In Search of the GAD CaM-binding Domain and Active Site in Mutant *vs.* Wild Type Tobacco Plants

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In Search of the GAD CaM-binding Domain and Active Site in Mutant vs. Wild Type Tobacco Plants

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Abstract

The phenotype of a recessive *t*emperature *d*ependent *s*hooty mutant (*tds*) of *Nicotiana tabacum* is expressed at low temperature (21°C) but at high temperature (30°C) the mutant phenotype is reversed to normal expression. The tds phenotype includes characteristics, such as, loss of apical dominance, thick, narrow leaves with abnormal mesophyll cells, and short internodes but the exact cause of this phenotype has not yet been determined (Samuelsen *et al.*, 1997). In plants γ-aminobutyric acid (GABA) is synthesized by glutamate decarboxylase (GAD), which is important in plant development, and is tightly controlled by Ca^{2+}/calmodulin (Yun and Oh, 1998). Calmodulin (CaM) binding to GAD is essential for regulating GABA and glutamate metabolism that leads to normal plant development (Baum *et al.*, 1996). It is possible that the mutation in the tds of *Nicotiana tabacum* is located in the sequence of the calmodulin-binding domain of the mutant GAD gene. Previous work by Rebecca Miller which attempted to amplify the entire GAD gene was inconclusive. For better amplification, primers were developed for a smaller sequence from the active site to the c-terminal calmodulin-binding domain of the GAD gene. Genomic DNA of mutant and wild type tobacco was used. Genomic DNA was tested with primers formed from a cDNA sequence. GeneSequer® (www.plantgdb.org) was used to try to make sure there were no large introns that would make amplification of the sequence difficult.

Introduction

At low temperature (21°C) the mutant phenotype is expressed in a recessive *t*emperature *d*ependent *s*hooty mutant (*tds*) of *Nicotiana tabacum* W-38 that includes thick, narrow leaves with abnormal mesophyll cells, short internodes, and loss of apical

dominance. However, it has been found that at high temperature (30°C) the mutant phenotype is reversed to a normal phenotype. The mutant tobacco plant is most commonly characterized by its long, narrow but thick leaves, and multiple shoot apices. They rarely flower but when they do they have a double flower appearance (Samuelsen *et al.*, 1997).

In the study by Samuelsen and coworkers (1997), the observation was first made that when growing mutant seedlings *in vitro* at 30°C they turned out normal compared to those grown in the greenhouse at 21°C. Normal wild type development resulted in mutants at 30°C but they were shorter than average wild type phenotype. At 21°C the abnormal development of mutants occurred. It was also observed that wild type plants flowered at low temperature but not high temperatures and the mutant plants flowered at high temperatures and were vegetative at low temperatures. Some suggestions as to why they are temperature dependent are that a temperature-sensitive lesion due to a point mutation may serve to decrease the thermal stability of gene products. Also, a homoeologous peptide may be compensating for the abnormal (or absence of) gene product at high temperatures but not low temperatures (Samuelsen *et al.*, 1997).

The decarboxylation of glutamate to CO_2 and GABA is catalyzed by GAD. GAD binds to calmodulin (CaM) which has only been shown to occur in plants. Severe morphological abnormalities occur in transgenic tobacco plants expressing a mutant petunia GAD lacking the CaM-binding domain. Such abnormalities include being shorter and having a more branched stem, slower leaf development, lack of pollen, extremely high GABA levels, and low glutamate levels (Baum *et al.*, 1996). The similarity to tds mutants is intriguing.

GAD activity in plants is characterized by the conversion of glutamic acid to GABA and binding of calmodulin. Baum and coworkers (1993) isolated the GAD gene from petunia DNA and were the first to report the isolation of a GAD gene from a plant and of a calmodulin-binding GAD from any organism. Their results indicated that intracellular signals via calmodulin play a role in regulating GABA synthesis in plants. The cloning of a plant GAD gene provides better tools to evaluate the role(s) of GABA in plants (Baum *et al.*, 1993).

Molecular evidence shows important roles in plant development from Ca^{2+}/calmodulin-dependent GAD and GABA. Only plant GADs have a calmodulin-binding domain at the c-terminus. Calmodulin (CaM) is a ubiquitous and multifunctional Ca^{2+} sensor that interacts with a wide array of cellular proteins to modulate their activity and functions in regulating diverse cellular processes (Yun and Oh, 1998). Isolated plant GAD is able to bind to calmodulin in the presence of Ca^{2+}. The role of calcium is that of a second messenger in plants and other organisms. In plants, it also mediates responses to a variety of environmental stimuli including mechanical stress and temperature shock. Furthermore it serves as an intracellular signal transducer by reversibly modulating the structure and function of Ca^{2+} binding proteins (CBP), such as calmodulin (which has four Ca^{2+} binding sites) by inducing conformational changes (Baum *et al.*, 1993).

In *Arabidopsis* at least 27 CBPs have been identified to interact with calmodulin in a Ca^{2+}-dependent manner. There have been phylogenic trees produced with five CBP families in several different plants. In each CBP gene family the members are highly conserved at the protein sequence level. Also, five GADs were found in *Arabidopsis* which most likely are part of a multigenic family (Reddy *et al.*, 2002).

In a study by Yun and Oh (1998), their results indicated presence of Ca^{2+}/calmodulin-dependent GADs in tobacco plants. In their study they isolated and characterized a 1771 bp tobacco cDNA clone, pNtGAD2 from Burley 21 tobacco plants. The molecular sequence of which and the biochemical characteristics of the pNtGAD2-encoded protein confirmed that it encodes a functional calmodulin-binding and Ca^{2+}/calmodulin-dependent tobacco GAD. Further, they also showed that tobacco GAD is a Ca^{2+}/calmodulin-dependent enzyme with calmodulin binding activity (Yun and Oh, 1998).

In animals GABA is a major transmitter in the central nervous system but its role in plants is still unclear. The synthesis of GABA by GAD is regulated by Ca^{2+}/calmodulin but GABA levels increase rapidly I response to oxygen, temperature, water and mechanical stresses along with elevated H^+ and substrate levels. The activity of GAD also increases in response to these conditions which in turn causes fluxes in cytosolic Ca^{2+} (Yun and Oh, 1998). GABA is a four-carbon non-protein amino acid. There are indications that stress initiates a signal-transduction pathway, which increases the cytosolic Ca^{2+}/calmodulin-dependent GAD activity and GABA synthesis. It may be necessary to generation of sense and anti-sense plants to find knockout mutants in an effort to determine the physiological function(s) of GABA in plants (Shelp *et al.*, 1999).

In the study by Baum and coworkers (1996) their results indicated that CaM binding to GAD is needed to regulate GABA and glutamate metabolism. Also, GAD activity needs to be regulated for normal plant development. Transgenic tobacco plants with insertion of a mutant petunia GAD that lacked the CaM-binding domain were used. When the mutant GAD was expressed it inhibited the formation of 500 kDa GAD

complexes, and the GAD activity was found to be Ca^{2+} independent, causing it to be abnormally regulated. Therefore the plant with the mutant petunia GAD lacking the CaM-binding demonstrated high GABA levels and low glutamate levels (Baum *et al.*, 1996).

A defect in the tds tobacco CaM-binding domain of GAD might be responsible for the mutant phenotype. Melanie Gries previously tested the hypothesis that tds may be due to lesions in GABA production. After taking samples from tds and wild type tobacco plant she found that the GABA levels were nearly three times higher in mutant plants than the wild type plants at low temperature. At high temperature, the mutants were found to have nearly identical levels of the GABA as the wild type. These findings support our hypothesis that a mutation in the tds of *Nicotiana tabacum* may be located in the sequence of the calmodulin-binding domain of the mutant GAD gene.

It is also possible that calmodulin is defective and the CaM-binding domain is intact in the tds. Plants have multiple CaM genes with numerous CaM isoforms. These might confer different Ca^{2+} sensitivity to CaM-binding enzymes or proteins. If one isoform of calmodulin that has high affinity for GAD is defective, this might have a similar effect as a mutated CaM-binding domain in GAD.

Van der Luit and coworkers (1999) studied the effect of cold shock and wind stimuli on Ca^{2+}signaling pathways. Specific Ca^{2+} signaling pathways are initiated by cold shock and wind stimuli. To determine if the initiated Ca^{2+} signaling pathways are spatially distinct, cells of tobacco seedlings were transformed with nucleoplasmin (a fusion protein)/aequorin construct and tested against these conditions. They found that nuclear Ca^{2+} concentration controlled separate Ca^{2+} signaling pathways. It was

specifically found to be controlling some components of the signaling pathways expressing *NpCaM-1*, a calmodulin transcript. *NpCaM-1* mRNA accumulations were induced by both cold shock and wind stimuli. It has been suggested that nuclear Ca^{2+} concentration could be exerting transcriptional regulation by Ca^{2+} or Ca^{2+}-sensitive protein kinases or by particular transcription factors directly interacting with Ca^{2+}/calmodulin (van der Luit *et al.*, 1999).

CaM regulates the activity of numerous target proteins, such as Ca^{2+}/CaM-dependent protein kinases (CaMKs) playing a vital role in transducing Ca^{2+} signals. The plant protein kinase, NtCaMK1 (isolated from a tobacco plant), has been shown to autophosphorylate and phosphorylate histone IIIs only when Ca^{2+}/CaM is present. Histones are small proteins that bind to DNA to pack it close together. Phosphorylation of histone III by kinases serves to show the effect of CaM on their activities. Therefore, CaM has a regulatory role on NtCaMK1 activity. Plants have different isoforms of CaM that respond to specific environmental stimuli (Ma *et al.*, 2004). Ma and coworkers (2004) tested three different CaM isoforms on NtCaMK1. Some activated it and some didn't. They concluded that NtCaMK1's protein kinase activity only occurs if Ca^{2+}/CaM are present and different isoforms of CaM differentially regulate it (Ma *et al.*, 2004).

Ling and coworkers (1994) determined the identity of a 62-kD CaM-binding protein from the root of fava bean seedlings and demonstrated through activity assays that GAD is a Ca^{2+}-CaM-stimulated enzyme. Therefore, there is a link between Ca^{2+} signaling and GABA synthesis. A peptide derived from the CaM-binding protein by tryptic digestion, consisted of a 13-residue sequence corresponding to a highly conserved

region of GAD. Future studies may aid in the understanding of stress-linked metabolism modulated by Ca^{2+}- mediated signal transduction in plant responses (Ling *et al.*, 1994).

Little is known about the biochemical pathway that CaM modulates. In plants only four CaM-stimulated enzymes have been well characterized and include: NAD kinase, Ca^{2+}-ATPase, nuclear nucleoside triphosphatase, and a slow vacuolar ion channel. It has been hypothesized that GABA is part of a metabolic mechanism for the control of pH, an amino acid metabolite, a nitrogen storage compound, and/or an insect neural inhibitor synthesized during plant stress (Ling *et al.*, 1994).

In plant leaves, petals, mesophyll cells, and cotyledons of various species activity of GAD has been measured. The CaM-GAD interactions have additional complexity resulting from the presence of the multiple CaM isoforms in plant systems. From the plant, *Arabidopsis*, six independent CaM cDNA isoforms have been characterized, each varying in amino acid sequence by up to four amino acids (Ling *et al.*, 1994).

Reddy and coworkers (2002) used a mixture of radiolabeled CaM isoforms from *Arabidopsis* to screen expression libraries prepared from various plant organs. They found about 14 previously unknown CaM-binding proteins (CBP). Among the large number of CBPs they found there were several specific to plants. Even though CaM is highly conserved between plants and animals, only a few CBPs are common to both. The recruitment in Ca^{2+}/CaM modulated signal transduction networks is entirely different in plants versus animals. It is suggested that plants have a unique set of CBPs specific to plant growth and development which function by mediating cellular activity (Reddy *et al.*, 2002).

Chen and coworkers (1994) conducted molecular studies of GAD expression using a cDNA coding for a 58-kD calcium-dependent CaM-binding GAD that they had previously isolated from petunia. This 58-kD CaM-binding GAD is expressed in all petunia organs tested including flowers and all floral parts, leaves, stems, roots, and seeds. They determined that petals of open flowers had the highest expression levels. Also, changes in the amount of 58-kD GAD coincided with developmental changes in GAD activity. Thus transcriptional and/or posttranslational processes play a role in developmentally regulating the expression of 58-kD CaM-binding GAD in petunia organs. Therefore, during petunia seed germination and organ development GAD gene expression is likely to play a role in controlling GABA synthesis rates (Chen *et al.*, 1994).

Yeutushenko and coworkers (2003) isolated the nucleotide sequence of two divergent GADs (GAD1 and GAD2) from a *Nicotiana tabacum* L. cv. Samsun NN leaf cDNA library. At least four tobacco GAD genes were suggested to exist based on further genomic DNA analysis, and they exist in pairs of highly identical genes (Yeutushenko *et al.*, 2003). A cDNA library consists of gene sequences derived from messenger RNA (mRNA) and therefore does not include introns (sequences of DNA spliced out during RNA synthesis), whereas genomic DNA libraries encompass the entire genome (including introns). Reddy and coworkers (2002), reported five GADs in *Arabidopsis*. Of these GADs, GAD2 was reported to be expressed in leaf, stem, flower, and root tissue. GAD2 has been located on chromosome one and has five introns located within the gene. One of these introns is about 3300bp (Reddy *et al.*, 2002).

The genomic sequence for *Arabidopsis* was published in 2000 ('The Arabidopsis Genome Initiative', 2000). Since there is no published genome for tobacco, there is interest in the *Arabidopsis* sequence in order to find a suitable sequence for making primers. Previous difficulty in amplifying the entire tobacco GAD gene may have been due to using genomic DNA and primers designed from cDNA. One must create primers that eliminate any large introns in order to make it possible to amplify and sequence. We are attempting to amplify only the DNA encoding the GAD active site and calmodulin-binding domain. Alignment of tobacco NtGAD1, NtGAD2, and NtGAD3 sequences with *Arabidopsis* genomic DNA (www.plantgdb.org) has shown five potential introns. Fortunately only two small introns fall within the region we plan to amplify. From the temperature dependent mutants that Samuelsen and coworkers identified, the identification and sequencing of a mutant GAD gene compared to the wild type GAD gene may determine the difference in mutant tobacco. A discovery of a gene structural difference effecting enzyme activity of GAD will provide data to support the regulation of plant development by the calmodulin-binding domain of GAD.

Materials and Methods

Purification of Burley 21 DNA

EPICENTRE® MasterPure™ plant leaf DNA purification kit was used to isolate the Burley 21 tobacco genomic DNA. A similar procedure was used to isolate W-38 DNA as well. The protocol was followed for EPICENTRE® plant leaf DNA purification with some modifications. To ensure no contamination, the following procedure was carried out under a laminar flow hood. Two leaf disks from Burley plants were punched into four 1.5 ml microfuge tubes by snapping the caps closed on the leaf. The leaf disks

were ground with 300 µl of Plant DNA Extraction Solution using a micro-pestle and incubated at 70°C for 30 minutes and then put on ice to chill for 10 minutes. The cellular debris was pelleted by centrifugation for 5 minutes at maximum speed. The supernatants were transferred to clean microfuge tubes and the centrifugation step was repeated to remove residual debris. Again, supernatants were transferred to clean microfuge tubes. An equal volume of isopropanol was added to the clarified supernatant and inverted 20 times to mix. The DNA was pelleted by centrifugation for 5 minutes at maximum speed. The supernatant was removed with a pipette. The pellets of DNA in all tubes were suspended in 100 µl of Cleanup Solution and vortexed for 20 seconds to ensure complete resuspension. To each tube of resuspended DNA 100 µl of isopropanol was added and mixed thoroughly by inversion. The DNA was pelleted by centrifugation for 5 minutes at maximum speed. The pellets were washed with 70% ethanol, which was carefully removed with a pipet and discarded. The DNA pellets were centrifuged for 30 seconds and any remaining ethanol was removed. The DNA in each tube was resuspended in 50 µl of nuclease free water.

Burley DNA Cleanup for PCR

All four tubes of Burley 21 DNA purified by the EPICENTRE® plant leaf DNA purification protocol were combined in a single microfuge tube. 2 µl of RNase A was added to the tube, inverted 20 times and incubated at 37°C for 15 minutes. 250 µl phenol and 250 µl chloroform (chloroform:isoamyl alcohol 24:1) were mix together in a microfuge tube. The RNase A treated DNA was added to it and inverted 15 times and centrifuged for 30 seconds at maximum speed. The top layer was removed and put into a new microfuge tube. 0.5 ml of chloroform was added to get rid of any phenol,

centrifuged for 30 seconds at maximum speed and again the top of the interface was removed and put into another microfuge tube. 100 µl of Clean-up solution and 100 µl of isopropanol were added and mixed by inverting 20 times. The DNA was pelleted by centrifugation for 5 minutes at maximum speed and the pellet was washed with 70% ethanol. The DNA was then resuspended in 100 µl of nuclease free water. W-38 DNA was cleaned up in a similar manner.

Primers Designed for Mutant and Wild Type GAD Amplification

The Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3_www_results.cgi) was used to create primers that encompassed the conserved active site and C-terminal binding domain within the GAD gene. The *Arabidopsis* genomic sequence was used to find the suitable sequence for making the primers because the tobacco genomic sequence is not published. Only two small introns were found in the region we were amplifying when using GeneSequer® (www.plantgdb.org) and aligning tobacco NtGAD1, NtGAD2, and NtGAD3 sequences with the *Arabidopsis* genomic DNA. Sigma Genosys synthesized the primers of interest. The 5'GAD3 primer sequence was 5'-CATGTGGATGCAGCAAGTGGTGG-3'. The 3'GAD3 primer sequence was 5'-TTAACAAACTCCATTAGTCTTC-3'. Due to unsuccessful PCR attempts to amplify the sequence, the primers were extended to include more terminal C's and G's for better annealing. The New 5' GAD primer sequence was 5'-TCCAATTCATGTGGATGCAG-3'. The New 3'GAD primer sequence was 5'-TCATCCAATTTTTCACTAGCAA-3'.

PCR Protocol

The final PCR reaction mixtures used were:

Reagent Added (µl)	Wild Type	Mutant	Burley 21	Control
Template	0.2(20ng)	0.5(20ng)	1(20ng)	0
Go Tag®	12.5	12.5	12.5	12.5
New 5'GAD	1	1	1	1
New 3'GAD	1	1	1	1
25 mM $MgCl_2$	1	1	1	1
Nuclease Free Water	9.3	9	8.5	9.5
Total Volume	25	25	25	25

The final PCR thermocycling settings used were:

Cycle Number	Step	Time (min.)	Temperature (°C)	Repeats
1	1	3	94	1
2	1	1	94	40
	2	2	55	
	3	2	72	
3	1	3	72	1

DNA Extraction from Agarose Gel

The protocol was followed to extract DNA from an agarose gel from the QIAEX II Handbook, February 1999 (QIAGEN). In a 1% agarose gel prepared by the above PCR protocol, 7 PCR reactions of the Burley 21 DNA was run. The DNA bands from the agarose gel were excised using a clean, sharp scalpel. The gel slices were weighed in two 1.5 ml microfuge tubes. 3 volumes of Buffer QX1 to 1 volume of gel were added. The QIAEX II was resuspended by vortexing for 30 seconds and then 20 µl of QIAEX II was added to each tube. The tubes were incubated for 10 minutes at 50°C to solubilize the agarose and bind the DNA. Every 2 minutes they were mixed by inverting 10 times to keep QIAEX II in suspension. The samples were centrifuged for 30 seconds and the supernatant was removed with a pipette. The pellets were washed with 500 µl of Buffer QXI by first resuspending them by vortexing and then centrifuging for 30 seconds and

removing all traces of supernatants with a pipette. The samples were washed twice in the same way with 500 µl of Buffer PE. The pellet was air-dried for 15 minutes. To elute DNA, 25 µl of dH_2O was added to each tube and DNA was resuspended by inverting tubes 10 times. The samples were incubated at room temperature for 5 minutes. They were then centrifuged for 30 seconds and supernatant was transferred to clean tubes and stored at -20°C.

Results

Primers to target the GAD gene of wild type and mutant tobacco were designed from the active site to the c-terminal calmodulin-binding domain. Since the published GAD sequence came from Burley 21 tobacco, it was used in order to optimize the PCR reaction with the idea that the wild type (W-38) and mutant (W-38) tobacco PCR would follow. Genomic Burley 21 DNA was successfully isolated (See Figure 1 in Appendix A). After many trials, a product with a single band was produced using Burley 21 with the final PCR conditions and the New5'GAD and New3'GAD primers (See Figure 2 in Appendix A). The same conditions were then tested on wild type and mutant W-38 tobacco but the gel did not show any bands produced for these tobacco plants (See Figure 3 in Appendix A).

An attempt was made to extract and sequence the DNA product from the single Burley 21 band to find out what the product is. The single PCR band around 3,000 bp was expected to be around 800 bp for product of the GAD gene sequence. Time ran out after two attempts failed to extract enough DNA from the bands produced by Burley 21 for sequencing.

Discussion

Trying to isolate a smaller more specific sequence of the GAD gene from the wild type and mutant tobacco still proved to be more difficult than expected. The NewGAD3' and NewGAD5' primers did amplify a single 3,000bp region in the Burley 21 DNA but did not result in the same amplification in the W-38 wild type or mutant tobacco DNA. Perhaps just amplifying the CaM-binding domain would be a next step to try. This might give us the information needed regarding the sequence of the CaM-binding domain. Since we were using cDNA primers, large introns could be interfering with amplifying genomic DNA sequences. To get better results we might have used a kit to isolate RNA and then reverse transcribe the genomic DNA into a cDNA sequence for PCR (rtPCR). This would take more time and be more costly. Ultimately it may be most favorable to wait until the genomic sequence of tobacco is published before attempting to amplify and sequence the GAD gene from wild type versus mutant plants.

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Appendix A

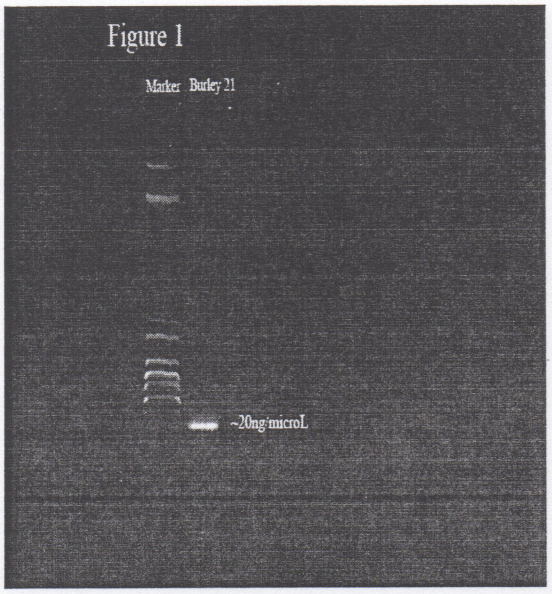

Figure 1: Band shows Burley DNA was successfully isolated and contained 20 ng/µL DNA.

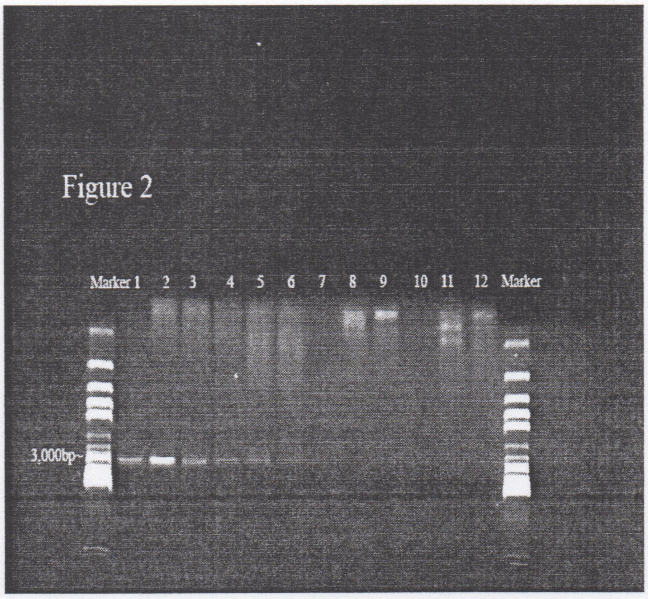

Figure 2: Trial 2 shows an isolated single strong band using Burley 21 DNA and final PCR conditions.

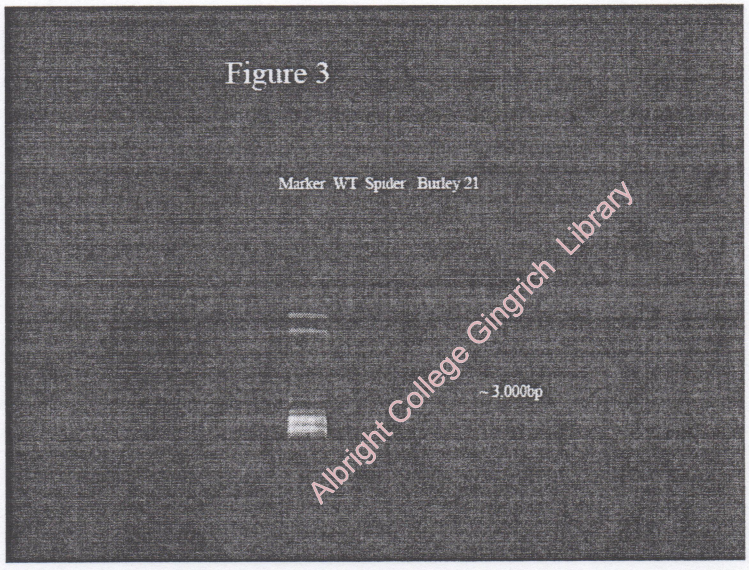

Figure 3: Same final PCR conditions ran on WT and Spider (mutant) tobacco DNA but only Burley 21 DNA shows a single band at 3,000 bp.