs have been achieved based on the vector pRTL2 (Figure 1) (Topfer et al, 1987; Carrington, unpublished). None of them contains the TL sequence

which is contained in pRTL2. According to the information available on pRTL2, there are four restriction enzyme sites, which are *Hin*dIII, *Sph*I, *Pst*I and *Hin*cII, upstream of the 35S promoter. Downstream of the termination signal there are three restriction enzyme sites, which are *Hin*dIII, *Sph*I and *Pst*I.

Antisense intermediate construct

cdn1 DNA was inserted into the expression cassette of pRTL2 in antisense orientation, resulting in antisense intermediate plasmid pIA (Figure 3). The transformants in INVαF' survived on LB plates and in liquid culture containing 50 μg/ml ampicillin. Sequence of this antisense construct using primer 2375 confirmed the junction where the 3' end of *cdn1* DNA links to the 35S promoter. Primer 2375 binds to the antisense strand and sits 331 nucleotides from the 3' end of *cdn1* cDNA. More than 500 nucleotides of good sequence were obtained, which included a 169-nucleotide sequence of pRTL2. There were 7 ambiguous nucleotides between the primer and the start of good sequence. The other sequencing effort using primer T1600, which binds to the sense strand of *cdn1* cDNA 272 nucleotides from the 5' end, confirmed the other junction where the 5' end of *cdn1* DNA links to the termination signal of the expression cassette. More than 380 nucleotides of clean sequence were obtained, with 4 ambiguous nucleotides between the primer and the first good sequence. The sequence information is appended (Figure 9). The construct then contains a 35S promoter with dual enhancer, coding region of *cdn1* in antisense orientation, and a termination signal for transcription.

Sense intermediate construct

The resulting sense intermediate construct pIS (Figure 4) contains a 35S promoter with dual enhancer, coding region of *cdn1* in sense orientation, and a termination signal. The two junctions of insert to vector have been confirmed by sequencing. Sequencing using primer T1600 confirmed the junction of the *cdn1* 5' end to the 35S promoter. There were

about 500 nucleotides of clean sequence obtained with about 10 nucleotides of ambiguity between primer and good sequence. The other junction, where the 3' end of *cdn1* cDNA joins the termination signal, was confirmed after the final binary vector was cloned. The sequence information is appended (Figure 10).

Empty intermediate construct

The TL sequence of pRTL2 has been deleted and the fragment left was religated. Using the M13 primer, antisense strand sequence information was obtained which included the entire termination signal and 150 nucleotides upstream, confirming that the religated vector contained neither *cdn1* cDNA nor TL sequence. The sequence information is appended (Figure 11). The religated junction is neither *Xho*I nor *Bam*HI, and the reason it religated is unclear. The *Xba*I site next to the *Bam*HI site was also destroyed. The residues left after digestion with *Xho*I and *Bam*HI and the real sequence after ligation are shown in Figure 5. The resulting empty vector pIE (Figure 6) contains an empty expression cassette.

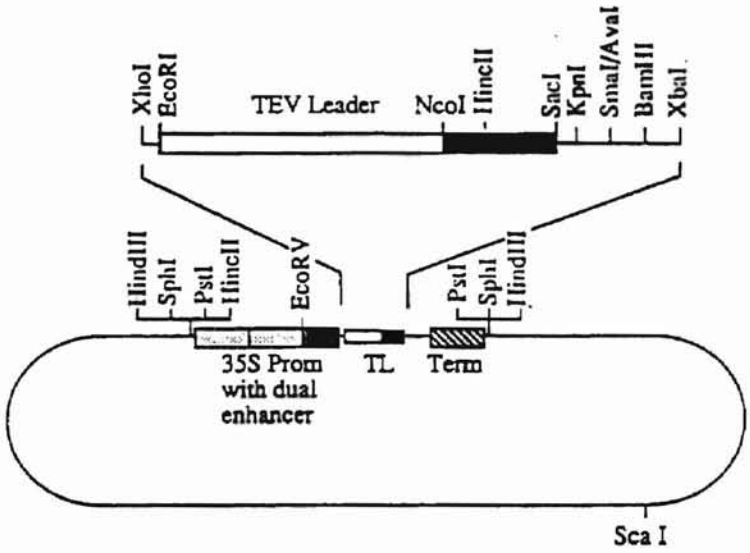


Figure 1. pRTL2 (Carrington, J., personal communication)

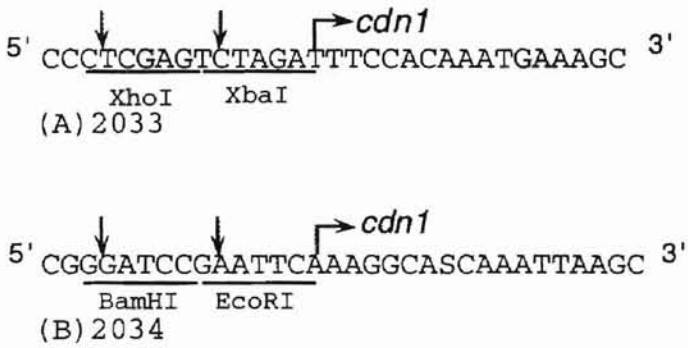


Figure 2. PCR primers used to amplify *cdn1* cDNA. S stands for G or C.

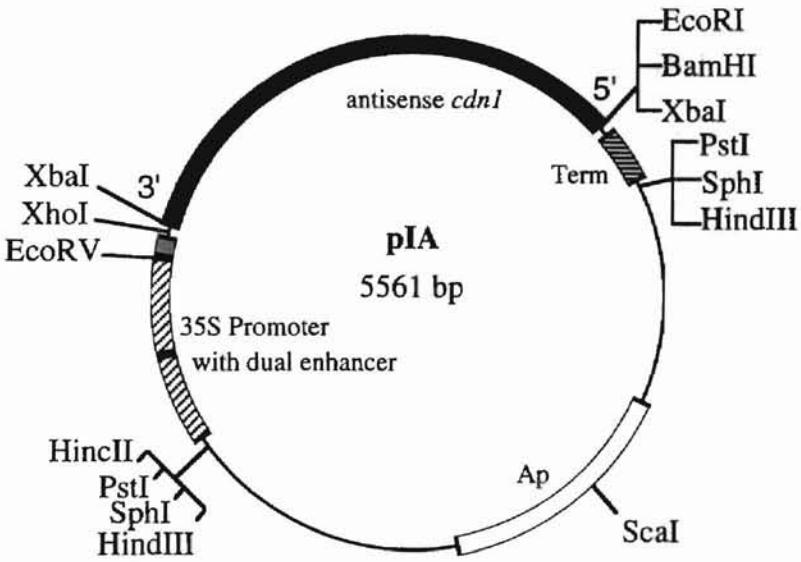


Figure 3. pIA, the intermediate plasmid of antisense *cdn1* construct. It contains antisense *cdn1* cDNA in the expression cassette of pRTL2.

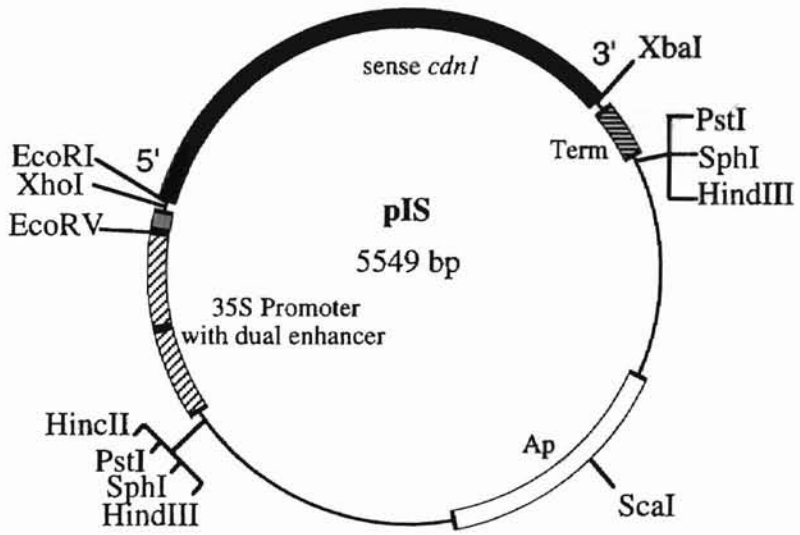
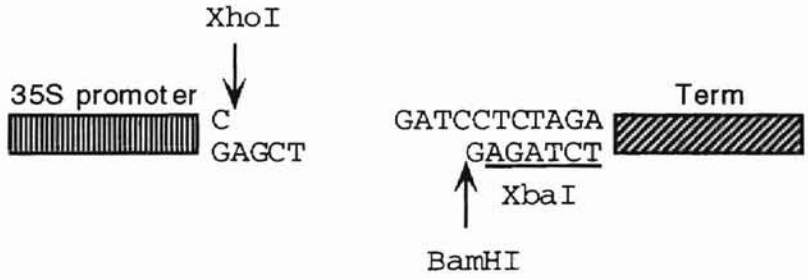
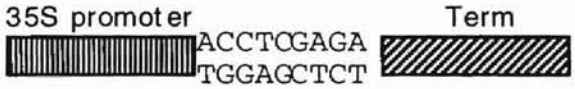


Figure 4. pIS, the intermediate plasmid of sense *cdn1* construct. It contains sense *cdn1* cDNA in the expression cassette of pRTL2.



A. Residues left after digestion with Xhol and BamHI (expected).



B. Actual sequence after ligation.

Figure 5. Diagrams of the ligated section in pIE.

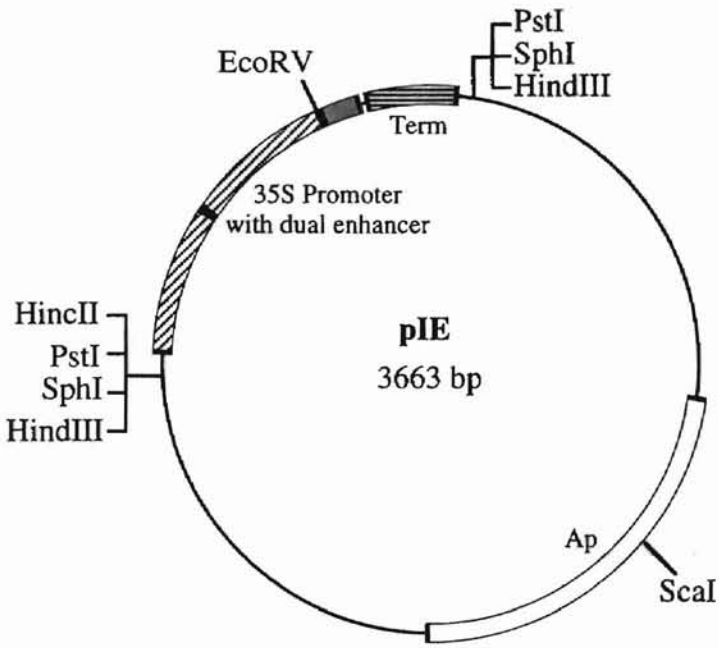


Figure 6. pIE, the intermediate plasmid of empty vector construct.

Chapter 3. Construction of Binary Plasmids Containing Expression Cassettes with *cdn1* cDNA in Sense and Antisense Orientation and Transformation of Tobacco

Introduction

The work described in this chapter can be divided into two parts. In the first part, the expression cassettes were inserted into polycloning sites of the plant transformation vector pBin19 (Bevan et al., 1984; Clontech, 1994), and the resulting plasmids were then transformed into *Agrobacterium tumefaciens* EHA105. At the same time, another plasmid pSW194, which contains a copy of the *virG* gene of pTiBo542, was also transformed into EHA105.

Agrobacterium strain EHA101 was recommended by Candace Haigler (Texas Tech. University, TX) and others performing cotton transformation. EHA105 is the same as EHA101 except that it lacks the kanamycin resistance gene on its Ti plasmid, which allows us to use kanamycin resistance as selectable marker on the binary vector.

During *Agrobacterium* infection of plant tissues, the *virG* gene induces the expression of other *vir* genes. Compared to the *virG* genes of other Ti plasmids, the *virG* gene from pTiBo542 was found to be a "superactivator" to induce the expression of other *vir* genes and hence was believed to increase the transformation efficiency of plants by *Agrobacterium* (Chen et al., 1991). Also, Hansen's group reported constitutive expression of the *virG* gene improves the transformation efficiency of *Agrobacterium* to plants (Hansen et al., 1994).

In the second part of this work, tobacco leaf pieces were infected with the recombinant *Agrobacterium*, and transformants were regenerated. Pathogen test on regenerated tobacco plants with *P. s. tabaci*, a bacterium that causes wildfire disease on tobacco, was also

performed. There are several reasons we began the research with transgenic plants by performing it on tobacco. First, we wanted to know if the antisense construct and the sense construct would interfere with normal metabolism and be detrimental to plants. Tobacco is a well established model for molecular biology study. There are well developed protocols for transformation and regeneration of tobacco explants. Also, the regeneration cycle of tobacco is much shorter compared to cotton. Because of these advantages of transformation in tobacco, we might gain experience that would influence our procedure with cotton. There was a further reason to do this experiment: The sesquiterpene cyclase of tobacco, 5-epi-aristolochene synthase, shares 50% identity and 66% similarity in amino sequence with CDN1 (Davis, 1998), so we may interfere with the phytoalexin production in tobacco by introduction of *cdn1* cDNA.

Materials and methods

Binary plasmids

E. coli strain mc1022 containing plasmid pBin19 was kindly given to us by Dr. Y. Huang (Department of Forestry, Oklahoma State University). pBin19 is an incP plasmid; it is 11.7 kb in length and contains kanamycin resistance genes outside and inside the T-DNA borders (Bevan et al., 1984). mc1022 was cultured in terrific broth or LB containing 50 μg/ml kanamycin overnight at 37°C; 6 × 2.4 ml overnight cultures were used to do plasmid preparation using a QIAprep kit, and plasmids were dissolved in 6 × 50 μl water. In order to prepare intact expression cassettes for insertion into pBin19, the expression cassettes needed to be cut from intermediate vectors by a restriction enzyme whose recognition site also exists in the polycloning site of pBin19. At the same time, this enzyme should not have recognition sites inside the expression cassette. *Pst*I is the only candidate that meets these criteria. Because there is more than one recognition site for restriction enzyme *Pst*I in pBin19, a partial digestion was done to get linear full-length pBin19. One μl of 1:100 diluted *Pst*I (Gibco) was incubated with 2-3 μg pBin19 in 40 μl

reaction solution for 10 min, and the reaction was stopped with EDTA. Linearized pBin19, (about 0.5 μg in each of 3 gels), was excised from the agarose gel and recovered with QIAquick gel extraction kit.

The expression cassettes in the three intermediate constructs, *i. e.*, pIA, pIS and pIE, were excised with *Pst*I and subsequently were ligated to pBin19. About 0.2 μg to 0.3 μg linearized pBin19 was used in each ligation reaction, and 0.3 to 0.6 μg insert DNA was used in each sense or antisense reaction, and about 0.2 μg insert DNA was used in the empty construct reaction. Ligation proceeded at 16°C overnight, and 1-3.5 μl of a ligation reaction was used to transform 25 μl XL-1 blue competent cells. Competent cells were prepared following a method described by Davis (Davis, 1998). Only transformants with an insertion in the polycloning site would appear white on an X-gal plate. White clones that survived on kanamycin-containing plates with X-gal were increased and tested for their restriction digestion patterns. The constructed binary plasmids were named pBA, pBS and pBE corresponding to different inserts in their expression cassettes, namely, antisense *cdn1* cDNA in pBA, sense *cdn1* cDNA in pBS, and nothing but an empty expression cassette in pBE.

The ligation junctions were further confirmed by sequencing using M13 reverse and M13 forward primers synthesized by the OSU Recombinant DNA/Protein Resource Facility. M13 reverse primer is a sense primer in pBin19; it locates 20 nucleotides upstream of the polycloning sites. M13 forward is an antisense primer, also in pBin19, 40 nucleotides downstream of polycloning sites.

Transformation into Agrobacterium

Helper plasmid pSW194 in *E. coli* strain E1028 was given kindly by Dr. S. Gelvin (Purdue University). pSW194 is an incW plasmid and codes for ampicillin and tetracycline resistance; a map of it is not available. I assumed it was the right vector without doing any digestion or other tests. One ml of overnight TB culture with 50 μg/ml ampicillin was used

to prepare plasmid using QIAprep kit; plasmid was dissolved in 50 µl H_2O, resulting in a concentration of about 0.14 µg/µl. About 0.56 µg plasmid was used in each transformation of *Agrobacterium.*

Agrobacterium tumefaciens EHA105 was from Dr. A. Burns (USDA, ARS, Stillwater, OK). EHA105 is a "hypervirulent, L, L-succinamopine helper strain" containing rifamycin resistance in its chromosome (Hood, E. E., 1993). It survived on a rifamycin-containing agar plate at 30°C but did not grow at 37°C.

The combination of one type of binary plasmid and pSW194 were transformed into EHA105 at the same time following the method described by An *et al.* (An, G., 1988). Twenty ml overnight cultured EHA105, O. $D._{600}$ 0.42, was chilled on ice for 30 min, cells were spun down and resuspended in 1 ml 20 mM $CaCl_2$, spun down again, resuspended in 800 µl 20 mM $CaCl_2$, and dispensed into ice-chilled tubes, 80 µl per tube. Different plasmid combinations were added to each tube, *i.e.* about 20-30 ng pBA and 560 ng pSW194 were added for the antisense construct, about 20-30 ng pBS and 560 ng pSW194 were added for the sense construct, and about 20-30 ng pBE and 560 ng pSW194 were added for the empty vector construct. Cells were then frozen in liquid nitrogen and thawed in 37°C water bath 5 minutes. Eight hundred µl YEP broth was added to each tube, followed by shaking at 180-200 rpm, 28°C for 2 hours. Cells were spun down and resuspended in 0.1 ml YEP and spread on agar plates containing 50 µg/ml ampicillin and 50 µg/ml kanamycin. Transformants were confirmed by comparing their PstI digestion patterns to both pSW194 and pBS on agarose gels.

Transformants from all three constructs were increased in 10 ml YEP broth containing 50 µg/ml ampicillin and 50 µg/ml kanamycin, at 27°C for 48 hours. Eight hundred and fifty µl of fresh culture was added to cryovials containing 150 µl sterile glycerol; glycerol stocks were then frozen in dry ice and transferred to -80°C freezer.

Plasmid prep from EHA105

Binary plasmids and pSW194 were prepared together from recombinant EHA105 using a QIAprep kit (Qiagen). Ti plasmid was isolated together with binary vectors and pSW194 following the protocol of Li (Li, X.-Q., 1995), basically a protocol of alkaline lysis.

Transformation and regeneration of tobacco

Seed of *Nicotiana tabacum* Burley 21 was from Dr. M. Daub (North Carolina State University) as a gift. Transformation and regeneration were done as described by Gallois (Gallois, et al, 1995). EHA105 was cultured 48 hours at 27°C and directly used to infect leaf pieces; its OD_{600} was 0.45 when used. About one month old Burley 21 leaves were sterilized by soaking in 11% bleach for 10 minutes, followed by 5 times of rinsing with sterile water. Leaf discs were cut with a razor blade or a 0.5-cm paper punch and were incubated with EHA105 culture liquid for several minutes. After excess liquid was blotted away, leaf discs were cultured on co-culture medium for 2 days, with 16 hours light per day. Then, leaf discs were simply transferred to selection and regeneration medium. Shoots appeared several weeks later. When shoots were about 5 mm long, they were excised and transferred to rooting medium. Once roots appeared, plants were transferred to soil. Seeds of the transgenic tobacco were harvested and stored at 4°C.

Genomic DNA extraction and PCR

Fresh leaves from plants about 1 month old were used to extract genomic DNA using the CTAB method as described by Doyle and Doyle (Doyle & Doyle, 1989); DNA was treated with RNase before final precipitation. Genomic DNA was then used as PCR template to verify the existence of a transgene. For antisense and sense constructs, PCR was performed with two internal primers of *cdn1* cDNA, 918 and 919. The program was:

1. 95°C 3 minutes
2. 95°C 45 seconds

3. 50°C 45 seconds
4. 72°C 1 minute
5. go to 2 for 35 cycles
6. 72°C 10 minutes
7. 4°C

For the empty vector construct, PCR used M13 forward and M13 reverse as primers. The program used above was not very specific with the M13 primers; it gave positive results to negative controls using non-transformed Burley 21 genomic DNA as template. So, a "touch down" program was used: DNA was denatured at 95°C, then in the replicating cycles, the annealing temperature started at 62°C for 2 cycles, dropped to 61 °C for 2 cycles, and beginning from 60°C dropped 1 degree per 5 cycles, and 29 cycles later, reached 55°C, then repeated 13 more cycles at this annealing temperature. The elongation temperature was 72°C for all cycles.

Pathogen tests on tobacco

In order to know the appropriate method, pathogen test was first performed on non-transgenic tobacco plants of Burley 21, which is resistant to *P. s. tabaci*, and Samsum, which is susceptible to *P. s. tabaci*. Inoculation was performed using a 1.5 ml syringe without a needle or a homemade "punch" which is a wood stopper with sixteen (4 x 4, 0.5 cm distance between each other) small nails standing on it. If a "punch" was used, nails were dipped into the inoculum for 10-20 seconds, and were punched on the leaves with a cardboard holding behind. *P. s. tabaci* JS-84-116 inoculum was prepared in five concentrations, 3×10^4 cfu/ml, 1×10^5 cfu/ml, 3×10^5 cfu/ml, 1×10^6 cfu/ml, and 3×10^6 cfu/ml. In the syringe inoculation, the concentration 1×10^5 gave the biggest visible difference between Burley 21 and Samsum. On the leaves inoculated with the "punch", the inoculum of concentration 3×10^6 gave the biggest difference. So inocula with the

concentrations of 1 x 10^5 and 3 x 10^6 were used to inoculate transgenic tobacco plants with syringe and "punch" respectively.

Results

Binary plasmids

Binary plasmids obtained from XL-1 blue transformants and pBin19 were digested with *Pst*I or *Bam*HI; fragments were separated on agarose gels. Following digestion with *Pst*I, pBin19 gave two bands on an agarose gel: about 4.8 kb and 2.0 kb. The 4.8 kb band looked four times darker than the 2.0 kb band. pBA and pBS each gave three bands on an agarose gel whose sizes were about: 4.8 kb, 2.9 kb, and 2.0 kb. The 4.8 kb band looked 4 times darker than the 2.0 kb band. *Bam*HI digestion of pBA gave 2 bands with sizes around 12 kb and 2.9 kb. From these results, I deduced *Pst*I fragments of pBin19 are about 4.8 kb + 4.8 kb + 2.0 kb; the 2.9 kb *Pst*I fragments in pBA and pBS were the insert containing the expression cassettes. *Pst*I digestion of pBE gave bands at 4.8 kb, 2.0 kb, and 0.9 kb on agarose gel. The 0.9 kb band was the empty expression cassette. The detailed sequence of pBin19 was obtained from GenBank and the information obtained is consistent with my deduction. The desired binary plasmid pBA is shown in Figure 7. *Pst*I digestion patterns were consistent with this diagram.

Using M13 reverse primer, more than 560 nucleotides of clear sequence were obtained from pBA. Within this sequence, 15 nucleotides were from pBin19, others were from the 35S promoter sequence. Another 550 nucleotides of sequence were obtained at the other end of the expression cassette using M13 forward primer, showing the other junction. This sequencing included the whole termination signal sequence and 180 nucleotides upstream, which was verified to be the sequence from the 5' end of *cdn1* cDNA. This again confirmed that the *cdn1* cDNA was in antisense orientation in the expression cassette, as proved before. The sequencing information indicated the expression cassette subcloned

from pIA was inserted into the polycloning site rather than the alternative *Pst*I sites in pBin19.

Similar results were obtained from sequencing pBS. The expression cassette from pIS was inserted into the *Pst*I site in the polycloning site of pBin19. M13 forward primer sits 40 nucleotides downstream of the termination signal (term), and gave minus strand sequence until 300 nucleotides upstream of term, proving the junction of term to pBin19 and the junction of 3' end of *cdn1* to term.

The same primers, M13 forward and M13 reverse, were used to sequence the junctions of the empty expression cassette to pBin19. The sequencing results showed that the empty expression cassette from pIE was inserted into the *Pst*I site in the polycloning site of pBin19 in opposite orientation to pBA and pBS. Also, sequencing using M13 forward primer included the whole term and about 150 nucleotides of upstream sequence from the 35S promoter, which again confirmed the direct linkage of the 35S promoter to the termination signal, as described in chapter 2.

Figure 8 summarizes the entire procedure of binary plasmid constructions. Because the accuracy of each ligation was important for the whole project, sequence information was obtained each time after ligation. Figures 9 to 11 align all the sequence information I obtained to the published sequences. Please note that none of the sequences was intended to get original information of the *cdn1* gene or other original DNA information, instead, I sequenced them to confirm the junctions of ligation. DNA was not quantitated in any of the reactions before sequencing (which obviously was not a good habit), and this resulted in some very crowded sequences, especially for the empty construct (indicated in the legend), although most sequences were clean. From the sequence information, there seemed to have been a two-nucleotide deletion at the 3' untranslated region in the antisense construct. There is also one nucleotide mismatch in the 5' non-translated region in the antisense construct and one extra nucleotide in the 5' non-translated region in the sense construct that does not agree with the published *cdn1* sequences (Davis, et al., 1998). However, there is

no other mismatch in the *cdn1* area around any junction. Actually, the clean sequences I obtained were part of the information that led to the published sequences. The group of van der Krol found that an antisense gene can down-regulate other genes that have only 80% homology with the antisense gene (van der Krol et al., 1990). However, since over-expression of the CDN1 enzyme was desired, errors in the sense construct are less tolerable. Since the 35S promoter sequence was never subjected to PCR in the entire construct procedure, the possibility of error in this sequence was small. There was no mismatch in this sequence between my sequences and published sequences.

Agrobacterium tumefaciens

Clones of *Agrobacterium tumefaciens* EHA105 that survived on ampicillin and kanamycin plates were increased, and plasmids were extracted with a QIAprep kit. Plasmid preparations from transformants possibly containing one of the three binary plasmids and pSW194, plus preparations of pSW194 and pBS, were incubated with *Pst*I, and patterns were compared on agarose gel (Figure 12). Both the antisense *A. tumefaciens* transformant and the sense *A. tumefaciens* transformant contained digestion patterns of pBA or pBS as described previously, as well as an additional band at the same position as *Pst*I-incubated pSW194. This band is at position corresponding to 12 kb; whether it is cut or uncut by *Pst*I is unknown to me. The empty construct contained the same pSW194 band and other bands that were expected from pBE, as described previously.

Plasmid preparations that were intended to harvest all plasmids from recombinant EHA105, including the Ti plasmid, were incubated with *Pst*I and separated on agarose gel (Figure 13). Besides the pattern of Figure 12, which was the *Pst*I digestion pattern of a binary plasmid plus pSW194, this gel also showed some other bands. These bands were all the same in the lanes of EHA105 transformed with the three different construct and their intensities were obviously different from bands of binary vector and pSW194. I assumed these bands were from Ti plasmid.

The recombinant EHA105 was used to infect tobacco and cotton explants in the downstream work; it is diagrammed in Figure 14 in terms of the plasmids it contains. Here briefly is described what each of the 4 genomic entities contributes to the transformation process: Genomic DNA of EHA105 is necessary for the life cycle of the *Agrobacterium*; "Ti" plasmid is the major helper plasmid for the transformation process as it contains all *vir* genes; pSW194 contains *virG* gene from pTiBo542, and is believed to further increase the efficiency of transformation and integration (Chen et al., 1991); the binary vector carries an expression cassette within the T-DNA borders, which will be integrated into plant genomic DNA by the actions of *vir* genes.

Transformation and regeneration

During the procedure when Burley 21 was incubated on selection and regeneration medium, leaf discs expanded extensively to 3 to 4 times larger than the original leaf disc and appeared "bleached". As a control, I transformed and regenerated shoots from leaf discs of Xanthi. The Xanthi leaf discs did not show any visible expansion nor did they appear "bleached". Shoots from both Burley 21 and Xanthi came out green. Among the three constructs, antisense was observed to have the highest transformation efficiency (not quantitated), empty the lowest. But all three provided an adequate number of putative transformants for analyses.

Twenty independent transformants from different leaf discs were obtained from each construct (*e.g.*, Figure 15). They survived on kanamycin plates as green shoots and rooted on kanamycin-containing agar. There was no visible difference between most of them and untransformed Burley 21 which was growing under the same conditions. Only one transformant (antisense construct No. 106) looked a little darker green than others. This abnormality may be due to mutation caused by "T-DNA" insertion. From the results of transformation of tobacco, we concluded our constructs were not detrimental to the plants.

Seeds of the transformed tobacco were harvested and stored at 4°C.

PCR

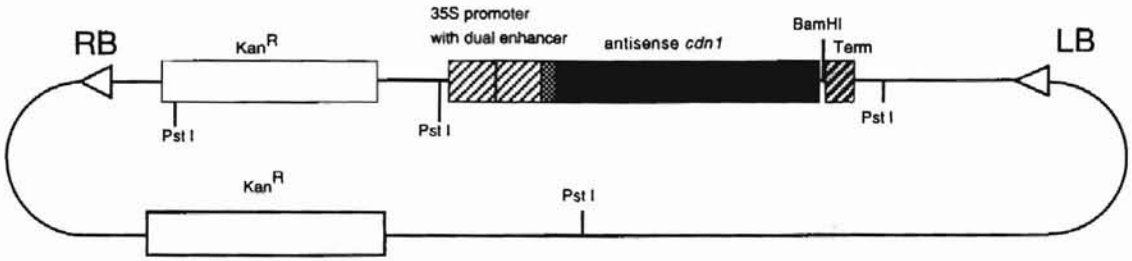
Using *cdn1* internal primers 919 and 918 and genomic DNA, 17 out of 18 antisense tobacco putative transformants showed the expected 1.2 kb fragment on agarose gel following PCR; 13 out of 14 sense transformants showed the same positive results (same gels as antisense) (Figure 16). The control using untransformed Burley 21 genomic DNA as template was negative in the same PCR. To confirm that the PCR products were *cdn1* DNA instead of DNA from tobacco, we sequenced PCR products and found they were *cdn1* DNA.

Using primers M13 forward and M13 reverse to do PCR, a 1.0 kb fragment was expected from empty construct transformants. This fragment is defined by 2 short sequences from pBin19 (about 20 bp at each end) plus the entire sequence from the empty expression cassette (0.95 kb). Six out of 7 empty vector transformants showed expected positive results in PCR, while the same conditions and same primers gave negative results with untransformed Burley 21 genomic DNA (Figure 16).

The other transformants have not been tested yet, although the genomic DNA from most of them has already been extracted and kept at -20°C.

Pathogen test on transgenic tobacco

The results of pathogen test with *P. s. tabaci* on transgenic tobacco plants were inconclusive. Small necrosis happened in all transgenic plants, but there were no clear differences between plants transformed with antisense construct, plants transformed with sense construct, and plants transformed with empty construct.



pBin19+antisense *cdn1* (14.6 kb)

Figure 7. Binary plasmid containing antisense *cdn1* cDNA.

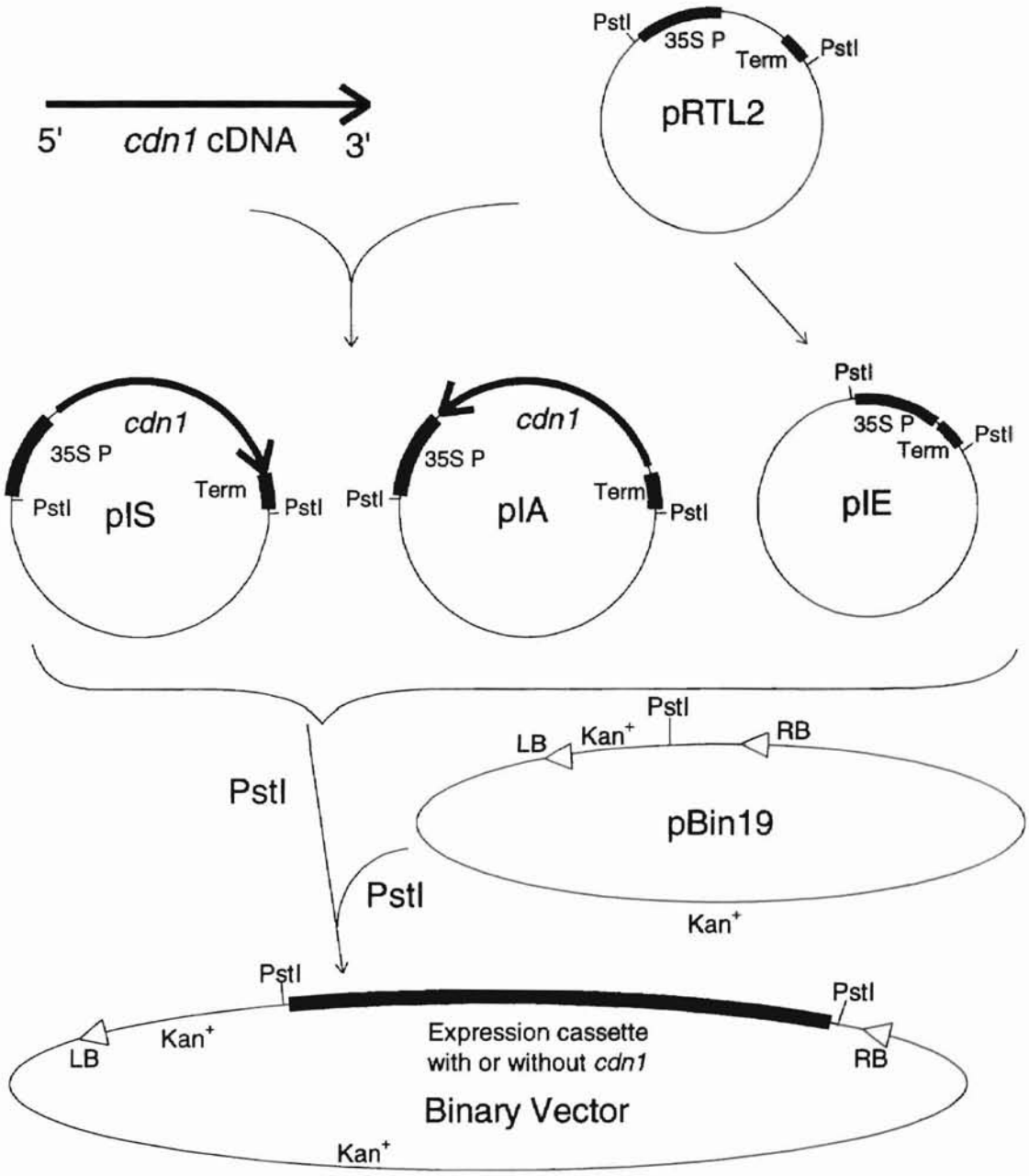
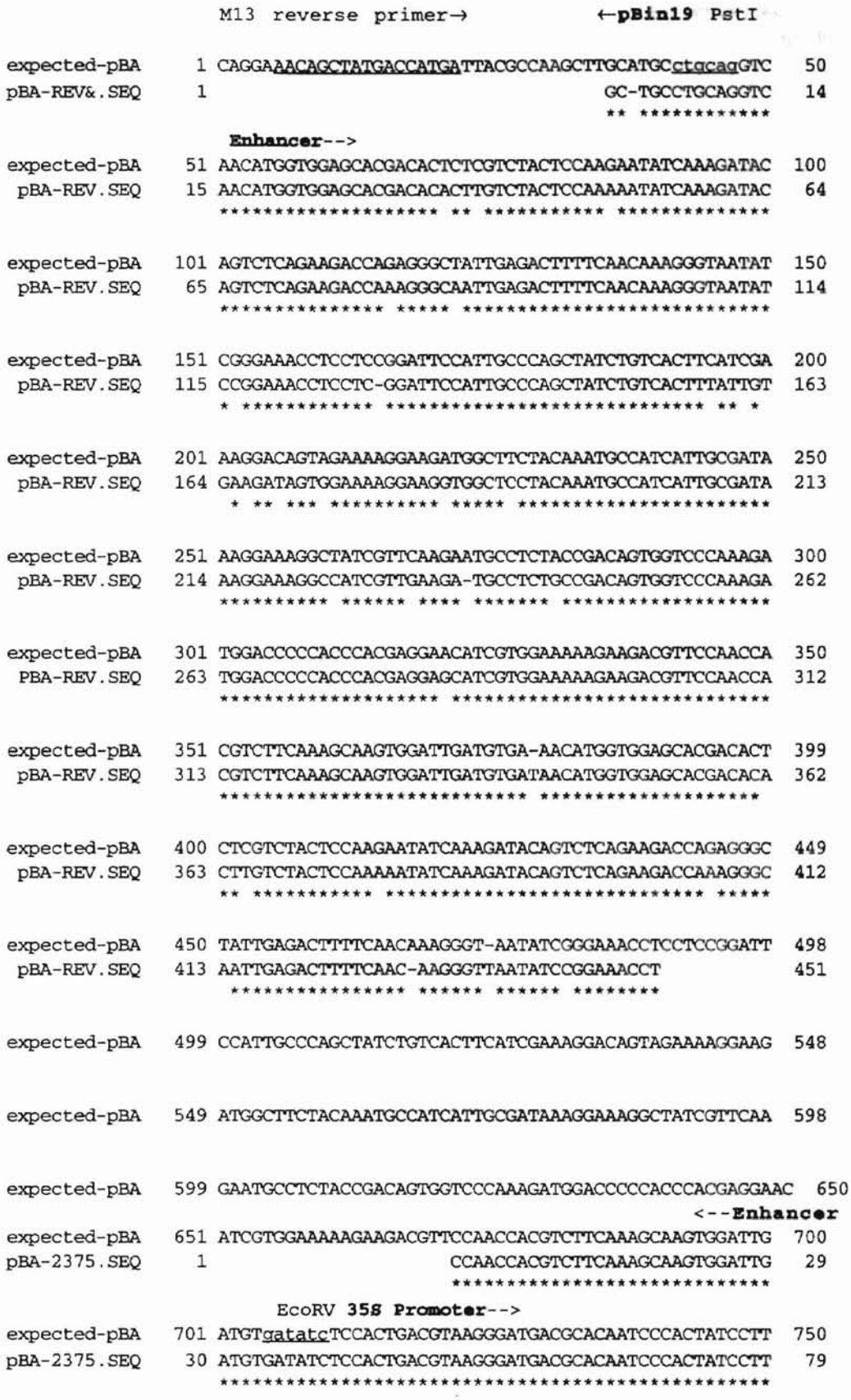


Figure 8. Summary of plasmid construction.

Figure 9. Alignment of pBA sequence with an expected sequence. Expected-pBA: Published sequences of pBin19, pRTL2 and *cdn1* cDNA. pBA-REV&.SEQ: Using M13 reverse primer, the sense strand of T-DNA in pBA was sequenced; pBA-2375.SEQ: Using primer 2375, the antisense strand of T-DNA in pBA was sequenced; pBA-UNI.SEQ: Using primer M13 forward, the antisense strand of T-DNA in pBA was sequenced. * indicates matching nucleotides. "C" in the brackets in the promoter sequence is the transcription start site.


M13 reverse primer→
←pBin19 PstI
expected-pBA 1 CAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGCctgcagGTC 50
pBA-REV&.SEQ 1 GC-TGCCTGCAGGTC 14
Enhancer-->
expected-pBA 51 AACATGGTGGAGCACGACACTCTCGTCTACTCCAAGAATATCAAAGATAC 100
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PBA-REV.SEQ 263 TGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCA 312
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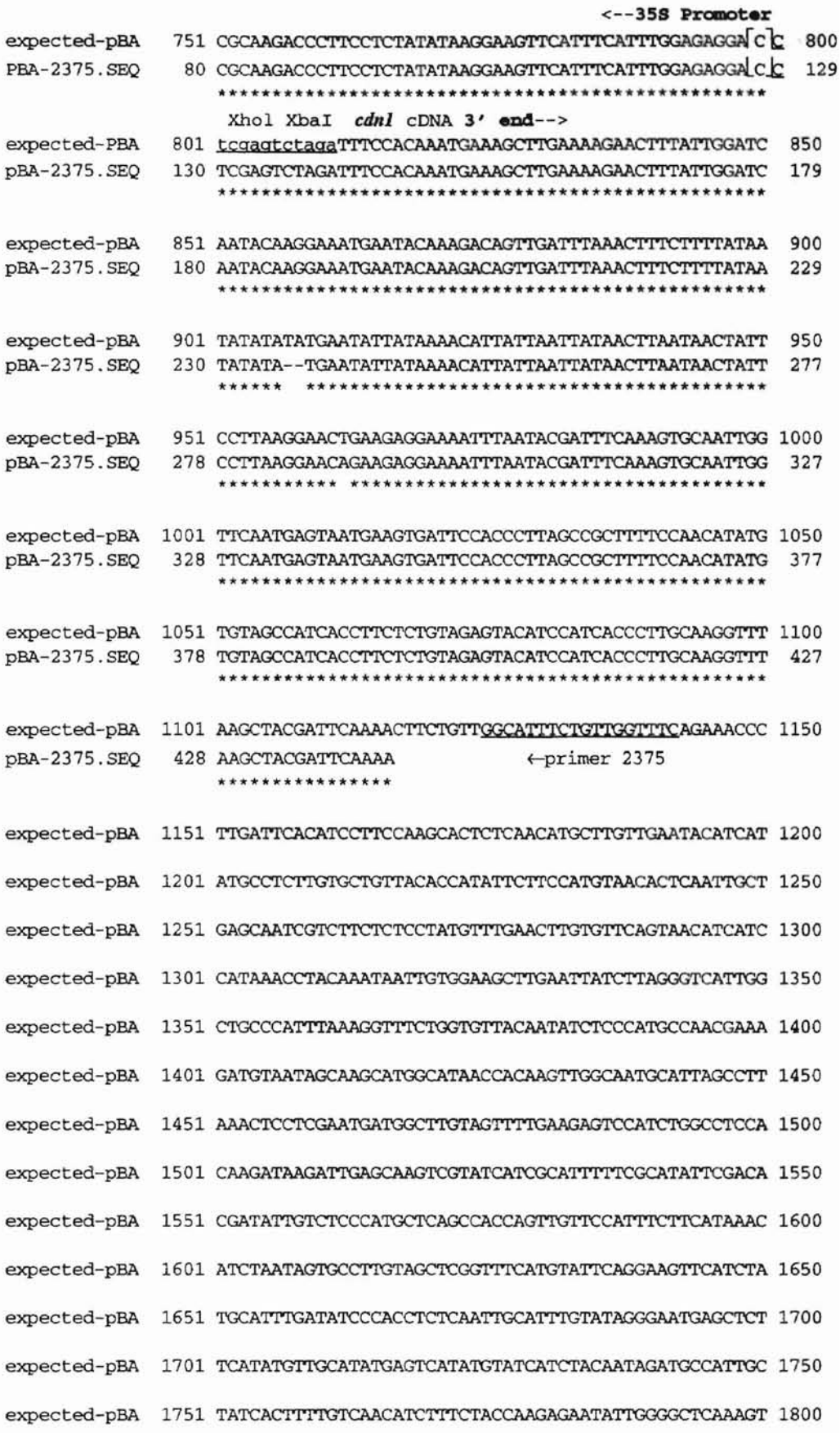
<--35S Promoter
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PBA-2375.SEQ 80 CGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACC 129
Xhol XbaI cdnl cDNA 3' end-->
expected-PBA 801 tcgagtctagaTTTCCACAAATGAAAGCTTGAAAAGAACTTTATTGGATC 850
pBA-2375.SEQ 130 TCGAGTCTAGATTTCCACAAATGAAAGCTTGAAAAGAACTTTATTGGATC 179
expected-pBA 851 AATACAAGGAAATGAATACAAAGACAGTTGATTTAAACTTTCTTTTATAA 900
pBA-2375.SEQ 180 AATACAAGGAAATGAATACAAAGACAGTTGATTTAAACTTTCTTTTATAA 229
expected-pBA 901 TATATATATGAATATTATAAAACATTATTAATTATAACTTAATAACTATT 950
pBA-2375.SEQ 230 TATATA--TGAATATTATAAAACATTATTAATTATAACTTAATAACTATT 277
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expected-pBA 1001 TTCAATGAGTAATGAAGTGATTCCACCCTTAGCCGCTTTTCCAACATATG 1050
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expected-pBA 1101 AAGCTACGATTCAAAACTTCTGTTGGCATTTCTGTTGGTTTCAGAAACCC 1150
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expected-pBA  1951 ACTCCAACAAAACCTTATTATGGGACTCAATGTCTTGGTATACTGAAAGA 2000

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expected-pBA  2401 CCAGTCCCTGGACTGAATCAATGAAGGCTAACTTTAGGGTTGAATTAGCC 2450

expected-pBA  2451 ATTGGTGCCACAATCATCTTCCTCACTTCTTCTTTCAATTGTTGGTGGCG 2500

expected-pBA  2451 ATTGGTGCCACAATCATCTTCCTCACTTCTTCTTTCAATTGTTGG-TGGC 2499

expected-pBA  2500 GTTTTTGAGTTTCAG-CATCAATATT-CTTGTCGGG-ACAATTG-AGGAA- 2545

expected-pBA  2546 -GAAATCTCCCCAAATGCTAGGCTGAAAATCGGCTTTGGGACGCATTTC 2593
pBA-UNI.SEQ     77                         CTGAAAATCGGCTTTGGGACGCATTTC  126
                                           ****************************

expected-pBA  2594 ATCCTTATTGGAAGAAAGGGGTGATGAAGAAGGCATTTGAGAAACTTGT 2642
pBA-UNI.SEQ    127 ATCCTTATTGGAAGAAAGGGGTGATGAAGAAGGCATTTGAGAAACTTGT  176
                   ************************************************
                                                    <--cdn1 cDNA 5' end
expected-pBA  2643 GAAGCCATTTCGATTGATTAAAAGCAAATATTGAAAAGCTTAATTTGTG 2691
pBA-UNI.SEQ    177 GAAGCCATTTCGATTGATCAAAAGCAAATATTGAAAAGCTTAATTTGTG  225
                   ***************** ******************************
                         EcoRI BamHI XbaI  Term -->
expected-pBA  2692 CCTTTgaattcggatcctctagaGTCCGCAAA--TCACCAGTCTCTCTCT 2739
pBA-UNI.SEQ    227 CCTTTGAATTCGGATCCTCTAGAGTCCGCAAAAATCACCAGTCTCTCTCT  275
                   ********************************  ****************

expected-pBA  2740 ACAAATCTATCTCTCTCTATTTT-CTCCAGAATAATGTGTGAGTAGTTCC 2788
pBA-UNI.SEQ    277 ACAAATCTATCTCTCTCTATTTTTCTCCAGAATAATGTGTGAGTAGTTCC  325
                   ********************** ***************************

expected-pBA  2789 CAGATAAGGGAATTAGGGTTCTTATAGGGTTTCGCTCATGTGTTGAGCAT 2838
pBA-UNI.SEQ    327 CAGATAAGGGAATTAGGGTTCTTATAGGGTTTCGCTCATGTGTTGAGCAT  375
                   ************************************************
```

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expected-pBA  2839 ATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATA 2888
pBA-UNI.SEQ    377 ATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATA  425
                   **************************************************

                                                <--Term PstI pBin19-->
expected-pBA  2889 AAATTTCTAATTCCTAAAACCAAAATCCAGTGACctgcagGTCGACTCTA 2938
pBA-UNI.SEQ    427 AAATTTCTAATTCCTAAAACCAAAATCCAGTGACCTGCAGGTCGACTCTA  475
                   **************************************************

expected-pBA  2939 GAGGATCCCCGGGTACCGAGCTCGAATTCACTGGCCGTCGTTTTAC 2984
pBA-UNI.SEQ    477 GAGGATCCCCGG-TACCGAGCTCGAAT       ←M13 forward primer
                   ************ **************
```

Figure 10. Alignment of pBS sequence with expected sequence. Expected-pBS: Published sequences of pBin19, pRTL2 and *cdn1* cDNA. pBS-REV.SEQ: Using M13 reverse primer, the sense strand of T-DNA in pBS was sequenced; pBS-T1600.SEQ: Using primer T1600, the antisense strand of T-DNA in pBS was sequenced; pBS-UNI.SEQ: Using M13 forward primer, the antisense strand of T-DNA in pBS was sequenced, this sequence was crowded, information was only to confirm the ligation junction. * indicates matching nucleotides. "C" in the brackets in the promoter sequence is the transcription start site.


M13 reverse primer→
←pBin19 PstI
expected-pBS 1 CAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGCctgcagGTC 50
pBS_REV.SEQ 1 CGCAAGCTTGCATGCCTGCAGGTC 24
Enhancer→
expected-pBS 51 AACATGGTGGAGCACGACACTCTCGTCTACTCCAAGAATATCAAAGATAC 100
pBS_REV.SEQ 25 AACATGGTGGAGCACGACACACTTGTCTACTCCAAAAATATCAAAGATAC 74
expected-pBS 101 AGTCTCAGAAGACCAGAGGGCTATTGAGACTTTTCAACAAAGGGTAATAT 150
pBS_REV.SEQ 75 AGTCTCAGAAGACCAAAGGGCAATTGAGACTTTTCAACAAAGGGTAATAT 124
expected-pBS 151 CGGGAAACCTCCTCCGGATTCCATTGCCCAGCTATCTGTCACTTCATCGA 200
pBS_REV.SEQ 125 CCGGAAACCTCCTC-GGATTCCATTGCCCAGCTATCTGTCACTTTATTGT 173
expected-pBS 201 AAGGACAGTAGAAAAGGAAGATGGCTTCTACAAATGCCATCATTGCGATA 250
pBS_REV.SEQ 174 GAAGATAGTGGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATA 223
expected-pBS 251 AAGGAAAGGCTATCGTTCAAGAATGCCTCTACCGACAGTGGTCCCAAAGA 300
pBS_REV.SEQ 224 AAGGAAAGGCCATCGTTGAAGA-TGCCTCTGCCGACAGTGGTCCCAAAGA 272
expected-pBS 301 TGGACCCCCACCCACGAGGAACATCGTGGAAAAAGAAGACGTTCCAACCA 350
pBS_REV.SEQ 273 TGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCA 322
expected-pBS 351 CGTCTTCAAAGCAAGTGGATTGATGTGA-AACATGGTGGAGCACGACACT 399
pBS_REV.SEQ 323 CGTCTTCAAAGCAAGTGGATTGATGTGATAACATGGTGGAGCACGACACA 372
expected-pBS 400 CTCGTCTACTCC-AAGAATATCAAAGATACAGTCTCAGAAGACCAGAGGG 448
pBS_REV.SEQ 373 CTTGTCTACTCCCAAAAATATCAAAGATACAGTCTC 408
expected-pBS 449 CTATTGAGACTTTTCAACAAAGGGTAATATCGGGAAACCTCCTCCGGATT 498
expected-pBS 499 CCATTGCCCAGCTATCTGTCACTTCATCGAAAGGACAGTAGAAAAGGAAG 548
expected-pBS 549 ATGGCTTCTACAAATGCCATCATTGCGATAAAGGAAAGGCTATCGTTCAA 598
expected-pBS 599 GAATGCCTCTACCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAAC 650
←Enhancer
expected-pBS 651 ATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTG 700
pBS-T1600.SEQ 1 TCCAACCACGT-TTCAAAGCAAGTGGATTG 29
EcoRV 35S promoter→
expected-pBS 701 ATGTgatatcTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTT 750
pBS-T1600.SEQ 30 ATGTGATATGTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTT 78
←35S Promoter
expected-pBS 751 CGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGA[C]C 800
pBS-T1600.SEQ 79 CGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGA[C]C 127

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                  ********** **********************************

                   XhoI EcoRI cdn1 cDNA 5' end→
expected-pBS    801 tcgaggaattcAAAGGCA-CAAATTAAGCTTTTCAATATTTGCTTTTAAT  849
pBS-T1600.SEQ   128 TCGAGGAATTCAAAGGCAGCAAATTAAGCTTTTCAATATTTGCTTTTAAT  176
                    ********** ********************************

expected-pBS    850 CAATCGAAATGGCTTCACAAGTTTCTCAAATGCCTTCTTCATCACCCCTT  899
pBS-T1600.SEQ   177 CAATCGAAATGGCTTCACAAGTTTCTCAAATGCCTTCTTCATCACCCCTT  225
                    **************************************************

expected-pBS    900 TCTTCCAATAAGGATGAAATGCGTCCCAAAGCCGATTTTCAGCCTAGCAT  949
pBS-T1600.SEQ   226 TCTTCCAATAAGGATGAAATGCGTCCCAAAGCCGATTTTCAGCCTAGCAT  275
                    **************************************************

expected-pBS    950 TTGGGGAGATTTCTTCCTCAATTGTCCCGACAAGAATATTGATGCTGAAA  999
pBS-T1600.SEQ   276 TTGGGGAGATTTCTTCCTCAATTGTCCCGACAAGAATATTGATGCTGAAA  325
                    **************************************************

expected-pBS   1000 CTCAAAAACGCCACCAACAATTGAAAGAAGAAGTGAGGAAGATGATTGTG 1049
pBS-T1600.SEQ   326 CTCAAAAACGCCACCAACAATTGAAAGAAGAAGTGAGGAAGATGATTGTG  375
                    **************************************************

expected-pBS   1050 GCACCAATGGCTAATTCAACCCTAAAGTTAGCCTTCATTGATTCAGTCCA 1099
pBS-T1600.SEQ   376 GCACCAATGGCTAATTCAACC                ←T1600 primer   402
                    **********************
expected-pBS   1100 GGGACTGGGTGTGAGTTACCATTTCACCAAGGAGATCGAAGATGAACTAG 1149

expected-pBS   1150 AGAATATCTACCATAACAACAATGATGCCGAGAACGACCTCTACACCACA 1199

expected-pBS   1200 TCCCTTCGATTCCGACTACTCCGAGAGCATGGATTCCATGTTTCATGCGA 1249

expected-pBS   1250 CGTATTCAACAAGTTTAAAGACGAGCAAGGGAATTTCAAGTCATCCGTGA 1299

expected-pBS   1300 CAAGCGATGTTCGAGGATTGTTGGAACTTTACCAAGCTTCCTATTTGAGG 1349

expected-pBS   1350 GTTCATGGGGAAGATATATTGGATGAAGCAATTTCTTTCACCAGCAACCA 1399

expected-pBS   1400 TTTAAGCCTTGCAGTAGCATCTTTGGACCATCCTTTATCCGAAGAGGTTT 1449

expected-pBS   1450 CTCATGCTTTGAAACAATCAATTCGAAGAGGCTTGCCAAGGGTTGAGGCA 1499

expected-pBS   1500 AGACACTATCTTTCAGTATACCAAGACATTGAGTCCCATAATAAGGTTTT 1549

expected-pBS   1550 GTTGGAGTTTGCTAAGATCGATTTCAACATGGTACAACTTTTGCATAGAA 1599

expected-pBS   1600 AAGAGCTAAGTGAGATTTCTAGGTGGTGGAAGGATTTAGACTTTCAAAGA 1649

expected-pBS   1650 AAGTTGCCATACGCAAGAGATAGAGTGGTTGAAGGCTATTTTTGGATCTC 1699

expected-pBS   1700 AGGAGTGTACTTTGAGCCCCAATATTCTCTTGGTAGAAAGATGTTGACAA 1749

expected-pBS   1750 AAGTGATAGCAATGGCATCTATTGTAGATGATACATATGACTCATATGCA 1799

expected-pBS   1800 ACATATGAAGAGCTCATTCCCTATACAAATGCAATTGAGAGGTGGGATAT 1849

expected-pBS   1850 CAAATGCATAGATGAACTTCCTGAATACATGAAACCGAGCTACAAGGCAC 1899
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expected-pBS  1900 TATTAGATGTTTATGAAGAAATGGAACAACTGGTGGCTGAGCATGGGAGA 1949

expected-pBS  1950 CAATATCGTGTCGAATATGCGAAAAATGCGATGATACGACTTGCTCAATC 1999

expected-pBS  2000 TTATCTTGTGGAGGCCAGATGGACTCTTCAAAACTACAAGCCATCATTCG 2049

expected-pBS  2050 AGGAGTTTAAGGCTAATGCATTGCCAACTTGTGGTTATGCCATGCTTGCT 2099

expected-pBS  2100 ATTACATCTTTCGTTGGCATGGGAGATATTGTAACACCAGAAACCTTTAA 2149

expected-pBS  2150 ATGGGCAGCCAATGACCCTAAGATAATTCAAGCTTCCACAATTATTTGTA 2199

expected-pBS  2200 GGTTTATGGATGATGTTACTGAACACAAGTTCAAACATAGGAGAGAAGAC 2249

expected-pBS  2250 GATTGCTCAGCAATTGAGTGTTACATGGAAGAATATGGTGTAACAGCACA 2299

expected-pBS  2300 AGAGGCATATGATGTATTCAACAAGCATGTTGAGAGTGCTTGGAAGGATG 2349

expected-pBS  2350 TGAATCAAGGGTTTCTGAAACCAACAGAAATGCCAACAGAAGTTTTGAAT 2399

expected-pBS  2400 CGTAGCTTAAACCTTGCAAGGGTGATGGATGTACTCTACAGAGAAGGTGA 2449

expected-pBS  2450 TGGCTACACATATGTTGGAAAAGCGGCTAAGGGTGGAATCACTTCATTAC 2499

expected-pBS  2500 TCATTGAACCAATTGCACTTTGAAATCGTATTAAATTTTCCTCTTCAGTT 2549

expected-pBS  2550 CCTTAAGGAATAGTTATTAAGTTATAATTAATAATGTTTTATAATATTCA 2599

expected-pBS  2600 TATATATATTATAAAAGAAAGTTTAAATCAACTGTCTTTGTATTCATTT 2648
 pBS-UNI.SEQ     1           ATAAAAGAAAGTTTAAATCAACTGTCTTTGTATTCATTT   40
                             ****************************************

                                                  ←cdn1 cDNA 3' end
expected-pBS  2649 CCTTGTATTGATCCAATAAAGTTCTTTTCAAGCTTTCATTTGTGGAAAt 2697
 pBS-UNI.SEQ    41 CCTTGTATTGATCCAATAAAGTTCTTTTCAAGCTTTCATTTGTGGAAAT   90
                   **************************************************

                    XbaI Term→
expected-pBS  2698 ctagaGTCCGCAAA--TCACCAGTCTCTCTCTACAAATCTATCTCTCTCT 2745
 pBS-UNI.SEQ    91 CTAGAGTCCGCAAAAATCACCAGTCTCTCTCTACAAATCTATCTCTCTCT  140
                   ************** **********************************

expected-pBS  2746 ATTTT-CTCCAGAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGG 2794
 pBS-UNI.SEQ   141 ATTTTTCTCCAGAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGG  190
                   ***** ******************************************

expected-pBS  2795 TTCTTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTAT 2844
 pBS-UNI.SEQ   191 TTCTTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTAT  240
                   **************************************************

expected-pBS  2845 GTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATTCCTAAA 2894
 pBS-UNI.SEQ   241 GTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATTCCTAAA  290
                   **************************************************

                          ←Term  PstI  pBin19→
expected-pBS  2895 ACCAAAATCCAGTGACctgcagGTCGACTCTAGAGGATCCCCGGGTACCG 2944
 pBS-UNI.SEQ   291 ACCAAAATCCAGTGACCTGCAGGTCGACTCTAGAGGATCCCCGG-TACCG  339
                   ********************************************* *****
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expected-pBS  2945 AGCTCGAATTCACTGGCCGTCGTTTTAC 2972
 pBS-UNI.SEQ   340 AGCTCGAAT-CACTGGC-C ←M13 forward primer 356
                   ********* *******
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Figure 11. Alignment of pBE sequence with expected sequence. Expected-pBE: Published sequences of pBin19 and pRTL2. pBE-REV.SEQ: Using M13 reverse primer, the sense strand of T-DNA in pBE was sequenced; pBE-UNI.SEQ: Using M13 forward primer, the antisense strand of T-DNA in pBE was sequenced. Both sequences were very crowded, should only be used to get information of the ligation.

```
                    M13 reverse primer→          ←pBin19 PstI Term→
expected-pBE      1 CAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGCctgcagGTC   50
 pBE-REV.SEQ      1                                AAGCTTGCATGCCTGCAGGTC   21
                                                   *********************

expected-pBE     51 ACTGGATTTTGGTTTTAGGAATTAGAAATTTTATTGATAGAAGTATTTTA  100
 pBE-REV.SEQ     22 ACTGGATTTTGGTTTTAGGAATTAGAAATTTTATTGATAGAAGTATTTTA   71
                    **************************************************

expected-pBE    101 CAAATACAAATACATACTAAGGGTTTCTTATATGCTCAACACATGAGCGA  150
 pBE-REV.SEQ     72 CAAATACAAATACATACTAAGGGTTTCTTATATGCTCAACACATGAGCGA  121
                    **************************************************

expected-pBE    151 AACCCTATAAGAACCCTAATTCCCTTATCTGGGAACTACTCACACATTAT  200
 pBE-REV.SEQ     122 AACCCTATAAGAACCCTAATTCCCTTATCTGGGAACTACTCACACATTAT  171
                    **************************************************

expected-pBE    201 TCTGGAGAAAA-TAGAGAGAGATAGATTTGTAGAGAGAGACTGGTGATTT  249
 pBE-REV.SEQ     172 TCTGGAGAAAAATAGAGAGAGATAGATTTGTAGAGAGAGACTGGTGATTT  221
                    *********** ***************************************
                              ←Term | 35S promoter→
expected-pBE    250 --GCGGACTCTAGAGGGTCCTCTCCAAATGAAATGAACTTCCTTATATAG  297
 pBE-REV.SEQ     222 TTGCGGACTCTCGAGG-TCCTCTCCAAATGAAATGAACTTCCTTATATAG  270
                      ********* **** **********************************

expected-pBE    298 AGGAAGGGTCTTGCGAAGGATAGTGGGATTGTGCGTCATCCCTTACGTCA  347
 pBE-REV.SEQ     271 AGGAAGGGTCTTGCGAAGGATAGTGGGATTGTGCGTCATCCCTTACGTCA  320
                    **************************************************
        ←35S promoter EcoRV Enhancer→
expected-pBE    348 GTGGAgatatcACATCAATCCACTTGCTTTGAAGACGTGGTTGGAACGTC  397
 pBE-REV.SEQ     321 GTGGAGATATCACATCAATCCACTTGCTTTGAAGACGTGGTTGGAACGTC  370
                    **************************************************

expected-pBE    398 TTCTTTTTCCACGATGTTCCTCGTGGGTGGGGGTCCATC-TTTGGGACCA  446
 pBE-REV.SEQ     371 TTCTTTTTCCACGATGCTCCTCGTGGGTGGGGGTCCATCCTTTGGGACCA  420
                    *************** ********************* **********

expected-pBE    447 CTGTCGGTAGAGGCATTCTTGAACG-ATAGCCTTTCCTTTATCGCAATGAT  496
 pBE-REV.SEQ     421 CTGTCGGCAGAGGCAT-CTTCAACGAATGGCCTTTCCTTTATCGCAATGAT  470
                    ******* ******** ******** ** ***********************

expected-pBE    497 GGCATTTGT-AGAAGCCATCTTCCTTTTCTACTGTCCTTTCGATGAAGTGACA  548
 pBE-REV.SEQ     471 GGCATTTGTTAGGAGCCACCTTCCTTTTCCACTATCTTC              509
                    ********* ** ***** ********** *** ** *

expected-pBE    549 GATAGCTGGG-CAATGG-AATCCGGAGGAGGTTTCCCG--ATATTACCCT  594
 pBE-UNI.SEQ      21 GATAGCTGGGGCAATGGGAATC-GGAGGGAGGTTTCCGGGATATTAACCC   69
                    ********** ****** **** *****  * ** ***  ****** **

expected-pBE    595 TT--GTTGAAAAG---TCTCAATAGCCCTCT--GGTCTTCT---GAGACT  634
 pBE-UNI.SEQ      70 TTTGGTTGGAAAAAGTTCTCAATTGCCCCTTTGGGTCTTTCTGGAGGACT  119
                    **  **** ***    ******* ****  *  ******       ****

expected-pBE    635 GTATCTTT--GATATTCTTGG-AGTAGA-CGAGAGTGT-CGTG-CTCCAC  678
 pBE-UNI.SEQ     120 GTATTCTTTGGATATTTTTGGGAGTAGAACAAGTGTGTTCGTGGCTCCAC  169
                    ****  **  ****** **** ****** * ** **** **** ******

expected-pBE    679 CATGTTTC--ACATCAATCCA-CTTGCTTTGAA--GACGTGGTTGG-AAC  722
```

```
pBE-UNI.SEQ    170 CATGTTATCAACATCAATCCAACTTGCTTTGGAAGGACGTGGTTGGGAAC  219
                   ******    *********** ********* *  *********** ***

expected-pBE   723 GTCTTCTTTTTCCACGATGTTCCTCGTGGGTGGGGGGTCCATCTTTGGGAC  772
pBE-UNI.SEQ    220 GTCTTCTTTTTCCACGATGCTCCTCGTGGGTGGGGGGTCCATCTTTGGGAC  269
                   ******************* ******************************

expected-pBE   773 CACTGTCGGTAGAGGCATTCTTGAACGATAGCCTTTCCTTTATCGCAATG  822
pBE-UNI.SEQ    270 CACTGTCGGCAGAGGCAT-CTTCAACGATGGCCTTTCCTTTATCGCAATG  318
                   ********* ******** *** ****** ********************

expected-pBE   823 ATGGCATTTGTAGAAGCCATCTTCCTTTTCTACTGTCCTTTCGATGAAGT  872
pBE-UNI.SEQ    319 ATGGCATTTGTAGGAGCCACCTTCCTTTTCCACTATCTTCACAATAAAGT  368
                   ************* ***** ********** *** ** *  * ** ****

expected-pBE   873 GACAGATAGCTGGGCAATGGAATCCGGAGGAGGTTTCCCGATATTACCCT  922
pBE-UNI.SEQ    369 GACAGATAGCTGGGCAATGGAATCCG-AGGAGGTTTCCGGATATTACCCT  417
                   ************************** *********** ***********

expected-pBE   923 TTGTTGAAAAGTCTCAATAGCCCTCTGGTCTTCTGAGACTGTATCTTTGA  972
pBE-UNI.SEQ    418 TTGTTGAAAAGTCTCAATTGCCCTTTGGTCTTCTGAGACTGTATCTTTGA  467
                   ****************** ***** *************************

                                                ←Enhancer PstI
expected-pBE   973 TATTCTTGGAGTAGACGAGAGTGTCGTGCTCCACCATGTTGACctgcagG 1022
pBE-UNI.SEQ    468 TATTTTTGGAGTAGACAAGTGTGTCGTGCTCCACCATGTTGACCTGCAGG  517
                   **** *********** ** ******************************

                    pBin19→
expected-pBE  1023 TCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCACTGGCCGTCGT 1072
pBE-UNI.SEQ    518 TCGACTCTAGAGGATCCCCGG-TACCGAGCTCGAAT-CACTGC-C       559
                   ********************* ************** *****  *

expected-pBE  1073 TTTAC 1077
pBE-UNI.SEQ ←M13 forward primer
```

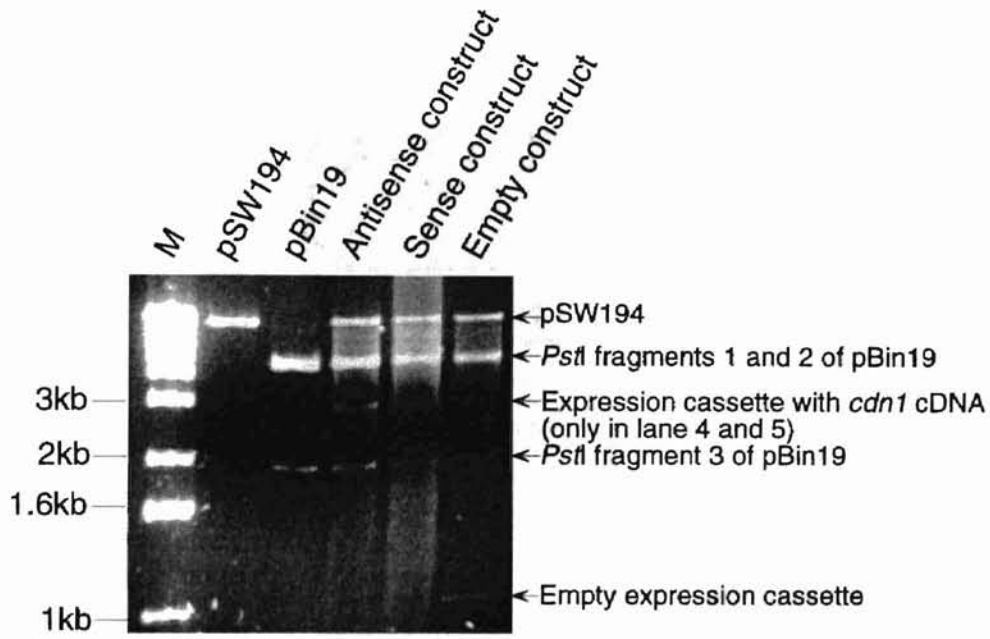


Figure 12. Verification of binary plasmid and pSW194 in recombinant EHA105. Plasmids were prepared using QIAGEN plasmid preparation kit which can only recover small plasmids (limit about 15kb). Plasmids were incubated with *PstI* at 37 degrees C and separated on 1.0% agarose gel.

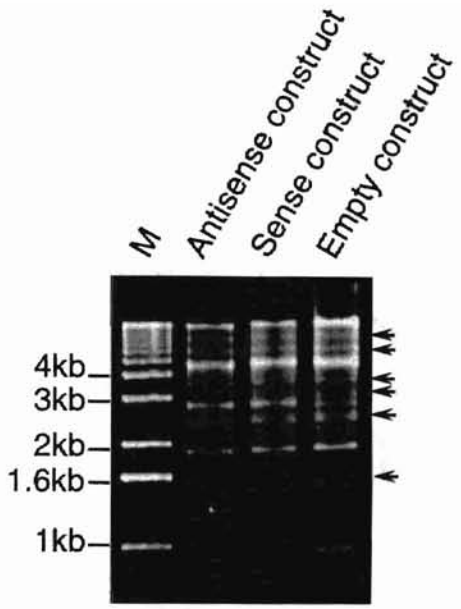


Figure 13. Evidence for the existence of Ti plasmid in recombinant EHA105. Plasmids were prepared following Li's protocol (Li, 1995), incubated with *PstI*, and separated on 1.0% agarose gel. The bands pointed to with arrows, presumably, were Ti plasmid fragments.

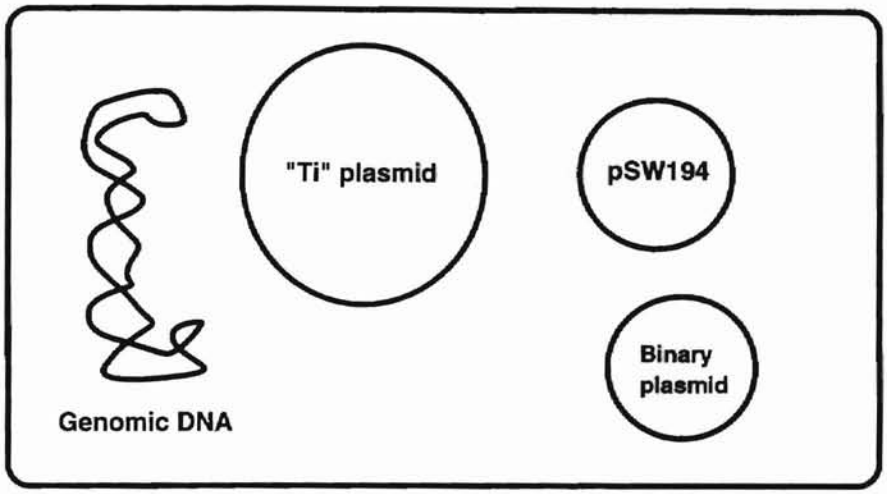


Figure 14. Sketch of recombinant EHA105 used for plant transformation. Genomic DNA confers rifamycin resistance. Helper plasmid pSW194 confers ampicillin and tetracyclin resistance. Binary plasmid confers kanamycin resistance. There is no resistance gene in "Ti" plasmid.

Figure 15. Transgenic Tobacco Plants.


3 21 96

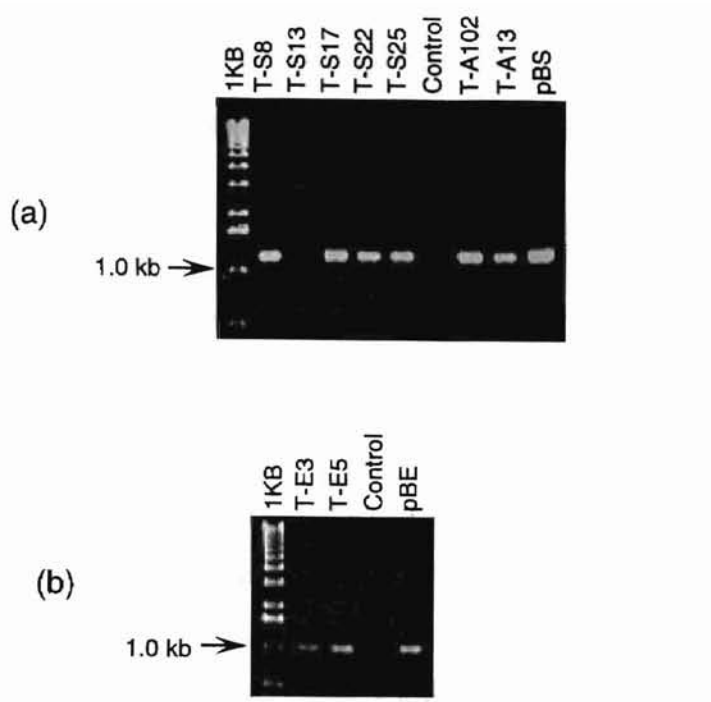


Figure 16. PCR screen for transgene in regenerated tobaco plants. (a) Sense and antisense plants were screened using two gene specific primers. A 1.2 kb band was amplified if the plant contained a transgene. (b) Plants transformed with an empty vector were screened using primers M13 forward and M13 reverse. A 1.0 kb band was amplified if the plant contained a transgene.

Chapter 4. Transformation of Cotton and Analysis of Transgenic Cotton

Introduction

Although embryogenic cultures have been achieved with bacterial-blight-resistant lines of cotton (*Gossypium hirsutum* L.), *e.g.* MAR line CABUCAG8US-1-88 (Trolinder, personal communication), the entire procedure of transformation MAR line with *Agrobacterium* followed by plant regeneration had not been tested. However the procedure has been tested and succeeds well on cotton Coker lines (*Gossypium hirsutum* L.) which are susceptible to bacterial blight. If we transform Coker, *e.g.* Coker 312-5a, with a transgene and cross the transgenic plants with a blight-resistant line, we should be able to obtain progenies which have both a transgene and a resistance gene.

The protocol that we used to transform and regenerate cotton was kindly suggested by Norma Trolinder (BioTex, Inc., Lubbock, TX). We are not at liberty to publish details of her procedure, but it is based on her earlier publications (Trolinder & Goodin, 1987; Trolinder & Goodin, 1988; Trolinder & Goodin, 1988b; Trolinder & Shang, 1991; Koonce et al., 1996). The procedure was laborious and time consuming, and was done by a group from Dr. M. Pierce and Dr. M. Essenberg's laboratory. I will only discuss the step of harvesting suspension cultures, which I worked on, and some of the results of the plant regeneration.

An efficient method was necessary to screen the large numbers of regenerated plants and identify those possessing a transgene. Based on other groups' experiences, we developed a protocol to purify genomic DNA from a fresh leaf disc and test the presence of a transgene by the polymerase chain reaction (PCR). This allowed us to test more than one

hundred regenerated plants in a short time. A small scale Southern analysis was also performed to show the incorporation of transgenes.

When plants are challenged with a non-host pathogen, which we call hetero-pathogen, a hypersensitive reaction can occur and phytoalexins may be produced. For example, when *Arabidopsis thaliana* was inoculated with a wheat pathogen, *Pseudomonas syringae* pv. *syringae*, phytoalexin accumulated during the hypersensitive reaction (Tsuji et al., 1991). Defense transcripts were also found to accumulate in bean in response to the hetero-pathogen *Pseudomonas syringae* pv. *tabaci* (Jakobek et al., 1993). When cotton line Ac44, susceptible to *Xcm*, was challenged with *Xanthomonas campestris pv. campestris*, which is a pathogen of cabbage, it responded with HR and phytoalexins accumulated (Essenberg et al., 1990). This result suggests that a defense pathway similar to what is turned on by *Xcm* in cotton lines resistant to bacterial blight is also turned on in blight-susceptible lines of cotton by hetero-pathogens.

The cotton line we transformed was susceptible to *Xcm*, which restricted us to testing the efficiency of interference of CDN1 by challenging the transgenic plants with a pathogen other than *Xcm*. Since the hetero-pathogen can induce the *cdn1* gene, we may inoculate the transgenic plant with a hetero-pathogen, and test the results of interfering with CDN1 on phytoalexin production. If the knockout is successful, there will be no phytoalexin or lower level of phytoalexin produced in some antisense transgenic plants than in control plants. And, if phytoalexin is very important for the plant's resistance to bacteria, these plants will be susceptible or be less resistant to that hetero-pathogen than control plants. Based on this hypothesis, we performed a hetero-pathogen test after the first generation of transgenic plants was obtained. In this test, we evaluated bacterial growth, visible response of the plant, and CDN1 enzyme activity. (Phytoalexin analysis may be performed by another member of the laboratory on a small number of promising plants).

In the study of Daub and Hagedorn (Daub & Hagedorn, 1980), a hetero-pathogen of bean, *P. s. pv. coronafaciens*, multiplied about 100 fold in bean leaves when the inoculum

concentration was 10^7 cfu/ml. We expected the growth pattern of *X. c.* pv. *vesicatoria* in cotton to be similar to *P. s. pv. coronafaciens* in beans. We used that information coupled with previous results on how an incompatible race of *Xcm* grows in highly resistant cotton to design our experiment based on this assumption, which was found later to be a mistake.

Materials and methods

Cotton seeds

G. hirsutum L. Coker 312-5a (V95-031) seed was obtained from Greg Cartwright (USDA-ARS, Lubbock, TX) and increased in Mexico in the 1995-1996 season. *G. hirsutum* L., MAR line CABUCAG8US-1-88 (I. P. S. #15) seed was obtained from Greg Cartwright (USDA-ARS, Lubbock, TX) and increased in a greenhouse (Stillwater, OK) during the summer of 1996.

Methods of suspension culture harvesting

After cotton tissues had been cultured in liquid medium for 4 to 6 weeks, or after they had grown to occupy more than half volume of the medium, tissues were harvested. Cells and medium were transferred to a 50 ml sterile plastic centrifuge tube with a 7 ml sterile plastic transfer pipette (Fisher 13-711-22), and big clumps of callus that could not go in the pipette were discarded. Fresh medium was added to the tube to make the total volume 50 ml. After the tissue mass had settled (5 to 10 min), supernatant was poured away, and tissue was resuspended in about 50 ml of fresh medium. After the tissue settled again, supernatant was poured away, and the tissue was resuspended in about 10 volumes of fresh medium. Using a 5 ml pipetter, 2 ml of this cell suspension was plated per plate on solidified medium. During the whole procedure, tissues were not allowed to sit more than 15 min without shaking or swirling. Usually, five to ten plates were prepared from each suspension culture flask. It worked well to harvest five cultures at a time.

From my experience, the suspension cultures that looked straw colored were good, and grew well in the next step. Cultures that looked brown but had a few green tissue masses had some growth in the next step too. But suspension cultures that were totally brown were not good. Cultures that did not grow in suspension were not good either, and these cultures were white and tended to looked rubbery and swollen. Cultures that looked very fine and gray were not good either.

PCR

Genomic DNA was prepared on a small scale following modification of a method of K. M. Haymes (Haymes, 1996). One or two leaf discs of fresh tissue from transgenic or non-transgenic cotton were ground to pulp (about 10 to 15 seconds) in a 1.5 ml eppendorf tube, using a pestle that fits the tube and that was attached to a benchtop power drill. CTAB buffer (250 µl, 100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2% CTAB; 0.4% β-mercaptoethanol) was added, and the tube was incubated at 65°C for 30 min to 1 hr. The solution was then extracted once with 250 µl chloroform-isoamyl alcohol (24:1) and centrifuged at 10 k × g for 3 min. Nucleic acid was precipitated from the supernatant with isopropanol. After centrifugation, the pellet was re-dissolved in 100 µl H_2O and incubated with RNase (final concentration 10 µg/ml) at 37°C for more than 20 min. Genomic DNA was precipitated by adding a half volume of 7.5 M NH_4Ac and 2 volumes of absolute ethanol, followed by centrifugation at 10 k × g for 15 min. Pellet was washed with 70% ethanol, dried and re-dissolved in 25 µl H_2O. One hundred to 500 ng of the product (0.5 µl to 1 µl) was added to a 25 µl PCR reaction.

PCR primers were synthesized by the OSU Recombinant DNA/Protein Resource Facility. Two internal primers, serial numbers 918 and 3188, which are specific to *cdn1* cDNA, were used to screen regenerated cotton. Primer 918 is a sense primer corresponding to nucleotides 433 to 453 of *cdn1*. Primer 3188 is an antisense primer

complementary to nucleotides 792-808 of *cdn1*. PCR was performed with the following program :

1. 94°C 2 min,
2. 94°C 45 sec,
3. 50°C 45 sec,
4. 72°C 50 sec,
5. go to step 2 , 35 more times,
6. 72°C 10 min,
7. 4°C.

Southern blot

Labeling and detection materials are from Dig DNA Labeling and Detection Kit (Boehringer Mannheim) unless stated otherwise. Two probes, both double stranded, were labeled with digoxigenin (Dig). Probe 1 was labeled by PCR with two *cdn1* cDNA specific primers, 918 and 3188, which border a 376 bp product. The template for probe 1 was 100 pg of plasmid pBA. The PCR program for labeling probe 1 was: 94°C 2 min; 36 cycles of, 94°C 40 sec, 48°C 40 sec, 72°C 3 min; 72°C 7 min; 4°C hold. Probe 2 was labeled by PCR with two primers, 3721 and 3748, which anneal to 35S promoter or enhancer element. Primer 3721 (complementary to nucleotides 782 to 799 in figures 9 and 10) anneals to the end of the 35S promoter sequences of pRTL2, while 3748 is a sense primer (nucleotides 68 to 85 in figures 9 and 10) corresponding to the sequence 20 nucleotides downstream from the beginning of the enhancer. To reduce the amount of non-specific products, template for labeling probe 2 was a 3.4 kb agarose-gel-purified *Bgl*II-*Xho*I fragment of pBA which includes the 35S promoter and enhancer element. This

combination of primers and template would give 2 products (because pRTL2 has double enhancer sequences), one 710 bp and the other 400 bp, with the 400 bp product dominant. Only the 400 bp product was used as probe 2. The PCR program for labeling probe 2 was the same as the program for probe 1 except that the annealing temperature was 42°C. Each PCR labeling mixture consisted of 2 mM $MgCl_2$, 1 μM of each primer, 0.2 mM each of dATP, dCTP, dGTP, 0.13 mM dTTP, 0.07 mM Dig-11-dUTP, and 5 units of Taq DNA polymerase in a 25 μl reaction (Promega). These PCR reactions gave approximately 0.5 to 1 μg of labeled products with an estimated labeling intensity of 8%. The PCR products from both probe labeling reactions were separated on agarose gel and the desired bands were excised and purified with QIAquick gel extraction kit.

Genomic DNA was prepared as described by Doyle (Doyle & Doyle, 1989). Ten μg of genomic DNA was digested overnight with 40 units of *Xba*I or *Eco*RI in 100 μl reaction, and extracted with phenol-chloroform. DNA was precipitated with 1 volume of 7.5 M ammonium acetate and 2 volumes of absolute ethanol, re-dissolved in 15 μl H_2O and separated on 0.8% agarose gel. The gel was immersed in denaturing solution (0.4 M NaOH, 0.5 M NaCl) for 40 min with gentle shaking, followed by immersing in neutralizing solution (0.5 M Tris, pH 7.5, 1.5 M NaCl) 40 min. DNA was transferred to nylon membrane (Amersham, Hybond-N^+) from the gel by the capillary method with 20x SSC (3M NaCl, 0.3M sodium citrate). DNA fragments were fixed to the membrane by applying 120,000 μjoul UV light in a Stratalinker (Bio-Rad). Hybridizations were performed at 65°C. Hybridization buffer consisted of 3-5 ng/ml probe, 5x SSC, 0.1% laurylsarcosine (Sigma), 0.2% SDS, 1% blocking reagent (Boehringer Mannheim). After hybridization, membranes were washed in stringent conditions which were at the same temperature as the hybridization and with the wash buffer of 0.1x SSC plus 0.1% SDS. Membranes were exposed to X-ray film (Kodak or Fuji) after incubation with anti-Dig antibody conjugated with alkaline phosphatase and then CPD™ substrate (Boehringer

Mannheim) according to manufacturer's instruction. Probes were stripped away by immersing the membrane in 50°C stripping solution (0.2 N NaOH plus 1% SDS) and shaking 20 min gently at room temperature. Membrane was then washed 5 min with 0.1% SSC plus 0.1% SDS, and was ready for next prehybridization and hybridization.

Hetero-pathogen test

Bacterium *X. c. pv. vesicatoria (Xcv)* was given to us by Dr. J. Anderson of the Horticulture and Landscape Architecture Department of OSU. It was increased in nutrient broth and stored in 15% glycerol at -80°C. Before inoculation, *Xcv* was cultured in 50 ml nutrient broth overnight at 28°C, with shaking at 200 rpm. This culture was diluted to 200 ml and grown until OD_{600} reached 0.20. Bacteria were diluted in sterile water saturated with $CaCO_3$. The final concentration of inoculum was 8.4×10^6 cfu/ml determined by diluting and plating on nutrient agar. Two youngest fully expanded leaves from each of 36 regenerated plants that were 3 weeks old were infiltrated through open stomata on the lower epidermis with a hand sprayer. (Ages of regenerated plants were based on the time they were in the soil; plants were potted in soil after the cuttings rooted in jars, which was about two weeks after cutting). A 0.68 cm^2 leaf disc was excised from each of 10 experimental plants after inoculation, rinsed and ground in 1 ml $CaCO_3$ solution, diluted 10-fold and 50 µl was plated on nutrient agar. The average of bacterial plate counts from these 10 leaf discs was converted to initial population per cm^2. Another leaf disc was harvested 8 or 12 days later from all 36 plants, rinsed and ground in 1 ml $CaCO_3$ solution, diluted variously, and 50 µl was plated to determine bacterial growth.

Enzyme assay

Enzyme assay was performed following Davis' method (Davis et al., 1996). Sixty-five hours after inoculation, one 0.68 cm^2 leaf disc per plant was harvested and stored in ice until homogenizing. Each leaf disc was ground using a hand pestle and mortar in 0.5 ml homogenization buffer (150 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 10% glycerol, 1 mM PMSF, 5 mM DTT, 0.1% Tween 80) on ice until no big pieces were visible and was ground for one more min after that. The crude extract was collected into an eppendorf tube and centrifuged 20 minutes at 14,000 g in a desktop centrifuge. Twenty-five µl of supernatant was added to 220 µl assay buffer (30 mM HEPES, 10% glycerol, 1 mM $MgCl_2$, 5 mM DTT), incubated at 30° for 5 min, followed by addition of 5 µl (E,E)-[1-^{3}H]FPP (Dupont NEN) which had a specific activity of 32 µCi μmol^{-1}. After FPP was added, the reaction was incubated at 30°C for 25 min, and the reaction was stopped by adding 3 ml hexane and vortexing 5 sec. The hexane phase (as much as can be withdrawn) was transferred to a test tube containing about 1 g silica powder, and mixed by pipetting. One ml hexane extract was added to 5 ml scintillation liquid and counted. The protein concentration in the crude extract was determined by the Bradford method (Davis, 1998).

Results

Transformation and regeneration

Transformations of Coker lines were started in several different sets. Many explants were thrown away during various stages of regeneration due to lack of enough space and personnel to maintain cultures. One set of explants transformed and regenerated (5aA1) was used to represent the regeneration rates because this was the first set transformed and received the most attention and had the least chance to be thrown away.

In the set 5aA1, 445 explants were infected with *Agrobacterium*, and 69 of these explants formed good callus and were cultured in suspension. Of these suspension

cultures, 48 were harvested and plated on solidified medium. Finally, 25 cell lines were generated, grown in jars, and also potted.

To date, ninety-nine cell lines of antisense plants, fifty-seven cell lines of sense plants, and twenty-eight cell lines of "empty" plants have been obtained. Most of them have two or more than two plants.

PCR

Using two gene specific primers, 918 and 3188, one fragment of about 680 bp was amplified from non-transgenic plant genomic DNA, which contains both exons and introns of the native *cdn1* gene. Based on the genomic sequence of *cdn1-B*, the only gene family member of *cdn1* whose genomic sequence had been obtained (Chen et al., 1996), there is one intron that is about 320 bp between primers 918 and 3188 in *cdn1* gene. The same set of primers was able to amplify a 376 bp fragment from plasmid pBA, which was the binary plasmid carrying the antisense cDNA of *cdn1* (Figure 17). These primers were expected to amplify two fragments from the genomic DNA of transgenic plants, and would be able to amplify only one fragment (680 bp) from regenerated cotton lines which had failed to integrate a transgene. We screened cotton lines which were regenerated after attempted transformation with the antisense construct by these methods. In 130 regenerated antisense plants (covering 58 independent cell lines), we were able to amplify two fragments from 114 plants, which included 51 cell lines, and only one fragment (680 bp) from 16 plants that covered 9 cell lines (Figure 17). These results suggest an 88% efficiency of the selection method was obtained.

Southern analysis

Four different transgenic plants were chosen to be analyzed by Southern blot. Genomic DNA from transgenic and non-transgenic plants was digested with *Eco*RI overnight, fractionated on 0.8% agarose gel, transferred to nylon membrane and probed

with digoxigenin labeled probe. The transferred DNA contained only one *Eco*RI cut site between the *cdn1* cDNA and the Term sequence, and it was outside the region detected by the probe. This means different-sized fragments would be detected for different positions of insertion of the transgene in the genome, and only one fragment would be detected per copy of the transgene. Every *Eco*RI fragment that contained a transgene would include the 35S promoter sequence and *cdn1* sequence. Using the 35S promoter and enhancer element as probe (probe 2), these different plants showed random insertions of the transgene with different numbers of copies (Figure 18). The copy number varied from 1 to 6 in these lines. One regenerated cell line, A1-57e, which was PCR negative for the transgene was also analyzed with Southern blot. Southern analysis confirmed that the result from PCR was correct (data not shown).

After detection with probe 2 (35S promoter and enhancer specific), the probe was stripped away from the membrane, and the same membrane was probed with probe 1 (*cdn1* gene specific probe). The same bands detected with probe 2 were also detected with probe 1, indicating 35S promoter and *cdn1* cDNA were still linked in the integrated gene (Data not shown). The stripping was complete because the probe 2-specific control DNA, which was the *Xba*I fragment containing 35S promoter but not *cdn1* sequence, was not detected in the second reaction.

Genomic DNA from the same plants was also digested with *Xba*I, which would excise the 1.7 kb *cdn1* cDNA (Figure 3) from the transferred DNA (if intact), and probed with a *cdn1* gene-specific probe (probe 1). The transgenic plants showed the intact cDNA that integrated (Figure 19). Of the four transgenic plants examined, A2-185b had multiple copies of the transgene; there was an extra band besides the expected 1.7 kb transgene band and other bands from the native *cdn1* genes (Figure 19), indicating some rearrangement of the transgene had happened in this cell line.

Gene family

Using probe 1, I was able to show the sesquiterpene cyclase gene family in non-transgenic cotton of the Coker line. Non-transgenic cotton plant genomic DNA was digested with *Eco*RI or *Xba*I, and probed with the *cdn1* gene specific probe (probe 1). After washing in highly stringent conditions, 12 bands were detected from both digests (Figure 20). From these results, I estimate there are about 12 sesquiterpene cyclase-like genes in the Coker line of *G. hirsutum.*

Enzyme assay

Sixty-five hours after inoculation with *Xcv*, a leaf disc was harvested and assayed for enzyme activity. Activities varied greatly from plant to plant even in "empty construct plants" (E plants). The average activities for the three constructs were (Figure 21): $(9.06\pm1.89) \times 10^{-2}$ nmol/(min cm^2) for 8 "Empty" plants (E plants); $(8.28\pm0.89) \times 10^{-2}$ nmol/(min cm^2) for 12 PCR positive antisense plants (A plants), which only showed 10% reduction of CDN1 activity of control E plants; $(1.28\pm0.13) \times 10^{-1}$ nmol/(min cm^2) for 14 sense plants (S plants), which is 143% of the average activity of E plants. Protein concentrations in the crude extracts were measured, although previous experience showed that the pigments in plant crude extracts affect absorbance generated by the protein coomassie blue binding reaction (Davis, E., Essenberg, M., & Pierce, M., not published). The average specific activities (nmol (+)-δ-cadinene produced min^{-1} μg protein^{-1}) were calculated based on protein concentration to be: $(2.63\pm0.46) \times 10^{-4}$ nmol/(min μg) for E plants, $(2.78\pm0.32) \times 10^{-4}$ nmol/(min μg) for A plants, $(3.85\pm0.39) \times 10^{-4}$ nmol/(min μg) for S plants. Protein contents measured from the A plants were less than those of the E plants on average. The results indicated that the knockout by antisense was not very successful, although in the sense construct, the CDN1 enzyme activity was increased by

43% on average. There was not much difference in the average enzyme level between A plants and E plants. The range of variation in A plants seemed to be smaller than in E plants (Figure 21 and 22); this is more obvious in Figure 22. Uninoculated control samples of two A plants and two E plants did not show any CDN1 activity. Of the S uninoculated samples, S3-248a did not have any enzyme activity, while S2-29f gave some activity, about one third of the average activity of E inoculated plants.

Two regenerated plants that were transformed with antisense construct but were transgene-negative in PCR reactions were also included in the experiments. The enzyme levels for these two plants were in the range of E plants.

Hypersensitive reaction and hetero-pathogen populations

The hypersensitive reaction varied from line to line. On day 8 after inoculation, a leaf disc was harvested from each plant to determine bacterial growth. We expected *Xcv* to multiply more than 100 fold. The leaf disc was ground in 1 ml $CaCO_3$ solution, diluted 500-fold and 10,000-fold in order to yield countable plates from any multiplication between 50-fold to 2000-fold, and 50 µl of each dilution was plated. We expected about 200 colonies from 10,000-fold dilution if the bacteria had multiplied 1000-fold and about 200 colonies from the 500-fold dilution if the bacteria had multiplied 50-fold. Most plates regardless of the dilution, had 0 or less than 5 colonies, showing the growth pattern of *Xcv* is not the same as we expected. By eight days the populations had in general not increased or had actually declined from the initial population level. In most transgenic plants the day 8 population was less than the day 0 average, although the plate counts were too low to give reliable numbers. Only plates from five plants had more than 20 colonies, telling us populations of *Xcv* in these five transformants were higher than the rest. Upon seeing these results, another leaf disc was harvested from each leaf on day 12 to count *Xcv* populations, and the homogenates were diluted 2- to 200-fold according to the results from day 8. The inoculated sections of leaf in those 5 lines which had higher *Xcv* populations

on day 8 were completely necrotic, too dry to count bacterial growth then, so they were not included in the day 12 analysis. The populations more reliably determined on day 12 were even lower than those estimated on day 8. The bacterial population density vs. enzyme activity is plotted in Figure 22. There was no obvious correlation between the bacterial populations and the enzyme levels.

Figure 23 and Figure 24 are pictures of inoculated leaves taken 9 days after inoculation. Two middle sections of each leaf were inoculated. In Figure 23, pictures are aligned in order of increasing bacterial population. All of them were based on the data of day 12 except the five plants that had higher bacterial populations in day 8, and the data from these five plants were from day 8. There seemed to be a trend that the extent of HR is positively correlated with the bacterial population density, which is a puzzle to me. In Figure 24 the leaves are aligned in order of increasing enzyme level at 65 hr. There was no clear correlation between HR and the enzyme level.

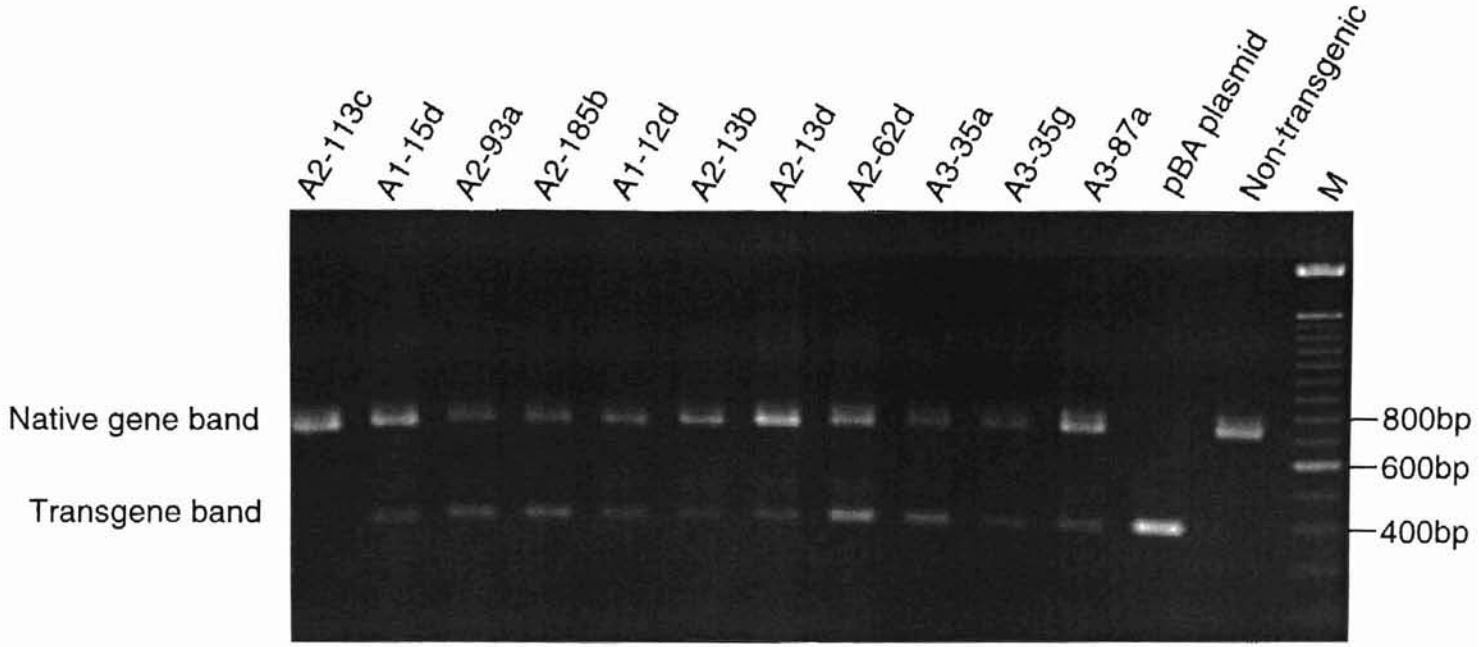


Figure 17. PCR screen for antisense transgene in regenerated cotton. PCR products were separated on 1.5% agarose gel. Lane 1 to lane 11 are transgenic plant candidates, lane 12 is a plasmid carrying the transgene, lane 13 is non-transgenic plant control. Lane 1 (A2-113c) is regenerated cotton without a transgene.

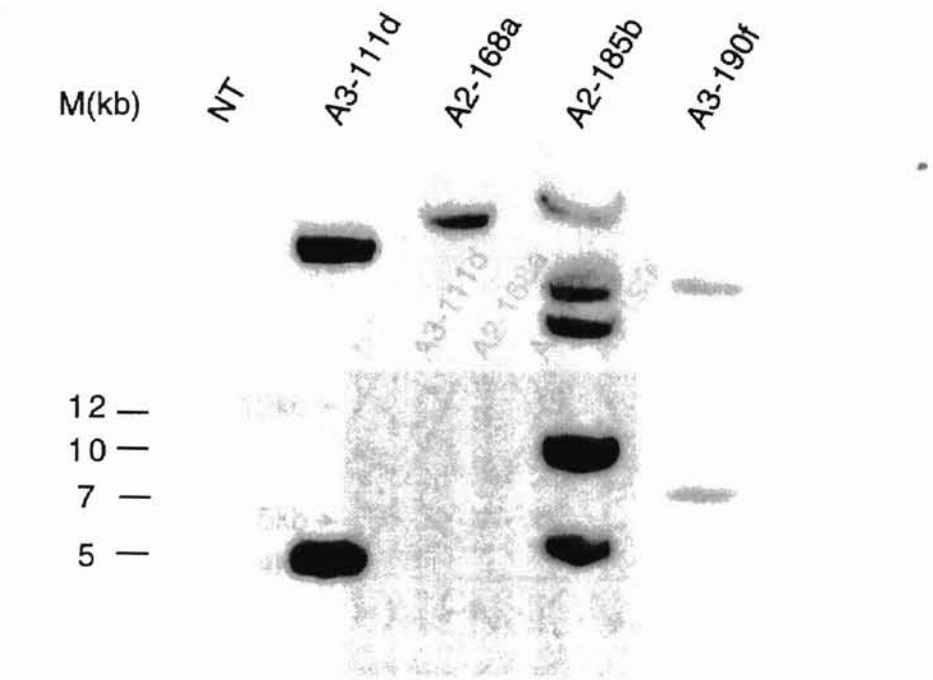


Figure 18. Southern analysis of selected transgenic plants. Genomic DNA was digested with *Eco*RI, separated on 0.8% agarose gel, transferred to nylon membrane, probed with a digoxigenin labeled fragment of 35S promoter and enhancer element. Lane 1 is non-transgenic plant, lane 2 to lane 5 are transgenic plants previously positive for transgene in PCR screen. In lane A2-185b, the second band from bottom looked two times darker than the others in the same lane, so it was counted to be two copies of transgene.

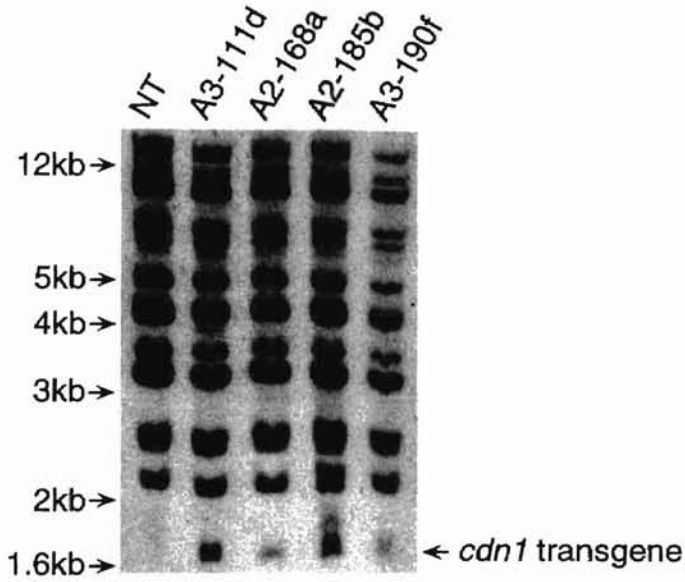


Figure 19. Southern blot showing the *cdn1* transgene (~1.7 kb) is intact. Genomic DNA was digested with *Xba*I, separated on 0.8% agarose gel, transferred to nylon membrane and probed with *cdn1* gene specific probe.

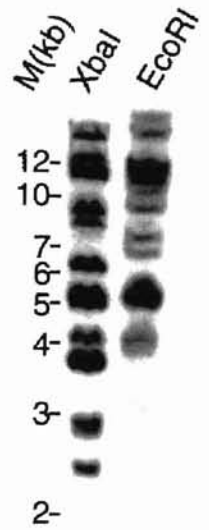
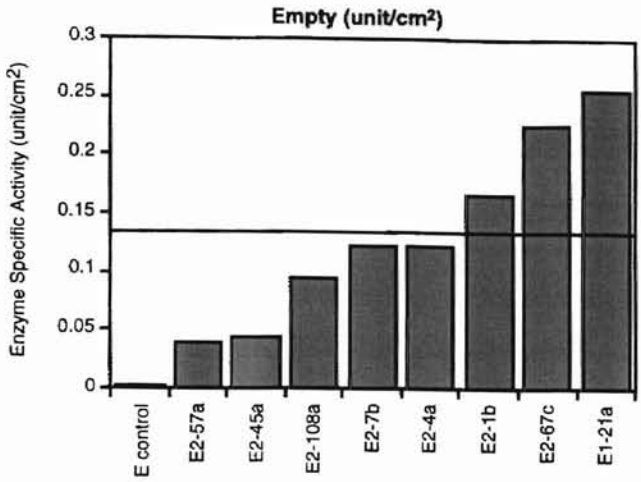


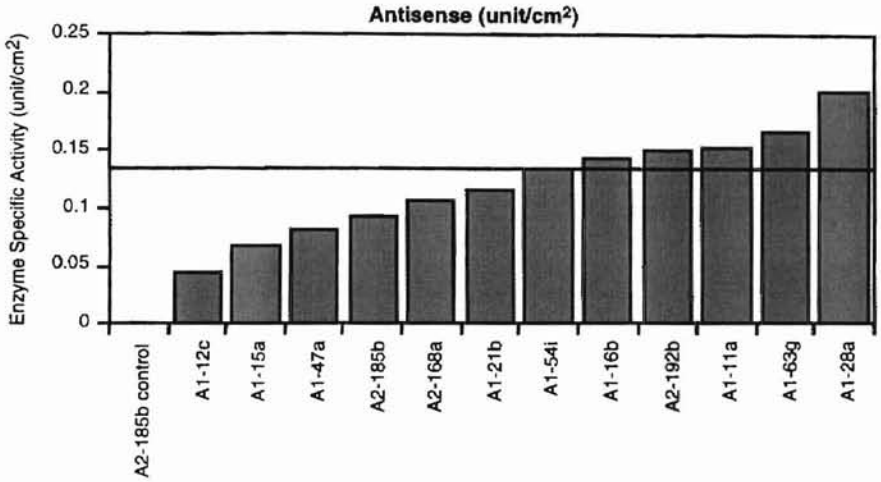
Figure 20. Gene family of *cdn1* gene. Genomic DNA from a Coker 312-5a non-transgenic plant was digested with XbaI or EcoRI, separated on an 0.8% agarose gel, transferred to nylon membrane, and probed with a digoxigenin labeled fragment of *cdn1* cDNA. Hybridization and washing were performed under stringent conditions.

Figure 21. CDN1 activities of transgenic plants, shown in unit/cm^2, one unit is defined as "catalyze formation of 1 nmol (+)-delta-cadinene per min." (a)Transgenic plants with empty construct; (b) Antisense transgenic plants; (c) Sense transgenic plants. The horizontal lines were the average enzyme level of E plants.

a


Empty (unit/cm²)
Enzyme Specific Activity (unit/cm²)
0.3
0.25
0.2
0.15
0.1
0.05
0
E control
E2-57a
E2-45a
E2-108a
E2-7b
E2-4a
E2-1b
E2-67c
E1-21a

b


Antisense (unit/cm²)
Enzyme Specific Activity (unit/cm²)
0.25
0.2
0.15
0.1
0.05
0
A2-185b control
A1-12c
A1-15a
A1-47a
A2-185b
A2-168a
A1-21b
A1-54i
A1-16b
A2-192b
A1-11a
A1-63g
A1-28a

c

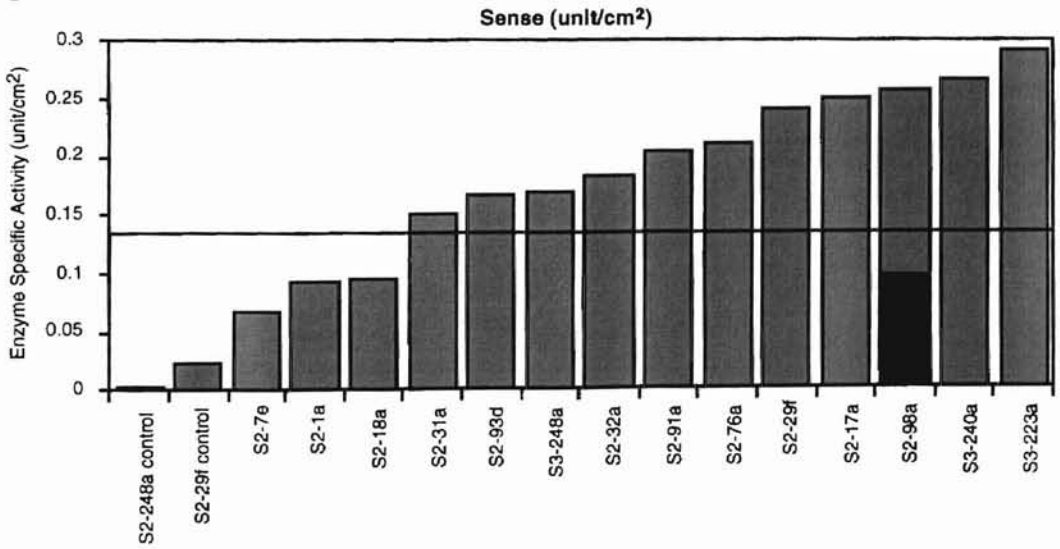
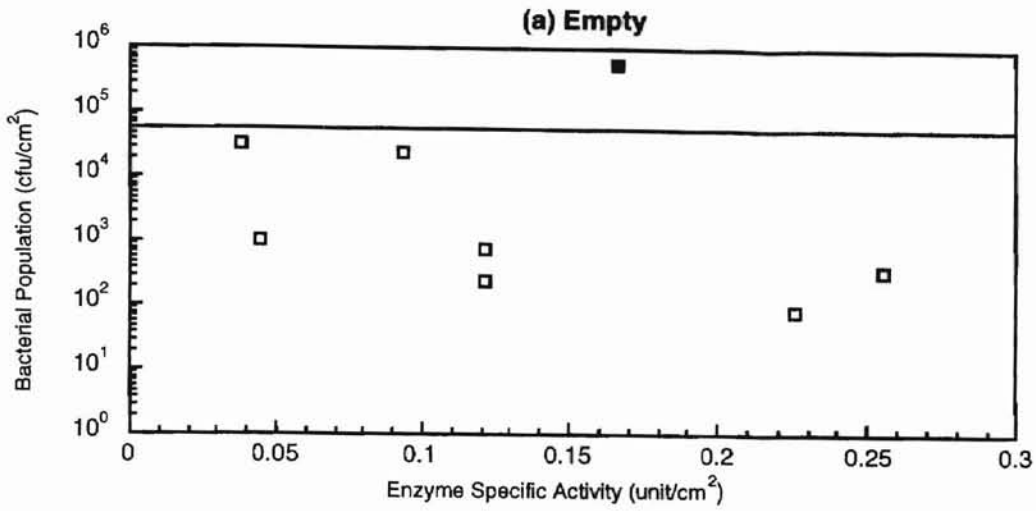
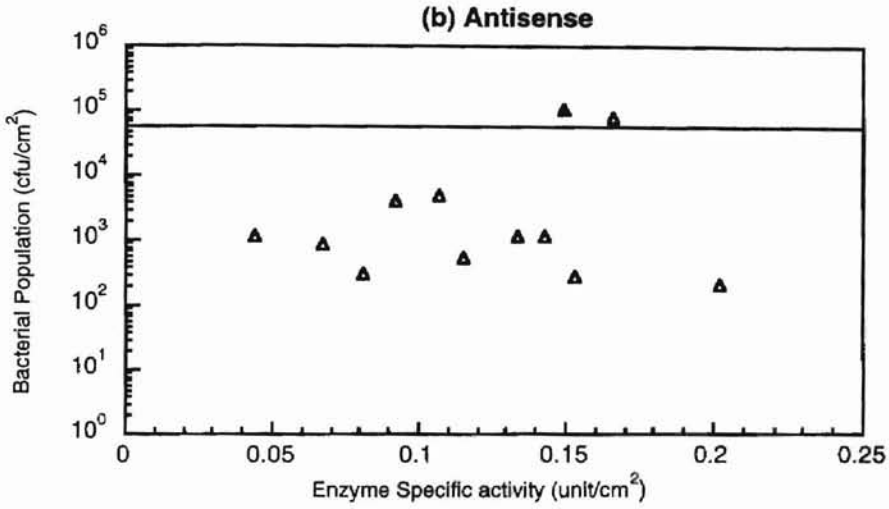

Sense (unit/cm²)
Enzyme Specific Activity (unit/cm²)
0.3
0.25
0.2
0.15
0.1
0.05
0
S2-248a control
S2-29f control
S2-7e
S2-1a
S2-18a
S2-31a
S2-93d
S3-248a
S2-32a
S2-91a
S2-76a
S2-29f
S2-17a
S2-98a
S3-240a
S3-223a

Figure 22. Bacterial populations plotted vs. Enzyme activities. Solid dots were the five plants which only had data from day 8, hollow symbols were data from day 12 of other plants. The horizontal lines show the average initial bacterial population.


(a) Empty
Bacterial Population (cfu/cm^2)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
0.05
0.1
0.15
0.2
0.25
0.3
Enzyme Specific Activity (unit/cm^2)


(b) Antisense
Bacterial Population (cfu/cm^2)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
0.05
0.1
0.15
0.2
0.25
Enzyme Specific activity (unit/cm^2)

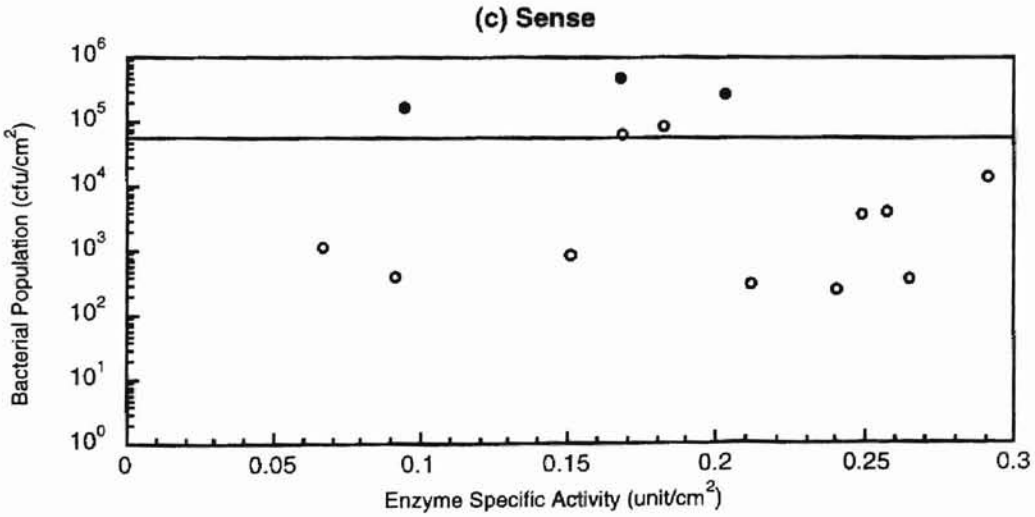

(c) Sense
Bacterial Population (cfu/cm^2)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
0.05
0.1
0.15
0.2
0.25
0.3
Enzyme Specific Activity (unit/cm^2)

Figure 23. Hypersensitive reaction of transgenic cotton 9 days after inoculation with *X. c.* pv. *vesicatoria.* Pictures of inoculated leaves were aligned by ascending bacterial population 8 or 12 days post inoculation.

Figure 24. Hypersensitive reaction of transgenic cotton 9 days after inoculation with *X. c.* pv. *vesicatoria.* Pictures of inoculated leaves were aligned by ascending enzyme activity at 65 hr post-inoculation.

Chapter 5. Discussion

Sense and antisense sequences of *cdn1* cDNA plus an expression cassette were inserted into binary vector pBin19 successfully. These binary plasmids, namely pBA, pBS, as well as the empty vector, pBE, were cloned into *Agrobacterium tumefaciens* EHA105. A helper plasmid pSW194 was co-transformed with the binary plasmid into EHA105. This helper plasmid was supposed to increase the efficiency of insertion which is believed to be the natural function of the Ti plasmid.

The Southern blot results (Figure 18) indicate that there are multiple copies of the transgene in most of the transgenic plants. This high integration rate may have been due to the use of the helper plasmid pSW194, which contains a copy of constitutively expressed *virG* gene and strongly induced the expression of other *vir* genes, and hence made the recombinant EHA105 "supervirulent".

The regeneration rates were obviously high; this may have been due to the method and medium we used, but it may also have been due to the intense care taken by the group directed by Dr. Pierce. Among the regenerated A plants, 88% showed positive for the transgene in the PCR reactions, indicating an 88% efficiency of the kanamycin selection.

In the enzyme assays after inoculation of transgenic plants with the hetero-pathogen *X. c.* pv. *vesicatoria*, there were obvious differences in the enzyme levels between the plants with the sense construct and the empty vector control plants. But it was disappointing to find that there was not much difference between plants with the antisense construct and the empty vector control plants. In other words, the intended knockout was not very successful. There are some possible reasons for this. The main reason may be the inefficient expression of the 35S promoter that we used in our constructs. Han's group

(Pih et al., 1996) studied three versions of cauliflower mosaic virus 35S promoter by fusing them to a GUS gene. Although there were few nucleotide differences between the sequences of these promoters, they found the 35S promoter from pRT101, from which the 35S promoter in pRTL2 (which we used) is derived, had very weak activity: GUS expression was less than 3 times that with no promoter, while the 35S promoter from pBI121 (Clontech) had about 7 times the activity of pRT101, and the third one, from pCaMVNEO, had about 14 times more activity than that of pRT101 (Han et al., 1996). Töpfer *et al.* also reported that CaMV 35S from pRT101 gave a low level of activity in tobacco. Han's group thought the lower activity of the promoter present in pRT101 was caused by the three nucleotide-substitution following the transcription start site (+1) due to the deletion of the native sequence and substitution with vector DNA, with the sequence CTC instead of ACG at +2 to +4. All other promoters studied by Han' group have ACG sequence from +2 to +4.

The unsuccessful knockout may also be due to the big family of the sesquiterpene cyclase genes. The Southern blot result showed that at least 12 gene members hybridized to the probe that is a fragment of *cdn1* cDNA under high stringency conditions. We do not know how many of them are expressed after induction by *Xcv*, but there are at least two expressed when cotton was challenged with *Xcm* (Davis, 1998).

I used crude extract of cotton foliage tissue to measure the protein concentration after the enzyme assay. The pigments interfere with the color reaction (Davis et al., not published), so the error caused by this may decrease the difference of enzyme level among the different constructs, which was already too small. That is why the enzyme activity was also expressed in nmol/(min cm^2).

The enzyme levels varied from plant to plant even in empty vector control plants. This may due to the regeneration of plants from undifferentiated calluses. Since plants included in the experiment of hetero-pathogen test and enzyme assay were all T1 plants from regeneration, there was much physical difference among the plants. We tried to minimize

physical differences due to culture history by cutting the explants to root at the same time and transplantation to the soil at the same time. There were, however, some cultural and physical differences including the time they were cultured in jars before they were excised from their mother plant, their height, their leaf numbers and the textures of their leaves. These differences may have contributed to some of the variations in response to bacteria, including the enzyme levels.

Although the knockout of *cdn1* with antisense DNA was not successful, we did get a range of different enzyme levels. I plotted the bacterial growth versus the enzyme levels; but observed no obvious correspondence between them. From our experimental results, the growth pattern of hetero-pathogen *Xcv* is not similar to *Xcm* in cotton resistant line (10^3-10^4 fold multiplication, Davis et al., 1996), neither is it the same *as P. s. pv. coronafaciens* in beans (100-fold multiplication, Daub et al., 1980), as we expected. Our results were more like those of hetero-pathogen test with *Arabidopsis thialiana* inoculated with a wheat pathogen (Tsuji et al., 1992). In their study, bacteria grew for 24 hr after inoculation, then declined rapidly.

We found *Xcv* declined in cotton foliage tissue between 8 days and 12 days after inoculation, but we did not test the *Xcv* growth curve throughout the post-inoculation period. Decline of the *Xcv* population may have happened at an earlier time before 8 days. Since most bacteria had been killed before we determined the population density, the results may not represent the actual growth or short-term survival capacity of *Xcv* in various transgenic cell lines. This experiment should only provide preliminary data for the next study.

In order to answer the question if *cdn1* is important, it is necessary to screen the transgenic plants and pick out the cell line(s) with good expression of the transgene. Only when the enzyme level is really low by the interference of antisense DNA, can we trust the correlative results. We are trying to find a good method to screen the large number of transgenic plants for the good expression.

The proposal to start the whole transgenic project was based on the reaction between *Xcm* and cotton, which was extensively studied in our lab. To answer if *cdn1* is important to resistance of cotton to bacterial blight, interference of *cdn1* activity in a resistant cotton line is needed. Currently, all transgenic lines of cotton in our lab are from Coker 312-5a, which is susceptible to bacterial blight. The reason to use the Coker line was to increase chance of success, because there is a successful protocol available for transformation and regeneration of Coker. Currently, these transgenic plants are planned for crossing with a bacterial blight resistant cotton line for the purpose of introducing both a transgene and resistance genes to the same line.

Since the group is considering a new construct to obtain high level expression of a transgene, I can make some suggestions: We may use a promoter that is commonly used and has rendered satisfying expression, for example, that of pBI121, or the promoter used by the group of Atanassova (Atanassova et al., 1995). We may also consider constructing a selectable gene or a reporter gene downstream of *cdn1* cDNA under the same promoter to allow us to select and prescreen the transgenic plants while they are still in various stages of regeneration. *i. e.*, by assaying the expression of the selectable gene or reporter gene, we will know the expression of the antisense gene in various regeneration stages without inoculation. The selectable gene will serve bifunctionally in this case, one allows selection, the other allows prescreening. There is a similar report on using this method (Delauney, A., 1988).

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