Construction of a Plasmid Containing the *bar* Gene For Use in Selection of Biolistically Transformed Orchids.

An Honors Thesis (HONRS 499)

by

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ABSTRACT

The long term goal in our lab is to develop a method to genetically engineer/transform orchid tissue. A gene gun has been constructed to transform orchid tissues which resist more conventional transformation techniques such as Agrobacterium tumefaciens mediated DNA uptake. The plasmid pG35barB, encoding the bar gene which confirms selection based on PPT resistance, has been successfully introduced into orchid tissues with this gene gun, and approximately 1 % of the tissues exhibited herbicide resistance. In order to mitigate viral symptoms in transformed orchids, PCR primers have been designed to amplify the Tobacco Mosaic Virus O-Strain coat protein gene from infected orchid tissues. The amplified fragment can then be ligated into pG35barB and used to biolistically transform orchid tissues. Transformants will be selected by resistance to phosphinothricin due to bar expression. These transformants will be challenged with the TMV-O virus to determine if viral symptoms are reduced as a result of TMV-O coat protein gene expression.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

A. tumefaciens	Agrobacterium tumefaciens
bp	Base Pairs
C	Degrees Celsius
CaMV 35S	Cauliflower Mosaic Virus 35S Promoter
cDNA	Complimentary Deoxyribonucleic Acid
CP	Coat Protein
DNA	Deoxyribonucleic Acid
dNTP	Deoxy-N Triphosphates
E. coli	Escherichia coli
ELISA	Enzyme-linked immunosorbent assay
g	Grams
min	Minute
ml	Mililiters
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
N_2	Nitrogen
ng	Nanograms
nm	Nanometers (Milimicrons)
orf	Open Reading Frames
PAT	Phosphinothricin Acetyltransferase
PCR	Polymerase Chain Reaction
PPT	Phosphinothricin
r.p.m.	Revolutions per Minute
RNA	Ribonucleic Acid
S. hygroscopicus	Streptomyces hygroscopicus
S. viridochromogenes	Streptomyces viridochromogenes
TMV-O	Tobacco Mosaic Virus - O Strain
ug	Micrograms
ul	Microliters
UV	Ultravioletv

INTRODUCTION

Ball State University is home to the Wheeler Orchid Collection. Due to its function as a plant rescue station for the United States customs service as well through the reception of numerous private donations, this collection holds some of the world's rarest orchids. In fact, some of the specimens are the only known representatives of their species. These orchids, as is true of many orchid species around the world, are confined to a greenhouse environment due to the massive destruction of orchid habitats, especially those in the Amazon Rain Forest. This greenhouse environment has some intrinsic hazards to the orchids.

In the native habitats, the orchids were very widely dispersed, and sap-transmitted viruses spread slowly. In overcrowded greenhouses, these viruses can spread rapidly, destroying the orchids [1]. Some of the orchids in the Wheeler collection are already infected and attempts have been made to isolate them from the larger portion of the collection. Additionally, fresh cutting edges are used for each plant, avoiding human-mediated sap transmission and limiting the spreading of the viruses. Unfortunately, the potential for an epidemic in the greenhouse still exists.

One of the infecting viruses is Odontoglossum Ringspot virus, also known as tobacco mosaic virus, O-strain [2]. The host symptoms for infection with this virus includes necrotic spots on the greenery and flower, ruining the commercial value of the infected orchid. Chronic infection can lead to death of the host.

Such destruction of the rare orchids is not an acceptable option, and for several years our lab has been attempting to halt the spread of the virus and reduce the deleterious host symptoms by cloning the virus's coat protein gene into orchid tissues.

Initially, our lab's transformation efforts focused on Agrobacterium tumefaciens. Although the transforming action of A. tumefaciens works naturally on dicots, some monocots have been transformed by allowing it to insert part of its Ti (tumor inducing) plasmid into the host's genome. No such transformation of orchids has been demonstrated to be definitively successful in our lab. Our efforts have now turned to the biolistic transformation of orchid tissues where DNA is shot into the tissue and homologously recombines into the genome. Plasmids have been constructed that are available commercially or from other research laboratories and have had screenable or selectable marker genes cloned into them for the express purpose of confirming transformation. One such plasmid, pG35barB, described by Rathore, et. al. [14] contains the bar gene, a gene which codes for resistance to PPT (phosphinothricin) via phosphinothricin acetyltransferase, cloned into it. This gene is especially useful because it can be used with selective media to identify transformants and to quantitatively determine stable gene expression in descendants of the transformants [14]. We intend to use this plasmid carrying the TMV-O coat protein gene to transform orchids (Figure 1). The CP gene will be PCR amplified out of infected orchid tissues and have appropriate endonuclease recognition sites placed on each end. The CP gene will then be

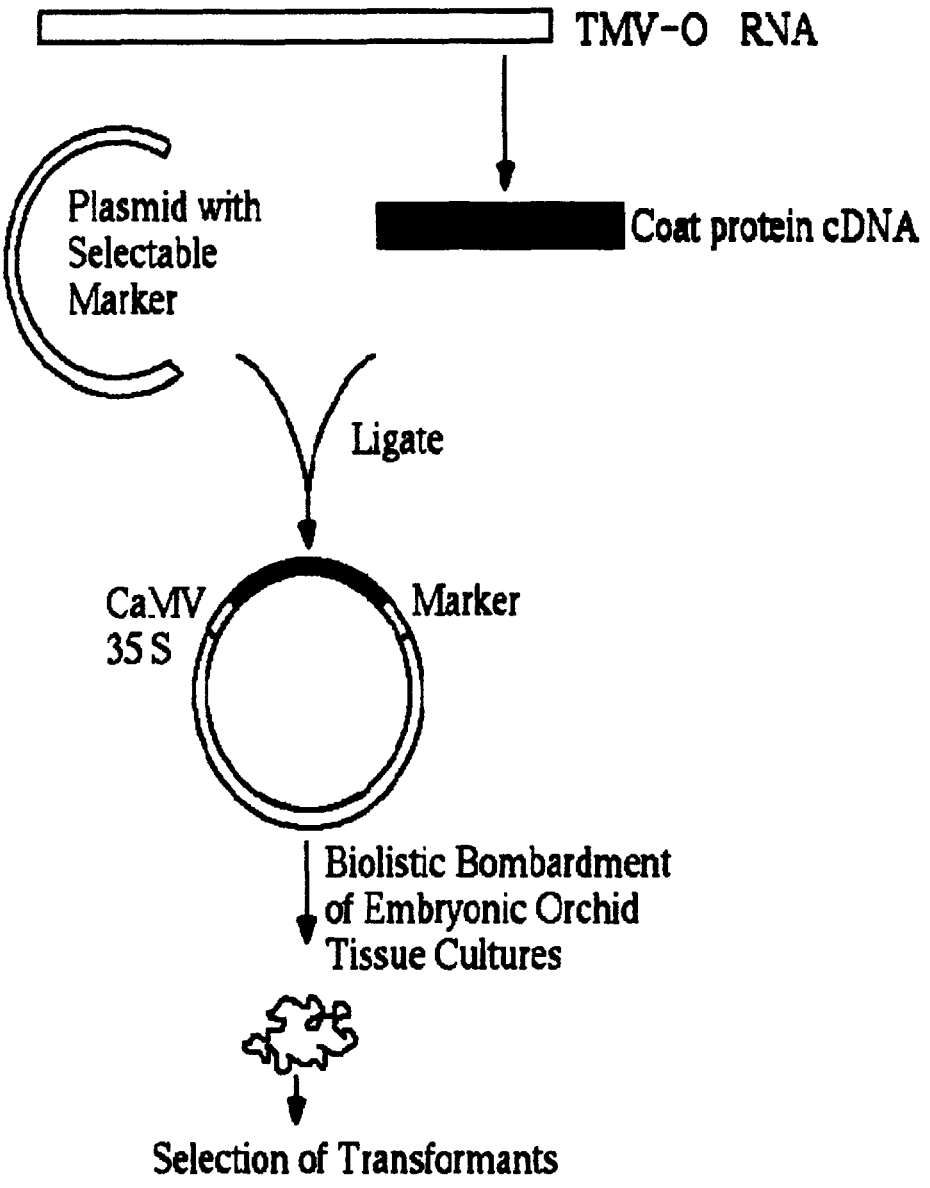

Figure 1. A schematic diagram of the future of this project including PCR amplification of the TMV-O CP gene, ligation into pG35barB, biolistic transformation of orchid tissues, and the selection of transformants prior to challenging with TMV-O.

digested and ligated into the plasmid which will be delivered to embryonic orchid tissue by tungsten particle bombardment. The tissues will be challenged with PPT, and those which remain healthy will be exposed to the virus. The virus symptoms will be scored on severity and the time between infection and symptom development. Less severe or acute symptoms will indicate symptom mitigation.

REVIEW OF THE LITERATURE

TMV-O. TMV-O is a single stranded sense RNA virus. The full viral particle is a cylindrical shape 300 nm long with a radius of 9 nm. This particle must be uncoated in the host plant in order for cDNA to be reverse transcribed and virus propagation to proceed. Studies have shown that infection of orchids with TMV-O leads to the formation of necrotic spots and plant death[11]. Greenhouse orchids are likely to be infected by the virus due to the wide species diversity in collection, the lack of acute symptoms, and the effortless transmission of the virus via nonsterile gardening tools [12].

Methods of Imparting Virus Resistance. Past studies on other TMV strains have found three different methods by which the virus's own genome can be used to impart resistance to the virus or virus symptoms. All three of these methods involve cloning the complimentary DNA (cDNA) of the infecting RNA virus or DNA of a protein of the infecting DNA virus into the genome of the target plant. The first of these three methods is to clone a replicase protein into the plant genome. The mRNA produced by the transformed plants, not the protein product, acts to block the action of the virus [3]. The exact mechanism of this protection has not been identified in the literature.

The second method is to clone the antisense cDNA of the viral coat protein gene into the target plant. This sequence is the compliment to the coding strand of cDNA. As such, the mRNA produced is complimentary to the mRNA transcribed from the coding strand. The antisense mRNA is able to complex with the sense mRNA,

blocking translation of the sense mRNA into the coat protein. Lacking this element of its structure, the virus is unable to complete its replication. This method has been successfully used in tobacco plants challenged with TMV as well as in other plants challenged with various viruses [4,5]. The mechanism of blocking mRNA with complimentary RNA is used for gene regulation in some prokaryotes [6].

The third method on which the remainder of this paper focuses is to clone the sense cDNA of the viral coat protein (CP) gene into the target plant. A three dimensional representation of the coat protein wherein alpha helices are represented by large, rigid cylinders and loops by smaller, more flexible cylinders is found in Figure 2. Tobacco plants producing the TMV CP have been shown to resist TMV for up to 30 days when untransformed plants developed symptoms in 3 to 4 days [7]. It has been discovered that the CP itself and not the mRNA acts to protect transformants [8]. The CP blocks the uncoating and subsequent transcription of viral particles by inhibiting the formation of striposomes, made up of viral particles bound to ribosomes. This inhibition is proposed to be achieved through blocking the ribosome with the overexpressed CP [9]. Since 3% of the viral particles still form striposomes, the full protection found in many transformants must be partially due to some other mechanism as yet not understood [10].

<u>Agrobacterium tumefaciens.</u> <u>A. tumefaciens</u> is a bacterium which, in nature, transform dicotyledon hosts with a portion of its own DNA (Figure 3). This portion of DNA resides, in the bacterium, as part of the Ti-plasmid or tumor-inducing plasmid. This plasmid

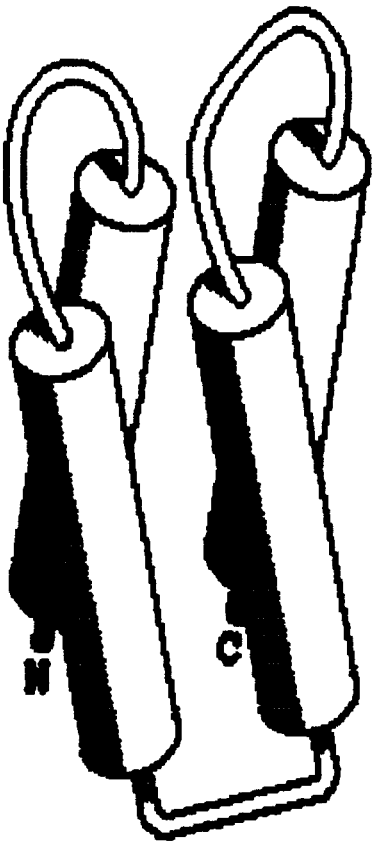

Figure 2. The Coat Protein of Tobacco Mosaic Virus, O-Strain.

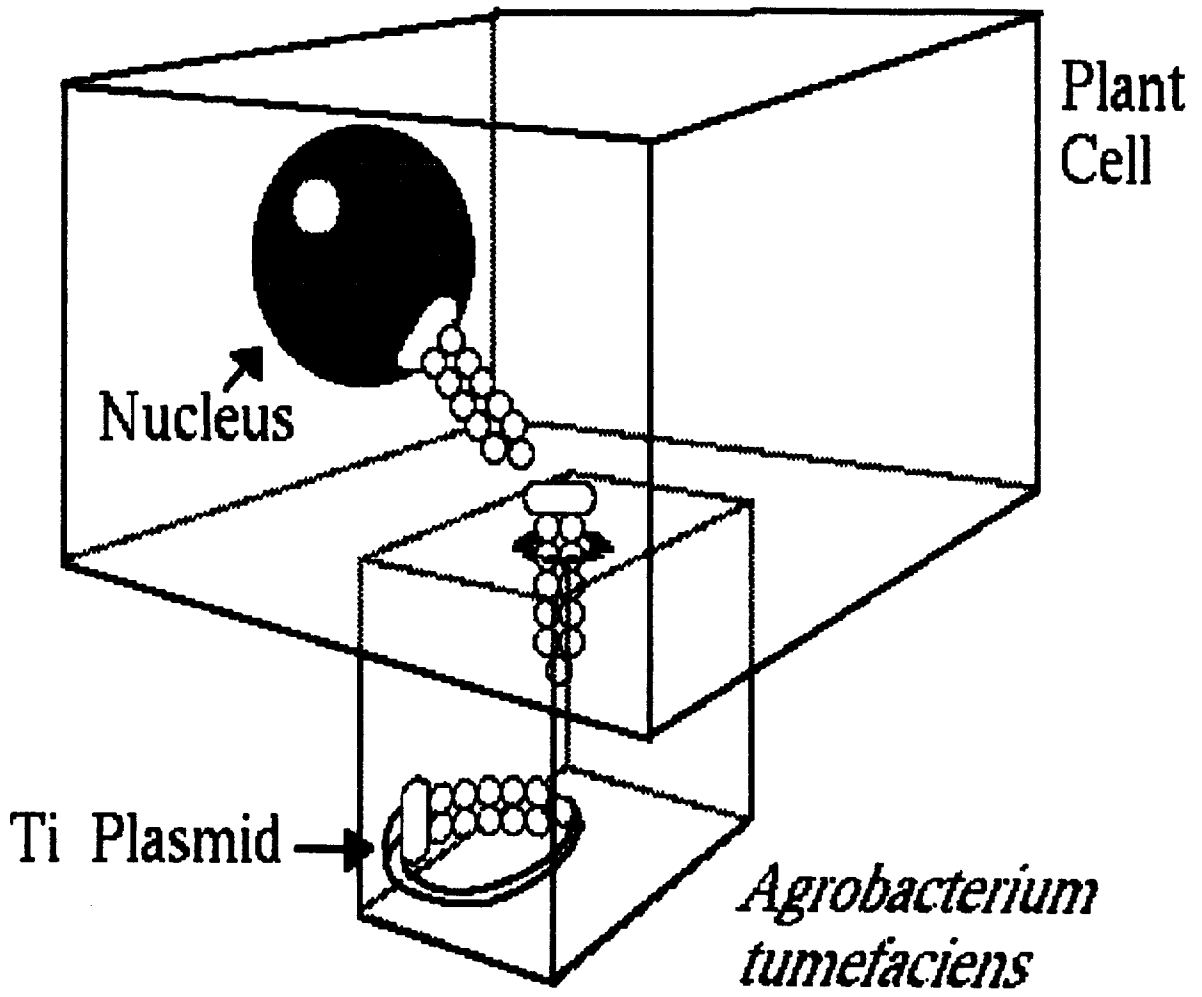

Figure 3. Action of *Agrobacterium tumefaciens* during cell transformation. A region of the Ti plasmid is being inserted into the plant cell's genome.

is named for its natural function of establishing a crown gall tumor on infected plants in which the bacterium can live. A portion of this plasmid, the T-DNA (transferred DNA), is incorporated into the host genome to induce the formation of the crown gall tumor [13]. However, successful use of this dicotyledon pathogen to transform orchids has never been reported.

Biolistic Transformation. Biolistic transformation refers to the delivery of DNA into target cells by accelerating DNA-coated tungsten particles to a high velocity and shooting them into tissues [15]. The tissues may become stably transformed presumably by homologous recombination of the transforming DNA into the target genome. Results reported by other investigators indicated that less than 1% of the target tissues express the genes being delivered into the cells [16,17]. Commercial "gene guns" can be very expensive; however, Takeuchi demonstrated that a very inexpensive gene gun could be made wherein the tungsten particles are accelerated in a stream of helium [18]. A similar gun constructed in our laboratory by Craig Reed is shown in Figure 4.

The selectable marker gene bar. Bialaphos is a tripeptide antibiotic produced by Streptomyces hygroscopicus and S. viridochromogenes made up of phosphinothricin (PPT) and two L-alanine residues [19]. PPT is used as the active ingredient in the broad-spectrum herbicides Basta and Ignite [14]. PPT, an analogue of glutamic acid, inhibits glutamine synthetase [20]. It is believed that this inhibition leads to an accumulation of ammonia, causing cell death [21]. Figure 5 shows the mechanism of action of the bar gene product, PAT. The bar gene, found in S.

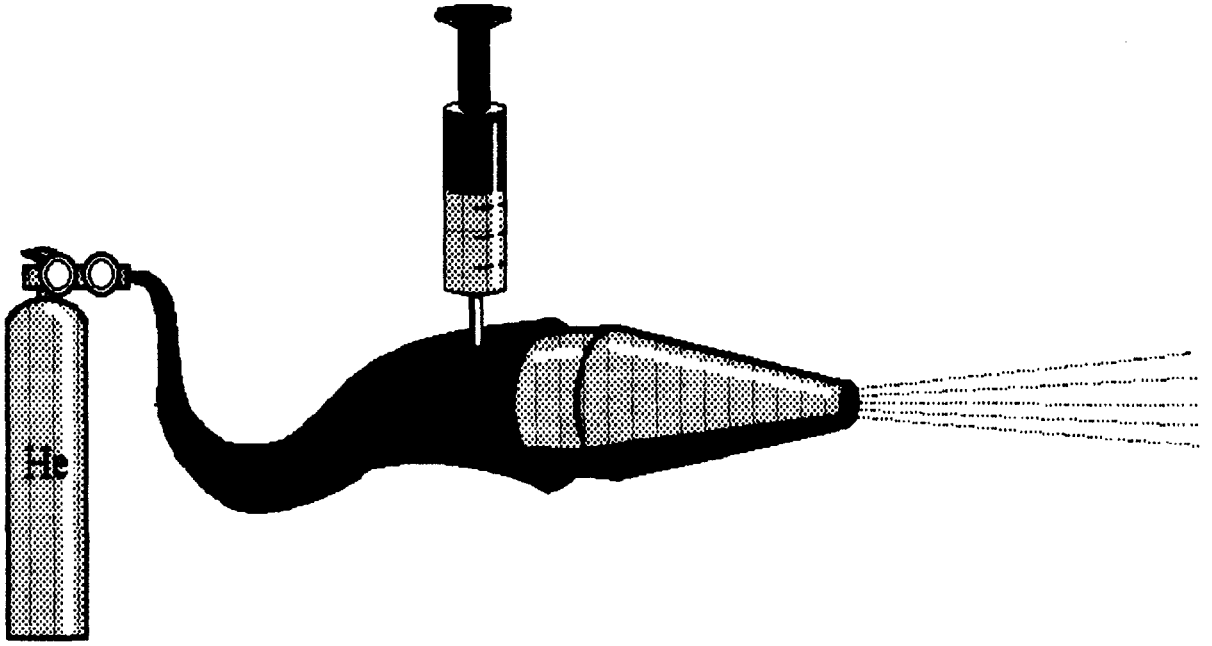

Figure 4. The "gene gun" that has been constructed in our lab. Tungsten particles coated with DNA are injected via a syringe into the helium stream.

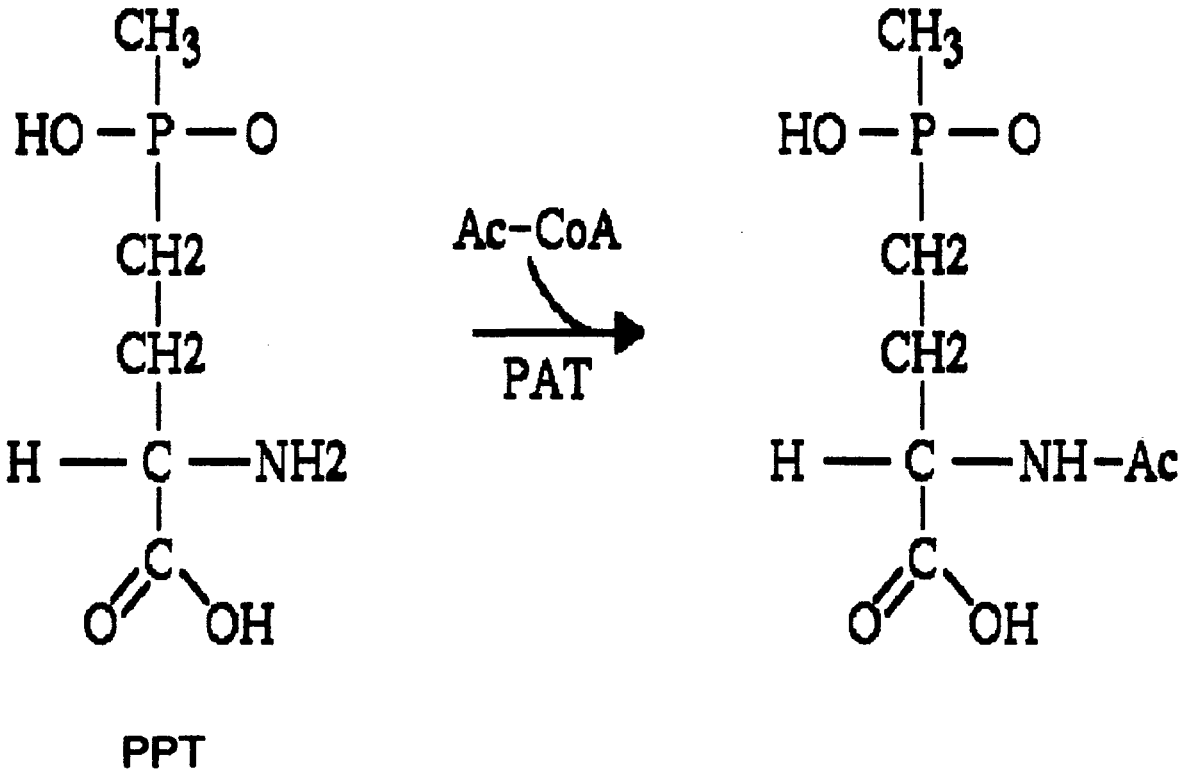

Figure 5. The action of the *bar* gene product PAT. The PPT is inactivated by the acetylation of its amine group.

hygroscopicus, is named for imparting bialaphos resistance [14]. It acts to protect S. hygroscopicus from the PPT it produces. A similar gene, pat, is produced by S. viridochromogenes [22]. The bar gene product, PAT, inactivates PPT by acetylating its amine group. This action makes the gene an ideal selectable marker [23].

The use of the bar gene as a selectable marker refers to the use of this gene to select transformants in molecular cloning. The gene can be placed adjacent to another gene which is to be inserted into a target genome but is not easily assayable. The potential transformants can then be challenged with PPT and any survivors are further tested for the second, harder to detect, gene or gene product [23]. The bar gene has been cloned into the plasmid pG35barB with 5′ modifications including the cauliflower mosaic virus 35 S promoter to enhance transcription [14]. Both a BamH I and a Xma I restriction site are found 5′ to the bar gene and 3′ to the CaMV 35 S promotor. This site is ideal for inserting genes to be used in transformation experiments with bar as a selectable marker. Previously in our laboratory, optimal acceleration conditions were determined using the "gene gun" and more than 1% of the tissues bombarded with the original plasmid were fully PPT resistant and presumably transformed. This research was conducted by Steven Parsons.

MATERIALS AND METHODS

Callus Tissue Culturing. Callus lines from Cattleya Chocolate Drop x Cattleytonia Kieth Roth (910531) developed in previous research were subcultured. The medium used contained one liter of Vacin and Went Basal Salt (Sigma) with the addition of 20 g of sucrose and 0.5 % Benzyl Adenine. The solution was aliquoted into twenty 50 ml aliquots in 250 ml Erlenmeyer flasks. Callus tissue was placed into sterile petri dishes under the hood, and sterile medium was added. The cultures were then grown under 24 hour light with constant agitation to prevent tissue differentiation [24].

A fungus infection contaminated the cultures which were cleaned as follows: Five ml of bleach was added to each 50 ml medium and the flasks were agitated for 20 minutes. Three ug/ml Antibiotic Antimitotic (Sigma) was added to each new flask and the cultures were sterilely transferred as previously described. The cultures are still being maintained for future transformation experiments.

Obtaining Virus Infected Tissue. A specimen of Daritanopsis Firecracker showing numerous necrotic spots had been previously ELISA [25] tested and determined to be infected by TMV-O (unpublished research by Herbert Saxxon of Ball State University). A section of infected leaf tissue was removed with a sterile razor blade, placed in a sealed plastic bag, and stored at -800 C.

PCR Primer Design. The primers were designed so that the TMV-O coat protein gene (sequence published by Wu [26]) would be amplified from the viral cDNA in the infected orchid tissue with the addition of restriction sites to facilitate ligation of the

amplified fragment into the plasmid pG35barB. This plasmid contains the selectable marker gene bar, which was given to our lab by Thomas Hodges of Purdue University, West Lafayette [14] (Figure 6).

Since it is desirable that the CP gene be placed 3′ to the CaMV 35 S promoter without disrupting the bar gene or the polyadnelyation tail, only one site for insertion is appropriate, a 5 bp region between unique BamH I and Xma I sites (Figure 7). The Xma I is better than Sma I due to its characteristic production of sticky ends rather than blunt ends.

The primers were designed such that the portions of the primers which are complimentary to the cDNA of TMV-O are each four codons in length, each beginning and ending with the beginning or ending of a codon. The region of the coat protein amplified is shown in Figure 8. This perfectly preserves the reading frame up to this point. The two primers are described as follows:

TMV1: Compliments the 3′ end of the coding strand.

TMV2: Identical to the 5′ end of the coding strand.

Each of these primers must have endonuclease sites added to facilitate the introduction of the PCR product into the plasmid. Therefore the following must be true:

TMV1: Xma I introduced 3′ to previously indicated sequence

TMV2: BamH I introduced 5′ to previously indicated sequence

In addition, to shift the reading frame of the insert, preserving the CamV promoter activity for both the TMV coat protein gene and the bar gene, TMV2 requires an insert of one or two base pairs 3′ to the BamH I site. Additional base pairs were added to

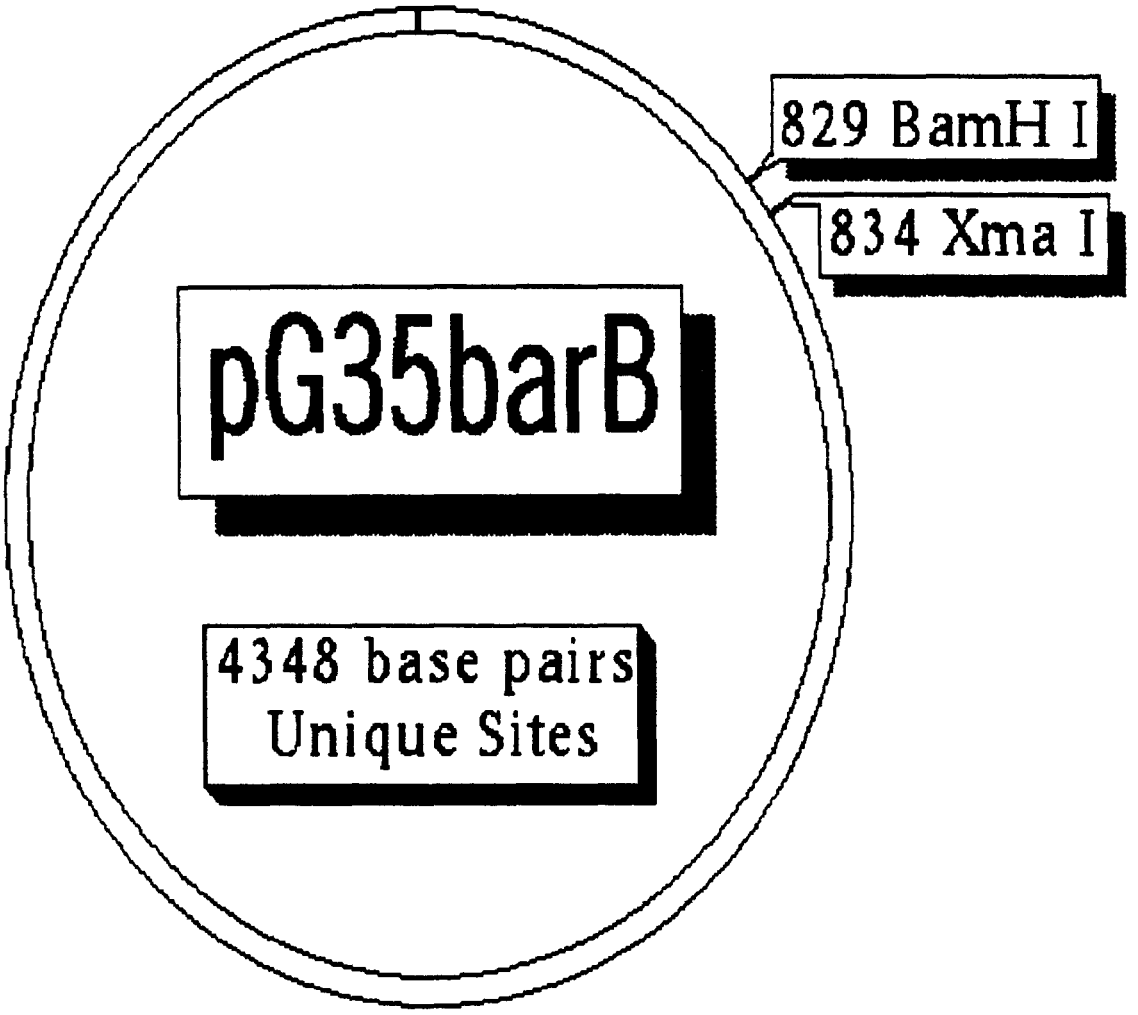

Figure 6. A scaled diagram of the plasmid being used, including the restriction sites which will be used to insert the TMV-O CP gene.

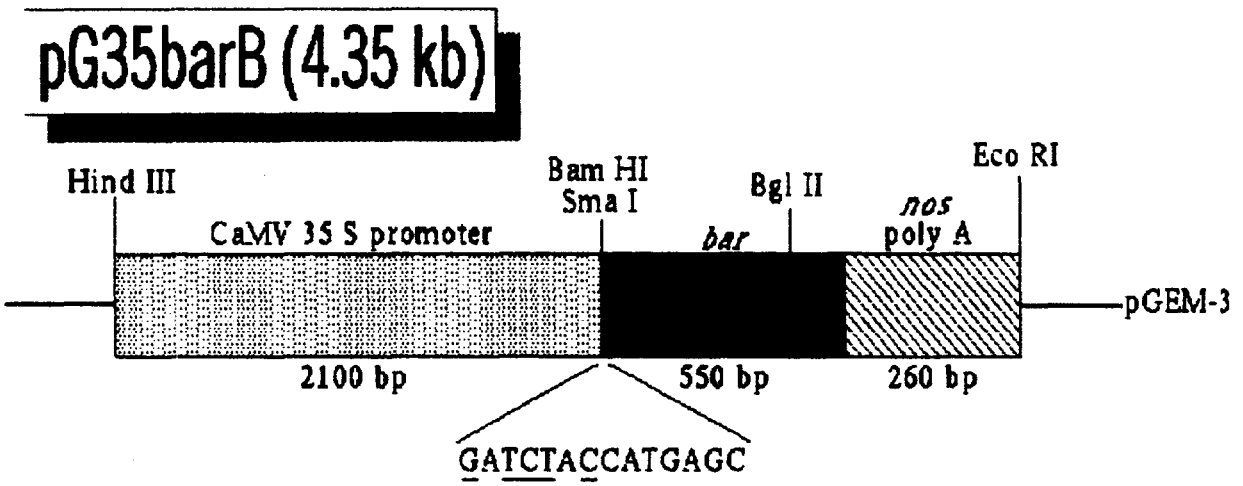

Figure 7. A schematic diagram of the plasmid which will have the TMV-O CP gene cloned in between the CaMV 35 S promoter and the *bar* gene.

2 5'	ACA	AUC	UGA	UUC	GUA	UUG	AAU	AUG M	UCU S	UAC Y	ACU T
36	AUU I	ACA T	GAC D	CCG P	UCU S	AAG K	CUG L	GCU A	UAU Y	UUA L	AGC S
70	UCG S	GCU A	UGG W	GCU A	GAC D	CCC P	AAU N	UCA S	CUA L	AUC I	AAC N
104	CUU L	UGU C	ACC T	AAU N	UCU S	CUG L	GGU G	AAU N	CAG Q	UUC R	CAA Q
138	ACA T	CAA Q	CAA Q	GCU A	CGA R	ACA T	ACU T	GUU V	CAA Q	CAG Q	CAG Q
172	UUU F	GCU A	GAU D	GUU V	UGG W	CAG Q	CCG P	GUU V	CCU P	ACU T	UUG L
206	GCC A	AGU A	AGG S	UUC R	CCU F	GCA A	GGC G	GCU A	GGU G	UAC Y	UUC F
240	AGA R	GAU D	UAU Y	CGC R	UAU Y	AUG D	GGU P	ACU I	UUU L	GAU D	ACU P
274	UUA L	AUA I	ACU T	UUC F	UUA L	AUG M	GGU G	ACU T	UUU F	GAU D	ACU T
308	CGU R	AAU N	AGA R	AUA I	AUC I	GAG E	GUA V	GAA E	AAU N	CCG P	CAG Q
342	AAU N	CCG P	ACA T	ACU T	ACG T	GAA E	ACA T	UUA L	GAU D	GCA A	ACU T
376	CGU R	AGA R	GUU V	GAU D	GAU D	GCA A	ACU T	GUA V	GCA A	AUA I	AGA R
410	UCU S	GCA A	AUA I	AAU N	AAU N	CUA L	UUA L	AAU N	GAG E	UUA L	GUU V
444	AGG R	GGA G	ACU T	GGU G	AUG M	UAC Y	AAU N	CAA Q	GUC V	UCA S	UUU F
478	GAG E	ACG T	AUG M	UCU S	GGA G	CUU L	ACU T	UGG W	ACC T	UCU S	UCC S
512	UAA STOP	UCA	UAU	GAG 3'	GAA CTT	AAU TTA	AAC TTG	GUU CAA	AGU 5'	GUU	GAA
546	CUA	UCC	GUG	GUG	CAU	ACG	AUA	AUG	CAU	AGU	3'

Figure 8. TMV-O RNA and amino acid sequence with the PCR primer regions of homology underlined.

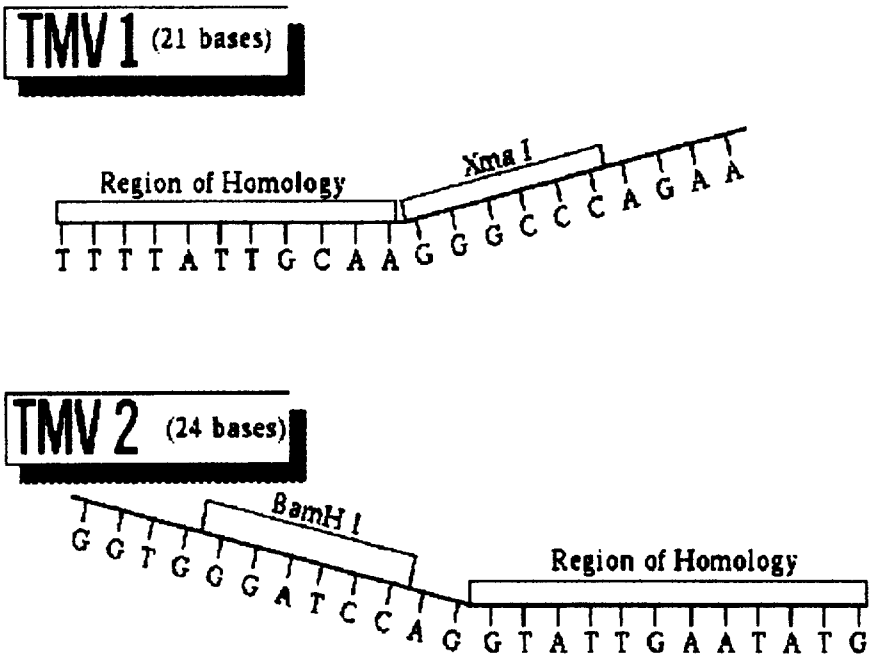

Figure 9. The PCR primers designed to amplify out the TMV-O CP gene while adding restriction sites for ligation into the plasmid pG35barB.

the flanking ends of the primers to facilitate endonuclease attachment. The Oligo primer analysis software package was used to determine which small modifications would minimize primer-primer interactions. The full primers are pictured in Figure 9.

CTAB Extraction of DNA. As part of its replication cycle, the RNA of TMV-O is reverse transcribed into cDNA. Presumably this DNA is abundant enough in infected plants to serve as a template for PCR amplification. The extraction was carried out by the method proposed by Doyle and Doyle and optimized in our lab for orchid tissue [27]. One fifth of a gram of virus infected orchid tissue was ground in liquid N_2. The ground tissue was incubated at 65 C for 30 minutes in 0.9 ul 8 X CTAB buffer (16 % w/v CTAB from Sigma, 1.4 M NaCl, 0.2% v/v 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0). Following incubation, the proteins were denatured by adding 0.4 ul of chloroform : isoamyl alcohol (24:1 v/v). The aqueous phase and a wash of the organic phase each had 260 ul of isopropanol added followed by a four hour incubation at -20 C and a ten minute centrifuge. The pellets were washed with 100 ul 80% ethanol, centrifuged for 10 minutes, dried and suspended in 50 ul TE each.

PCR Amplification of CTAB Extracted DNA. Each reaction well contained 5 ul of the DNA template, 0.5 ug of each primer, 1 ul of each dNTP, 1 unit of Taq polymerase, 5 ul 25 mM MgCl2, 5 ul 10 X Thermocycling Buffer (Sigma), and enough water to fill to a total volume of 50 ul. The negative control had no template DNA. The PCR consisted of forty cycles of 94 C, one minute; 60 C, one minute; and 72 C, 2 minutes. This was followed by a 10 minute

extension of 72 C.

RT-PCR. When PCR using a DNA template failed to amplify the CP gene, it was decided that the DNA template must be reversed transcribed in the laboratory. The orchid and viral RNA in the tissue sample was isolated as described by Chomczynsk [28] after grinding the tissues in liquid nitrogen. Two ug of the RNA was then heated at 70 C for 5 minutes, relaxing the RNA secondary structure. The samples were cooled on ice, and 28 ul of the master mix (50 mM Tris pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM MgCl2, 2 mM dNTPs, 0.5 ug of the 3′ primer, 1 unit RNAsin, 300 units M-MLV-RT enzyme) was added to each reaction tube. The negative control had no RNA template. After one hour at 37 C, the samples were heated to 95 C to inactivate the reverse transcriptase. Fifty ul of the PCR master mix [20 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 0.5 ug 5′ primer, and 1 unit Taq polymerase] was added to each tube and the PCR (30 cycles of 94 C, 1 minute; 42 C, 1 minute; and 72 C, 1 minute followed by a 10 minute extension at 72 C) was carried out.

Reamplification of RT-PCR product. The Sigma PCR product Wizard miniprep was used to remove the primers, leaving only DNA of at least 500 bp in length. The remaining DNA was used as the template for the reamplification. Each reaction well contained 0.5 ug of each primer, 1 ul of each dNTP, 1 unit of Taq polymerase, 5 ul 25 mM MgCl2, 5 ul 10 X Thermocycling Buffer (Sigma), and enough water to fill to a total volume of 50 ul. The negative control had no template DNA. The experimental tubes had 30 ul, 10 ul, and 1 ul of template respectively. The PCR consisted of forty cycles of 94

C, one minute; 60 C, one minute; and 72 C, 2 minutes. This was followed by a 10 minute extension of 720 C.

Agarose Gel Electrophoresis of PCR products. The gels were prepared at 1.5% in TBE buffer. The gel was prestained with ethidium bromide. qx174/Hae III was used as a standard marker. The gels were electrophoresed at 50 volts in TBE buffer. The gel pictured was photographed with a Polaroid camera using an orange filter. The resulting photograph was scanned into a Gateway 2000 computer using the Photoshop software for PC.

RESULTS AND DISCUSSION

PCR of CTAB extracted DNA. The electrophoresis of the PCR products and subsequent visualization with ethidium bromide staining and an ultraviolet (UV) light source showed no bands other than the smear at the bottom of the lane that is characteristic of primers. No other definite bands or smears could be seen to indicate the presence of large DNA fragments. This initial PCR which was based on the supposition that the cDNA could be found in the tissues did not succeed in producing any bands. This led us to believe that the gene must only be locatable in its RNA form. For this reason we tried RT-PCR to reverse transcribe the RNA and then amplify the cDNA construct.

RT-PCR. UV spectroscopy indicated that some RNA was present prior to the reverse transcription, but it was unclear if any DNA strands were present prior to PCR. The PCR products were separated by agarose gel electrophoresis. The experimental lanes showed a very faint band of approximately 500 bp in length, the size of the CP gene we were trying to amplify, but it was too faint to reproduce in a photograph.

Reamplification of RT-PCR product. We reamplified this RT-PCR product to intensify the band, but it showed up in all of the lanes, including the control (Figure 10). We repeated the procedure to check our results, but the band still appeared in the control lanes. We concluded that we were unsuccessful in amplifying the desired RNA.

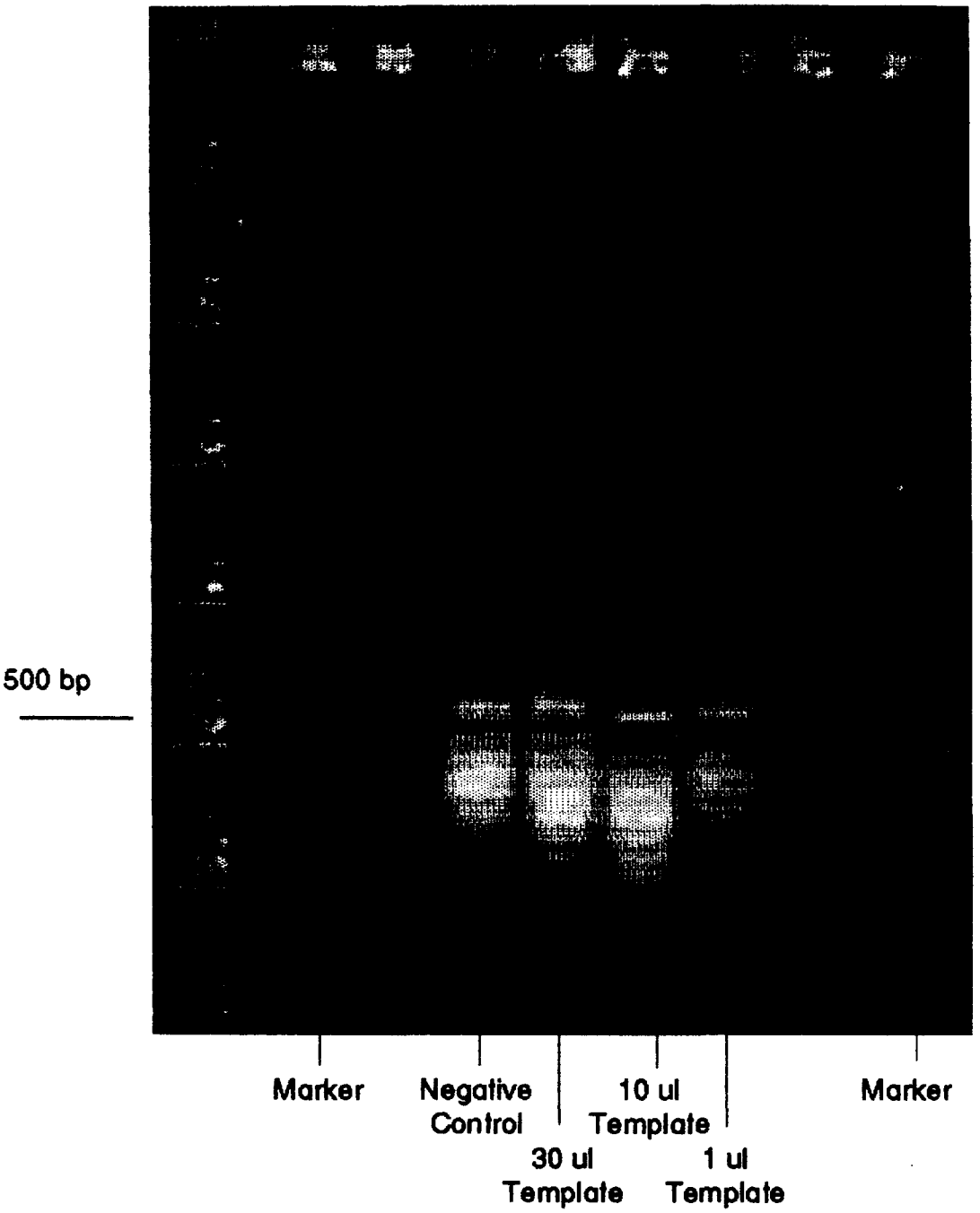

Figure 10. The agarose gel electrophoresis of the reamplified RT-PCR products.

CONCLUSIONS

The most probable source of error which could have caused our lack of positive results is the synthesis of the primers within our own lab. To date, none of the primers we have produced have given any definitive results. The decision has been made to order the primers from a biological supply company. When the new primers arrive, we will repeat the CTAB extraction and PCR. If this fails to work, we will once again proceed to RT-PCR, using a higher concentration of RNA this time. Once we have isolated the CP gene, we will clone it into pG35bar B. We will use the new plasmid to biolistically bombard the callus tissue, following the protocol which has already been successfully used in our lab with pG35bar B. The tissues will be exposed to PPT, and any survivors will be made to mature into adult orchids and challenged with TMV-O. Once the full protocol for introducing the TMV-O CP gene into orchid tissues is developed, we will be able to use it to clone other genes into the orchid or other plants by way of biolistic bombardment with the selectable marker *bar*. These genes can code for resistance to other viruses or unique traits which would enhance the esthetic quality of the orchids.

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