VECTOR TOBACCO (USA) LTD.
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01-121-01p
Revised

August 31, 2001

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PETITION FOR DETERMINATION OF REGULATORY STATUS

Dear Dr. Firko:

Enclosed please find six copies of a revised petition for determination of the regulatory status for the *Nicotina tabacum* cultivar "Vector 21-41" that has been genetically modified in order to reduce the levels of nicotine present in the plant, and which is currently deemed a "regulated article". Dr. Susan Koehler reviewed the original application (Petition Number 01-121-01p) for completeness and the additional information and clarification APHIS requested are incorporated into the petition. Based on the data and information contained in the enclosed petition, we contend that there is no longer "reason to believe" that the modified tobacco plant should be deemed to be a regulated article. As discussed further in our petition, Vector 21-41 does not present a plant pest risk and is not otherwise deleterious to human health or the environment.

The enclosed petition does not contain any Confidential Business Information.

The undersigned certifies that, to the best of his knowledge and belief, this petition includes all data, information, and views relevant to the position of the undersigned, which is the subject of the petition.

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Application for Determination of Nonregulated Status for Vector 21-41 Reduced-Nicotine Tobacco

Petition Number: 01-121-01p

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Abbreviations and Scientific Terms

BP	Basepair
DAO	Diamine oxidase
GUS	β-glucuronidase
Kan	Kanamycin
KB	Kilo basepair
mg	Milligrams
MH	Maleic hydrzide
MS	Murashige and Skoog
NAB	N-Nitrosoanabasine
NAD	Nicotinamide adenine dinucleotide
NAT	N-Nitrosanatabine
NAMN	Nicotinic acid mononucleotide
ng	Nanogram
NNK	N-nitrosonicotine keytone
NNN	N-Nitrosonornicotine
NOS	Nopaline synthase
NPTII	Neomycin phosphotransferase
NtQPT1	The tobacco gene encoding quinolinic acid phosphoribosyltransferase
ODC	Ornithine decarboxylase
Orthene	Insecticide
pg	Picograms

PMTase	Putrescine methyltrasnferase
ppm	Parts per million
QPTase	Quinolinic acid phosphoribosyltransferase
Ti plasmid	Tumor-inducing plasmid of *Agrobacterium tumefaciens*
TPM	Total particulate matter
TSNA	Tobacco Specific Nitrosamines

I. RATIONALE FOR DEVELOPMENT OF REDUCED-NICOTINE TOBACCO, VECTOR 21-41

Vector 21-41, a transgenic tobacco producing very low nicotine levels, represents the first successful attempt to remove nicotine from tobacco leaf through genetic engineering. Attempts to reduce nicotine through traditional genetic manipulation have only reduced nicotine levels about ten-fold. Nicotine removal by way of chemical processes result in a less than desirable product for processing. Molecular biological technologies used to develop Vector 21-41 reduce nicotine levels about 20-fold and produce a full-flavor product. This transgenic tobacco line was developed in Dr. Mark A. Conkling's laboratory in the Department of Genetics at North Carolina State University, and is a result of a research program designed to understand the function of genes expressed exclusively in tobacco roots. Analysis of one root-specific cDNA clone (*NtQPT1*) revealed that it encoded quinolinic acid phosphoribosyltransferase (QPTase). QPTase is one of the key enzymes in nicotine biosynthesis. Nicotine is synthesized in the roots and is then transported to the leaves. Analysis of the *NtQPT1* promoter revealed that it would direct transgene expression specifically in the root cortex. In order to reduce nicotine levels, the *NtQPT1* promoter was used to direct antisense expression of the *NtQPT1* cDNA. Tobacco plants carrying this transgene were screened for reduced nicotine levels and the transgenic line designated *Vector 21-41* exhibited the lowest levels. Field grown Vector 21-41 has nicotine levels ranging from 700 to 1500 parts per million (ppm) (see Table 4 page 21 of Petition for Deregulation) as opposed to normal tobacco's 20,000 to 40,000 ppm levels (Tso, 1972). Additionally, the levels of tobacco-specific nitrosamines (TSNAs) were reduced to undetectable levels in Vector 21-41 leaves and 15- to 50-fold in tobacco smoke (see Table 6 page 34 of Petition for Deregulation).

Nicotine is widely believed to be the addictive component of tobacco. It also serves as the precursor for the production of Tobacco Specific NitrosAmines (TSNAs), which are potent mutagens and potential carcinogens. Nicotine addiction has been studied extensively and researchers generally agree that smoking can be greatly reduced if nicotine delivery can be reduced below addictive levels. Tobacco products made from Vector 21-41 deliver nicotine below the addictive levels suggested by Benowicz and Henningfield (1994). With these low nicotine levels, this product is targeted toward helping smokers control their use of tobacco. In limited, extended-use smoking panels, many participants believed the cigarettes made from Vector 21-41 tobacco have either helped them quit or believe they will eventually be able to quit. Additional more extensive trials, conducted by Dr. Jed Rose of Duke University Medical School, are underway to confirm and quantify this observation.

II. THE BIOLOGY OF TOBACCO

Tobacco, *Nicotiana tabacum*, is the primary species of the genus *Nicotiana* to be commercialized for the production of smoking and other tobacco products. Various *Nicotiana* species have been smoked and/or chewed by indigenous peoples of North and South America since pre-Columbian times. *N. tabacum* and, to a much lesser extent, *N. rustica* are the only species to be domesticated for commercialization. No *Nicotiana* species is found on the Federal noxious weed list (www.fs.fed.us/r9/weed/sec1C.html).

Tobacco has also been used extensively as an "experimental plant". Pioneering studies on phytohormones, photoperiodism, plant tissue and organ culture, protoplast fusion, organelle transfer, disease resistance, and, more recently, transformation/regeneration have all been conducted using tobacco. Because of the ease of tissue culture and regeneration, tobacco is arguably the easiest plant for transgenic analysis. As such, it has become the model transgenic plant system in both industry and academia to test new genetic constructs in greenhouse and field trials. The Animal and Plant Health Inspection Service (APHIS) has received many notifications of genetically modified tobacco lines.

Although tobacco can be transformed by DNA-mediated transformation and micro-projectile bombardment, the principle means of transformation has been *Agrobacterium*-mediated transformation. Typically, relatively few copies of the transforming DNA are integrated into the tobacco genome following *Agrobacterium*-mediated transformation. In addition to the DNA sequences of interest, *Agrobacterium*-mediated transformation introduces the left- and right-border sequences of the Agrobacterium T-DNA and a selectable marker. Selectable markers include bacterial genes conferring resistance to antibiotics (such as kanamycin or methotrexate) or bacterial or plant genes conferring resistance to herbicides (such as glyphosate).

A. The Genus *Nicotiana*

The characteristic feature of *Nicotiana* species is the presence of nicotine and other alkaloids. The genus *Nicotiana* is classified into four subgenera, 14 sections, and 66 species. Forty-five of the species are indigenous to North and South America, nineteen indigenous to Australia, one indigenous to Polynesia and Tonga, and one indigenous to Africa. Among the species for which somatic chromosome numbers have been determined, four species have 18 chromosomes, two species have 20 chromosomes, 27 species have 24 chromosomes, four species have 32 chromosomes, three species have 32 chromosomes, two species have 36 chromosomes, two species have 38 chromosomes, five species have 40 chromosomes, one species has 44 chromosomes, and eight species have 48 chromosomes. *N. tabacum* (2N=48) is an amphidiploid species resulting from a hybridization of two progenitor species believed to be *N. sylvestris* (2N=24) and *N. tomentosiformis* (2N=24) (Smith, 1979).

Of the 45 *Nicotiana* species indigenous to North and South America, only five are found in the United States (in *Illustrated Book of the Genus Nicotiana,*1990). *N. trigonophylla* (2N=24) is found along the Mexican border in Texas, New Mexico, and Arizona. It is cross-compatible with *N. tabacum* in laboratory experiments. *N. repanda* (2N=48) is found in Texas along the gulf coast. It is cross-compatible with *N. tabacum* with the progeny exhibiting cytoplasmic male-sterility. *N. attenuata* (2N=24) is found in the western United States from Mexico to Canada. It has not been reported to be cross-compatible with *N. tabacum. N. bigelovii* (2N=48) is found along the Pacific coast of California. It is cross-compatible with *N. tabacum* with the progeny exhibiting cytoplasmic male-sterility. *N. cleavelandii* (2N=48) is found along the Pacific coast of southern California. It has not been reported to be cross-compatible with *N. tabacum.*

B. *Nicotiana tabacum*

Tobacco, *N. tabacum*, originated in northern Argentina and southern Bolivia as an amphidiploid species resulting from a hybridization of two progenitor species believed to be *N. sylvestris* (2N=24) and *N. tomentosiformis* (2N=24) (Smith, 1979). Tobacco has been domesticated and is the primary *Nicotiana* species used commercially. The tobacco flower is tubular and is about 5 cm long. Because tobacco is self-compatible, it reproduces primarily by self-fertilization. Tobacco is not wind pollinated, therefore the only potential means for pollen to outcross naturally is by insect vectors. Studies measuring the frequency of outcrossing among populations of tobacco have been conducted (Litton and Stokes, 1964; McMurtrey, et al., 1960). These studies show that when two populations of tobacco are grown adjacent to one another, flower at the same time, and the flowers allowed to mature and produce seeds that less than 2% of the seeds result from cross-fertilization. Paul, Lewis, and Dunwell (1991) measured the frequency at which homozygous kanamycin-resistant transgenic tobacco plants would outcross to non-transgenic plants at distances of one, ten, and twenty-one meters. Approximately 103,000 seedlings were screened and 39 kanamycin resistance progeny were observed (0.038% outcrossing frequency). The percentages of capules that contained kanamycin-resistant progeny decreased as distance from the transgenic donors; 1 meter 7/44, 10 meters 4/322, 21 meters 1/373. Because the insect vectors that cross-fertilize tobacco do not travel long distances, certified seed production requires a separation of 1320 feet from other tobacco varieties.

The commercial production of tobacco leaf severely limits the ability of the tobacco to out-cross. In this process, flowers are not allowed to develop. Typically, seedlings are grown in seedbeds or in greenhouse hydroponic systems for about 45 days. The seedlings are clipped periodically during this time and are then transplanted into the field at a density of about 7000 plants per acre. Approximately 50 days after transplant, flowers begin to develop. Developing flowers and the apical meristem are removed in a process called topping. Topping releases the plant from apical dominance and induces the production of nicotine and of axillary buds (suckers). Suckers are removed manually or their development retarded by the application of chemicals such as maleic hydrazide (MH). The tobacco plants enter senescence and typically the leaves are harvested about 90 days after transplant. Tobacco leaves are then either air-cured (Burley varieties) or are cured by heating (flue-cured varieties).

C. Weediness of *Nicotiana* spp.

No *Nicotiana* species (including tobacco) are found on the Federal noxious weed list (www.fs.fed.us/r9/weed/sec1C.html). Tobacco is an annual plant. Seeds are shed as the seed-pods mature and can germinate immediately; *i.e.*, the seeds do not have a dormancy period. Thus, any volunteer seedlings will emerge in the fall and will be killed by the winter temperatures.

D. Modes of Gene Escape in Tobacco

Genetic material of *N. tabacum* may escape from a planting site by vegetative material, by seed, or by pollen. Vegetative propagation is not a common method by which tobacco can reproduce in the wild. The dispersal of tobacco seed from a cultivated transgenic crop would be exceptionally rare. As mentioned earlier (Section II.B), in the commercial production of tobacco leaf the developing flower is removed. This releases the plant from apical dominance and

induces the production of nicotine and of axillary buds (suckers). Suckers are removed manually or their development retarded by the application of chemicals, and thus will not produce flowers.

The escape of transgenic pollen to fertilize neighboring tobacco fields will also be a highly unlikely event. Tobacco reproduces primarily by self-fertilization. Under ideal conditions for cross-pollination (*i.e.*, transgenic and non-transgenic plants adjacent to one another, both flowering at the same time, and the flowers of both being allowed to fully develop and produce seeds), less than 2% of seeds would result from cross-pollination (See Section II.B). Commercial tobacco crops are grown for leaf production and, thus, will have their flowers removed. This will drastically reduce chances of out-crossing. Secondly, unlike corn, the tobacco crop is the vegetative parts of the plant (leaves and stems) and, thus, any out-crossing would not affect the current crop. The vast majority of tobacco farmers (>90%) do not keep seed back, but use certified seed each year. Certified seed is produced with an isolation distance of at least 1320 feet, and thus, would not be pollinated by transgenic varieties. Certified tobacco seed production is generally limited to the areas of the US used for commercial production of tobacco. For example, Rickard Seeds and Newton Tobacco Seeds are located in Kentucky and GoldLeaf Seeds is located in South Carolina. No wild cross-compatible *Nicotiana* species are found in these areas.

Identity preservation of Vector 21-41 is critical to the success of Vector Tobacco. As such, Vector Tobacco will take all measures possible to preserve the genetic purity of its seed stock. It is our intention to produce all seed in greenhouses. Greenhouses producing Vector 21-41 seeds will have only Vector 21-41 tobacco plants within the structure; *i.e.*, no other tobacco varieties will be grown within the structure. Greenhouses producing seeds will be isolated from commercial tobacco fields by at least 1320 feet. Greenhouse vents will be screened with 52/52 mesh screening and will follow insect control practices that will prevent insects from carrying pollen into or out of the greenhouses.

E. Characteristics of the Nontransformed Cultivar

Vector 21-41 was developed by transforming the tobacco cultivar Burley 21 LA with a DNA construct containing the *NtQPT1* promoter directing the expression of antisense transcripts of the *NtQPT1* cDNA (details are provided in Section IV). Burley 21 LA is a Low-Alkaloid variety of the commercial cultivar Burley 21. Legg and co-workers (1969) incorporated genes (now called *nic1* and *nic2*) from low alkaloid content Cuban cigar cultivars into the Burley 21 cultivar by hybridizing the two cultivars followed by a series of twelve back-crosses. Collins and co-workers (1974) compared Burley 21 and Burley 21 LA lines and found that they did not differ in days to flower, number of leaves, leaf size, and plant height. Legg and co-workers (1970) registered the Burley 21 LA germplasm for research purposes. No tobacco cultivar nor sexually compatible *Nicotiana* species are considered to be noxious weeds.

F. Nicotine Biosynthesis in Tobacco

Alkaloids are secondary metabolites that contain nitrogen in their cyclic organic structures. Alkaloids have been used by man as stimulants, drugs, narcotics, and poisons. Most alkaloids have unknown function for the plants that produce them, although some alkaloids act as phytoalexins or are involved in plant-pest interactions. Nicotine is the predominant alkaloid found in tobacco. During the domestication of tobacco, varieties producing high levels of

nicotine were typically selected. Commercial varieties typically produce nicotine at levels between 2% and 4% of dry weight (20,000 to 40,000 ppm) (Tso, 1972). Nicotine has been found in other plant species and families including some non-solanacious plants, however, the amount synthesized is much lower than that of *N. tabacum* (Leete, 1983).

Studies have been carried out to establish the nicotine biosynthetic pathway (Leete, 1983; Hashimoto and Yamada, 1994) by administering labeled compounds, studying tissue cultured plants, and most recently, using molecular techniques. Nicotine is composed of pyridine and N-methylpyrrolidine rings linked together and is produced by the condensation of two metabolites, nicotinic acid and N- methylpyrroline, that are synthesized by two separate pathways (Waller and Dermer, 1981). (See Figure 1) Nicotinic acid is synthesized in the pyridine nucleotide cycle pathway (Wagner et al., 1986; Wagner and Wagner, 1985), whereas N-methylpyrroline is synthesized from ornithine or arginine via putrescine (Tiburcio and Galston, 1986).

The enzymatic activities of the enzymes involved in nicotine biosynthesis have been assayed in different organs of the tobacco plant, in dedifferentiated and differentiated tobacco tissue, in tobacco root cultures, and in plants with different rates of nicotine production (Wagner et al., 1986). The regulatory step enzyme for the methylpyrroline pathway is PMTase, which controls the transition from primary to secondary metabolism. For the other pathway, the regulatory step enzyme is QPTase, which is the entry point for the pyridine-nucleotide cycle (Wagner ct al., 1986). Several additional less stringent regulatory steps that help to complete the concerted regulatory system are observed.

Few enzymes in the nicotine biosynthetic pathway have been isolated and characterized. In particular, there is a lack of information regarding the enzyme responsible for the condensation of nicotinic acid and N-methylpyrrolinium cation. A crude enzyme extract has been isolated from *Nicotiana* species that can catalyze the formation of nicotine from nicotinic acid and N-methylpyrrolinium chloride in the presence of oxygen (Friesen and Leete, 1990). Despite the importance of this enzyme activity, further characterization has not been reported.

The Nicotine Biosynthetic Pathway in Tobacco

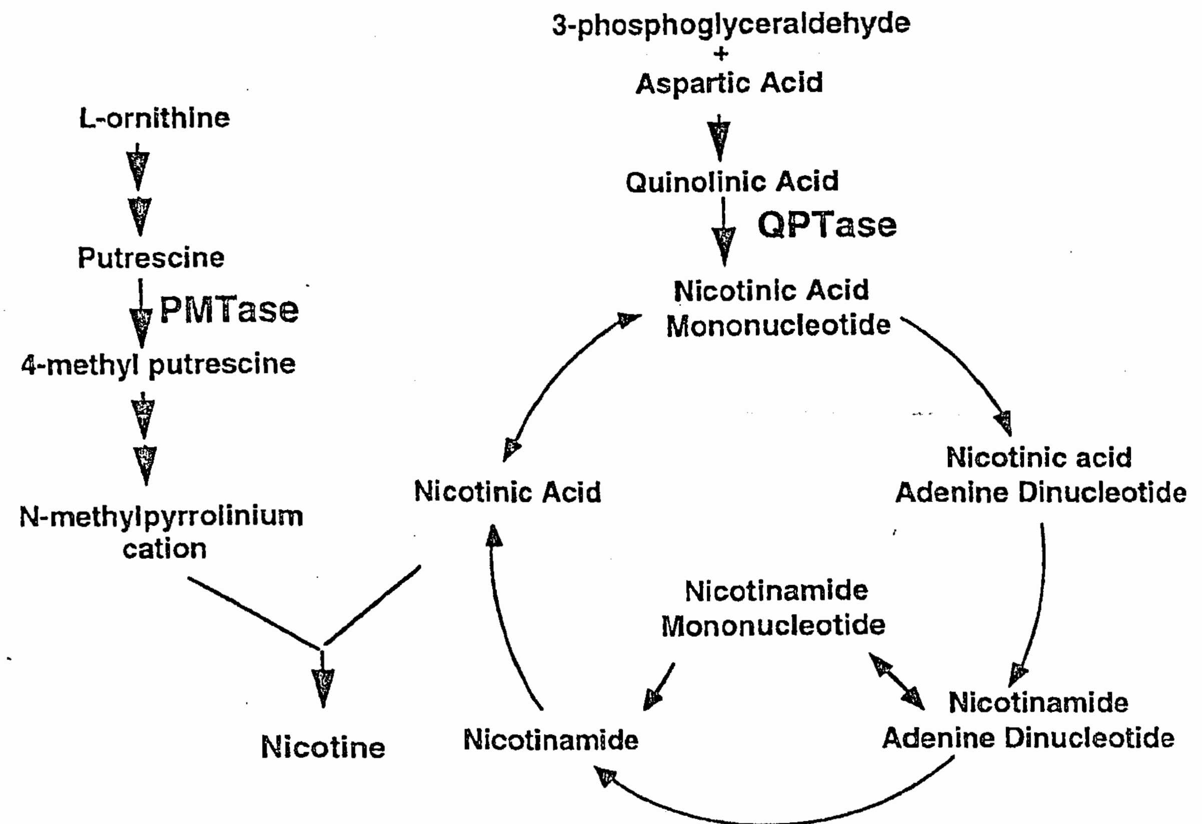


Figure 1. The Nicotine Biosynthetic Pathway in Tobacco
The synthesis of nicotine requires precursors from two different pathways. One precursor, N-methylpyrrolinium cation is provided by the methylpyrroline pathway, while the other precursor, nicotine acid, is the product of the pyridine nucleotide cycle. PMTase (putrescine methyl transferase) and QPTase (quinolinate phosphoribosyltransferase) are highlighted. They are the rate-limiting enzymes in this pathway.

1. The pyridine-nucleotide cycle

Nicotinic acid is the source of the pyridine ring of the nicotine (Dawson et al., 1956). The pyridine-nucleotide cycle has two possible pathways leading to nicotinic acid (Wagner and Wagner, 1985; Wagner et al., 1986). One enzymatic route takes advantage of the fact that nicotinamide adenine dinucleotide (NAD) is involved in a rather rapid turnover, as the enhanced nicotine production stimulates the synthesis and degradation of NAD. The second route is a direct one, using one enzymatic step that transforms nicotinic acid mononucleotide (NAMN) to nicotinic acid. The study of nicotine biosynthesis had led to the discovery of a new pathway that exists in all higher plants for the formation of nicotinic acid. 3-phosphoglyceraldehyde or its

equivalent condenses with aspartic acid or its equivalent to form quinolinic acid (Dalton, 1980). Quinolinic acid phosphoribosyltransferase (QPTase), which converts quinolinic acid to NAMN, serves as the entry-point enzyme into the pyridine nucleotide cycle that supplies nicotinic acid (Wagner and Wagner, 1985). The pyridine-nucleotide cycle, provides NAD and nicotinic acid, is a part of primary metabolism but also provides components for the secondary metabolism such as nicotine synthesis in the root of tobacco (Waller and Dermer, 1981).

2. The methylpyrroline pathway

The other precursor for nicotine is the N-methylpyrrolinium cation. In this part of the biosynthetic pathway, nicotine and tropane alkaloid share a common early pathway originating from ornithine and/or arginine by the action of ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) and diverging at the N- methylpyrrolinium cation. The early pathway involves the symmetric diamine putrescine, that is found ubiquitously in plants and usually is metabolized to conjugated forms or to the polyamines, spermidine and spermine. Putrescine N-methyltransferase (PMTase) is the first committed enzyme in alkaloid biosynthesis and this step of the reaction is unique to alkaloid-producing plants. The PMT cDNA has been isolated from tobacco (Hibi et al., 1994). The PMT transcript can only be detected in tobacco roots. The deduced amino acid sequence of PMT cDNA encodes a 41 kD protein, PMTase. PMTase catalyzes the formation of N-methylputrescine which is then oxidatively deaminated to 4-aminobutanol by diamine oxidase (DAO). 4-aminobutanol spontaneously cyclizes to the N-methylpyrroliniu cation, a direct precursor for nicotine (Feth et al., 1986).

G. Genetic Control of Nicotine Biosynthesis

Genetic control of total alkaloid production in the tobacco plants has received considerable attention. Some studies indicate that differences in alkaloid levels among flue-cured varieties are inherited quantitatively (Legg et al., 1965). Experiments and observations by Valleau (1949) showed that large differences in alkaloid content were controlled by a rather simple genetic system. This hypothesis has been supported further by the identification of the *Nicl* and *Nic2* loci. Hybrid lines were developed by incorporating genes from low alkaloid content Cuban cigar cultivars into Burley 21 cultivars by a series of 12 back-crosses (Legg et al., 1969). Detailed genetic analyses of parental, F1, F2, B1, and B2 generations of lines differing in alkaloid content were conducted. Legg and co-workers (1969) concluded that the low alkaloid lines differed from standard cultivars at two non-linked loci, *Nicl* (formerly known as A) and *Nic2* (formally known as B). *Nic* gene actions are semidominant and primarily additive. The genotypes of standard cultivars are *Nicl/Nicl Nic2/Nic2* and those low nicotine lines are *nicl/nicl nic2/nic2*. *Nicl/Nicl nic2/nic2* lines are high intermediates and *nicl/nicl Nic2/Nic2* lines are low intermediates (Legg and Collins, 1971). The *Nicl* locus has an approximately 2.4-fold stronger effect than the *Nic2* locus. Doubled haploid tobacco breeding lines of these four alkaloid genotypes are established (Collins et al., 1974). These lines are not different in days to flower, number of leaves, leaf size, and plant height. However, the *nicl/nicl nic2/nic2* line is more susceptible to insect damage (Legg et al., 1970) and has longer maturity dates (Miller et al., 1982).

Different nicotine content among commercial varieties of tobacco indicates that genes in addition to *Nicl* and *Nic2* may be involved in the inheritance of alkaloid production. However, the other genes have only minor effects in low nicotine plants (Legg et al., 1969). The enzyme

activities for the enzymes that participate in nicotine biosynthesis have been analyzed in the four homozygous allelic Burley 21 combinations producing high, high intermediate, low intermediate, and low nicotine levels (Saunders and Bush, 1979). PMTase and QPTase activities in root tissues of these four genotypes are proportional to leaf nicotine level. No differences were observed for the QPTase activity in leaves of these four genotypes (Saunders and Bush, 1979). Both *Nicl* and *Nic2* affect PMTase and QPTase activities in roots, thus are involved in regulation of nicotine synthesis. A cDNA encoding PMT was isolated by subtraction hybridization of the wild-type line (*Nic/Nicl Nic2/Nic2*) and the low nicotine mutant line (*nicl/nicl nic2/nic2*) (Hibi et al., 1994). *PMT* transcript levels in roots were correlated to nicotine levels in leaves in the *nic* mutants (Hibi et al., 1994). Similarly, *NtQPT1* transcript levels correlate to nicotine levels in the *nic* mutants (Song, 1997).

H. Spatial and Temporal Regulation of Nicotine Biosynthesis

As with many other metabolic pathways, the nicotine synthetic pathway is regulated spatially and temporally. Nicotine biosynthesis is also affected by internal and external stimuli such as plant hormones, light, nutrients, and stress.

As demonstrated by the classical reciprocal grafting experiments between a nicotine producing plant, tobacco, and nonproducing plant, tomato (Dawson, 1941), nicotine is synthesized in the root and is translocated to the aerial parts of the plant. This is confirmed by the observations that the enzymes involved in nicotine biosynthesis, PMTase, DAO, and QPTase have high enzyme activities in tobacco roots, but low enzyme activities in leaves. Accordingly, the transcripts of *PMT* and *NtQPT1* are found exclusively in the root (Hibi et al., 1994; Song, 1997). The alkaloid-synthesizing function of plant roots shows remarkable specificity. Recent development of plant organ culture technologies and characterization of several biosynthetic enzymes have confirmed the root-specific localization of alkaloid biosynthesis.

Nicotine biosynthesis can be detected immediately after the germination of the seed. Nicotine formation is observed in connection with the protein metabolism of the aleurons in the radicle and the hypocotyl. The cotyledons remain free of nicotine. Later when the hypocotyl elongates, nicotine levels decrease, while the roots continue to synthesize nicotine (Tso, 1972). Nicotine synthesis is associated with young, developing root tissue. The root-synthesized nicotine transports to leaves via the xylem (Tso, 1972). The leaves, however, not only accumulate the nicotine, but can also transform it into a wide array of products including nor-nicotine and Tobacco-Specific NitrosAmines (TSNA's), all of which are derived from nicotine synthesized in the roots (Bush et al., 1993).

Every step in tobacco production that affects plant metabolism will influence the level of nicotine content in the leaves. Tobacco type, distribution in the plant, cultural practices, ripening, and fertilizer treatment are among some prominent factors (Tso, 1972). Experiments with cell suspension culture, callus culture and root culture of *Nicotiana* species have shown that light inhibits nicotine accumulation in the culture (Hobbs and Yeoman, 1991). Various environmental factors, plant growth regulators, combined with the growth stage and growth conditions of tobacco affect tobacco nicotine biosynthesis in roots.

Nicotine biosynthesis is regulated by topping, wounding, and plant growth regulators. Removal of flower heads and several young leaves (topping), is a common practice among tobacco growers. Topping eliminates apical dominance and increases nicotine content in tobacco leaves. Topping induces PMTase and QPTase activities in roots, and thus increases nicotine synthesis levels (Saunders and Bush, 1979; Wagner et al., 1986). Topping rapidly induces the PMT transcript levels in roots. Exogenous supply of auxin down-regulates PMT transcript in cultured tobacco roots (Hibi et al., 1994). Auxin has long been known to down-regulate nicotine content in tobacco. In tobacco tissue culture, kinetin promotes nicotine production in the absence of auxin. Auxin strongly inhibits nicotine formation, without affecting tissue growth (Tabata et al., 1971). The nicotine content of the *N. rustica* root culture decreases in the presence of auxin (Rhodes et al., 1994).

Topping not only removes the apical dominance, but also wounds the plant. Leaf damage has been shown to increase rapidly the concentration of different types of secondary metabolites found in undamaged leaves. Many of these damage-induced increases have proven defense-related roles. Baldwin (1991) reported that large increases observed for the concentration of nicotine in *N. sylvestris* plants in response to leaf damage and these alkaloidal responses reduced the growth of a tobacco herbivore and protected alkaloid induced tissues in laboratory feeding trials. They demonstrated that this increase of nicotine content was due to the increased amounts of nicotine entering the in the xylem stream. This indicates that the wound-induced nicotine accumulation in leaves is due to the increased nicotine synthesis in the roots of the wounded plants.

I. Identification of the root-specific gene, *NtQPT1*

NtQPT1 (originally called *TobRD2*) is a tobacco gene isolated as part of a screen for genes expressed specifically in roots of tobacco. Using a combination of differential screening and subtractive hybridization, Conkling and co-workers (1990) isolated a collection of genes expressed exclusively in tobacco roots. They subsequently demonstrated that root-specific expression was regulated, at least in part, transcriptionally and that the genes were members of small gene families.

When blots of genomic DNA digested with *Hin*dIII, *Bam*HI, and *Eco*RI are probed with the *NtQPT1* cDNA (then called *TobRD2*) and washed under moderate stringency (0.2X SSC at 42°C), two strongly hydridizing bands and several weakly hybridizing bands are visible (see Fig. 5 of Conkling, et al. 1990). Probing genomic and cDNA libraries with the *NtQPT1* cDNA identified numerous independent clones. Restriction and sequence analyses demonstrated that these clones resulted from two tobacco genes, *NtQPT1* and *NtQPT2*. The coding regions of these genes are highly similar (>90% nucleic acid identity) as are the promoter sequences (>80% identity for the first 1 kb). RNA blot analysis of RNA isolated from tobacco roots, leaves, and tissue culture cells, demonstrates that RNA hybridizing to *NtQPT1* is found only in the roots (see Fig. 2 of Conkling, et al. 1990). Experiments in which four-times more RNA from leaves and stems (relative to roots) were blotted, hybridized, and overexposed showed that leaves and stems had less than 1% the level of RNA relative to roots (Song, 1997).

The *NtQPT1* promoter directs transgene expression only in tobacco roots (see Fig. 4 of the Petition for Deregulation) and in the same tissue as the endogenous *NtQPT1* gene (see Fig. 2

and 3 of the Petition for Deregulation). Tobacco is an amphidiploid species, containing diploid genomes of its two progenitor species, *N. sylvestris* and *N. tomentosiformis*. Both *N. sylvestris* and *N. tomentosiformis* produce nicotine and, therefore, must have QPTase activity. The data are most consistent with the hypothesis that *NtQPT1* and *NtQPT2* are homeologous genes, *i.e.*, one gene from each of the progenitor species.

Because of the requirement for nicotinamide adenine dinucleotide (NAD), all cells of an organism must have QPTase activity (see Fig. 1 of the Petition for Deregulation). Therefore, there must be gene(s) encoding QPTase that are expressed consitutively throughout the plant. We believe that the weakly hybridizing bands present on genomic blots (see Fig. 5 of Conkling, et al. 1990) may represent these genes.

J. Analysis of Tissue-Specific Expression of the *NtQPT1* Gene

The analysis of the tissue-specific expression of the NtQPT1 gene was part of a Ph.D. thesis (Song, W. 1997 "Molecular Characterization of Two Root-Specific Genes; *TobRB7* and *NtQPT1*." Department of Genetics, North Carolina State University) and has not been published otherwise.

In situ hybridizations to root sections demonstrated that *NtQPT1* is expressed in the root cortex (Fig. 2). A genomic fragment encoding the *NtQPT1* gene was isolated and approximately 5kb sequenced. The 5' and 3' untranslated sequences of the cDNA are identical to that of the genomic fragment, implying that this gene encodes the cDNA. Transcriptional fusions of the *NtQPT1* promoter sequence were fused to GUS and transformed into tobacco *via* *Agrobacterium*-mediated transformation. Histochemical staining of transformants (Fig. 3) revealed root cortex-specific expression in a pattern mimicking that observed by *in situ* analysis of *NtQPT1* transcripts (Fig. 2). These data demonstrate that the *NtQPT1* promoter contains all of the sequences necessary to direct transgene expression in the same tissues as the endogenous gene.

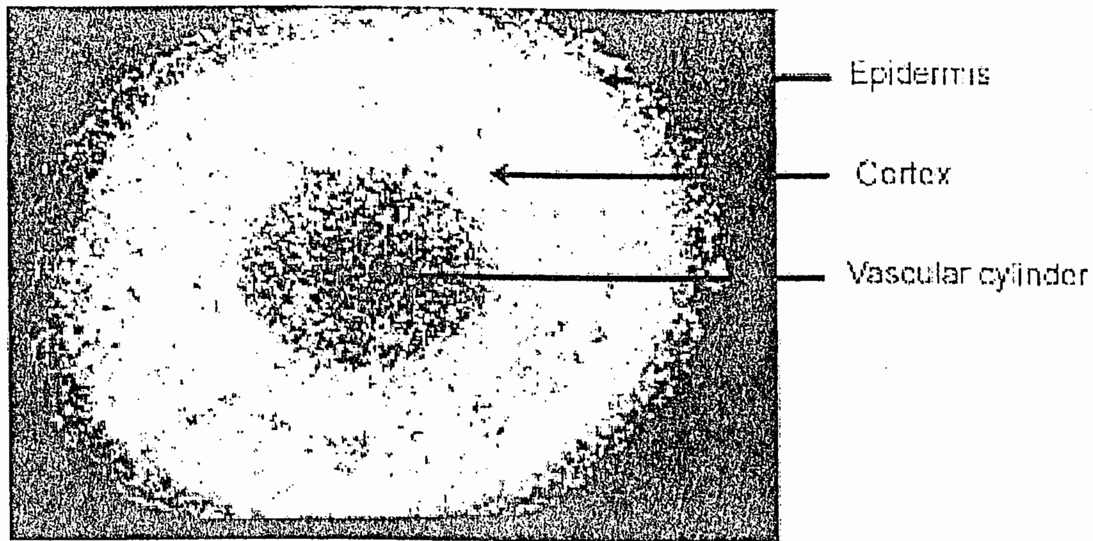


Figure 2: ***In situ*** **localization of** ***NtQPT1*** **mRNAin paraffin section of tobacco roots.** Dark-field image of a cross-section of a mature tbacco root probes with ^{32}P-labled "antisense" *NtQPT1* cRNA. Siver grains over regions containing *NtQPT1* mRNA appear white.

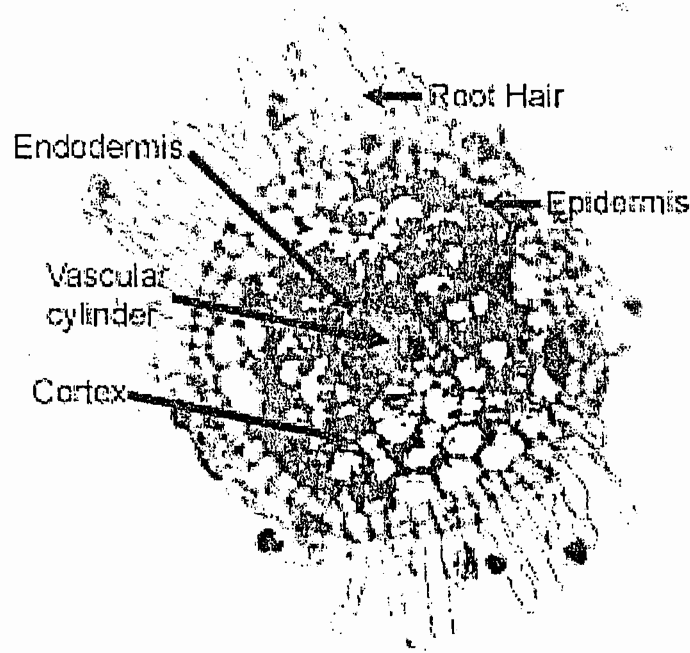


Figure 3: Histochemical localization of GUS activity in transgenic roots.

To characterize the *NtQPT1* promoter, a 5'-nested deletion series containing approximately 2.0, 1.5, 1.4, 1.25, 0.9, 0.8, 0.7, 0.6, 0.5, 0.3. 0.2 and 0.13 kb of 5' flanking sequence were transformed into tobacco and analyzed for GUS expression (Fig. 4). Promoters of 0.6 kb and greater direct root cortex-specific GUS expression at extremely high levels (about four-times that

of the CaMV 35S promoter). An apparent enhancer element resides between -600 bp and -500 bp. Promoters of 130 bp direct root cortex-specific expression at lower levels.

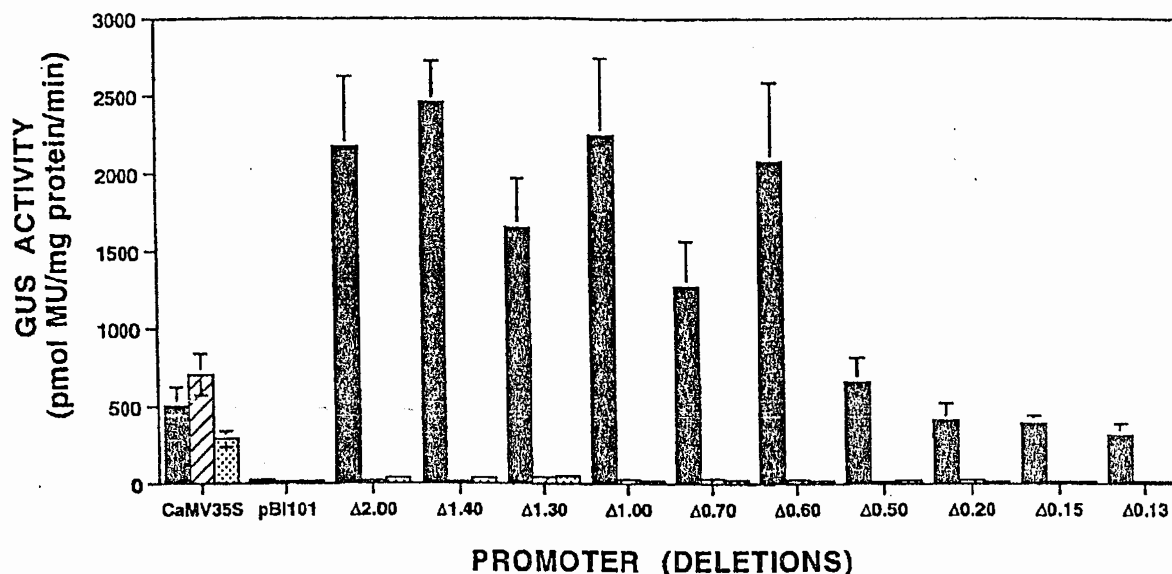


Figure 4: GUS activities in roots (solid bars), leaves (cross-hatched bars), and stems (stippled bars) of transgenic tobacco plants carrying the CaMV 35S promoter (CaMV 35S), the promoterless GUS (pBI101), and 5' nested deletions of the *NtQPT1* promoter (Δ2.00, Δ1.40, ...) fused to GUS. For each construct at least 20 independent transformants were assayed.

K. Functional Characterization of *NtQPT1*

The functional characterization of *NtQPT1,* its inferred amino acid sequence, and the mutant complementation analysis were part of a Ph.D. thesis (Song, W. 1997 "Molecular Characterization of Two Root-Specific Genes; *TobRB7* and *NtQPT1*." Department of Genetics, North Carolina State University) and have not been published otherwise.

Comparisons of the *NtQPT1* amino acid sequence with the GenBank database revealed limited similarity (15%) to a bacterial protein that encodes quinolinate phosphoribosyl transferase (QPTase). Quinolinate phosphoribosyl transferase is required for *de novo* NAD biosynthesis in both prokaryotes and eukaryotes. In tobacco, high levels of QPTase were detected in roots, but not in leaves. To determine unequivocally if *NtQPT1* encodes QPRTase, we sought to complement a bacterial strain (TH265) that is mutant in quinolinate phosphoribosyl transferase (*nadC⁻*). This mutant cannot grow on medium lacking nicotinic acid. However, expression of the NtQPT1 protein in this bacterial strain conferred the *NadC⁺* phenotype, confirming that *NtQPT1* encodes QPTase. Figure 5 illustrates the ability of *NtQPT1* to complement the *nadC⁻* mutation.

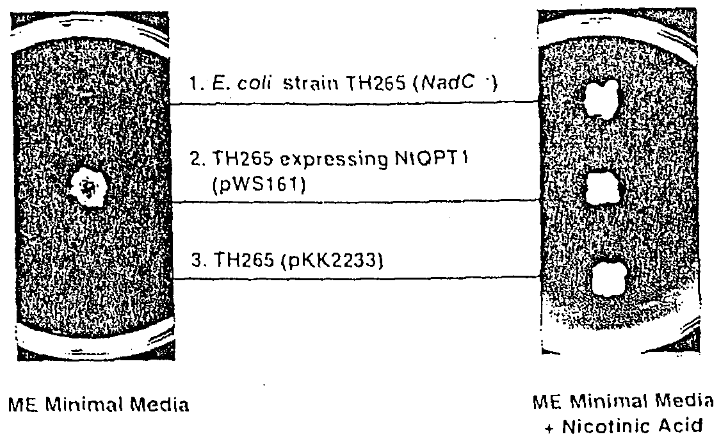


Figure 5: Complementation of *E. coli* strain TH266 (*nadC*⁻) by expression of the *NtQPT1* cDNA.
Competent TH265 cells were transformed by an expression vector carrying *NtQPT1* cDNA (pWS161) or the expression vector only (pKK2233) and the transformants, together with nontransformed TH265, were patched on minimal medium (ME) with or without nicotinic acid (0.0002%).

To determine if *NtQPT1* is a key regulatory gene in the nicotine biosynthetic pathway, we examined the effects of *Nic1* and *Nic2* mutants and of topping on *NtQPT1* steady-state mRNA levels. If *NtQPT1* is, in fact, involved in nicotine biosynthesis, we would predict that *NtQPT1* mRNA levels would be lower in $nic1^-/nic2^-$ double mutants. Total RNA was isolated from root tissue samples of wild-type ($Nic1^+/Nic2^+$), intermediate ($nic1^-/Nic2^+$ and $Nic1^+/nic2^-$), and low-nicotine ($nic1^-/nic2^-$) tobacco lines using Tri-Reagent (Molecular Research Center, Inc. Cincinnati, OH). Five micrograms of isolated RNA was fractionated by electrophoresis through a 1% agarose gel containing 1.1 M formaldehyde and transferred to a nylon membrane. Blots were probed with ^{32}P-labeled DNA fragments (labeled with a Random Primed DNA Labeling kit from Boehringer Mannheim Biochemical, Indianapolis, IN). The nylon membrane was incubated in hybridization solution [1% (w/v) BSA, 1 mM EDTA, 0.5 M Na-PO_4 (pH 7.2), 7% (w/v) SDS, 5 X 10^5 cpm/ml probe] at 65°C for 16 hours followed by two washes (0.1X SSC, 0.1% (w/v) SDS; 65°C; 30 minutes each, 1X SSC = 150 mM sodium chloride, 15 mM sodium citrate). Equal loading and transfer were checked by successively probing the membrane with a maize rDNA probe. Relative intensitites of the transcript levels were measured by densitometry and normalized against the rRNA intensities.

Nicotine levels of the near isogenic low nicotine tobacco varieties (Figure 6) are presented as the mean of relative levels obtained from several published reports (Legg and Collins, 1971; Saunders and Bush, 1979; Miller et al., 1982).

NtQPT1 mRNA transcript levels in $nic1^-/nic2^-$ double mutants were approximately 25% that of wild-type (Fig. 6). Further, there was a close correlation between nicotine levels and *NtQPT1* transcript levels in these near isogenic lines (Fig. 6).

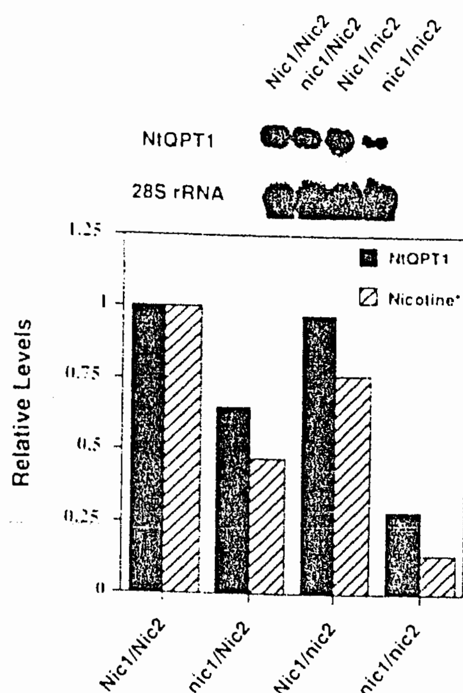


Figure 6: NtQPT1 transcript levels are regulated in the *nic* mutant lines. Relative levels of the *NtQPT1* transcript (solid bars) and nicotine content (cross-hatched bars) in wild-type (Nic1+/Nic2+) and the various low nicotine genotypes. Total RNA was isolated from the roots of hydroponically grown tobacco lines having the genotypes: **Nic1/Nic2:** (*Nic1/Nic1 Nic2/Nic2*), **nic1/Nic2.** (*nic1/nic1 Nic2/Nic2*), **Nic1/nic2.** (*Nic1/Nic1 nic2/nic2*), and **nic1/nic2.** (*nic1/nic1 nic2/nic2*).

To determine whether *NtQPT1* transcript levels responded to topping, tobacco plants were grown from seeds in soil for one month and transferred to pots containing sand. Plants were grown in a greenhouse until they began setting flowers (two to three additional months). Flower heads and the top two nodes from sixteen plants were then removed and at each time point (0, 1, 3, 6, and 24 hours) roots from four of the topped plants were harvested. For the control, roots from four that had not been topped were harvested at the appropriate time points. Total RNA was isolated from root tissue samples using Tri-Reagent (Molecular Research Center, Inc. Cincinnati, OH). Five micrograms of isolated RNA was fractionated by electrophoresis through a 1% agarose gel containing 1.1 M formaldehyde and transferred to a nylon membrane. Blots were probed with ^{32}P-labeled DNA fragments (labeled with a Random Primed DNA Labeling kit from Boehringer Mannheim Biochemical, Indianapolis, IN). The nylon membrane was incubated in hybridization solution [1% (w/v) BSA, 1 mM EDTA, 0.5 M Na-PO_4 (pH 7.2), 7% (w/v) SDS, 5 X 10^5 cpm/ml probe] at 65°C for 16 hours followed by two washes (0.1X SSC, 0.1% (w/v) SDS; 65°C; 30 minutes each, 1X SSC = 150 mM sodium chloride, 15 mM sodium citrate). Equal loading and transfer were checked by successively probing the membrane with a maize rDNA probe. Relative intensitites of the transcript levels were measured by densitometry and normalized against the rRNA intensities. The data demonstrate that *NtQPT1* transcript

levels resond to topping and that within six hours of topping the mRNA levels increased about eight-fold (Fig. 7).

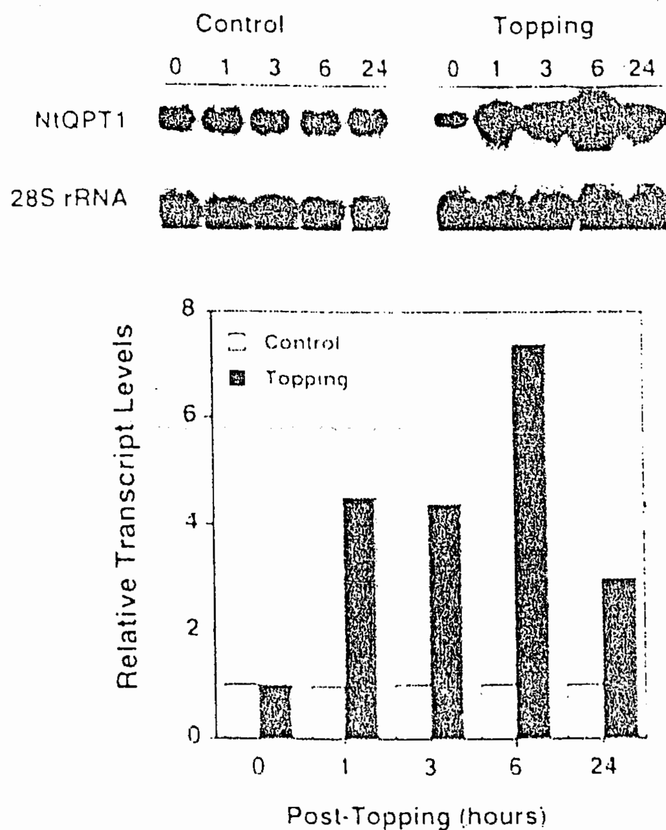


Figure 7: Relative *NtQPT1* transcript levels at various times after topping (solid bars). Transcript levels of control plants (not topped) are shown by open bars.

In summary, *NtQPT1* is expressed in tobacco roots (Fig. 3) and encodes quinolinate phosphoribosyl transferase (QPTase) (Fig. 5). Expression of *NtQPT1* is regulated by *Nic1* and *Nic2* (Fig. 6). Topping (removal of apical dominance) up-regulates expression of *NtQPT1* (Fig. 7). Nicotine is synthesized in tobacco roots. QPTase is a key regulatory enzyme in nicotine biosynthesis (Fig. 1). Saunders and Bush (1979) measured QPTase activities and nicotine levels in low nicotine tobacco lines (*Nic1* and *Nic2* mutants) and observed a strict correlation of QPTase activity and nicotine content. Nicotine biosynthesis is stimulated by topping. Therefore, *NtQPT1* is a key regulatory gene in the nicotine biosynthetic pathway.

III. DESCRIPTION OF THE TRANSFORMATION SYSTEM

The vector system, pBin 19, (Bevan, 1984) used to transform genes into tobacco is a binary system based on the Ti plasmid of *Agrobacterium tumefaciens*. pBin19 has been sequenced and is in the GenBank database, accession number U09365. Although some of the DNA sequences used in the transformation process were derived from the plant pathogen *A. tumefaciens*, the genes causing crown gall disease were first removed, and therefore, the recipient plant does not have crown gall disease. Figure 8 illustrates the plasmid vector, pYTY32 [pBin19 carrying the *NtQPT1* antisense expression cassette]. Table 1 lists the genetic elements present in pYTY32 and their origins. pYTY32 was transformed into the disarmed *Agrobacterium tumefaciens* strain LBA4404 (Hoekema, et al. 1983) via electroporation. [LBA4404 carries a Ti-plasmid that has had the T-DNA region completely removed. It provides the *vir* gene products in trans for plant transformation.] Tobacco leaf disks were transformed and transgenic plants regenerated according to the procedure of An and co-workers (1986). Transformed plant cells, callus, seedlings, and plantlets were selected on MS medium containing kanamycin at 300 µg/ml.

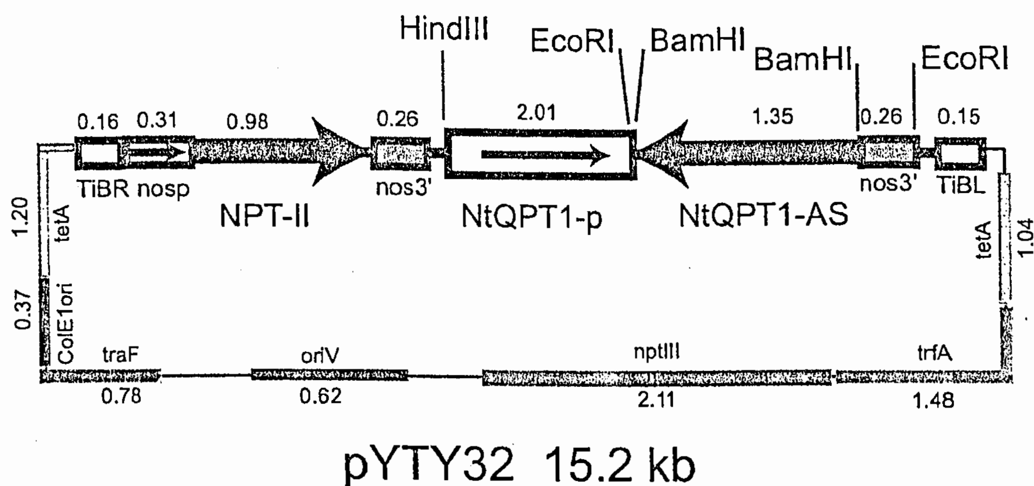


Figure 8: The plasmid pYTY32.
pYTY32 was produced by cloning the *NtQPT1* antisense expression cassette (the *NtQPT1* promoter driving expression of antisense trasncripts of the *NtQPT1* cDNA terminated by the *nos* terminator) into pBin19. The genetics elements of pBin19 were obtained from its sequence (Accession # U09365). Sizes, in kb, of the genetic elements are shown. The direction of the sense orientation of structural genes within the T-DNA is shown.

Table 1. Genetic elements in pYTY32:

T-DNA SEQUENCES

Genetic element	Origin	Size (kb)	Function
T-Border-Right (TiBR)	*A. tumefaciens*	0.16	Right border of T-DNA
Nos Promoter (nosp)	*A. tumefaciens*	0.31	Transcribe the npt-II gene
Nos Terminator (nos3')	*A. tumefaciens*	0.26	Terminate transcription
Neomycin phosphotransferase (npt-II)	*E. coli* Tn5	0.98	Kanamycin resistance/ Plant selectable marker
NtQPT1 Promoter (NtQPT1-p)	*N. tabacum*	2.01	Transcribe NtQPT1 antisense
NtQPT1 cDNA Antisense (NtQPT1-AS)	*N. tabacum*	1.35	Supress QPTase expression
T-Border-Left (TiBL)	*A. tumefaciens*	0.15	Left border of T-DNA

pBin19 SEQUENCES OUTSIDE OF T-DNA.

Genetic element	Origin	Size (kb)	Function
tetA	*P. aeruginosa*	2.24	None. T-DNA disrupts gene
trfA	*E. coli* pRK2	1.48	Plasmid transfer/conjugation
nptIII	*S. faecalis*	2.11	Kanamycin resistance/ Bacteria selectable marker
oriV	*E. coli* pRK2	0.62	Wide host range origin of replication
traF	*E. coli* pRP4	0.78	Plasmid transfer/conjugation
ColE1 Ori	*E. coli* pcolE1	0.37	Origin of replication

IV. DONOR GENES AND REGULATORY SEQUENCES

A. Quinolinic acid PhosphoribosylTransferase (*NtQPT1*) Antisense Gene

As described in **Sections B.9.** and **B.10.**, *NtQPT1* is a tobacco gene expressed specifically in the cortex cells of the root. *NtQPT1* encodes QPTase and its expression is up-regulated and down-regulated by endogenous tobacco genes, phytohormone treatments, topping and wounding

in a fashion that parallels the synthesis of nicotine. The 2.0 kb *NtQPT1* promoter will direct transgene expression at high levels (Figure 4) in the same tissues as the endogenous *NtQPT1* gene (Fig. 2, 3, and 4).

In order to reduce expression of the endogenous *NtQPT1* gene, an antisense strategy was used. Antisense RNA is complementary to the mRNA transcribed by the endogenous gene. By a molecular mechanism that is not yet understood, expression of an antisense transcript in the same cell expressing sense transcripts of the endogenous gene can down-regulate the production of the gene product encoded by the endogenous gene. Antisense suppression of gene expression requires a high degree of sequence complementarity between the antisense and sense transcripts. In order to reduce the expression of QPTase (and therefore, the levels of nicotine), the full-length *NtQPT1* cDNA was cloned in the antisense orientation behind the 2.0 kb *NtQPT1* promoter. The antisense transcript was terminated by the *nos* termination/polyadenylation sequences described earlier. This cassette (*NtQPT1* promoter – *NtQPT1* cDNA antisense – *nos* terminator) was cloned into pBin19.

B. The Selectable Marker Gene: Neomycin Phosphotransferase

In addition to the *NtQPT1* antisense gene, the *nptII* gene from the *E. coli* transposon Tn5 has been introduced into transgenic tobacco for use as a selectable marker. The gene encodes neomycin phosphotransferase that confers resistance to the common aminoglycoside antibiotic kanamycin. The DNA sequence has been determined (Beck, et al. 1982). Expression of the *nptII* gene is under the control of the *nos* promoter and the *nos* terminator.

The *nptII* gene was the first selectable marker for plant transformation to be approved for human consumption (U.S. FDA, 1994). The biosafety of the use of kanamycin-resistance as a selectable marker in transgenic plants has been discussed extensively (Nap, et al., 1992; Redenbaugh, et al. 1994). The FDA evaluated the potential for horizontal transfer of the *npt-II* gene to soil or intestinal (human and animal) microorganisms. They concluded that the probability of gene transfer was substantially less than the occurrence of kanamycin-resistance arising by mutation (U.S. FDA 1998). Similarly, horizontal gene transfer to the epithelial cells lining the intestinal lumin was not observed (Hoskins, 1978; U.S. FDA 1998). The *npt-II* gene product has no significant homology with proteins listed as food allergens or toxins and is instable to proteolytic enzymes, heat, and stomach acid conditions. For these reasons, FDA had no allergenicity or toxicity concerns (U.S. FDA 1998).

Neither the *NtQPT1* antisense gene nor the *nptII* selectable marker has any inherent plant pest characteristics, and neither poses a risk to plant health when introduced into transgenic plants.

C. Other Sequences

pYTY 32 is a binary Agrobacterium transformation plasmid based on pBin19. Because it utilizes the Agrobacterium *vir* gene products to transfer DNA from the bacterium to the plant genome, the right and left T-DNA border sequences are included. The complete nucleotide sequence of the T-DNA region of pBIN19 (3212 bp) is given in GenBank Accession number U12540.

V. GENETIC ANALYSIS AND AGRONOMIC PERFORMANCE

A. Transformation, Screening and Production of Homozygous Vector 21-41 Seeds

The low-nicotine tobacco variety, **Vector 21-41**, was developed by transforming Burley 21 LA with the binary Agrobacterium vector, pYTY32. pYTY32 carries the 2.0 kb *NtQPT1* root-cortex specific promoter which drives the antisense expression of the *NtQPT1* cDNA terminated by the nopaline synthase terminator (*nos* 3)' from *Agrobacterium tumefaciens* T-DNA. The selectable marker for this construct was neomycin phosphotransferase (*nptII*) from *E. coli* Tn5 whose expression is directed by the *nos* promoter from *Agrobacterium tumefaciens* T-DNA. *nptII* confers resistance to kanamycin. Transformed cells, tissues, and seedlings were selected by their ability to grow on MS medium containing 300 μg/ml kanamycin.

pYTY32 was transformed into the disarmed *Agrobacterium* strain LBA4404 via electroporation. Plasmid DNA isolated from the transformed Agrobacterium was digested with diagnostic restriction endonucleases and compared with the plasmid DNA used to transform the *Agrobacterium*. The restriction patterns were identical, suggesting that no gross rearrangements of the plasmid had occurred. Axinic leaf disks of Burley 21 LA were transformed with *Agrobacteium* carrying pYTY32 according to An *et al.* (1986). Transformed callus and then shoots were selected on MS medium containing 300 μg/ml kanamycin. Shoots were rooted on MS medium containing 300 μg/ml kanamycin.

One hundred independent pYTY32 transformants of Burley 21 LA (T_0) were allowed to self. Progeny of the selfed-plants (T_1) were germinated on medium containing kanamycin and the segregation of kanamycin resistance scored. One hundred to 200 seeds were surface sterilized and allowed to germinate on MS medium containing 300 μg/ml kanamycin. Cotyledons of kanamycin-resistant progeny (containing the transgene) are green while those of kanamycin-senstive progeny (lacking the transgene) are yellow. Progeny of 67 of the 100 primary transformants segregated 3:1 for kanamycin resistance (a single locus). The remaining 33 progeny segregated at ratios of 15:1 or higher (two or more loci). T_1 progeny segregating 3:1 (resulting from transformation at a single locus) were subjected to further analysis. Nicotine levels of T_1 progeny segregating 3:1 were measured qualitatively using a micro-assay. T_1 progeny of transformant # 41 exhibited the lowest nicotine levels and were further characterized. Homozygous progeny of transformant #41 and their descendants were named **"Vector 21-41"**.

One hundred ninety three T_1 progeny of tranformant #41 were surface sterilized and germinated on MS medium containing 300 μg/ml kanamycin. Segregation of the kanamycin-resistant phenotype was assayed (Table 2).

Table 2: T_0 Segregation analysis of transformant # 41.

Class	Observed (*o*)	Expected (*e*) (3:1)	Deviation (*d*)	d^2	d^2/e
Green (kan^r)	148	145	+3	9	0.06
Yellow (kan^s)	45	48	-3	9	0.19

$$\chi^2 = \Sigma d^2/e = 0.25$$

The analysis is consistent with the hypothesis that the transgene conferring kanamycin-resistance in transformant #41 segregates 3:1 and, thus, is a single locus.

Twelve T_1 progeny ($T_1$1-12) of transformant #41 were germinated on soil and allowed to produce seed. T_2 seeds were surface sterilized and plated on MS medium containing kanamycin. Table 3 illustrates the results of this analysis.

Table 3: Segregation analysis of T_1 generation of transformant #41.

F1 plant #	kan^r	kan^s	χ^2	Interpretation
T_1-1	184	0		Homozygous
T_1-2	197	23	24.8	Heterozygous
T_1-3	157	0		Homozygous
T_1-4	175	38	5.6	Heterozygous
T_1-5	0	257		Nontransgenic
T_1-6	158	57	0.22	Heterozygous
T_1-7	0	137		Nontransgenic
T_1-8	121	55	3.65	Heterozygous
T_1-9	219	53	4.34	Heterozygous
T_1-10	0	243		Nontransgenic
T_1-11	87	40	2.67	Heterozygous
T_1-12	150	58	0.92	Heterozygous

These data are consistent with the trangene segregating as a single locus. Plants T_1-1 and T_1-3 appear to be homozygous for the transgene. Plants T_1-2, T_1-4, T_1-6, T_1-8, T_1-9, T_1-10, and T_1-11 are heterozygous for the transgene. Plants T_1-5, T_1-7, and T_1-10 do not carry the transgene and are nontransgenic.

Nicotine levels in several generations of progeny of transformant #41 were quantified at the Southern Research and Testing Laboratories in Wilson, NC using gas chromatography/flame ionization detection. Table 4 summarizes these results.

Table 4: Nicotine levels in generations of Vector 21-41 tobacco.

Generation	Where Grown	Topped	Leaf Position Number	Zygosity	Nicotine (ppm)
T_1-1	Greenhouse	No	3	Homozygous	70
T_1-2	Greenhouse	No	3	Heterozygous	180
T_1-3	Greenhouse	No	3	Homozygous	100
T_1-4	Greenhouse	No	3	Heterozygous	200
T_1-5	Greenhouse	No	3	Nontransgenic	8070
T_1-6	Greenhouse	No	3	Heterozygous	180
T_1-7	Greenhouse	No	3	Nontransgenic	8020
T_1-8	Greenhouse	No	3	Heterozygous	250
T_1-9	Greenhouse	No	3	Heterozygous	170
T_1-10	Greenhouse	No	3	Nontransgenic	9000
T_1-11	Greenhouse	No	3	Heterozygous	220
T_1-12	Greenhouse	No	3	Heterozygous	190
T_2-1	Greenhouse	No	3	Homozygous	100
T_2-3	Greenhouse	No	3	Homozygous	120
T_3	Greenhouse	No	3	Homozgous	190
T_4	Greenhouse	No	3	Homozygous	190
T_4	Field (NC)	Yes	Whole Plt.	Homozygous	1440
T_4	Field (Arg)	Yes	WholePlt.	Homozygous	700
T_4	Field (Arg)	Yes	Whole Plt.	Homozygous	1000
T_4	Field (LA)	Yes	1-8	Homozygous	1490

The data presented in Tables 2, 3, and 4 are consistent with the hypothesis that the transgene conferring kanamycin-resistance is a single locus and with the hypothesis that the transgene conferring lower nicotine levels co-segregates with kanamycin-resistance.

B. Genomic DNA Gel Blot (Southern) Analysis of Vector 21-41

The identity of the genetic material that was integrated into the genome of Vector 21-41 was analyzed by DNA gel blot analysis. Because tobacco contains two endogenous *NtQPT* genes (tobacco is an amphidiploid species), probes to the *NtQPT1* antisense transgene were not used. Instead a radionucleotide-labeled probe of the 800 bp *npt-II* gene was used. Genomic DNA (20ug) was digested with *Hind*III, *Eco*RI, and *Bam*HI restriction enzymes that cut within the T-DNA (see Figure 8), size-fractionated by electrophoresis on an 0.8% agarose gel, and transferred to a nitrocellulose filter according to Sambrook et al. (1989). The filter was probed with ^{32}P labeled DNA from the *npt*-II gene. Figure 9 demonstrates that for each enzyme, two DNA fragments hybridized. These bands result from the restriction site within the T-DNA and a restriction site in the flanking genomic sequence. This result demonstrates that there are two transgenic inserts at the locus segregating for the transgene.

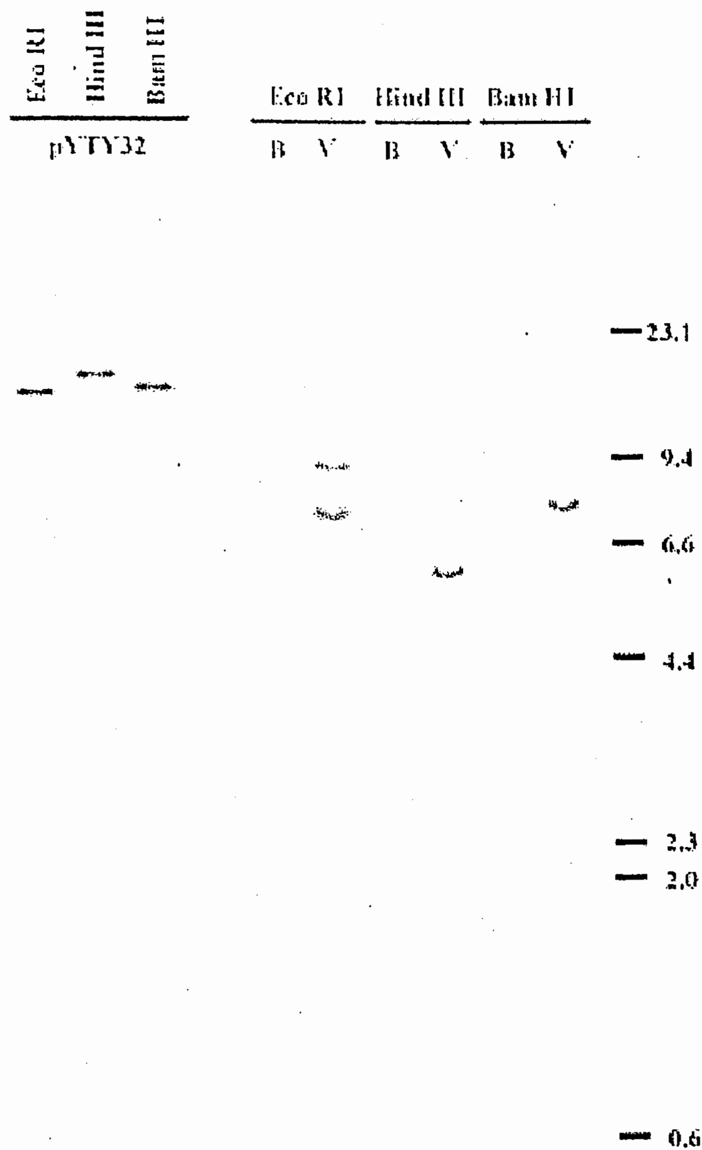
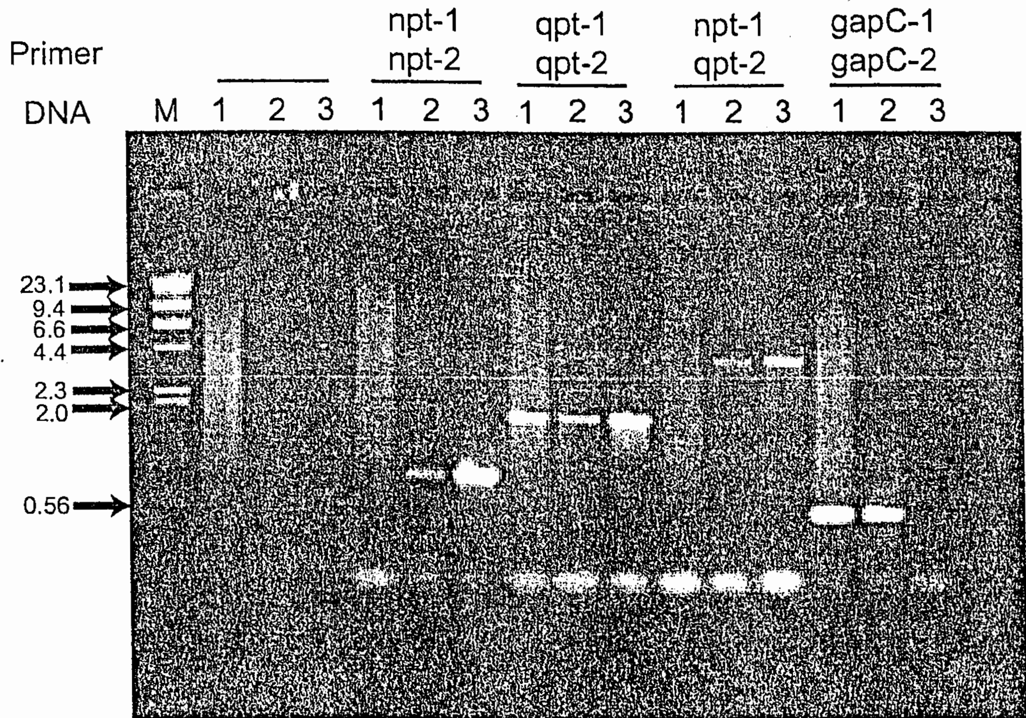
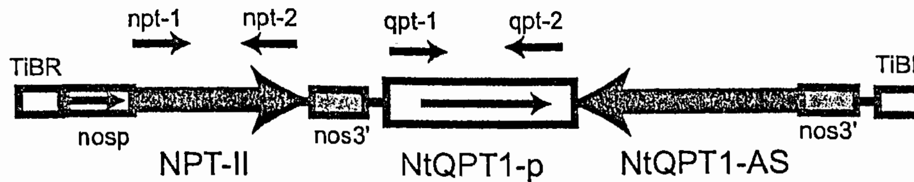


Figure 9: Genomic DNA blot of Vector 21-41.
15 μg of genomic DNA isolated from leaves of Vector 21-41 (V) and untransformed Burley 21 LA (B) was digested to completion with *Eco*RI, *Hin*dIII, and *Bam*HI, fractionated on an 0.8% agarose gel, blotted to a nylon membrane, and hybridized with the *npt*-II gene (0.98 kb) that had been labeled with ^{32}P. 108 pg of pYTY32 plasmid DNA digested with *Eco*RI, *Hin*dIII, and *Bam*HI was included as a single copy control.

To determine if the transgenes had rearranged during the transformation process, PCR primers were designed and used to amplify genomic DNA from untransformed Burley 21 LA, Vector 21-41, and the pYTY32 plasmid. Figures 10 and 11 illustrates the results of these analyses.

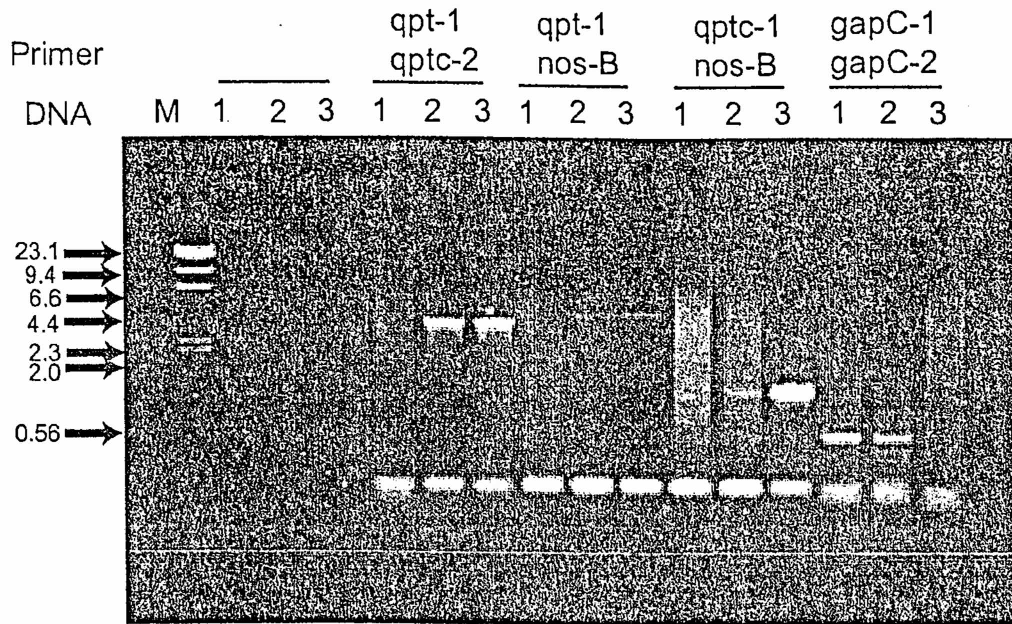


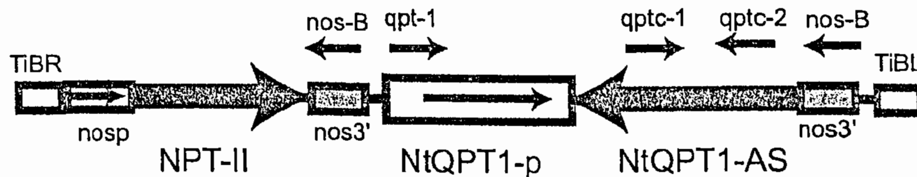


DNA's
M Marker - Lambda digested with HindIII
1 Untransformed Burley 21 LA
2 Vector 21-41
3 pYTY 32

Figure 10: PCR analysis of the transgene in Vector 21-41.
Genomic DNA (100 ng) from untransformed Burley 21 LA (1), Vector 21-41 (2), and pYTY32 (100 pg) (3) were used as templates for PCR amplifications (30 cycles) using the primers shown.

The data in Figure 10 show that untransformed Burley 21 LA does not contain an *npt*-II gene (Primers npt-1 and npt-2) and that the *npt*-II amplification product of Vector 21-41 and pYTY32 are the same size, *i.e.*, no rearrangement. Amplification of the DNA with primers within the *NtQPT1* promoter (qpt-1 and qpt-2) give the same size bands, demonstrating that the *NtQPT1* promoter is present in all DNA's and that the NtQPT1 promoter clone had not rearranged from the promoter in the tobacco genome. Amplification of the DNA with primers npt-1 and qpt-2 demonstrate that untransformed Burley 21-41 does not have the this genetic element and that the amplification products of Vector 21-41 and pYTY32 are the same size, *i.e.*, no rearrangements. The gapC primers are PCR primers directed against the endogenous glyceraldehyde phosphate dehydrogenase gene (GapC) and were included as negative controls (for pYTY32) and positive controls (for untransformed Burley 21 LA) DNA's.





DNA's
- M Marker - Lambda digested with HindIII
- 1 Untransformed Burley 21 LA
- 2 Vector 21-41
- 3 pYTY 32

Figure 11: PCR analysis of the transgene in Vector 21-41.
Genomic DNA (100 ng) from untransformed Burley 21 LA (1), Vector 21-41 (2), and pYTY32 (100 pg) (3) were used as templates for PCR amplifications (30 cycles) using the primers shown.

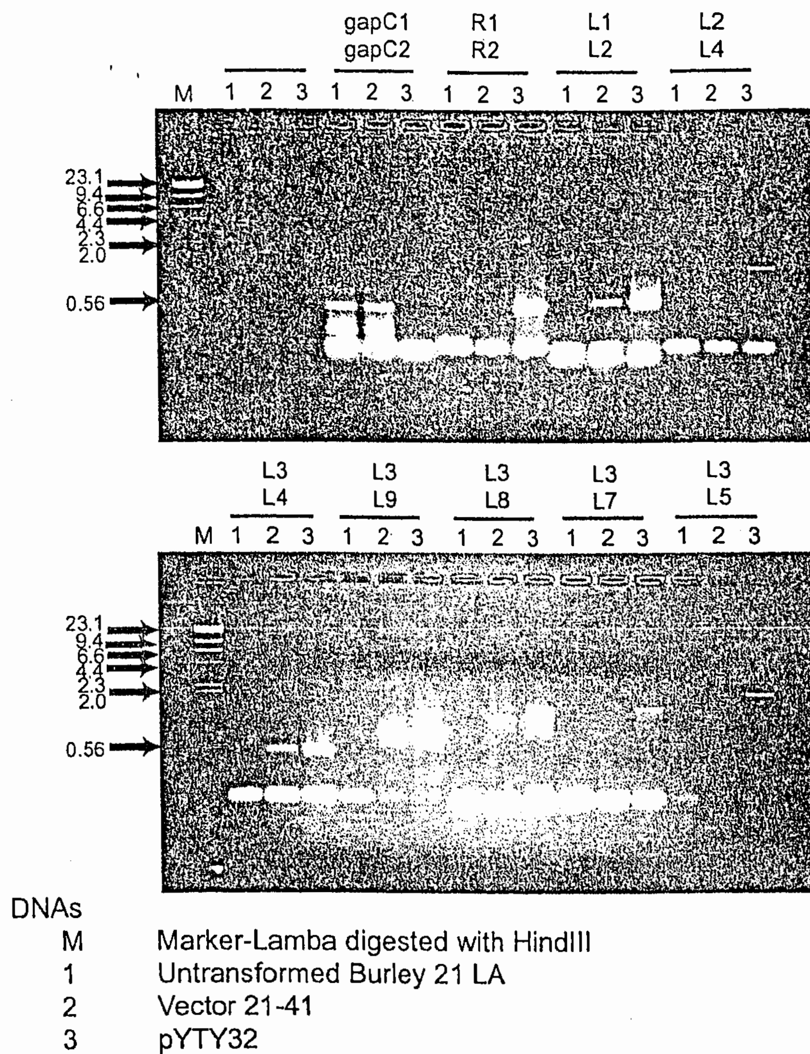
Figure 11 analyzes the *NtQPT1* antisense construct. Amplifications of the DNA's with the primers qpt-1 and qptc-2 demonstrate that the *NtQPT1* promoter is adjacent to, and in the proper orientation with, the antisense *NtQPT1* cDNA. The amplification products of Vector 21-41 and pYTY32 DNA's are the same size, *i.e.*, no rearrangement. Amplification of the DNA's with the primers qpt-1 and nos-B illustrates that the *NtQPT1* prmoter is adjacent to, and in the proper orientation with, the *nos* terminator. The amplification products of Vector 21-41 and pYTY32

DNA's are the same size, *i.e.*, no rearrangement. Amplification of the DNA's with the primers qptc-1 and nos-B illustrates that the *NtQPT1* antisense cDNA is adjacent to, and in the proper orientation with, the *nos* terminator. The amplification products of Vector 21-41 and pYTY32 DNA's are the same size, *i.e.*, no rearrangement. Untransformed Burley 21-41 DNA does not amplify with any of these primer sets. Because these primer sets are specific for the pYTY32 transgene (unlike npt-1 and npt-2 primers), these primers would provide diagnostic products for the potential release of the *NtQPT1* antisense transgene. The gapC primers are PCR primers directed against the endogenous glyceraldehyde phosphate dehydrogenase gene (GapC) and were included as negative controls (for pYTY32) and positive controls (for untransformed Burley 21 LA) DNA's.

Taken together, the data in Figures 10 and 11 demonstrate that no major rearrangements of the *npt*-II and *NtQPT1* antisense transgenes occurred during transformation/regeneration of Vector 21-41.

C. Analysis of Transfer of pYTY32 DNA Sequences Flanking the T-DNA into Vector 21-41.

The possible transfer of pYTY32 DNA sequences that flank the T-DNA into Vector 21-41 was assayed by PCR analysis (Figure 12). PCR primers were designed that would amplify an approximately 600 bp DNA fragment immediately adjacent to either the T-DNA right border (primers R1 and R2) or the T-DNA left border (primers L1 and L2). Genomic DNA (100 ng) from untransformed Burley 21 LA and Vector 21-41 and from the plasmid pYTY32 (100 ng) were used as templates for PCR amplifications using the primer sets (primers R1 and R2 and primers L1 and L2). No amplification products were observed when genomic DNA from Burley 21 LA or Vector 21-41 served as templates for primers R1 and R2 (pYTY32 template amplified the predicted 600 bp product). This indicates that pYTY32 sequences adjacent to the T-DNA right border were not transferred into Vector 21-41. However, a amplification product of approximately 600 bp was observed when Vector 21-41 genomic DNA was amplified using primers L1 and L2. This amplification product was the same size as the product obtained from pYTY32. To determine if the entire 3' half of the tetA gene [The T-DNA of pBin19 disrupts the tetA gene into a 5' half of approximately 1.2 kb and a 3' half of approximately 1.0 kb.] a primer, L4, was designed. When amplified using primers L2 and L4, the predicted 1.3 kb amplification product was observed. In order to determine the limits of the flanking sequences that were transferred, a primer, L3, was designed that primed in the region between L2 and L4. This primer was used in combination with a series of primers (L9, L8, L7, and L5). Figure 12 demonstrates that amplification products are observed in Vector 21-41 for the primer sets L3/L9 and L3/L8, but not for L3/L7 and L3/L5. [When pYTY32 DNA is amplified, the predicted amplification products are observed for all primer sets.] Therefore, somewhere between 2.1 kb and 2.4 kb of pYTY32 sequences flanking the T-DNA left border are transferred in Vector 21-41. This would transfer the 3' half of the *tetA* gene and about one half of the *trfA* gene. Thus, although sequences outside of the pYTY32 T-DNA are transferred to Vector 21-41, these sequences do not include the complete coding regions of any genes.



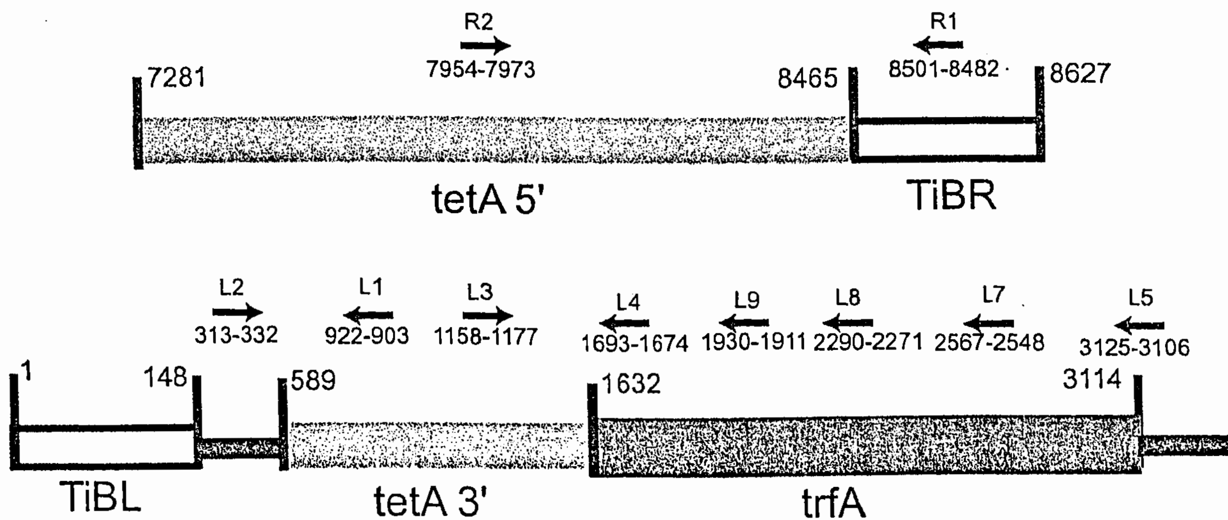


Figure 12: PCR analysis of the transgene in Vector 21-41.
Genomic DNA (100 ng) from untransformed Burley 21 LA (1), Vector 21-41 (2), and pYTY32 (100 pg) (3) were used as templates for PCR amplifications (30 cycles) using the primers shown.

D. The Arrangement of Transgenes within Vector 21-41.

Genetic evidence presented in Tables 2, 3, and 4 suggest that the trangene conferring kanamycin resistance segregates as a single locus. Quantitation of nicotine levels among T_1 progeny of transformant #41 suggests that the low nicotine phenotype co-segregates with the kanamycin-resistance phenotype (Table 4). DNA blot data (Fig. 9) demonstrate that Vector 21-41 contains two copies of the transgene. Analysis for rearrangement of the transgenes present in Vector 21-41 demonstrate that no rearrangments of the T-DNA had occurred (Fig. 10 and 11). Finally, approximately 2.1 to 2.4 kb of pYTY32 DNA sequences flanking the left T-DNA border were transferred to Vector 21-41 (Fig. 12). An interpretation consistent to all these observations is presented in Figure 13. Briefly, Vector 21-41 contains two T-DNA inserts arranged as tandem, inverted repeats with the *npt*-II genes oriented towards the flanking genomic DNA. The 2.1 to 2.4 kb of DNA sequences of pYTY32 that flank the T-DNA would be positioned between these inverted repeats. To test this model, a PCR primer (TBL-1) at the insertion site of the left border that would prime was designed. This primer would prime at the TiB-L border adjacent to the tetA sequence. The qptc-1 primer (see Figure 11) primes DNA synthesis towards the left border from within the *NtQPT1* antisense gene. If the two T-DNA inserts are arranged as inverse tandem repeats within the Vector 21-41 genome, an amplification product is predicted when these two primers are used in a PCR reaction. Figure 13 presents the results of such an amplification.

Taken together, genetic and molecular evidence is consistent with the hypothesis that Vector 21-41 contains two copies of the T-DNA region of pYTY32. These copies are arranged as inverted, tandem repeats. Approximately 2.1 kb of pBin19 sequences (that carry the 3' half of the *tetA* gene and about one half of the *trfA* gene) is located between the inverted T-DNA repeats (see Figure 13).

Figure 13: Amplification between the two tandem inverted T-DNA repeats: PCR primers TBL-1 and qptc-1 were used to amplify geneomic DNA (100 ng) isolated from untransformed Burley 21 LA (1) and Vector 21-41 (2).

E. Expression of the Inserted Genes

NtQPT1 antisense

Antisense transcripts of the *NtQPT1* gene are not expected to direct the synthesis of a polypeptide. Expression of the antisense *NtQPT1* transcript is directed by the *NtQPT1* promoter. The *NtQPT1* promoter directs transgene expression in the same tissues (the root cortex) as the endogenous *NtQPT1* gene (Fig. 2 and 3). Because double-stranded RNA molecules are preferentially degraded, we would anticipate that the RNA levels from both the antisense *NtQPT1* transgene and the endogenous *NtQPT1* gene would be degraded. This would be reflected by lowered levels of QPTase activity and lower nicotine levels. The nicotine levels of Vector 21-41 are more than ten-fold lower than its untransformed parent (Table 2). Because the transgenic antisense *NtQPT1* RNA is expressed in the same tissues as the endogenous *NtQPT1* gene and are degraded, we could not measure antisense expression levels.

NPTII

NPTII expression levels were measured in leaves, roots, and stems using a NPTII ELISA kit (Agdia, Elkhart, IN). Four leaves, the stem, and the roots were sampled from ten T_4 Vector 21-41 plants at the 6-leaf stage.and NPTII levels quantified by ELISA. NPTII levels in leaves averaged 2.83 ± 0.81 ng/mg protein, in stems averaged 3.79 ± 1.31 ng/mg protein, and in roots averaged 2.02 ± 0.54 ng/mg protein.

F. Characteristics of Low-Nicotine Tobacco, Vector 21-41

The agronomic performance of Vector 21-41 was determined in a field test conducted at the NCDA Central Crops Research Station (Clayton, NC) (Field Test Report: No-Nic-FT1 99-180-05n). T_4 progeny homozygous for the transgene were compared to the Burley 21 LA parent and a control transformant. The design was three treatments (Vector 21-41, a Burley 21 LA transformed line carrying only the *NtQPT1* promoter [Promoter-Control], and untransformed Burley 21 LA [Wild-type]), 15 replicates, 10 plants per replicate. During the test, Orthene was applied two times at a rate of 1 lb/acre to control insects. This is the application rate used on all tobacco field tests at the NCDA Central Crop Research Station during the 2000 season. No difference was observed in insect damage of the low nicotine line, Vector 21-41, and the Promoter Control and Wild-Type lines following this insect management protocol. The flower buds and top two nodes of flowering tobacco plants were removed (topping) prior to the flower opening (about 60 days after transplant). Suckers were controlled by MH treatment. Approximately 20 days after topping, the field was harvested. The field was harvested by leaf priming (lower third, middle third, and top third). Leaves were air cured and weighed. Samples for chemical analysis were reconstituted from cured leaf by adding appropriate quantities of the three primings. A variety of agronomic and chemical traits (listed below) were assayed during the trial. The data are summarized in Table 5.

Table 5.

	TREATMENT		
	Vector 21-41	Promoter Control	Wild-type
Days from transplant until flowering (days)	57.1 ± 3.6*	56.7 ± 3.4*	57.6 ± 3.4*
Height at flowering (cm)	118.6 ± 20.1*	112.1 ± 21.4*	110.8 ± 19.5*
Leaf number at flowering	22.8 ±1.4*	22.6 ± 4.9*	23.4 ± 1.9*
Leaf size (Length) (cm)			
5th leaf	37.1 ± 4.7*	39.4 ± 4.0*	38.3 ± 4.2*
10th leaf	35.6 ± 3.6*	38.3 ± 4.3*	36.8 ± 3.3*
15th leaf	30.7 ± 3.5*	31.7 ± 2.5*	32.8 ± 4.3*
Leaf size (Width) (cm)			
5th leaf	15.6 ± 1.8*	16.0 ± 2.7*	17.0 ± 2.9*
10th leaf	14.7 ± 2.3*	15.8 ± 1.8*	15.6 ± 1.6*
15th leaf	13.7 ± 2.5*	13.4 ± 2.0*	13.2 ± 2.3*
Internodes (mm)	66.0 ± 13.4*	57.7 ± 12.5*	54.6 ± 5.3*
Yield (kg/ha)	890.3 ± 70.7*	780 ± 68.5*	809.2 ± 71.2*
Nicotine (ppm)	1440 ± 660**	19,120 ± 8990*	21,540 ± 9340*
Nor-Nicotine (ppm)	400±100**	1560 ± 220*	1270 ± 520*
Total Alkaloids (ppm)	2300 ± 700**	20,700 ± 9300*	23,100 ± 940*
% Total Nitrogen	2.53 ± 0.78*	2.96 ± 0.42*	2.64 ± 0.91*
% Reducing Sugars	10.29 ± 0.89**	5.87 ± 2.04*	5.51 ± 2.40*

Data from 2000 field trial at Central Crops Research Station, Clayton, NC.
15 replicates/10 plants per replicate. Data were analyzed using the F-test.
*= No significant difference, ** = Significant at the 1% level.

The data presented in the Field Report (99-180-05n) contained a 10-fold less calculation error for the % nicotine and the % nor-nicotine. This error has been corrected in Table 5.

For all traits measured there was no significant difference for the Promoter Control and Wild-Type lines. The low nicotine, transgenic line, Vector 21-41, exhibited significantly lower nicotine, nor-nicotine, and total alkaloid percentages than the Promoter Control and Wild-Type lines. Reducing sugars in the Vector 21-41 line were about two-fold higher than the Promoter Control and Wild-Type lines.

Cigarettes were produced using the Vector 21-41 tobacco from the 2000 field trial. Labstat International, Inc. quantified Tobacco Specific NitrosAmine (TSNA) levels in mainstream smoke and compared them to TSNA levels of a standard, full-flavor cigarette. Table 6 presents those data.

Table 6:

Yields of Tobacco Specific Nitrosamines
in Mainstream Tobacco Smoke:
'Standard' Conditions

Brand No.	Wt. Of Cig. [mg/cig]	Puff Count (/cig)	TPM [mg/cig]	NNN [ng/cig]	NAT [ng/cig]	NAB [ng/cig]	NNK [ng/cig]
V-21	892	7.7	17.6	29.3	13.2	2.93	17.7
V-21	896	7.6	15.9	27.4	12.3	2.78	18.7
V-21	863	7.0	16.1	31.9	12.4	2.75	16.7
V-21	896	7.6	15.9	25.8	10.5	3.15	18.2
V-21	881	7.3	15.0	28.5	9.04	2.51	14.3
Average	885	7.4	16.1	28.5	NQ	NQ	NQ
Std Dev	14.1	0.3	0.9	2.3	NQ	NQ	NQ
Coeff Var	1.6	3.8	5.9	8.0	NA	NA	NA
Std	1024	7.5	21.5	435	(590)*	103	348
Std	1023	7.6	20.8	424	531	100	349
Std	1040	7.9	23.0	441	517	98.3	351
Std	1018	7.4	20.2	410	526	92.8	355
Std	1035	7.3	18.7	449	501	114	355
Average	1028	7.5	20.9	432	519	102	352
Std Dev	9.3	0.2	1.6	15.4	13.1	7.9	3.3
Coeff Var	0.9	2.7	7.6	3.6	2.5	7.8	1.0

( )* DENOTES AN OUTLIER

Note: A result of BDL (Below Method Detection Limit) indicates that no analyte was detected.
A result of NQ indicates that the analyte was detected, but was below the practical limit of quantitation (LOQ).

NA = Not Applicable

Method Detection Limits

	Wt.of Cig. (mg/cig)	Puff Count (/cig)	TPM [mg/cig]	NNN [ng/cig]	NAT [ng/cig]	NAB [ng/cig]	NNK [ng/cig]
MDL	NA	NA	NA	3.11	3.79	2.30	5.79
LOQ	NA	NA	NA	10.4	12.6	7.67	19.3

V-21 = Vector 21-41
Std. = A full-flavor generic cigarette produced by Liggett.
TPM = Total Particulate Matter
NNN = N-Nitrosonornicotine
NAT = N-Nitrosoanatabine
NAB = N-Nitrosoanabasine
NNK = N=Nitrosonicotine keytone [4(Methylnitrosamino)-1-(3-pyridyl)-1-butanone]

VI. ENVIRONMENTAL CONSEQUENCES OF INTRODUCTION OF THE TRANSFORMED CULTIVAR

A. Nicotine

Nicotine is a secondary metabolite produced in the roots of *Nicotiana* species that is subsequently transported to the leaves. Nicotine production is commonly believed to have evolved as a means to protect the plant from insect herbivores. Insect management practices in greenhouses often employ nicotine sprays. Insect herbivores (such as aphids, white flies, and horn worms) have evolved resistance to nicotine at the levels commonly produced by tobacco (~30,000 ppm) and thus, insect management practices (often systemic insecticides) are commonly used during field production.

Nicotine content of tobacco varies naturally depending upon a variety of factors, including; 1) Natural variation among varieties (*e.g.*, burley vs. flue-cured or low-alkaloid vs. normal lines); 2) Seasonal variation in rainfall and temperature; 3) Soil nitrogen levels; 4) Plant spacing; and 5) Developmental stages of the plants.

B. Low-Nicotine Transgenic Plants

Because Vector 21-41 was developed using antisense technology in which expression of the endogenous tobacco *NtQPT* genes is lowered by the expression of antisense *NtQPT1* transcripts, it is difficult to envision an environmental consequence for Vector 21-41. Antisense technology requires a significant level of complementarity between the antisense and sense transcripts. Thus, should the transgene somehow be transferred horizontally, it is highly unlikely that it would have any effect in the recipient organism. As mentioned earlier, nicotine levels in "normal" tobacco vary significantly. For example, young tobacco plants have very low nicotine levels in their leaves and low alkaloid tobacco lines have nicotine levels of about 10% of wild type. If Vector 21-41 is grown with normal insect management practices, the yield per acre is equivalent to its non-transgenic parent (Burley 21LA) (Table 2). A field experiment designed to compare Vector 21-41 with Burley 21LA grown in the absence of systemic insecticides failed due to insect pressure; both the transgenic and parental plants were destroyed by insects, primarily tobacco horn worms which devastated the leaf lamina and by tobacco bud worms which destroyed the apical meristem. This experiment was conducted at the Central Crops Research Station, Clayton, NC at the same time as the 2000 field trial (99-180-05n). Insect pressure was reported to be very high that summer. The experimental design was similar to that of the 99-180-05n trial, but with ten replicates of ten plants each for the three treatments (Vector 21-41, Promoter Control, and Wild-Type and the plants were grown without the use of any insecticides. Seedlings were transplanted on the same day as 99-180-05n. Within two weeks of transplant, insect damage was evident. Within six weeks, the leaf lamina of all plants (Vector 21-41 and the controls) had been completely stripped and the apical meristem removed. This halted plant development. At the end of the season, the plants were 30-40 cm high and contained only the stem and leaf midribs. There was no difference in insect predation observed between Vector 21-41 and controls.

Although nicotine may have evolved as a defense against insect herbivores, insect pests of tobacco have evolved resistance to the nicotine levels common in commercial tobacco production. Much of the damage may occur early in plant development when nicotine levels are

low. There are three general approaches to insect control; a regular spray schedule (every 10 to 14 days), insecticide treatment when insects are observed, and an integrated pest management (IPM) protocol (Miller and Fowlkes, 1999). IPM methods are recommended and require monitoring the field for insect damage. Wireworms and cutworms can be controlled by the incorporation of insecticides into the soil prior to transplant. Foliar or systemic insecticides will control tobacco fleas, budworms, hornworms, and to some extent aphids. Using conventional insect management practices, yields of Vector 21-41 leaf equaled those of the controls. Thus, Vector 21-41 is not more prone to insect predation than other tobacco varieties.

C. Neomycin Phosphotransferase

Neomycin phosphotransferase has undergone extensive testing as a selectable marker for transgenic plants (Nap, et al., 1992; Redenbaugh, et al. 1994). On September 4, 1998, the U.S. FDA Center for Food Safety and Applied Nutrition published a report entitled, "Guidance for Industry: Use of Antibiotic Resistance Marker Genes in Transgenic Plants" (http://www.cfsan.fda.gov/~dms/opa-armg.html) which evaluated the safety of neomycin phosphotransferase. To summarize briefly, the FDA evaluated the potential for horizontal transfer of the *nptII* gene to soil or intestinal (human and animal) microorganisms. They concluded that the probability of gene transfer was substantially less than the occurrence of kanamycin-resistance arising by mutation. Similarly, horizontal gene transfer to the epithelial cells lining the intestinal lumin was not observed. The *nptII* gene product has no significant homology with proteins listed as food allergens or toxins and is instable to proteolytic enzymes, heat, and stomach acid conditions. For these reasons, FDA had no allergenicity or toxicity concerns.

Kanamycin and other aminoglycoside antibiotics are not used as herbicides in agriculture. Horizontal transfer of the *nptII* gene to other crops and/or plants is not expected to in any way increase their competitiveness or weediness.

D. Transfer of the New Genes to *Nicotiana* Species.

Among cultivated plants, tobacco is uniquely positioned to greatly decrease (or eliminate) the possibility of transfer of the new genes.

1. Tobacco leaves, and not seeds, are the product. Thus, in the unlikely event of pollen transfer (see below), the new genes would not be present in the product.
2. A vast majority of tobacco producers plant certified seed and do not save seed back.
3. Tobacco flowers are self-compatible and are tubular. Thus, greatly favoring self-pollination.
4. Tobacco is not wind pollinated and pollinating insect vectors travel only short distances (<1320 feet).
5. During tobacco production, flowers are removed prior to anthesis, pollen dehiscence, and seed production (topping).
6. Tobacco is grown in regions of the U.S. in which wild, cross-compatible species are not found (see Section II.1).
7. Transfer of the genes conferring kanamycin-resistance and lower nicotine levels to other tobacco varieties (or wild relatives) is not expected to confer any competitive advantage. In fact, reduction of nicotine would be expected to reduce resistance to

insect herbivores and therefore, tobacco carrying the transgene would be expected to be less competitive in the absense of insect management practices.

Taken together, it is highly unlikely that the new genes contained in Vector 21-41 will be transferred to other wild or cultivated *Nicotiana* species.

VII. ADVERSE CONSEQUENCES: NEW CULTIVAR INTRODUCTION

No adverse consequences of the new cultivar introduction are foreseen. No deleterious effects on the plants, non-target organisms, or the environment were observed in the field trial nor are predicted. There is no perceived environmental impact from reduced nicotine levels. The modification gives no increase in weediness, no increased possibility of out-crossing to other species, and no change in biodiversity. Lowering nicotine content of the tobacco did not, in any way, increase the competitiveness of the plant. Nicotine levels in Vector 21-41 were reduced by the introduction of a tobacco gene construct (the *NtQPT1* promoter direction expression of antisense transcripts of the *NtQPT1* cDNA) into tobacco. Expression of the antisense transcript is limited to the root tissue in which the endogenous *NtQPT1* sense gene is expressed.

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APPENDICES

Acknowledged Notifications of Vector 21-41

Report of Field Trial 99-180-5n

APHIS RELEASE NOTIFICATIONS

FILING	TO	TIME PERIOD	APHIS ID NO.	REPORT
06/22/99	NC	08/01/99-11/31/99	99-180-05n	ATTACHED
01/22/01	PA	05/01/01-09/30/01	01-024-26n	IN PREPARATION
02/28/01	PA	05/01/01-09/30/01	01-061-01n	IN PREPARATION
03/21/01	TX	05/01/01-09/30/01	01-081-03n	NOT PLANTED
04/03/01	MS	05/01/01-09/30/01	01-094-07n	IN PREPARATION
04/10/01	IL	05/12/01-10/31/01	01-102-04n	IN PREPARATION
04/11/01	MS	05/10/01-10/10/01	01-102-01n	IN PREPARATION
04/11/01	LA	05/07/01-10/10/01	01-102-03n	IN PREPARATION
04/11/01	PA	05/10/01-10/10/01	01-102-05n	IN PREPARATION
04/23/01	LA	05/10/01-10/10/01	01-114-02n	IN PREPARATION
04/25/01	MS	05/10/01-10/10/01	01-116-01n	IN PREPARATION
04/26/01	IA	06/01/01-10/01/01	01-117-03n	IN PREPARATION
04/27/01	PA	05/01/01-09/30/01	01-122-01n	IN PREPARATION
05/22/01	HI	06/01/01-06/30/02	01-143-07n	
06/05/01	IL	06/01/01-10/31/01	01-158-01n	IN PREPARATION

North Carolina State University is a land-grant university and a constituent institution of The University of North Carolina

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December 11, 2000

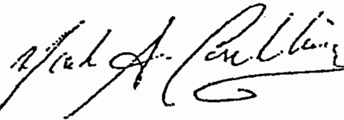
Ms. Anita Drummond
USDA, APHIS, PPQ
Unit 147, Room 5B33
4700 River Road
Riverdale, MD 20737

Dear Ms. Drummond:

Enclosed please find the report of the field test (99-180-05n) of our transgenic tobacco that had been modified to reduce nicotine. The test was performed at the NCSU/NCDA Central Crops Research Station in Clayton, NC.

If you have any questions, please contact me.

Sincerely yours,



Mark A. Conkling
Associate Professor of Genetics
(919) 515-5734
mark_conkling@ncsu.edu

cc: Dr. Johnny Wynne
Dr. John Bunch

Field Test Report: No-Nic-FT1 99-180-05n

Final

Mark A. Conkling
North Carolina State University

The low nicotine tobacco variety, **Vector Burley 21-41**, was developed by transforming Burley 21 LA with the binary Agrobacterium vector, pYTY32. pYTY32 carries the 2.0 kb *NtQPT1* root-cortex specific promoter which drives the antisense expression of the *NtQPT1* cDNA terminated by the nopaline synthase (*nos*) 3' from *Agrobacterium tumefaciens* T-DNA. (*NtQPT1* is a tobacco gene that encodes quinolinate phosphoribosyl transferase, an enzyme involved in nicotine biosynthesis.) The selectable marker for this construct was neomycin phosphotransferase (*nptII*) from *E. coli* Tn5 whose expression is directed by the *nos* promoter from *Agrobacterium tumefaciens* T-DNA. *nptII* confers resistance to kanamycin. Transformed cells, tissues, and seedlings were selected by their ability to grow on MS medium containing 300 μg/ml kanamycin.

One hundred independent pYTY32 transformants of Burley 21 LA (T_0) were allowed to self. Progeny of the selfed plants (T_1) were germinated on medium containing kanamycin and the segregation of kanamycin resistance scored. T_1 progeny segregating 3:1 resulted from transformation at a single locus and were subjected to further analysis.

Nicotine levels of T_1 progeny segregating 3:1 were measured qualitatively using a micro-assay. T_1 progeny that had less than 10% of the nicotine levels of the Burley 21 LA parent were allowed to self to produce T_2 progeny. Homozygous T_2 progeny were identified by germinating seeds on medium containing kanamycin and selecting ones in which 100% of the progeny were resistant to kanamycin (heterozygous progeny would segregate 3:1). Nicotine levels in homozygous and heterozyous T_2 progeny were qualitatively using the micro-assay and again showed levels less than 10% of the Burley 21 LA parent. Leaf samples of homozygous T_2 progeny were sent to the Southern Research and Testing Laboratory in Wilson, NC for quantitative analysis of nicotine levels using Gas Chromatography/Flame Ionization Detection. Homozygous T_2 progeny of transformant #41 gave the lowest nicotine levels (~70 ppm) and was renamed **Vector Burley 21-41.** Vector Burley 21-41 plants were allowed to self (producing T_3 progeny). T_3 progeny were grown and nicotine levels assayed qualitatively and quantitatively. T_3 progeny were allowed to self, producing T_4 progeny. Samples of the bulked seeds of the T_4 progeny were grown and nicotine levels tested.

Field trials of Vector Burley 21-41 T_4 progeny were performed at the Central Crops Research Station (Clayton, NC) and compared to the Burley 21 LA parent. The design was three treatments (Vector Burley 21-41, a Burley 21 LA transformed line carrying only the *NtQPT1* promoter [Promoter-Control], and untransformed Burley 21 LA [Wild-type]), 15 replicates, 10 plants per replicate.

Methods of observation:
Investigators observed the field trial at least once each week. During times at which flowers were developing (~days 45 to 70 after transplant), the field was monitored every two days to remove flowers. During the test, Orthene was applied two times at a rate of 1 lb/acre to control insects. No difference was observed in insect damage of the low nicotine line, Vector Burley 21-41, and the Promoter Control and Wild-Type lines following this insect management protocol. A variety of agronomic and chemical traits (listed below) were assayed during the trial.
The data are summarized below:

	TREATMENT		
	Vector Burley 21-41	Promoter Control	Wild-type
Days from transplant until flowering (days)	57.1 ± 3.6*	56.7 ± 3.4*	57.6 ± 3.4*
Height at flowering (cm)	118.6 ± 20.1*	112.1 ± 21.4*	110.8 ± 19.5*
Leaf number at flowering.	22.8 ±1.4*	22.6 ± 4.9*	23.4 ± 1.9*
Leaf size (Length) (cm)			
5th leaf	37.1 ± 4.7*	39.4 ± 4.0*	38.3 ± 4.2*
10th leaf	35.6 ± 3.6*	38.3 ± 4.3*	36.8 ± 3.3*
15th leaf	30.7 ± 3.5*	31.7 ± 2.5*	32.8 ± 4.3*
Leaf size (Width) (cm)			
5th leaf	15.6 ± 1.8*	16.0 ± 2.7*	17.0 ± 2.9*
10th leaf	14.7 ± 2.3*	15.8 ± 1.8*	15.6 ± 1.6*
15th leaf	13.7 ± 2.5*	13.4 ± 2.0*	13.2 ± 2.3*
Internodes (mm)	66.0 ± 13.4*	57.7 ± 12.5*	54.6 ± 5.3*
Yield (kg/ha)	890.3 ± 70.7*	780 ± 68.5*	809.2 ± 71.2*
% Nicotine (X 10^2)	1.44 ± 0.66**	19.12 ± 8.99*	21.54 ± 9.34*
% Nor-Nicotine (X 10^2)	0.4 ± 0.1**	1.56 ± 0.22*	1.27 ± 0.52*
% Total Alkaloids	0.23 ± 0.07**	2.07 ± 0.93*	2.31 ± 0.94*
% Total Nitrogen	2.53 ± 0.78*	2.96 ± 0.42*	2.64 ± 0.91*
% Reducing Sugars	10.29 ± 0.89**	5.87 ± 2.04*	5.51 ± 2.40*

Data from 2000 field trial at Central Crops Research Station, Clayton, NC.
15 replicates/10 plants per replicate. Data were analyzed using the F-test.
*= No significant difference, ** = Significant at the 1% level.

For all traits measured there was no significant difference for the Promoter Control and Wild-Type lines. The low nicotine, transgenic line, Vector Burley 21-41, exhibited significantly lower nicotine, nor-nicotine, and total alkaloid percentages than the Promoter Control and Wild-Type lines. Reducing sugars in the Vector Burley 21-41 line were about two-fold higher than the Promoter Control and Wild-Type lines.

At the conclusion of the field trial, the field was disked and the emergence of volunteers monitored. This monitoring will continue during the 2001 growing season.

No deleterious effects on the plants, non-target organisms, or the environment were observed. This result was expected because the transgene primarily affects alkaloid (nicotine and nor-nicotine) levels. Nicotine content of tobacco varies naturally depending upon a variety of factors, including; 1) Natural variation among varieties (*e.g.*, burley vs. flue-cured or low-alkaloid vs. normal lines). 2) Seasonal variation in rainfall and temperature. and 3) Developmental stages of the plants. The leaves of Vector Burley 21-41 still contain nicotine at around 0.01% (dry weight). There is no perceived environmental impact from reduced nicotine levels. The modification gives no increase in weediness, no increased possibility of out-crossing to other species, and no change in biodiversity. Lowering nicotine content of the tobacco did not, in any way, increase the competitiveness of the plant.

Dec. 11, 2000

Mark A. Conkling

Date

01-121-01p
addendum

VECTOR TOBACCO (USA) LTD.
Dr. Mark Conkling
Vice President–Genetic Research

19 October 2001

Susan Koehler, Ph.D.
Senior Biotechnologist
Permits and Risk Assessments
USDA/APHIS
4700 River Road, Unit 133
Riverdale, MD 20737-1236

Subject: Petition 01-121-01p for a Determination of Nonregulated Status for the Reduced Nicotine Tobacco Vector 21-41

Dear Dr. Koehler:

Please find below responses to each of the points raised in your letter dated September 26, 2001. Additional field data from this year's trials and supplementary information supporting a determination of nonregulated status are included in some of the responses and at the end of this letter. Also enclosed are twenty bound color copies and one unbound color copy of the petition.

1. What is the sample size for Fig. 6 on page 14 in the revised petition? Was the probe used for quantifying NtQPT1 transcript specific for NtQPT1 or will it also hybridize with NtQPT2 transcript?

   RESPONSE:
   Roots were harvested from four plants of each of the genotypes [wild-type (*Nic1*$^+$/*Nic2*$^+$), intermediate (*nic1*$^-$/*Nic2*$^+$ and *Nic1*$^+$/*nic2*$^-$), and low-nicotine (*nic1*$^-$/*nic2*$^-$)], pooled, and total RNA extracted using Tri-Reagent. Blots were probed with the full-length *NtQPT1* cDNA that had been labeled with ^{32}P. This probe will cross-hybridize with the *NtQPT2* transcript under the conditions used.

2. The text on page 19 that refers to the data presented in Table 2 needs to cite Table 2. In addition, Table 3 on page 20 is mistakenly referenced in the preceding paragraph as Table 2.

   RESPONSE:
   These corrections have been made.

10/22/01
MF?

710 West Main Street • Durham, NC 27701 • Telephone: (919) 683-8944 • Fax: (919) 683-8966 • Email: mconkling@vectob.com

VECTOR TOBACCO (USA) LTD.

3. Explain why the segregation ratios for kanamycin resistance for some of those listed as heterozygous in Table 3 don't seem to fit the 3:1 ratio, e.g., T_1-2, T_1-4, and T_1-9. It seems that for T_1-2 that the segregation ratio is more consistent with a 15:1 ratio. Could it be a result of different segregation of the endogenous wild-type *NtQPT* loci? The paragraph between Tables 2 and 3 of the original submission was inadvertently omitted from the revised petition and should still be included.

<u>*RESPONSE:*</u>

Segregation of T_1 progeny was used to determine to the number of loci conferring kanamycin-resistance (Table 2). The segregation ratio for kanamycin-resistance and sensitivity matches a 3:1 ratio almost perfectly ($\chi^2 = 0.25$ when the expected ratio equals 3:1) and, thus, the transgene segregates as a single locus. The χ^2 analysis tests a hypothesis of the observed number versus the expected number. If we hypothesized that the transgene integrated into two loci, the expected ratio would be 15:1 and the $\chi^2 = 96.75$. 96.75 is much higher than 0.25 and thus, the hypothesis that the transgene integrated into two loci is rejected. In Table 3, the segregation of the T_2 progeny of twelve T_1 plants is listed. Plants T_1-2, T_1-4, T_1-6, T_1-8, T_1-9, T_1-11, and T_1-12 were heterozygous for the transgene. Chi-squared analyses of these progeny, with the exception of T_1-2, showed that the observed segregation was consistent with the hypothesis of that the transgene integrated into one locus. The chi-squared analysis of T_1–2 for a single locus was 24.8 and for two loci was 6.2. The ratio observed for T_1-2 does not fit either hypothesis. The cause of this aberrant ratio is not understood. Be that as it may, the segregation ratios for the T_1 progeny (Table 2) and six of seven heterozygous T_2 progeny (Table 3) is consistent with the hypothesis that the transgene inserted at a single locus. This genetic interpretation is supported by physical evidence presented in Figures 9 and 13 that demonstrate that two T-DNA copies had integrated into the tobacco as an inverted tandem repeat. This repeat would segregate as a single locus.

The paragraph between Tables 2 and 3 of the original submission was omitted from the revised petition because it did not explain the data presented in Tables 2 and 3. It has been re-written as follows:

Nicotine levels of T_1 progeny segregating 3:1 were measured qualitatively using a micro-assay. T_1 progeny of transformant #41 exhibited the lowest nicotine levels and were characterized further. Twelve T_1 progeny of transformant #41 (designated T_1-1 to T_1-12) were germinated without kanamycin selection and grown in a greenhouse. At flowering, leaf samples were taken from the third leaf position and nicotine levels quantified at the Southern Research and Testing Laboratory using gas chromatography/flame ionization detection. These plants were not topped and seeds were harvested from each T_1 plant. T_2 progeny of these T_1 plants were germinated on kanamycin to determine which T_1 plants were homozygous (T_1-1 and T_1-3) heterozygous (T_1-2, T_1-4, T_1-6, T_1-8, T_1-9, T_1-11, and T_1-12), and did not carry the transgene (T_1-5, T_1-7, and T_1-10) (see Table 3). Nicotine levels of T_1

plants carrying the transgene ranged from 70 ppm to 250 ppm, while nicotine levels for plants not carrying the transgene ranged from 8020ppm to 9000 ppm (Table 4). Thus, low nicotine levels co-segregated with the transgene that conferred kanamycin-resistance. T_2 progeny from the homozygous plants T_1-1 and T_1-3 (designated T_2-1 and T_2-3, respectively) were grown in the greenhouse. At flowering, leaf samples were taken from the third leaf position of five plants and pooled. Nicotine levels were quantified and exhibited levels of 100 and 120 ppm, respectively. The plants were not topped and seeds were harvested. Homozygous seeds from T_2-1 (designated **Vector 21-41**) were used to generate the T_3 generation. T_3 progeny were germinated without kanamycin selection and grown in the greenhouse. At flowering, leaf samples were taken from the third leaf position of ten plants. The samples were pooled and nicotine levels quantified yielding a level of 190 ppm. Approximately 1,000 T_3 plants were allowed to flower and set seeds. Seeds from these plants were pooled for the T_4 generation. Plants from the T_4 generation were planted in the greenhouse. At flowering, leaf samples were taken from the third leaf position of five plants and pooled. The pooled sample was extracted three independent times, and the nicotine levels of each extraction measured (average 190 ppm; standard deviation 13 ppm).

Nicotine levels of cured leaves from topped T_4 plants grown in fields in various locations were measured. The nicotine levels of Vector 21-41 and of the Burley 21 LA parent were measured in a field trial (99-180-05n) and are shown in Table 5 and summarized in Table 4 [Field (NC)]. In this case, plants were grown in 15 replicates with ten plants per replicate. The average nicotine content of Vector 21-41 plants was 1440 ppm (±660) whereas the average nicotine content of the Burley 21 LA parent was 23,100 ppm (±9,340). Vector 21-41 (T_4 generation) was grown in fields in Argentina in 1999 and 2000 and had nicotine levels of 700 ppm and 1000 ppm. In Table 4, preliminary data for nicotine levels for the lowest priming (leaves at position numbers 1 through 8) of Vector 21-41 grown in Louisiana (01-114-02n) are shown to be 1490 ppm. Subsequently, nicotine levels have been measured from whole plant samples of crops grown at eight different locations in Louisiana. In these measurements, three independent samples from each location were extracted and nicotine levels quantified at the Vector Tobacco laboratories in Durham, NC. The average nicotine contents (± standard deviation) are 950 ppm (±110), 1719 (±20), 1305 (±48), 1072 (±103), 1138 (±10), 968 (±12), 832 (±14), and 1101 (±20). Thus, the low nicotine phenotype of Vector 21-41 is stable through the T_4 generation. T_4 progeny average 1143 ppm with a standard deviation of 295.

4. Does leaf number in Table 4 mean the number of leaves or the position of the leaf on the plant?

RESPONSE:

"Leaf Number" means the position of the leaf on the plant. The lowest leaf is #1. The column heading in Table 4 has been re-labeled to "Leaf Position Number".

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4. Were the samples for nicotine analysis for T_2-1 and T_2-3, T_3, and T_4 in the greenhouse just the 3rd leaf from one plant or the 3rd leaf pooled from several homozygous progeny from the preceding generation? If the ppm value is the mean of several samples, please indicated this and provide the standard deviation and range of values observed.

RESPONSE:

Details of the experimental design summarized in Table 4 are presented above in the response to question #3.

T_2 progeny from the homozygous plants T_1-1 and T_1-3 (designated T_2-1 and T_2-3, respectively) were grown in the greenhouse. At flowering, leaf samples were taken from the third leaf position of five plants and pooled. Nicotine levels were quantified and exhibited levels of 100 and 120 ppm, respectively.

Homozygous seeds from T_2-1 (designated **Vector 21-41**) were used to generate the T_3 generation. T_3 progeny were germinated without kanamycin selection and grown in the greenhouse. At flowering, leaf samples were taken from the third leaf position of ten plants. The samples were pooled and nicotine levels quantified yielding a level of 190 ppm.

Approximately 1,000 T_3 plants were allowed to flower and set seeds. Seeds from these plants were pooled for the T_4 generation. Plants from the T_4 generation were planted in the greenhouse. At flowering, leaf samples were taken from the third leaf position of five plants and pooled. The pooled sample was extracted three independent times, and the nicotine levels of each extraction measured (average 190 ppm; standard deviation 13 ppm; range 177 ppm to 203 ppm).

4. How come the nicotine levels of T_3 and T_4 are closer to those listed as heterozygous?

RESPONSE:

I am not convinced that we can distinguish heterozygous and homozygous plants based on nicotine content. There is plant to plant variation in nicotine levels that can result from environmental factors, such as drought or water stress, nitrogen levels, temperature, wounding, sunlight, etc. Section II, H of the Petition details spatial, temporal, and environmental influences on nicotine biosynthesis. The T_3 and T_4 plants were grown at different times of year, relatively few plants were sampled, and the samples were pooled prior to nicotine analysis.

4. Why are the levels higher for field grown plants?

RESPONSE:

We cannot compare nicotine levels between greenhouse-grown and field-grown plants presented in Table 4. First, all the greenhouse-grown plants were not topped while those grown in the field were topped. [Topping induces nicotine biosynthesis.] Secondly, only the leaf at the third position was sampled for greenhouse grown plants, while the entire plant was

sampled from those grown in the field. [Nicotine levels increase in leaves with higher leaf positions.] Thirdly, the greenhouse-grown and field-grown plants were subjected to vastly different environments, including (but not limited to) insect predation, water and drought stress, nitrogen levels, planting media (MetroMix vs. soil), light quality, temperatures, and mechanical wounding. All these environmental factors affect nicotine biosynthesis.

4. Are there greenhouse or field data for the Burley 21 LA line?

   RESPONSE:

   For greenhouse grown plants, nicotine levels were measured from the non-transgenic segregants T1 of transformant #41. The average nicotine content was 8363 ppm (±552), while the average nicotine content of all generations of transformant # 41 (T_1 to T_4) is 166 ppm (±53) (Table 4).

   For field grown plants, Vector 21-41 (T_4 generation) was compared directly with its parent, Burley 21 LA, and a transgenic "promoter control" transformant in the field trial, 99-180-05n, conducted in North Carolina (Table 5). This trial consisted of 15 replicates with 10 plants per replicate. The average nicotine content of Burley 21 LA was 21,540 ppm (±9340), the average nicotine content of the promoter control was 19,120 ppm (±8990), and the average nicotine content of Vector 21-41 was 1440 ppm (±660).

   I am convinced that Vector 21-41 plants carrying the transgene have significantly lower nicotine levels (in both the greenhouse and the field) than either the parent Burley 21 LA or non-transgenic segregants of the original transformant #41.

5. For Fig. 9 in the revised petition, what generation of plants was used and were the samples pooled from several plants or a single plant?

   RESPONSE:

   For the DNA blot analysis, DNA was extracted from five, T_4-generation plants at the 6-leaf stage. Two leaves from each of these plants were harvested and pooled. DNA was extracted from the pooled leaves.

6. In Fig. 13, the direction of the arrow for the qptc-1 primer placed over the left NtQPT1-AS appears to be in the reverse orientation. Please fix this. Another error has been noted in top of pg. 18, par.1. In order to reduce the expression of QPTase (and therefore, the levels of nicotine), the full-length NtQPT1 cDNA was cloned in the antisense orientation behind the 2.0 kb NtQPT1 **promoter."**

   RESPONSE:

   These corrections have been made in the petition.

7. Based on the PCR analysis provided, we do not expect that portions of the plasmid that include *nptIII* or *traF* genes are present in Vector 21-41, however because portions adjacent to the left T-DNA border are present, it is possible that some kind of rearrangement of the plasmid occurred during integration and portions of these genes may be integrated in another portion of the genome. Please provide PCR or Southern Blot data that indicate whether sequences including the translation start site are present or if a complete copy of these sequences is present. Also please indicated whether the probe used for *nptII* could cross hybridize with the *ntpIII* gene from the plasmid backbone.

RESPONSE:

The possibility of either *nptIII* or *traF* integrating into the genome was addressed by PCR analysis. For both genes, PCR primers that prime towards the 3' end of the gene (encoding the C-terminus of the protein) and cover the start of translation were used. These "forward" primers were used in combination with two "reverse" PCR primers that hybridize within the gene and at the end of the gene and prime towards the 5' end of the gene. The template DNAs for the amplifications were genomic DNA (100 ng) from untransformed Burley 21-41 (1) Vector 21-41 (2), and pYTY32 (100 pg) (3). The gapC primers are PCR primers directed against the endogenous glyceraldehydes phosphate dehydrogenase gene (GapC) and were included as negative controls (for pYTY32) and positive controls (for untransformed Burley 21 LA and Vector 21-41 genomic DNAs). Figure 1 *RESPONSE* illustrates the PCR strategy and its results.

nptIII: According to the pBin19 sequence (Accession #U09365) the *nptIII* gene begins at nucleotide number 965 and extends to nucleotide number 3078. The "forward primer", nptIII-F, hybridizes to nucleotide numbers 965 to 984 and primes towards the 3' end of the gene.

The "reverse primer", nptIII-R1, hybridizes to sequences number 1289 to 1260 and primes towards the 5' end of the gene. In combination with nptIII-F, this primer would amplify a DNA fragment predicted to be 325 base pairs.

The "reverse primer", nptIII-R2, hybridizes to sequences number 2125 to 2105 and primes towards the 5' end of the gene. In combination with nptIII-F, this primer would amplify a DNA fragment predicted to be 1161 base pairs.

Figure 1 *RESPONSE* illustrates that the primer-pairs nptIII-F and nptIII-R1 and nptIII-F and nptIII-R2 amplify pYTY32 DNA and yield the predicted-sized amplification products. No DNA fragments were amplified from either untransformed Burley 21 or Vector 21-41 genomic DNAs.

traF: According to the pBin19 sequence (Accession #U09365) the *traF* gene begins at nucleotide number 10,982 and extends to nucleotide number 11,765. The "forward primer", traF-F, hybridizes to nucleotide numbers 10,985 to 11,004 and primes towards the 3' end of the gene.

The "reverse primer", traF-R1, hybridizes to sequences number 11,584 to 11,565 and primes towards the 5' end of the gene. In combination with nptIII-F, this primer would amplify a DNA fragment predicted to be 600 base pairs.

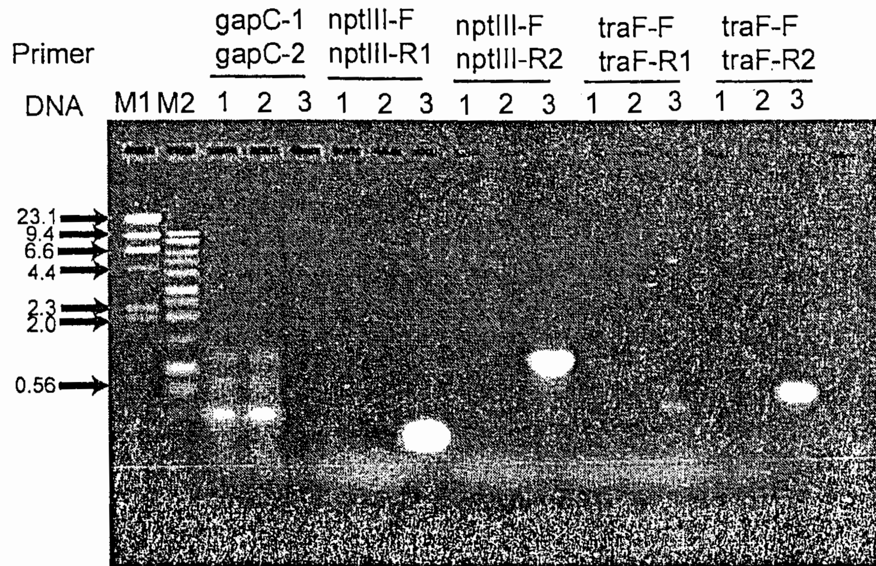
The "reverse primer", traF-R2, hybridizes to sequences number 11,748 to 11,729 and primes towards the 5' end of the gene. In combination with traF-F, this primer would amplify a DNA fragment predicted to be 781 base pairs.

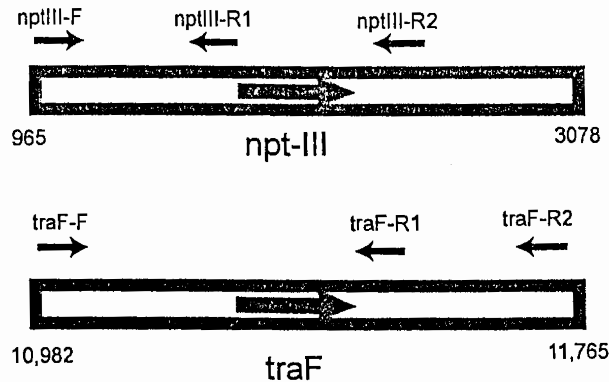
Figure 1 RESPONSE illustrates that the primer-pairs traF-F and traF-R1 and traF-F and traF-R2 amplify pYTY32 DNA and yield the predicted-sized amplification products. No DNA fragments were amplified from either untransformed Burley 21 or Vector 21-41 genomic DNAs.

The gapC primers amplified the predicted sized fragments from both untransformed Burley 21 LA and Vector 21-41 genomic DNAs, but not from pYTY32 DNA.

The results illustrated in Figure 1 *RESPONSE* demonstrate that sequences from the *nptIII* and the *traF* genes of the pYTY32 backbone have not integrated into the Vector 21-41 genome.

The *npt-II* and *npt-III* genes are not homologous and would not cross-hybridize under the conditions used.





DNA's

M1	Marker - Lambda digested with HindIII
M2	Marker - Promega 1 kb Ladder
1	Untransformed Burley 21 LA
2	Vector 21-41
3	pYTY 32

Figure 1-RESPONSE: PCR analysis of the transgene in Vector 21-41. Genomic DNA (100 ng) from untransformed Burley 21 LA (1), Vector 21-41 (2), and pYTY32 (100 pg) were used as templates for PCR amplification (30 cycles) using primers to npt-III and traF genes.

8. Please provide a more clear explanation on how the mistake in the field data report for nicotine and nor-nicotine values were corrected in Table 5. Why are reducing sugars evaluated, and what is the significance of the difference in reducing sugars between Vector 21-41 and the wild-type, e.g., what is the normal range of reducing sugars in tobacco and what, if any, effect would it have on the plant posing a plant pest risk or non-target effect?

RESPONSE:

When reviewing data from the 2000 field test (99-180-5n) we discovered a ten-fold calculation error had been made in the "% Nicotine" and "% Nor-Nicotine" entries (Vector 21-41, Promoter Control, and Wild-Type) in the "Final Field Test Report". The levels presented in the table of the report (pg. 3) are ten-fold lower than the actual levels. In Table 5 of the Petition for Deregulation this ten-fold error has been corrected. In Table 5, we converted *% Nicotine* to *ppm* (parts per million) to be consistent with the text of the petition and the units generally used to describe nicotine content in tobacco.

Reducing sugars are evaluated as part of the tobacco seed certification process. Reducing sugars will impact the flavor characteristics of the processed leaf and how that leaf will be used in the smoking product. Percent reducing sugars vary greatly, about 100-fold, among different tobacco types. For example, flue-cured, type 13 varieties have about 22.09% (dry weight) reducing sugars, Oriental varieties (blends of Macedonia, Smyrna, and Samsun types) have about 12.30%, and Maryland, type 12 varieties have about 0.21% (Leffingwell, J.C., Leaf Chemistry, Basic Chemical Constituents of Tobacco Leaf and Differences among Tobacco Types, in *Tobacco Production, Chemistry, and Technology*, D.L. Davis and M.T. Nielsen eds. Blackwell Science Ltd., Oxford England, 1999). A search of the literature failed to retrieve any report of changes in plant risk or non-target effects associated with different levels of reducing sugars. The increase to 10.3% in reducing sugars observed for Vector 21-41 is still well within the range of reducing sugars observed among tobacco varieties. It is difficult to visualize a circumstance where increasing the reducing sugar content of Vector 21-41 to approximately 10% would have any environmental impact greater than growing "normal" flue-cured tobacco varieties having reducing sugar levels of approximately 20%.

With the data currently available, it is difficult to speculate "Why" the reducing sugars are elevated in Vector 21-41 relative to Burley 21 LA. However, different tobacco varieties (for example flue-cured varieties) have reducing sugar levels in the 20% range. Until more antisense, low nicotine transgenic lines are examined, it is too early to say if the reduction in nicotine is causally linked to increased reducing sugars. It is interesting that Mark Stitt and co-workers have examined the interaction of carbon fixation pathways and nitrogen assimilation pathways (Current Opin. Plant Biol., 1: 197-200, 1998; Curr. Opin. Plant Biol.. 2: 178-186, 1999) and have shown that the pathways are linked. If a pathway that uses an excess of nitrogen (the nicotine biosynthetic pathway) were inhibited, one would predict additional energy going into the carbon fixation pathway, thereby increasing reducing sugars.

9. Please provide us with a copy of the reference for Miller and Fowlkes, 1999, and include the full citation for the references. Also, please provide us with a copy of the relevant pages from Tso, 1972 indicated on page 1.

   RESPONSE:
   Citation of the Miller and Fowlkes reference has been added to the petition and the requested materials have been provided.

10. Data or information about disease resistance was not included. Please provide any data or observations you have made of Vector 21-41 on resistance or susceptibility to specific diseases or types of diseases.

   RESPONSE:
   Vector 21-41 was grown in a field trial with its parent Burley 21 LA and a control transformant of Burley 21 LA that did not reduce nicotine levels (Table 5). Plants were monitored at least once per week throughout the entire growing season. As flowers were emerging (about 50 days after transplant), the field was monitored at least three times per week. No differences in disease resistance or susceptibility were observed among the three lines. Comparisons of the yields of these lines support these observations; yields of Vector 21-41 were slightly higher than non-transformed Burley 21 LA or the transgenic control (Table 5).

   Observations from the 2001 trials in Pennsylvania show that Vector 21-41 is susceptible to blue mold (*Peronospora tabacina*) infection. However, the parent Burley 21 LA and Burley 21 are also highly susceptible to blue mold infection. In addition, blue mold occurred on other tobacco varieties in most of the tobacco production regions of North America during the 2001 growing season (http://www.ces.ncsu.edu/depts/pp/bluemold/fcst2001/exec2001.htm).

   Although not observed in the field trials, Burley 21 is highly susceptible to black shank (*Phytophthora nicotinanae*). There is no reason to believe that the genetic manipulations of Vector 21-41 would alter its black shank susceptibility.

   Over 600 farm field trials in Pennsylvania, Illinois, Louisiana, Mississippi, and Iowa were planted with Vector 21-41 tobacco this year. Tobacco is an intensively managed agricultural crop requiring frequent monitoring and attention. As part of a normal program of good agricultural practice, these fields were monitored for disease and insect pests by the farm owners and workers, Vector Tobacco agronomists, and contracted consultants during the growing season. No exceptional disease or pest susceptibility, other than the blue mold mentioned above, were observed. In addition, no abnormal plant morphology or phenotypes due to presence of the transgenes were observed.

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Changes in disease resistance or susceptibility directly attributable to the reduced nicotine content of Vector 21-41 (relative to its non-transformed parent) are difficult to envision. Resistance genes are reasonably well understood, and have been introgressed into commercial tobacco cultivars through many years of plant breeding. These genes do not alter nicotine content. Additionally, the nicotine content of tobacco varies tremendously during plant development. Young plants have low nicotine levels.

Additional information on plant pest risk and other potential adverse consequences

In addition to the information contained in Section VI of the Petition, the experimental data, field observations, and traditional tobacco agricultural practices discussed below further support the determination of nonregulated status of the reduced-nicotine tobacco, Vector 21-41.

Vector 21-41 does not pose a plant pest risk. *Nicotiana tabacum* has been highly domesticated for commercialized production of tobacco leaf, is self-pollinating, and has high seed germination rates. The reduced nicotine phenotype of Vector 21-41 is unlikely to affect weediness. To determine if reducing nicotine content in some way significantly increased the numbers of seeds produced, we counted the numbers of seeds per pod produced by greenhouse grown Vector 21-41 plants and compared those numbers to other tobacco varieties (see Table 1-*RESPONSE* below). The average number of seeds per pod for Vector 21-41 was 1370. This number is similar to other varieties measured.

Table 1 - *RESPONSE*: Seeds per pod.

Variety	Average	Std. Deviation	Range	Number of pods
Vector 21-41	1370	307	789-1660	20
Burley 21 LA	1426	572	603-2176	8
SR1	1097	469	689-2365	12
Ti121	1630	444	989-2130	8
Ti57	1582	434	930-2062	10
Tn90	1199	237	869-1512	8

Seed pods were harvested from greenhouse grown plants. Seeds were counted from individual seed pods using a BioRad FluorS Max-2 digital camera and the "Colony Count" software. Sensitively was set at 6.1.

Preliminary data from 53 farms in Pennsylvania growing Vector 21-41 in 2001 are presented in Table 2 - *RESPONSE*: 2001 Field data. These data further demonstrate that the transgenes in Vector 21-41 do not result in unexpected or variable agronomic traits. There is no significant difference in the traits measured from these 53 different farms. The differences compared to the data in Table 5 of the petition are due to different growing conditions in 2001 vs. 2000 and in Pennsylvania vs. North Carolina.

Table 2 - Response: 2001 Field Data

APHIS #	# OF FARMS	DAYS FROM TRANSPLANT TO FLOWERING	HEIGHT AT FLOWERING (cm)	LEAF # AT FLOWERING	LEAF SIZE LENGTH (cm)			LEAF SIZE WIDTH (cm)			INTERNODE (mm)		
					5th	10th	15th	5th	10th	15th	5th	10th	15th
01-024-26n	17	65.22±8.14	88.3±14.5	17.39±1.65	56.1±4.37	53.46±6.93	37.36±10.08	30.58±4.37	25.23±4.95	15.08±5.38	46.1±16.4	44.8±20.7	41.6.±15.9
01-061-01n	19	63.46±8.27	91.67±11.96	17.47±1.56	56.18±7.01	54.63±6.68	40.08±12.50	31.01±4.21	25.51±6.60	16.03±4.93	45.2±11.2	48.8±08.0	46.3±12.6
01-102-05n	17	64.56±5.56	91.89±14.35	16.88±1.14	56.03±5.51	52.79±6.93	37.84±9.27	30.13±3.75	24.36±4.69	15.29±1.18	45.9±07.4	48.8±06.8	45.8±11.8

2001 Preliminary Data From 53 Field Trials in Pennsylvania.
5 replicates/10 plants per replicate.

The experience from the over 600 farm field trials conducted in five states this year indicate that existing approved amounts of pesticides labeled for use on tobacco can control insect, disease, and weed problems. The specific rates of insecticide, fungicide, and herbicide application for any one field depended on traditional agronomic factors, e.g., the geographic location (state), climate (temperature and rainfall), and time of transplanting but were always within approved label amounts.

As noted in Sections V and VI of the petition and several points in this response letter, there are no notable differences between Vector 21-41 and other nontransgenic tobacco varieties, other than nicotine levels. The genes engineered into the plant, an antisense construct of a tobacco nicotine biosynthetic enzyme and the kanamycin resistance selectable marker, do not impart any toxic properties on the transgenic tobacco plant. There is no reason to believe Vector 21-41 tobacco would have any harmful effects on beneficial insects, other plants, or any threatened or endangered species.

The transgenic tobacco line, Vector 21-41 will not result in altered environmental or other effects as compared to its non-transgenic counterpart. The biology of tobacco, traditional agronomic practices used to grow tobacco, and the specific genetic modifications introduced into Vector 21-41 discussed in the Petition and in this response letter are the basis for this conclusion. The major points are summarized below:

- No altered weediness – Neither reduced nicotine nor kanamycin resistance affect weediness. Kanamycin is not used as a herbicide and reduced nicotine will not give the plants any competitive advantage.

- No transfer to related species – In normal cultivation practices tobacco plants are topped before flower matures. This will eliminate possible outcrossing. Additionally, extensive data show that tobacco grown in the field primarily self-fertilizes.

- No altered plant pest potential – Reduced nicotine will not make the plants more resistant to plant pests. No differences were observed (compared to unmodified tobacco) during field trials performed in the United States.

- No potential impact on non-target organisms – Nicotine is a secondary metabolite in tobacco. No non-target organisms require nicotine, and thus its reduction is benign.

- No potential impact on biodiversity – Because the modification we have introduced does not confer any resistance to plant herbicides, pests, or pathogens, it should have no impact on biodiversity.

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The responses and additional information in this letter further support a determination that Vector 21-41 does not present a plant pest risk and is not otherwise deleterious to the environment. The petition and this response letter justify approval of Vector Tobacco's petition for determination of nonregulated status for the reduced nicotine tobacco line Vector 21-41.

Sincerely,

Mark A. Conkling, Ph.D.
Vice President - Genetic Research