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TRANSGENIC PLANTS CONTAINING MOLECULAR DECOYS THAT ALTER PROTEIN CONTENT THEREIN

Related Applications

This application claims the benefit of provisional application serial number 60/229,198, filed August 30, 2000, the disclosure of which is incorporated by reference herein in its entirety.

Field of the Invention

The present invention describes a process for the production of transgenic plants such as transgenic tobacco plants with altered protein content therein, leading to altered phenotypes such as reduced nicotine levels, along with transgenic plants so produced and seed for such plants.

Background of the Invention

The production of tobacco with decreased levels of nicotine is of interest, given concerns regarding the addictive nature of nicotine. Additionally, tobacco plants with extremely low levels of nicotine production, or no nicotine production, are attractive as recipients for transgenes expressing commercially valuable products such as pharmaceuticals, cosmetic components, or food additives. Various processes have been designed for the removal of nicotine from tobacco. However, most of these processes remove other ingredients from tobacco in addition to nicotine, thereby adversely affecting the tobacco. Classical crop breeding techniques have produced tobacco plants with lower levels of nicotine (approximately 8%) than that found in wild-type tobacco plants. Tobacco plants and tobacco having even further reductions in nicotine content are desirable.

Nicotine is formed primarily in the roots of the tobacco plant and is subsequently transported to the leaves, where it is stored (Tso, *Physiology and*

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Biochemistry of Tobacco Plants, pp. 233-34, Dowden, Hutchinson & Ross, Stroudsburg, Pa. (1972)). Nicotine is produced by the condensation of two precursors, nicotinic acid and N-methylpyrolinium, that arise from two separate biosynthetic pathways (*see* **Figure 1**)(Bush and Saunders (1977) *Proc. Am. Chem. Soc. Symp.,* New Orleans, pp. 389-425; Hashimoto and Yamada (1994) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 45, 257-285; Waller and Dermer (1981) *In: The Biochemistry of Plants: A Comprehensive Treatise*, P.K. Stumpf and E.E. Conn, eds. Academia Press, pp. 317-395). The pyridine nucleotide cycle synthesize nicotinic acid (Wagner *et al.* (1986) *Planta* **167**, 226-232; Wagner and Wagner (1985) *Planta* **165**, 532-537), whereas N-methylpyrrolinium cations are synthesized from ornithine or arginine via putrescence (Leete (1980) *In: Encyclopedia of Plant Physiology, Secondary Plant Products*, Vol. 8, E.A. Bell and B.V. Charlwood, eds, Springer-Verlag, pp. 65-91; Tiburcio and Galston (1986) *Phytochemistry*, **25**, 107-110). Reciprocal grafting experiments have demonstrated that nicotine is synthesized in roots and transported through the xylem to leaves and other plant organs (Dawson (1941) *Science*, **94**, 396-397).

Two regulatory loci (*Nic1* and *Nic2*) regulate nicotine production. Legg *et al.* ((1969) *J. Hered.*, **60**, 213-217) incorporated genes from low alkaloid content Cuban cigar cultivars into Burley 21 cultivars. These investigators showed that the low alkaloid lines differed from standard cultivars at two loci, *Nic1* (formerly identified as *A*) and *Nic2* (formerly identified as *B*). These two loci are unlinked and the gene action is semi-dominant and primarily additive (Legg *et al.* (1969) *J. Hered.*, **60**, 213-217). Collins *et al.* ((1974) *Crop Sci.*, **14**, 77-80) prepared doubled haploid tobacco breeding lines of these four alkaloid genotypes. The genotype of standard cultivars is *Nic1/Nic1 Nic2/Nic2* and that of low nicotine lines is *nic1/nic1 nic2/nic2*. *Nic1/Nic1 nic2/nic2* is a high intermediate and *nic1/nic1 Nic2/Nic2* is a low intermediate (Legg and Collins (1971) *Can. J. Genet. Cytol.* **13**, 287-291). These lines are similar in days-to-flower, number of leaves, leaf size, and plant height. Enzyme analyses of roots of single and double *Nic* mutants show that the activities of two enzymes, quinolinate phosphoriboxyl transferase (QPTase) and putrescence methyl transferase (PMTase), are directly proportional to levels of nicotine biosynthesis (Saunders and Bush (1979) *Plant Physiol* **64**:236). Both *Nic1* and *Nic2* affect PMTase and QPTase

activities in roots, and thus, regulate nicotine synthesis (Leete (1983) *In: Alkaloids: Chemical and Biological Perspectives*, S.W. Pelletier, ed. John Wiley & Sons, pp. 85-152).

Hibi *et al.* ((1994) *Plant Cell*, **6**, 723-735) isolated the cDNA encoding PMTase, *PMT*, and showed that *PMT* transcript levels are regulated by *Nic1* and *Nic2*. The QPTase cDNA and genomic clones (*NtQPT1*) have also been isolated and the transcript levels of *NtQPT1* are also regulated by *Nic1* and *Nic2* (Song, W., Mendu, N., and Conkling, M.A.. (1999) *Plant Cell*, in preparation). Thus, it appears that the *Nic* genes regulate nicotine content by regulating the transcript levels of genes encoding the two rate-limiting enzymes, PMTase and QPTase. Further, *Nic1* and *Nic2* have been shown to be positive regulators of *NtQPT1* transcription and that promoter sequences upstream of the transcription initiation site contain the *cis*-acting sequences necessary for *Nic* gene product activation of *NtQPT1* transcription. Because expression of QPTase and PMTase are coordinately-regulated by the *Nic* gene products, it likely that the *Nic* gene products also directly regulate transcription of the *PMT* gene.

One approach for reducing the level of a biological product, such as nicotine, is to reduce the amount of a required enzyme (i.e. QPTase and PMTase) in the biosynthetic pathway leading to that product. Where the affected enzyme naturally occurs in a rate-limiting amount (relative to the other enzymes required in the pathway), any reduction in that enzyme's abundance will decrease the production of the end product. If the amount of the enzyme is not normally rate-limiting, its presence in a cell must be reduced to rate-limiting levels in order to diminish the pathway's output. Conversely, if the naturally-occurring amount of enzyme is rate limiting, then any increase in the enzyme's activity will result in an increase in the biosynthetic pathway's end product. The modification of nicotine levels in tobacco plants by antisense regulation of putrescence methyl transferase (PMTase) expression is proposed in US Patents 5,369,023 and 5,260,205 to Nakatani and Malik. PCT application WO 94/28142 to Wahad and Malik describes DNA encoding PMT and the use of sense and antisense PMT constructs. Additionally, PCT Application WO98/56923 to Conkling et al. describes DNA encoding a plant quinolate phosphoribosyl transferase (QPRTase) enzyme, constructs comprising such DNA, and

methods of altering QPRTase expression to increase or decrease nicotine production in plants. Despite previous efforts and successes, there remains a need for new approaches to reduce the production of gene products in plants (e.g., nicotine).

Summary of the Invention

A first aspect of the present invention is an isolated nucleic acid molecule (*e.g.,* a plasmid) comprising, consisting essentially of, or consisting of a *cis*-acting regulatory element, and the use of such an isolated nucleic acid for the production of a transgenic plant or host cell having altered levels (*e.g.,* increased or decreased levels) of a protein of interest therein. A particular example is a *Nic* gene product responsive element (e.g., a DNA sequence that binds to a *Nic* gene product) such as *(a)* isolated nucleic acids having a sequence according to **SEQ ID NO: 1** or a fragment thereof consisting essentially of or consisting of, desirably, at least 20-455 consecutive nucleotides, preferably, at least 30-400 consecutive nucleotides, more preferably, 50-350 consecutive nucleotides, and, most preferably, 100-300 or 200-400 consecutive nucleotides; and *(b)* isolated nucleic acids that hybridize to the complement of **SEQ ID NO:1** and bind or are otherwise responsive to a *Nic* gene product (*i.e.,* increase or decrease transcription of an operatively associated gene and hence increase or decrease the level of the encoded protein of interest in the host cells).

The *Nic* gene product responsive element can also be obtained from the sequence disclosed in U.S. Patent No. 5,459,252, herein expressly incorporated by reference in its entirety. In some embodiments, the *Nic* gene product responsive element resides between -1000 and -600 or -700 bp of the *NtQPT1* promoter, the sequence of which is disclosed in U.S. Patent No. 5,459,252. Accordingly, some embodiments involve a 300-400 nucleotide long fragment of the *NtQPT1* promoter that corresponds to the sequence of the *NtQPT1* promoter between -1000 and -600 or -700, as disclosed in U.S. Patent No. 5,459,252.

A second aspect of the present invention is a recombinant nucleic acid construct comprising containing a *cis*-acting regulatory element such as a *Nic* gene product responsive element as described above, along with the use of such a recombinant nucleic acid for the production of a transgenic plant or host cell as described herein. The construct may be a vector, such as a ballistic nucleic acid

transfer particle or an *Agrobacterium* vector. Plant cells containing such constructs, and preferably multiple copies thereof, are also an aspect of the invention.

A further aspect of the present invention is a method of making a transgenic tobacco plant having reduced nicotine content and/or tobacco specific nitrosamines (TSNA)s. The method comprises introducing an exogenous nucleic acid construct comprising a *Nic* gene product responsive element as described above into said at least one tobacco plant cell to produce at least one transformed tobacco plant cell. The at least one transformed tobacco plant cell contains the exogenous nucleic acid in an amount or copy number sufficient to reduce the nicotine and/or TSNA level of a tobacco plant regenerated from that cell or cells as compared to the nicotine and/or TSNA level that would be present in the absence of the exogenous nucleic acid. The method may further include generating a tobacco plant from the transformed plant cells, and (optionally) collecting tobacco leaves, stems, or seed from the tobacco plant. Thus, tobacco plants, including the leaves, stems, and seeds, generated from said method are also aspects of the present invention.

A further aspect of the present invention is a tobacco plant having reduced levels of nicotine and/or TSNAs therein, the plant comprising cells containing an exogenous nucleic acid, which exogenous nucleic acid comprises a *Nic* gene product responsive element as described above. The exogenous nucleic acid is contained in the cells in a copy number sufficient to reduce the nicotine level of that tobacco plant as compared to the nicotine level that would be present in that plant in the absence of the exogenous nucleic acid. Again, the leaves, stems, and seeds of such plant are also aspects of the present invention.

Tobacco products including, but not limited to, smoking materials (e.g., cigarettes, cigars, pipe tobacco), snuff, chewing tobacco, gum, lozenges that are prepared from said transgenic tobacco plants are also embodiments of the invention. Preferably these tobacco products are manufactured from harvested tobacco leaves and stems that have been cut, dried, cured, and/or fermented according to conventional techniques in tobacco preparation. However, modified techniques in curing and tobacco processing can also be implemented to further lower the levels of TSNAs. In some embodiments, the tobacco that is made substantially free of nicotine and/or TSNAs is prepared from a variety of Burley tobacco (e.g., Burley 21), Oriental

tobacco, or Flue-cured tobacco. It should be understood, however, that most tobacco varieties can be made to be nicotine and/or TSNA free using the embodiments described herein.

Additional embodiments include tobacco products that have been carefully blended so that desired levels of nicotine and/or TSNAs are obtained. For example, tobacco having a reduced level of nicotine and/or TSNAs, prepared as described above, can be blended with conventional tobacco so as to obtain virtually any amount of nicotine and/or TSNAs. Further, two or more varieties of tobacco having a reduced level of nicotine and/or TSNAs can be blended so as to achieve a desired amount of nicotine and/or TSNAs. In this manner, differences in variety, flavor, as well as amounts of nicotine and/or TSNAs can be incrementally adjusted. These blended tobacco products can be incorporated into tobacco use cessation kits and programs designed to reduce or eliminate nicotine dependence and carcinogenic potential. Such kits and programs are also embodiments of the invention.

More embodiments of the invention concern methods to reduce the carcinogenic potential of tobacco products, including cigarettes, cigars, chewing tobacco, snuff and tobacco-containing gum and lozenges. Some methods, for example involve the preparation of tobacco having a reduced amount of nicotine and/or TSNAs and the manufacture of tobacco products containing said tobacco. Accordingly, the transgenic tobacco plants, described above, are harvested, cured, and processed into tobacco products. These tobacco products have a reduced carcinogenic potential because they are prepared from tobacco that has a reduced amount of nicotine and/or TSNAs.

Yet another aspect of the invention concerns the reduction of the amount of TSNAs and metabolites thereof in humans who smoke, consume or otherwise ingest tobacco. This method is practiced by providing a tobacco product having a reduced amount of TSNAs, as described above, to said humans, thereby lowering the carcinogenic potential of such product in said humans.

More generally, the present invention provides a method of making a plant having increased or reduced content of a protein of interest therein, wherein the protein of interest is regulated by a *cis*-acting element selected from the group consisting of *(i)* a *cis*-acting activating element that binds an activator compound,

which activator compound increases expression of said protein of interest in said plant, and *(ii)*, a *cis*-acting repressor element that binds a repressor compound, which repressor compound decreases expression of said protein of interest in said plant. The method comprises introducing an exogenous nucleic acid construct comprising said *cis*-acting element into at least one plant cell to produce at least one transformed plant cell, with the at least one transformed plant cell containing the exogenous nucleic acid in a copy number sufficient to increase or reduce the level of said protein of interest in a plant regenerated from said cells as compared to the amount of said protein of interest that would be present in the absence of said exogenous nucleic acid.

The present invention thus generally provides a plant (and parts thereof) having increased or reduced levels of a protein of interest therein, the plant comprising cells containing an exogenous nucleic acid, which exogenous nucleic acid comprises a *cis*-acting element selected from the group consisting of *(i)* a *cis*-acting activating element that binds an activator compound, which activator compound increases expression of said protein of interest in said plant, and *(ii)*, a *cis*-acting repressor element that binds a repressor compound, which repressor compound decreases expression of said protein of interest in said plant; the cells containing the exogenous nucleic acid in a copy number sufficient to increase or reduce the level of the protein of interest in the plant as compared to the amount of the protein of interest that would be present in the absence of the exogenous nucleic acid.

Thus the present invention provides a general method of decreasing expression of a protein of interest in a (prokaryotic or eukaryotic) host cell, wherein transcription of the protein of interest is enhanced by a *cis*-acting activating element that binds an activator compound, which activator compound increases expression of the protein of interest in the host cell. The method comprises the steps of: *(a)* providing a decoy recombinant nucleic acid construct comprising the *cis*-acting activating element; and *(b)* introducing the decoy construct into the host cell in an amount sufficient to bind the activator compound and reduce expression of the protein of interest.

Further, the present invention provides a general method of increasing expression of a protein of interest in a host cell, wherein transcription of the protein of interest is reduced by a *cis*-acting repressor element that binds a repressor compound, which repressor compound reduces expression of said protein of interest in said host

cell. The method comprises the steps of: *(a)* providing a decoy recombinant nucleic acid construct comprising said *cis*-acting activating element; and *(b)* introducing said decoy construct into said host cell in an amount sufficient to bind said repressor compound and increase expression of said protein of interest.

The foregoing and other aspects of the present invention are explained in greater detail in the drawings herein and the specification set forth below.

Brief Description of the Drawings

Figure 1 depicts the biosynthetic pathway leading to nicotine biosynthesis. Enzyme activities known to be regulated by *Nic1* and *Nic2* are QPTase (quinolinate phosphoribosyl transferase) and PMTase (putrescence methyl-transferase). QPTase and PMTase are the rate-limiting enzymatic steps in nicotine biosynthesis and thus, nicotine levels are directly proportional to the QPTase and PMTase activities.

Figure 2 shows a diagrammatic representation of the *NtQPT1* gene and the *NtQPT1* promoter-*uidA* chimeras. The site of transcription initiation is indicated (+1) and the arrow indicates the *NtQPT1* transcript. Ten exons are presented as crosshatched bars. The deletion series of the promoter is also shown as solid bars truncated from the 5' end of the promoter. Sizes of promoter fragments fused to the *uidA* gene, which encodes β-glucuronidase (GUS) are indicated (i.e. Δ2.0, Δ1.4, etc.) in kilobase pairs (kb). Chimeric *NtQPT1* promoter-*uidA* fusions were cloned into pBI101.

Figure 3 shows β-glucuronidase (GUS) activity in roots, leaves, and stems of transgenic tobacco plants carrying the CaMV 35S promoter (CaMV 35S), the promoterless GUS (pBI101), and 5' nested deletions of the *TobRD2* (gene encoding *NtQPT1*) promoter fused to GUS. Sizes of promoter fragments fused to the *uidA* gene are indicated (i.e. Δ2.0, Δ1.4, etc.) in kilobase pairs (kb). For each construct at least 20 independent transformants were assayed.

Detailed Description of Preferred Embodiments

The term "plants" as used herein refers to vascular plants. Exemplary plants include, but are not limited, to corn (*Zea mays*), canola (*Brassica napus*, *Brassica rapa* ssp.), alfalfa (*Medicago saliva*), rice (*Oryza sativa*), rape (*Brassica napus*), rye

(*Secale cereale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), sunflower (*Helianthus annus*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Cofea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), apple (*Malus pumila*), blackberry (*Rubus*), strawberry (*Fragaria*), walnut (*Juglans regia*), grape (*Vitis vinifera*), apricot (*Prunus armeniaca*), cherry (*Prunus*), peach (*Prunus persica*), plum (*Prunus domestica*), pear (*Pyrus communis*), watermelon (*Citrullus vulgaris*), duckweed (*Lemna*), oats, barley, vegetables, ornamentals, conifers, and turfgrasses (*e.g.*, for ornamental, recreational or forage purposes). Vegetables include Solanaceous species (*e.g.*, tomatoes; *Lycopersicon esculentum*), lettuce (e.g., *Lactuea sativa*), carrots (*Caucus carota*), cauliflower (*Brassica oleracea*), celery (*apium graveolens*), eggplant (*Solanum melongena*), asparagus (*Asparagus officinalis*), ochra (*Abelmoschus esculentus*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), members of the genus *Cucurbita* such as Hubbard squash (*C. Hubbard*), Butternut squash (*C. moschata*), Zucchini (*C. pepo*), Crookneck squash (*C. crookneck*), *C. argyrosperma* , *C. argyrosperma* ssp *sororia*, *C. digitata*, *C. ecuadorensis*, *C. foetidissima*, *C. lundelliana*, and *C. martinezii*, and members of the genus *Cucumis* such as cucumber (*Cucumis sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamental plants include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia* hybrida), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherima*), and chrysanthemum. Conifers, which may be employed in practicing the present invention, include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*);

Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*). Turfgrass include but are not limited to zoysiagrasses, bentgrasses, fescue grasses, bluegrasses, St. Augustinegrasses, bermudagrasses, buffalograsses, ryegrasses, and orchardgrasses. Also included are plants that serve primarily as laboratory models, *e.g.*, *Arabidopsis*. Preferred plants for use in the present methods include (but are not limited to) legumes, solanaceous species (*e.g.*, tomatoes), leafy vegetables such as lettuce and cabbage, turfgrasses, and crop plants (*e.g.*, tobacco, wheat, sorghum, barley, rye, rice, corn, cotton, cassava, and the like), and laboratory plants (*e.g.*, *Arabidopsis*).While any plant may be used to carry out the present invention, tobacco plants are particularly preferred.

Plant parts that can be collected from the plants of the present invention (*e.g.*, cut or harvested) include, for example, fruits, flowers, seed, roots, tubers, leaves, stems, bark, wood, etc. Note that when reference is made to a particular protein being increased or reduced in a plant, the amount of that protein may be altered throughout the plant, or only in a particular part of the plant.

In overview, in an illustrative embodiment of the invention, nicotine is produced in tobacco plants by the condensation of nicotinic acid and N-methylpyrrolinium cation. The biosynthetic pathway resulting in nicotine production is illustrated in **Figure 1**. Two regulatory loci *(Nic1* and *Nic2)* act as co-dominant regulators of nicotine production. Enzyme analyses of roots of single and double *Nic* mutants show that the activities of two enzymes, quinolate phosphoribosyl transferase (QPTase) and putrescence methyl transferase (PMTase), are directly proportional to levels of nicotine biosynthesis. A comparison of enzyme activity in tobacco tissues (root and callus) with different capacities for nicotine synthesis shows that QPTase and PMTase activity are strictly correlated with nicotine content (Wagner and Wagner, *Planta* **165**:532 (1985)). Saunders and Bush *(Plant Physiol* 64:236 (1979) showed that the level of QPTase in the roots of low nicotine mutants is proportional to the levels of nicotine in the leaves.

The present invention is, in one preferred embodiment, based upon an isolated nucleic acid (e.g., **SEQ ID NO:1** or a fragment thereof consisting of, desirably, at

least 20-450 consecutive nucleotides, preferably, at least 30-400 consecutive nucleotides, more preferably, 50-350 consecutive nucleotides, and, most preferably, 100-300 or 200-400 consecutive nucleotides) that is or contains at least one *cis*-acting regulatory element, which exists upstream of the plant quinolate phosphoribosyl transferase (QPTase) and putrescence methyl transferase (PMTase) coding sequences. Another example is the *Nic* gene product responsive element obtained from the sequence disclosed in U.S. Patent No. 5,459,252, herein expressly incorporated by reference in its entirety. In some embodiments, the *Nic* gene product responsive element resides between -1000 and -600 or -700 bp of the *NtQPT1* promoter. Accordingly, some embodiments involve a 300-400 nucleotide long fragment of the *NtQPT1* promoter that corresponds to the sequence of the *NtQPT1* promoter between -1000 and -600 or -700, as disclosed in U.S. Patent No. 5,459,252.

Thus, in some embodiments, the embodied nucleic acids have a structure that promotes an interaction with one or more transcription factors (e.g., *Nic1* and *Nic2)*, which are involved in initiating transcription of QPTase and/or PMTase. Accordingly, said nucleic acids are said to be or contain at least one transcription factor (e.g., *Nic1* and *Nic2)* binding sequences, which are also referred to as "*cis*-acting regulatory elements." By introducing multiple copies of these cis-acting regulatory elements (e.g., sequences that interact with *Nic1* and/or *Nic2)* into a plant cell, the ability of the transcription factor to initiate transcription of the targeted gene (e.g., QPTase and/or PMTase genes) can be reduced or squelched.

As QPTase and PMTase activities are strictly correlated with nicotine content, construction of transgenic tobacco plants in which QPTase or PMTase levels are lowered in the plant roots (compared to levels in wild-type plants), as decribed above, result in plants having reduced levels of nicotine. Without wishing to be bound by any particular theory, it is contemplated that the creation of tobacco plants, tobacco, and tobacco products that have a reduced amount of nicotine will also have a reduced amount of TSNA. That is, by removing nicotine from tobacco plants, tobacco, and tobacco products, one effectively removes the alkaloid substrate for TSNA formation. It was found that the reduction of nicotine in tobacco was directly related to the reduction of TSNAs. Unexpectedly, the methods described herein not only produce

tobacco with a reduced addictive potential but, concomitantly, produce a tobacco that has a lower carcinogenic potential.

It should be emphasized that the phrase "a reduced amount" is intended to refer to an amount of nicotine and or TSNA in a transgenic tobacco plant, tobacco, or a tobacco product that is less than what would be found in a tobacco plant, tobacco, or a tobacco product from the same variety of tobacco processed in the same manner, which was not made transgenic for reduced nicotine and/or TSNA. Thus, in some contexts, wild-type tobacco of the same variety that has been processed in the same manner is used as a control by which to measure whether a reduction in nicotine and/or TSNA has been obtained by the inventive methods described herein.

Wild type tobacco varies significantly in the amount of TSNAs and nicotine depending on the variety and the manner it is grown, harvested, and cured. For example, a Burley tobacco leaf has 30,000 parts per million (ppm) nicotine and 8,000 parts per billion (ppb) TSNA; a Flue-Cured Burley leaf has 20,000 ppm nicotine and 300 ppb TSNA; and an Oriental cured leaf has 10,000 ppm nicotine and 100 ppb TSNA. A tobacco plant or portion thereof having a reduced amount of nicotine and/or TSNA, according to the invention, can have no detectable nicotine and/or TSNA, or may contain some detectable amounts of one or more TSNA and/or nicotine so long as the amount of nicotine and/or TSNA is less than that found in a control plant of the same variety. That is, a Burley tobacco leaf embodiment of the invention having a reduced amount of nicotine can have between 0 and 30,000 ppm nicotine and 0 and 8,000 ppb TSNA desirably between 0 and 20,000 ppm nicotine and 0 and 6,000 ppb TSNA more desirably between 0 and 10,000 ppm nicotine and 0 and 5,000 ppb TSNA preferably between 0 and 5,000 ppm nicotine and 0 and 4,000 ppb TSNA more preferably between 0 and 2,500 ppm nicotine and 0 and 2,000 ppb TSNA and most preferably between 0 and 1,000 ppm nicotine and 0 and 1,000 ppb TSNA. Embodiments of Burley leaf prepared by the methods described herein can also have between 0 and 1000 ppm nicotine and 0 and 500 ppb TSNA and some embodiments of Burley leaf prepared by the methods described herein have virtually no detectable amount of nicotine or TSNA.

Similarly, a Flue-cured tobacco leaf embodiment of the invention having a reduced amount of nicotine can have between 0 and 20,000 ppm nicotine and 0 and

300 ppb TSNA desirably between 0 and 15,000 ppm nicotine and 0 and 250 ppb TSNA more desirably between 0 and 10,000 ppm nicotine and 0 and 200 ppb TSNA preferably between 0 and 5,000 ppm nicotine and 0 and 150 ppb TSNA more preferably between 0 and 2,500 ppm nicotine and 0 and 100 ppb TSNA and most preferably between 0 and 1,000 ppm nicotine and 0 and 50 ppb TSNA. Embodiments of flue-cured tobacco prepared by the methods described herein can also have between 0 and 500 ppm nicotine and 0 and 25 ppb TSNA and some embodiments of flue-cured tobacco prepared by the methods described herein have virtually no detectable amount of nicotine or TSNA.

Further, an Oriental cured tobacco embodiment of the invention having a reduced amount of nicotine can have between 0 and 10,000 ppm nicotine and 0 and 100 ppb TSNA desirably between 0 and 7,000 ppm nicotine and 0 and 75 ppb TSNA more desirably between 0 and 5,000 ppm nicotine and 0 and 50 ppb TSNA preferably between 0 and 3,000 ppm nicotine and 0 and 25 ppb TSNA more preferably between 0 and 1,500 ppm nicotine and 0 and 10 ppb TSNA and most preferably between 0 and 500 ppm nicotine and no TSNA. Embodiments of Oriental cured tobacco prepared by the methods described herein can also have between 0 and 250 ppm nicotine and no TSNA and some embodiments of Oriental cured tobacco prepared by the methods described herein have virtually no detectable amount of nicotine or TSNA.

The present invention provides methods and nucleic acid constructs for producing such transgenic plants, as well as such transgenic plants. Such methods include the development of transgenic cassettes that will reduce (or eliminate) nicotine biosynthesis. Tobacco plants are transformed with an excess number of DNA sequences (*cis*-acting elements) from the promoters of genes encoding, but not limited to, QPTase and PMTase that are regulated in nicotine biosynthesis. These *cis*-acting elements are preferably integrated into the plant genome so as to allow for transfer to successive generations. Typically, the *Nic1* and *Nic2* DNA-binding proteins that interact with these *cis*-acting DNA sequences are expressed at relatively low levels in the cell, thus the excess of transgenic *cis*-acting elements will compete with the endogenous elements associated with the genes encoding, but not limited to, QPTase and PMTase for available *Nic1* and *Nic2*. Accordingly, these *cis*-acting DNA sequences (and those of other *cis*-acting elements) are referred to herein as

"decoys" or "molecular decoys". The competition decreases occupancy of *trans*-acting DNA-binding proteins on their cognate *cis*-acting elements, thereby down-regulating the synthesis of nicotine biosynthesis enzymes.

The present invention also provides DNA molecules of *cis*-acting elements of QPTase or PMTase, and vectors comprising those DNA molecules, as well as transgenic plant cells and plants transformed with those DNA molecules and vectors. Transgenic tobacco cells and plants of this invention are characterized by lower nicotine content than untransformed control tobacco cells and plants.

Tobacco plants with low levels of nicotine production, or substantially no nicotine production, are attractive as recipients for transgenes expressing commercially valuable products such as pharmaceuticals, cosmetic components, or food additives. Tobacco is attractive as a recipient plant for a transgene encoding a desirable product, as tobacco is easily genetically engineered and produces a very large biomass per acre; tobacco plants with reduced resources devoted to nicotine production accordingly will have more resources available for production of transgene products. Methods of transforming tobacco with transgenes producing desired products are known in the art; any suitable technique may be utilized with the low nicotine tobacco plants of the present invention.

Tobacco plants according to the present invention with reduced QPTase and PMTase expression and reduced nicotine levels will be desirable in the production of tobacco products having reduced nicotine and/or TSNA content. The tobacco plants described herein are suitable for conventional growing and harvesting techniques (e.g. topping or no topping, bagging the flowers or not bagging the flowers, cultivation in manure rich soil or without manure) and the harvested leaves and stems are suitable for use in any traditional tobacco product including, but not limited to, pipe, cigar and cigarette tobacco, and chewing tobacco in any form including leaf tobacco, shredded tobacco, or cut tobacco.

It is also contemplated that the low nicotine and/or TSNA tobacco described herein can be processed and blended with conventional tobacco so as to create a wide-range of tobacco products with varying amounts of nicotine and/or nitrosamines. These blended tobacco products can be used in tobacco product cessation programs so as to slowly move a consumer from a high nicotine and TSNA product to a low

nicotine and TSNA product. For example, a smoker can begin the program smoking blended cigarettes having 10mg of nicotine and 1.5mg of nitrosamine, gradually move to smoking cigarettes with 7mg of nicotine and 1mg of nitrosamine, followed by cigarettes having 5.0mg nicotine and 0.5mg nitrosamine, followed by cigarettes having 2.0mg nicotine and 0.25mg nitrosamine, followed by cigarettes having 1.0mg nicotine and no TSNA until the consumer decides to smoke only the cigarettes having virtually no nicotine and nitrosamines or quitting smoking altogether. Accordingly, the blended cigarettes described herein provide the basis for an approach to reduce the carcinogenic potential in a human in a step-wise fashion.

1. Nucleic acids encoding *cis*-acting elements such as *Nic* gene product responsive elements.

Any of a variety of *cis*-acting elements can be used in carrying out the present invention, depending upon the particular application of the present invention. Examples of *cis*-acting elements (and corresponding transcription factors) that may be used, alone or in combination with one another, in practicing the present invention include, but are not limited to, AS-1 and ASF-1 (see U.S. Patents 4,990,607 and 5,223,419), the AATT repeat element and PABF (see U.S. Patents 5,834,236 and 6,191,258), a wounding-responsive *cis*-acting element from potato (Siebert et al., *Plant Cell* 1:961-8 (1989)), an embryo-specific *cis*-acting element from bean (Bustos et al, *Plant Cell* 1:839-853 (1989)), a root-specific *cis*-acting element from the tobacco RB7 promoter (US patent 5,459,252 and Yamamoto et al., *Plant Cell* 3:371-382 (1991)), a positive poly(dA-dT) regulatory element and binding protein and negative CCCAA repeat element and binding protein (Wang et al., *Mol. Cell Biol.* 12:3399-3406 (1992)), a root-tip regulatory element from the tobacco phytochrome A1 promoter of tobacco (Adam et al., *Plant Mol Biol* 29:983-993 (1995)), an anaerobiosis-reponsive element from the maize glyceraldehyde-3-phosphate dehydrogenase 4 gene (Geffers et al., *Plant Mol Biol* 43:11-21 (2000)), and a seed-specific regulatory region from an *Arabidopis oleosin* gene (see US patent 5,792,922), all of which are hereby expressly incorporated by reference in their entireties.

The status of the art is such that large databases list identified cis-acting regulatory regions (e.g., Plant Cis-acting Regulatory elements, "PLACE", with some

1,340 entries, see http://www.dna.affrc.go.jp/hotdocs/PLACE/, and Plant Cis-acting Regulatory Elements "PlantCARE", which lists some 159 plant promoter, see http://sphinx.rug.ac.be:8080/PlantCARE/. The listed cis-acting regulatory elements in these databases and the cis-acting regulatory elements that are provided in Raumbauts et al., *Nucleic acids Research* 27:295-296 (1999), and Higo et al., *Nucleic acids Research* 27:297-300 (1999) can be used with embodiments of the invention. Accordingly, the databases and references above are hereby expressly incorporated by reference in their entireties. Additional examples of cis-acting regulatory regions, which can be used with embodiments of the invention include: Lacombe E, Van Doorsselaere J, Boerjan W, Boudet AM, Grima-Pettenati J, Characterization of cis-elements required for vascular expression of the cinnamoyl CoA reductase gene and for protein-DNA complex formation Plant J 23: 663-676 (2000); Tilly JJ, Allen DW, Jack T The CArG boxes in the promoter of the Arabidopsis floral organ identity gene APETALA3 mediate diverse regulatory effects Development 125: 1647-1657 (1998); Cordes S., Deikman J., Margossian L.J., Fischer R.L. Interaction of a developmentally regulated DNA-binding factor with sites flanking two different fruit-ripening genes from tomato Plant Cell 1(10):1025-1034 (1989); Hagen G., Martin G., Li Y., Guilfoyle T. Auxin-induced expression of the soybean GH3 promoter in transgenic tobacco plants" Plant Mol. Biol. 17:567-569 (1991); Pastuglia M., Roby D., Dumas C., Cock J.M., Rapid induction by wounding and bacterial infection of an S gene family receptor-like kinase in Brassica oleracea, Plant Cell 9:1-13 (1997); Grierson C, Du JS, Zabala MT, Beggs K, Smith C, Holdsworth M, Bevan M Separate cis sequences and trans factors direct metabolic and developmental regulation of a potato tuber storage protein gene Plant J 5:815-826 (1994); MBSI, Petunia hybrida MYB binding site involved in flavonoid biosynthetic gene regulation, Koes R.E., Spelt C.E., van Den Elzen P.J.M., Mol J.N.M. Cloning and molecular characterization of the chalcone synthase multigene family of Petunia hybrida, Gene 81:245-257 (1989); Inaba T., Nagano Y., Sakakibara T., Sasaki Y., Identification of a cis-regulatory element involved in phytochrome down-regulated expression of the pea small GTPase gene pra2, Plant Physiol. 120:491-499(1999); DRE, Arabidopsis thaliana cis-acting element involved in dehydration, low-temperature, salt stresses, Yamaguchi-Shinozaki K., Shinozaki K., Arabidopsis DNA encoding two desiccation-responsive

rd29 genes, Plant Physiol. 101:1119-1120 (1993); Rushton P.J., Torres J.T., Parniske M., Wernert P., Hahlbrock K., Somssich I.E., Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes, EMBO J. 15(20):5690-5700 (1996); MSA-like cis-acting element involved in cell cycle regulation, Ito M., Criqui M.C., Sakabe M., Ohno T., Hata S., Kouchi H., Hashimoto J, Fukuda H., Komamine A., Watanabe A. Cell-cycle regulated transcription of A- and B-type plant cyclin genes in synchronous cultures, Plant J. 11:983-992 (1997), all of which are hereby expressly incorporated by reference in their entireties. In general, preferred are elements that are not critical to sustaining life of the host cell (*e.g.*, not associated with "housekeeping genes" that are essential for basic cell functions), but are functionally associated with regulating transcription of a gene or family of genes that result in a non-lethal phenotypic change in the plant.

Nic gene product responsive elements can be isolated by screening the promoter region of genes that are transcriptionally activated by the *Nic* gene product in the same manner as described herein, or can be identified by hybridization to **SEQ ID NO: 1** herein and subsequent screening for the ability to bind the *Nic* gene product in the manner described below.

Nucleic acid sequences employed in carrying out the present invention include naturally occurring or synthetic fragments with sequence similarity to **SEQ ID NO:1** or a fragment thereof consisting of, desirably, at least 20-455 consecutive nucleotides, preferably, at least 30-400 consecutive nucleotides, more preferably, 50-350 consecutive nucleotides, and, most preferably, 100-300 or 200-400 consecutive nucleotides. This definition is intended to encompass natural allelic variations of DNA of **SEQ ID NO:1** or said fragments. Thus, DNA sequences that hybridize to DNA of **SEQ ID NO:1,** or the complement thereof, may also be employed in carrying out the present invention. Preferred embodiments include fragments of **SEQ ID NO: 1**, or other *Nic* gene product responsive elements (*i.e.*, elements that bind to the complement of **SEQ ID NO:1**), that retain the ability to bind the *Nic* gene product. Such fragments will, in general, be continuous fragments or portions of the naturally occurring construct that are at least 20, 40 or 60 nucleotides in length. Conditions which permit other DNA sequences with sequence similarity to **SEQ ID NO:1** can be determined in a routine manner. For example, hybridization of such sequences may be

carried out under conditions of reduced stringency or even stringent conditions (e.g., conditions represented by a wash stringency of 0.3 M NaCl, 0.03 M sodium citrate, 0.1% SDS at 60°C or even 70°C to DNA with the sequence given herein as **SEQ ID NO:1** using a standard *in situ* hybridization assay. *See* J. Sambrook et al., Molecular Cloning, A Laboratory Manual (2d Ed. 1989)(Cold Spring Harbor Laboratory)). In general, such sequences will be at least 65% similar, 75% similar, 80% similar, 85% similar, 90% similar, or even 95% similar, or more, with the sequence given herein as **SEQ ID NO:1**. Determinations of sequence similarity are made with the two sequences aligned for maximum matching; gaps in either of the two sequences being matched are allowed in maximizing matching. Gap lengths of 10 or less are preferred, gap lengths of 5 or less are more preferred, and gap lengths of 2 or less still more preferred.

The DNA sequence of the present invention may consist essentially of the sequence provided herein (**SEQ ID NO:1**), or equivalent nucleotide sequences representing alleles or polymorphic variants of these genes, or coding regions thereof.

Use of the phrase "substantial sequence similarity" in the present specification and claims means that DNA, RNA or amino acid sequences which have slight and non-consequential sequence variations from the actual sequences disclosed and claimed herein are considered to be equivalent to the sequences of the present invention. In this regard, "slight and non-consequential sequence variations" mean that "similar" sequences (i.e., the sequences that have substantial sequence similarity with the DNA, RNA, or proteins disclosed and claimed herein) will be functionally equivalent to the sequences disclosed and claimed in the present invention. Functionally equivalent sequences will function in substantially the same manner to produce substantially the same compositions as the nucleic acid and amino acid compositions disclosed and claimed herein.

Additional nucleic acid sequence for use with aspects of the invention include the *Nic* gene product responsive element, which can be obtained from the sequence disclosed in U.S. Patent No. 5,459,252, herein expressly incorporated by reference in its entirety. In some embodiments, the *Nic* gene product responsive element resides between -1000 and -600 or -700 bp of the *NtQPT1* promoter. Accordingly, some embodiments involve a 300-400 nucleotide long fragment of the *NtQPT1* promoter

that corresponds to the sequence of the *NtQPT1* promoter between -1000 and -600 or -700, as disclosed in U.S. Patent No. 5,459,252.

DNA sequences provided herein can be transformed into a variety of host cells, as discussed below. A variety of suitable host cells, having desirable growth and handling properties, are readily available in the art.

Use of the phrase "isolated" or "substantially pure" in the present specification and claims as a modifier of DNA, RNA, polypeptides or proteins means that the DNA, RNA, polypeptides or proteins so designated have been separated from their *in vivo* cellular environments through the efforts of human beings. As used herein, a "native DNA sequence" or "natural DNA sequence" means a DNA sequence which can be isolated from non-transgenic cells or tissue. Native DNA sequences are those which have not been artificially altered, such as by site-directed mutagenesis. Once native DNA sequences are identified, DNA molecules having native DNA sequences may be chemically synthesized or produced using recombinant DNA procedures as are known in the art. As used herein, a native plant DNA sequence is that which can be isolated from non-transgenic plant cells or tissue. As used herein, a native tobacco DNA sequence is that which can be isolated from non-transgenic tobacco cells or tissue

2. Nucleic acid constructs and transfer vectors.

Nucleic acid constructs, or "cassettes," of the present invention include a *cis*-acting element such as a *Nic* gene product responsive element as described above, typically as a recombinant construct in a linear or circular nucleic acid that serves as a transfer vector for introducing the *Nic* gene product into plant cells.

The construct or cassette may be provided in a DNA construct which also has at least one replication system. For convenience, it is common to have a replication system functional in *Escherichia coli,* such as ColE1, pSCl0l, pACYC184, or the like. In this manner, at each stage after each manipulation, the resulting construct may be cloned, sequenced, and the correctness of the manipulation determined. In addition, or in place of the *E. coli* replication system, a broad host-range replication system may be employed, such as the replication systems of the P-1 incompatibility plasmids, e.g., pRK290. In addition to the replication system, there will frequently be at least one

marker present, which may be useful in one or more hosts, or different markers for individual hosts. That is, one marker may be employed for selection in a prokaryotic host, while another marker may be employed for selection in an eukaryotic host, particularly the plant host. The markers may be protection against a biocide, such as antibiotics, toxins, heavy metals, or the like; may provide complementation, by imparting prototrophy to an auxotrophic host; or may provide a visible phenotype through the production of a novel compound in the plant.

Nucleic acid constructs of the present invention may include one or more matrix attachment regions positioned 5', 3', or both 5' and 3' to the *cis*-acting element(s) to enhance the stability and/or hereditability thereof, as described in U.S. Patents Nos. 5,773,689 to Thompson et al., 5,773,695 to Thompson et al., 6,245,974 to Michalowski et al., 6,239,328 to Thompson et al., 6,100,448 to Thompson et al., and 6,037,525 to Thompson et al., the disclosures of which are incorporated by reference herein in their entirety.

The various fragments comprising the various constructs, cassettes, markers, and the like may be introduced consecutively by restriction enzyme cleavage of an appropriate replication system, and insertion of the particular construct or fragment into the available site. After ligation and cloning the DNA construct may be isolated for further manipulation. All of these techniques are amply exemplified in the literature as exemplified by J. Sambrook et al., Molecular Cloning, A Laboratory Manual (2d Ed. 1989)(Cold Spring Harbor Laboratory).

Vectors which may be used to transform plant tissue with nucleic acid constructs of the present invention include both ballistic vectors and *Agrobacterium* vectors, as well as vectors suitable for DNA-mediated transformation. These are discussed in greater detail below.

The nucleic acid constructs molecules and vectors used to produce the transformed cells and plants of this invention may further comprise a dominant selectable marker gene. Suitable dominant selectable markers for use in tobacco include, *inter alia*, antibiotic resistance genes encoding neomycin phosphotransferase (NPTII), hygromycin phosphotransferase (HPT), and chloramphenicol acetyltransferase (CAT). Another well-known dominant selectable marker suitable for use in tobacco is a mutant dihydrofolate reductase gene that encodes methotrexate-

resistant dihydrofolate reductase. DNA vectors containing suitable antibiotic resistance genes, and the corresponding antibiotics, are commercially available.

3. Plant transformation, regeneration and propagation.

Transformed cells are selected out of the surrounding population of non-transformed cells by placing the mixed population of cells into a culture medium containing an appropriate concentration of the antibiotic (or other compound normally toxic to the cells) against which the chosen dominant selectable marker gene product confers resistance. Thus, only those plant cells that have been transformed will survive and multiply.

Methods of making recombinant plants of the present invention, in general, involve first providing a plant cell capable of regeneration (the plant cell typically residing in a tissue capable of regeneration). The plant cell is then transformed with a DNA construct comprising a cassette of the present invention (as described herein) and a recombinant plant is regenerated from the transformed plant cell. As explained below, the transforming step is carried out by techniques as are known in the art, including but not limited to bombarding the plant cell with microparticles carrying the transcription cassette, infecting the cell with an *Agrobacterium tumefaciens* containing a Ti plasmid carrying the cassette, or any other technique suitable for the production of a transgenic plant.

Microparticles carrying a DNA construct of the present invention, which microparticle is suitable for the ballistic transformation of a plant cell, are also useful for making transformed plants of the present invention. The microparticle is propelled into a plant cell to produce a transformed plant cell, and a plant is regenerated from the transformed plant cell. Any suitable ballistic cell transformation methodology and apparatus can be used in practicing the present invention. Exemplary apparatus and procedures are disclosed in Sanford and Wolf, U.S. Patent No. 4,945,050, and in Christou et al., U.S. Patent No. 5,015,580 (the disclosures of all U.S. Patent References cited herein are to be incorporated herein by reference). When using ballistic transformation procedures, the cassette may be incorporated into a plasmid capable of replicating in or integrating into the cell to be transformed. Examples of microparticles suitable for use in such systems include 1 to 5 micrometer (μm) gold

spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

Numerous *Agrobacterium* vector systems useful in carrying out the present invention are known. For example, U.S. Patent No. 4,459,355 discloses a method for transforming susceptible plants, including dicots, with an *Agrobacterium* strain containing the Ti plasmid. The transformation of woody plants with an *Agrobacterium* vector is disclosed in U.S. Patent No. 4,795,855. Further, U.S. Patent No. 4,940,838 to Schilperoort et al. discloses a binary *Agrobacterium* vector (i.e., one in which the *Agrobacterium* contains one plasmid having the vir region of a Ti plasmid but no T region, and a second plasmid having a T region but no vir region) useful in carrying out the present invention.

As a high copy number of decoy sequences must typically be present in the genome, tandem copies of the *cis*-acting element(s) could be inserted into an *Agrobacterium* vector, but the preferred method of plant transformation is by particle bombardment which introduces multiple copies of the transgenic DNA into the plant genome. The actual number of the *cis*-acting element (whether each individually present on a vector such as a plasmid, counting multiple copies on a single vector or plasmid, or combinations thereof) that must be inserted into the host cells (and progeny or daughter cells thereof) to obtain increased or decreased levels of the protein of interest in the cells and plants of the invention will depend in part upon the particular element, but in general will be at least 20, 30 or 50 to about 500, 1,000 or 2,000, or more.

Plant species may be transformed with the DNA construct of the present invention by the DNA-mediated transformation of plant cell protoplasts and subsequent regeneration of the plant from the transformed protoplasts in accordance with procedures well known in the art. Fusion of tobacco protoplasts with DNA-containing liposomes or via electroporation is known in the art. (Shillito et al., "Direct Gene Transfer to Protoplasts of Dicotyledonous and Monocotyledonous Plants by a Number of Methods, Including Electroporation", *Methods in Enzymology* **153**, 313-36 (1987)).

As used herein, "transformation" refers to the introduction of exogenous DNA into cells, so as to produce transgenic cells stably transformed with the exogenous

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DNA. By "stably transformed" is meant that the exogeneous nucleic acid is passed to daughter or progeny cells of the initially transformed cells, and preferably passed to or inherited by progeny plants of the transformed plants (including sexually and asexually reproduced progeny plants).

Transformed cells are induced to regenerate intact plants through application of cell and tissue culture techniques that are well known in the art. The method of plant regeneration is chosen so as to be compatible with the method of transformation. After regeneration of transgenic plants from transformed cells, the introduced DNA sequence is readily transferred to other plant varieties through conventional plant breeding practices and without undue experimentation.

For example, to analyze the segregation of the transgenic DNA, regenerated transformed plants (R_0) may be grown to maturity, tested for levels of the protein of interest, and selfed to produce R_1 plants. A percentage of R_1 plants carrying the transgenic DNA are homozygous for the transgenic DNA. To identify homozygous R_1 plants, transgenic R_1 plants are grown to maturity and selfed. Homozygous R_1 plants will produce R_2 progeny where each progeny plant carries the transgenic DNA; progeny of heterozygous R_1 plants will segregate 3:1.

Nicotine serves as a natural pesticide which helps protect tobacco plants from damage by pests. It may therefore be desirable to additionally transform low or no nicotine plants produced by the present methods with a transgene (such as *Bacillus thuringiensis)* that will confer additional insect protection.

A preferred plant for use in the present invention is any species of the genus *Nicotiana,* or tobacco, including *N tabacum, N rustica and N glutinosa.* Any strain or variety of tobacco may be used.

Any plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a vector of the present invention. The term "organogenesis," as used herein, means a process by which shoots and roots are developed sequentially from meristematic centers; the term "embryogenesis," as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed.

Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, callus tissue, existing meristematic tissue (e.g., apical meristems, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

Plants of the present invention may take a variety of forms. The plants may be chimeras of transformed cells and non-transformed cells; the plants may be clonal transformants (e.g., all cells transformed to contain the cassette); the plants may comprise grafts of transformed and untransformed tissues (e.g., a transformed root stock grafted to an untransformed scion in citrus species). The transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, first generation (or T1) transformed plants may be selfed to give homozygous second generation (or T2) transformed plants, and the T2 plants further propagated through classical breeding techniques. A dominant selectable marker (such as *npt*II) can be associated with the construct to assist in breeding.

In some preferred embodiments of the invention, to help insure that a sufficient number of decoy or *cis*-acting elements are inserted in cells and retained over many cell divisions to produce a transgenic plant with altered levels of protein or proteins therein, biolistic transformation is used as described above, circular DNA or plasmids are used to carry the *cis*-acting decoy segments as described above, the circular DNA or plasmids that are used are relatively small (*e.g.*, they consist of less than 10,000 or less than 6,000 base pairs), and a high molar ratio of the *cis*-acting element to selectable marker (*e.g.*, 10 to 1) is inserted into the host cells.

As used herein, a crop comprises a plurality of plants of the present invention, and of the same genus, planted together in an agricultural field. By "agricultural field" is meant a common plot of soil or a greenhouse. Thus, the present invention provides a method of producing a crop of plants having altered levels of a protein of interest, (*e.g.*, QPTase and PMTase activity and thus having decreased nicotine levels), compared to a similar crop of non-transformed plants of the same species and variety.

While the invention describes methods to reduce nicotine levels in transgenic tobacco, this method can also be employed to phenocopy mutations in *trans*-acting transcriptional activators and repressors without cloning their respective genomic loci.

Promoter regions of a gene can be analyzed, using technology known by those skilled in the art, to define regions of the promoter that respond to transcription factors. Typically, this is done by deletion analysis of the promoter. Nested deletions of the promoter are fused to a reporter gene and expression of the reporter gene is monitored in transgenic organisms. Isolation of transcription factors using current technologies is very difficult; the present invention circumvents the necessity of cloning the cognate transcription factors for applications in which it is desirable to disrupt any set of genes that are coordinately regulated by one or more transcriptional activators. Conversely, the process would up-regulate the expression of any set of genes that are coordinately regulated by one or more transcriptional repressor.

As noted above, the present invention could be employed to disrupt gene expression and down-regulate the expression of a protein of interest that is under the control of a *cis*-acting activating element in a variety of host cells, including plant (particularly vascular plant such as monocot and dicot), animal (avian, mammalian), fungi, or bacteria cells, both *in vivo* and *in vitro*. In bacteria and fungi, multicopy plasmids can be used to increase copies of molecular decoy present in the cell.

The examples which follow are set forth to illustrate the present invention, and are not to be construed as limiting thereof.

EXAMPLE 1

Localization of *cis*-acting Element in *NtQPT1* Promoter

To characterize the minimal sequence required for the *NtQPT1* *cis*-acting element, the promoter region of the *NtQPT1* gene was isolated, truncated at the 5' end, and fused to the gene encoding β-glucuronidase (GUS) to assess function as a specific enhancer of nicotine production. The *NtQPT1* gene was isolated and sequenced. The start of the transcript was determined by comparing the *TobRD2* cDNA sequence to the genomic locus sequence. Sequence located 5' of the transcription start site was defined as promoter sequence. Using PCR primers and the promoter as a template, truncations were made at the 5' end of the promoter to determine minimal *cis*-acting enhancer sequence (*see* **Figure 2**). The truncations were fused to the *uidA* gene, which encodes GUS. The fusion gene was inserted into

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a vector and transformed by standard methods of ballistic transformation into *Nicotiana tabacum* Burley 21.

GUS activity was assessed by dividing plants into roots, stems, and leaves. Each plant tissue, transformed with a different *NtQPT1* truncation construct, was ground with a morter and pestle, proteins were extracted with $NaPO_4$ buffer, pH 7.0, X-Glc (100 ug/mL) was added, and the assay was carried out at 37°C for 30 min. GUS activity was measured at 595 nm). For each construct at least 20 independent transformants were assayed. A mean and standard deviation was determined. GUS activity in truncations was compared to a CaMV 35S–GUS fusion and a promoterless GUS (pBI101) control. Maximal GUS activity, representing *NtQPT1* expression, was obtained when –586 to –2000 bp was fused to the *uidA* gene (**Figure 3**). Shorter promoters from –1 to –586 did not support high levels of *uidA* expression. Therefore, the *NtQPT1* *cis*-acting element is located between –586 and –2000 bp 5' of the transcription start site.

EXAMPLE 2

Localization of *Nic* Gene Product Binding Site in *NtQPT1* Promoter

The *NtQPT1* promoter deletion series fused to the *uidA* reporter gene (encoding GUS) was transformed into *nic*$^-$/*nic*$^-$ homozygous *N. tabacum* plants. R_0 transformants having the transgenic DNA at a single locus were crossed with *nic*$^-$/*nic*$^-$ or *Nic*$^+$/*Nic*$^+$ homozygous plants to give homozygous (*nic*$^-$/*nic*$^-$) and heterozygous (*Nic*$^+$/*nic*$^-$) progeny carrying the transgene (*NtQPT1* promoter-*GUS*) at the same chromosomal location. GUS activity was quantified in multiple progeny from multiple, independent transformants and compared between Nic$^+$ and nic$^-$ phenotypes (**TABLE 1**). Ratios greater than 1.5 were determined to contain the *cis*-acting elements responding to *Nic* gene product activation.

TABLE 1. Regulation of *NtQPT1* Promoter-Directed GUS Expression by the *Nic* Gene Products in Tobacco.

Promoter	Independent Transformants	GUS activity in Nic$^+$/nic$^-$ [2]	GUS activity in nic$^-$/nic$^-$ [2]	GUS ratio of Nic/nic
2.0 (2010)[1]	2	111.7 (7)	21.2 (5)	5.3

		46.6 (6)	11.9 (8)	5.6
1.3 (1306)[1]	2	92.4 (6) 93.9 (7)	16.6 (4) 12.9 (5)	5.5 7.3
1.0 (1042)[1]	3	55.7 (6) 74.1 (6) 78.0 (5)	18.3 (4) 27.5 (7) 17.2 (7)	3.0 2.7 4.5
734	1	5.5 (5)	3.5 (5)	1.5
586	3	47.55 (5) 24.3 (3) 29.1 (33)	44.9 (5) 16.0 (36) 30.3 (19)	1.06 1.5 0.97
535	3	71.6 (10) 54.0 (5) 51.9 (5)	50.3 (5) 40.7 (3) 67.8 (5)	1.4 1.3 0.8
CaMV 35S	4	32.7 (4) 44.8 (6) 54.8 (5) 9.7 (4)	19.6 (4) 47.6 (3) 40.6 (5) 8.6 (3)	1.7 0.94 1.3 1.1

GUS activity is expressed as pmol MU/μg protein/min. [1]Actual promoter size (bp) is indicated in parenthesis. [2]Number in parenthesis indicates the number of plants tested.

These experiments demonstrated that binding of *Nic* gene products is located between approximately –1000 and -600 or -700 bp of the *NtQPT1* promoter as determined by GUS activity in *Nic*$^+$/*nic*$^-$ and *nic*$^-$/*nic*$^-$ plants.

EXAMPLE 3

Regulation of *NtQPT1* Gene Expression Using Molecular Decoys

Nucleotide sequence located between –1000 and -600 or -700 bp of the *NtQPT1* promoter is inserted in tandem arrays into a plant-*Agrobacterium* shuttle vector and subsequently transformed into tobacco via methods known to one skilled in the art. Plants stably transformed with said vector are assessed for the level of expression of *NtQPT1* and for nicotine and/or TSNA content. These experiments will demonstrate that tobacco transformed with molecular decoys that interact with *Nic* gene products will exhibit a reduced amount of nicotine and/or TSNA. Plants with multiple tandem insertions of the molecular decoy which have reduced *NtQPT1* expression and reduced nicotine levels are used for expression of commercially valuable products and production of tobacco products having reduced nicotine and/or TSNA content.

EXAMPLE 4

Regulation of ASF-1 Binding Using a TGACG Molecular Decoy

The nucleotide sequence TGACG is inserted in tandem arrays into a plant-*Agrobacterium* shuttle vector and transformed into a plant such as pea via methods known to one skilled in the art. Plants stably transformed with said vector have reduced binding activity of *trans*-acting DNA binding factor ASF-1 which recognizes the sequence motif TGACG which is found in plant genes such as histone genes (Mikami et al., (1987) FEBS Lett. 223:273); enzyme genes for agropine biosynthesis (Velten et al., EMBO J. 3:2723-30); the octopine synthase gene (Ellis et al., EMBO J. 6:3203); and the mannopine synthase gene (DeRita and Gelvin, (1987) Mol. Gen. Genet. 207:233); as well as the CaMV35S gene, histone H3 gene and nopaline synthase gene.

EXAMPLE 5

Regulation of Spatial and Temporal Expression of Beta-Phaseolin Using Molecular Decoys

The nucleotide sequence corresponding to UAS1 (-295 to –109) of the beta-phaseolin gene is inserted in tandem arrays into a plant-*Agrobacterium* shuttle vector and transformed into a bean plant via methods known to one skilled in the art. Plants stably transformed with said vector have reduced binding activity of *trans*-acting DNA binding factor PvALF which recognizes the sequences CATGCAAA and CATGCATG located in UAS1 (Bobb et al. (1997) *Nucleic Acids Res* **25**(3):641-7). Plants with reduced binding of PvALF would have reduced expression of seed-specific expression of beta-phaseolin primarily in cotyledons and shoot meristem (Bustos et al. (1991) *EMBO J.* **10**(6):1469-1479).

Transformation of tandem arrays of nucleotide sequences corresponding to the vicilin-box (GCCACCTCAA; **SEQ ID NO:2**) and site B (CACACGTCAA; **SEQ ID NO:3**) of the beta-phaseolin gene into a bean plant results in the reduced binding activity of *trans*-acting DNA binding factors ROM1 and ROM2 leading to premature onset of beta-phaseolin expression. ROM1 and ROM2 proteins function as repressors of beta-phaseolin and phytohemagglutinin L-subunit expression to block onset of seed

maturation (U.S. Patent No. 6,160,202 to Bustos; Chern et al. (1996) *Plant Cell* **8**:305-321; Chern et al. (1996) *Plant J.* **10**:135-148).

EXAMPLE 6

Regulation of Plant Gene Expression Using Molecular Decoys

Transformation of tobacco plants with tandem arrays of the root-specific *cis*-acting element from the tobacco RB7 promoter (US Patent No. 5,459,252 to Conkling et al.; Yamamoto et al. (1991) *Plant Cell* **12**:3399-3406), which codes for a structural gene, results in the reduced binding activity of the *trans*-acting DNA binding factor of the RB7 *cis*-acting element.

Likewise, similar tandem arrays of the following *cis*-elements are transformed into the plants to reduce binding activity of the corresponding *trans*-acting DNA binding factors: the AATT *cis*-acting repeat element and its corresponding PABF *trans*-acting factor (see US Patent Nos. 5,834,236 and 6,191,258); the positive poly(dA-dT) regulatory element and binding protein and negative CCAA repeat element and binding protein (Wang et al. (1992) *Mol. Cell Biol.* **12**:3399-3406); the root-tip regulatory element from the tobacco phytochrome A1 promoter of tobacco (Adam et al. (1995) *Plant Mol. Biol.* **29**:983-993); the anaerobiosis-responsive element from the maize glyceraldehyde-3-phosphate dehydrogenase 4 gene (Geffers et al. (2000) *Plant Mol. Biol.* **43**:11-21); and the seed-specific regulatory region from an *Arabidopsis* oleosin gene (see US Patent No. 5,792,922).

EXAMPLE 7

Tobacco Having Reduced Nicotine and/or TSNA Levels Generated Using Molecular Decoys

Multiple copies of an approximately 300 or 400 nucleotide long fragment of the *NtQPT1* promoter (e.g., including nucleotide sequence located between –1000 and -600 or -700 bp of the *NtQPT1* promoter, such as **SEQ ID NO:1**) are affixed to microparticles (e.g., by precipitation) that are suitable for the ballistic transformation of a plant cell (e.g., 1 to 5 μm gold spheres). The microparticles are propelled into tobacco plant cells (e.g., Burley 21 LA) so as to produce transformed plant cells, and plants are regenerated from the transformed plant cells. Burley 21 LA is a variety of

Burley 21 with substantially reduced levels of nicotine as compared with Burley 21 (*i.e.*, Burley 21 LA has 8% the nicotine levels of Burley 21, see Legg *et al.*, *Can J Genet Cytol*, 13:287-91 (1971); Legg *et al.*, *J Hered*, 60:213-17 (1969))

Any suitable ballistic cell transformation methodology and apparatus can be used. Exemplary apparatus and procedures are disclosed in Sanford and Wolf, U.S. Patent No. 4,945,050, and in Christou et al., U.S. Patent No. 5,015,580, both of which are herein expressly incorporated by reference in their entireties. Optionally, the transformed nucleic acid can include a gene encoding a selectable marker (e.g., a marker that allows for positive or negative selection of transformants) or the molecular decoys can be co-transferred with a selectable marker gene. In this manner, positive transformants can be easily identified.

Transformed cells, tissues, and seedlings are grown on Murashige-Skoog (MS) medium (with or without the selection compound, e.g., antibiotic, depending on whether a selectable marker was used. One-hundred independent transformants of Burley 21 LA (T_0) are allowed to self. Progeny of the selfed plants (T_1) are germinated. Nicotine levels of T_1 progeny are measured qualitatively using a micro-assay technique. Approximately ~200 mg fresh tobacco leaves are collected and ground in 1 ml extraction solution. (Extraction solution: 1 ml Acetic acid in 100 ml H_2O) Homogenate is centrifuged for 5 min at 14,000 x g and supernatant removed to a clean tube, to which the following reagents are added: 100 µL NH_4OAC (5 g/100 ml H_2O + 50 µL Brij 35); 500 µL Cyanogen Bromide (Sigma C-6388, 0.5 g/100 ml H_2O + 50 µL Brij 35); 400 µL Aniline (0.3 ml buffered Aniline in 100 ml NH_4OAC + 50 µL Brij 35). A nicotine standard stock solution of 10 mg/ml in extraction solution is prepared and diluted to create a standard series for calibration. Absorbance at 460 nm is read and nicotine content of test samples are determined using the standard calibration curve.

T_1 progeny that have less than 10% of the nicotine levels of the Burley 21 LA parent are allowed to self to produce T_2 progeny. Homozygous T_2 progeny are then identified. Nicotine levels in homozygous and heterozygous T_2 progeny are also qualitatively determined using the micro-assay. Leaf samples of homozygous T_2 progeny can also be sent to the Southern Research and Testing Laboratory in Wilson, NC for quantitative analysis of nicotine levels using Gas Chromatography/Flame

Ionization Detection (GC/FID). Homozygous T_2 progeny of will have nicotine levels that are substantially reduced as compared to the untransformed tobacco (e.g., ~70 ppm). Because the nicotine levels in such plants are substantially reduced, the TSNA levels in these plants is concomitantly reduced.

These experiments will demonstrate that tobacco transformed with molecular decoys that interact with *Nic* gene products will exhibit a reduced amount of nicotine and/or TSNA. Plants with multiple tandem insertions of the molecular decoy which have reduced *NtQPT1* expression and reduced nicotine levels are used for expression of commercially valuable products and production of tobacco products having reduced nicotine and/or TSNA content.

EXAMPLE 8

Low Nicotine and TSNA blended Tobacco

The following example describes several ways to create tobacco products having specific amounts of nicotine and/or TSNAs through blending. Some blending approaches begin with tobacco prepared from varieties that have extremely low amounts of nicotine and/or TSNAs. By blending prepared tobacco from a low nicotine/TSNA variety (e.g., undetectable levels of nicotine and/or TSNAs) with a conventional tobacco (e.g., Burley, which has 30,000 parts per million (ppm) nicotine and 8,000 parts per billion (ppb) TSNA; Flue-Cured, which has 20,000 ppm nicotine and 300 ppb TSNA; and Oriental, which has 10,000 ppm nicotine and 100 ppb TSNA), tobacco products having virtually any desired amount of nicotine and/or TSNAs can be manufactured. Tobacco products having various amounts of nicotine and/or TSNAs can be incorporated into tobacco use cessation kits and programs to help tobacco users reduce or eliminate their dependence on nicotine and reduce the carcinogenic potential.

For example, a step 1 tobacco product can be comprised of approximately 25% low nicotine/TSNA tobacco and 75% conventional tobacco; a step 2 tobacco product can be comprised of approximately 50% low nicotine/TSNA tobacco and 50% conventional tobacco; a step 3 tobacco product can be comprised of approximately 75% low nicotine/TSNA tobacco and 25% conventional tobacco; and a step 4 tobacco product can be comprised of approximately 100% low nicotine/TSNA

tobacco and 0% conventional tobacco. A tobacco use cessation kit can comprise an amount of tobacco product from each of the aforementioned blends to satisfy a consumer for a single month program. That is, if the consumer is a one pack a day smoker, for example, a single month kit would provide 7 packs from each step, a total of 28 packs of cigarettes. Each tobacco use cessation kit would include a set of instructions that specifically guide the consumer through the step-by-step process. Of course, tobacco products having specific amounts of nicotine and/or TSNAs would be made available in conveniently sized amounts (e.g., boxes of cigars, packs of cigarettes, tins of snuff, and pouches or twists of chew) so that consumers could select the amount of nicotine and/or TSNA they individually desire. There are many ways to obtain various low nicotine/low TSNA tobacco blends using the teachings described herein and the following is intended merely to guide one of skill in the art to one possible approach.

To obtain a step 1 tobacco product, which is a 25% low nicotine/TSNA blend, prepared tobacco from an approximately 0 ppm nicotine/TSNA tobacco can be mixed with conventional Burley, Flue-cured, or Oriental in a 25%/75% ratio respectively to obtain a Burly tobacco product having 22,500 ppm nicotine and 6,000 ppb TSNA, a Flue-cured product having 15,000 ppm nicotine and 225 ppb TSNA, and an Oriental product having 7,500 ppm nicotine and 75 ppb TSNA. Similarly, to obtain a step 2 product, which is 50% low nicotine/TSNA blend, prepared tobacco from an approximately 0 ppm nicotine/TSNA tobacco can be mixed with conventional Burley, Flue-cured, or Oriental in a 50%/50% ratio respectively to obtain a Burly tobacco product having 15,000 ppm nicotine and 4,000 ppb TSNA, a Flue-cured product having 10,000 ppm nicotine and 150 ppb TSNA, and an Oriental product having 5000 ppm nicotine and 50 ppb TSNA. Further, a step 3 product, which is a 75%/25% low nicotine/TSNA blend, prepared tobacco from an approximately 0 ppm nicotine/TSNA tobacco can be mixed with conventional Burley, Flue-cured, or Oriental in a 75%/25% ratio respectively to obtain a Burly tobacco product having 7,500 ppm nicotine and 2,000 ppb TSNA, a Flue-cured product having 5,000 ppm nicotine and 75 ppb TSNA, and an Oriental product having 2,500 ppm nicotine and 25 ppb TSNA.

It should be appreciated that tobacco products are often a blend of many different types of tobaccos, which were grown in many different parts of the world

under various growing conditions. As a result, the amount of nicotine and TSNAs will differ from crop to crop. Nevertheless, by using conventional techniques one can easily determine an average amount of nicotine and TSNA per crop used to create a desired blend. By adjusting the amount of each type of tobacco that makes up the blend one of skill can balance the amount of nicotine and/or TSNA with other considerations such as appearance, flavor, and smokability. In this manner, a variety of types of tobacco products having varying level of nicotine and/or nitrosamine, as well as, appearance, flavor and smokeability can be created.

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein. All references cited herein are hereby expressly incorporated by reference.

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THAT WHICH IS CLAIMED IS:

1. An isolated nucleic acid selected from the group consisting of :

(a) an isolated nucleic acid consisting essentially of the nucleotide sequence of SEQ ID NO:1 or a fragment thereof, wherein said fragment is between about 20-455 consecutive nucleotides;

(b) an isolated nucleic acid that hybridizes to the complement of SEQ ID NO :1 or to the complement of a fragment thereof of (a); and

(c) an isolated nucleic acid that is at least 90% identical to the nucleic acid of (a), wherein said nucleic acid is capable of binding to or is otherwise responsive to a *Nic* gene product.

2. The isolated nucleic acid according to claim 1 consisting essentially of the sequence given herein as SEQ ID NO :1 or a fragment thereof, wherein said fragment is between about 20-455 consecutive nucleotides.

3. The isolated nucleic acid according to claim 1 consisting essentially of the nucleotide sequence given herein as SEQ ID NO :1.

4. The isolated nucleic acid according to claim 1, wherein said nucleic acid is DNA.

5. A recombinant nucleic acid construct comprising the nucleic acid of claim 1, wherein said nucleic acid is joined to said recombinant nucleic acid construct and said recombinant nucleic acid construct does not contain *NtQPT1* coding sequence.

6. The recombinant nucleic acid construct according to claim 5, wherein said recombinant nucleic acid construct is linear.

7. The recombinant nucleic acid construct according to claim 5, wherein said recombinant nucleic acid construct is circular.

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8. A microparticle comprising the nucleic acid of claim 1, wherein said nucleic acid is joined to said microparticle.

9. The recombinant nucleic acid construct according to claim 5, wherein said recombinant nucleic acid construct is an *Agrobacterium* vector.

10. A plant cell transformed with the nucleic acid according to claim 1.

11. A method of making a transgenic tobacco plant having a reduced amount of nicotine, comprising:

introducing a nucleic acid consisting essentially of a *Nic* gene product responsive element into at least one tobacco plant cell so as to produce at least one transformed tobacco plant cell,

said at least one transformed tobacco plant cell containing said nucleic acid in a copy number sufficient to reduce the amount of nicotine in a tobacco plant regenerated from said cell as compared to the amount of nicotine that would be present in the absence of said nucleic acid; and

regenerating said at least one transformed tobacco plant cell so as to obtain said tobacco plant.

12. The method of claim 11, further comprising:

collecting leaves from said tobacco plant, said tobacco leaves containing a reduced amount of nicotine as compared to the amount of nicotine that would be present in said tobacco plant in the absence of said nucleic acid.

13. The method according to claim 11, further comprising:

collecting tobacco seed from said tobacco plant, said tobacco seed containing said nucleic acid in a copy number sufficient to reduce the amount of nicotine of a tobacco plant regenerated from said seed as compared to the amount of nicotine that would be present in the absence of said nucleic acid.

14. The method according to claim 11, wherein said *Nic* gene product responsive element is selected from the group consisting of :

(a) an isolated nucleic acid consisting essentially of the nucleotide sequence of SEQ ID NO:1 or a fragment thereof, wherein said fragment is between about 20-455 consecutive nucleotides;

(b) an isolated nucleic acid that hybridizes to the complement of SEQ ID NO:1 or to the complement of a fragment thereof of (a); and

(c) an isolated nucleic acid that is at least 90% identical to the nucleic acid of (a), wherein said nucleic acid is capable of binding to or is otherwise responsive to a *Nic* gene product..

15. The method according to claim 11, wherein said *Nic* gene product responsive element consists essentially of a nucleic acid consisting of the nucleotide sequence of SEQ ID NO :1.

16. The method according to claim 11, wherein said nucleic acid is contained within a recombinant nucleic acid construct, wherein said isolated nucleic acid is joined to said recombinant nucleic acid construct, said recombinant nucleic acid construct does not contain *NtQPT1* coding sequence, and said recombinant construct is linear or circular.

17. The method according to claim 11, wherein said nucleic acid is DNA.

18. The method according to claim 11, wherein said introducing step comprises ballistic transformation.

19. The method according to claim 11, wherein said introducing step comprises *Agrobacterium* transformation.

20. A tobacco plant produced by the method of claim 11.

21. A tobacco leaf collected from the tobacco plant of claim 20.

22. A tobacco seed collected from the tobacco plant of claim 20.

23. A tobacco plant having a reduced amount of nicotine therein, said plant comprising cells containing an exogenous nucleic acid, wherein said exogenous nucleic acid consists essentially of a *Nic* gene product responsive element; said exogenous nucleic acid contained in said cells in a copy number sufficient to reduce the amount of nicotine in said tobacco plant as compared to the amount of nicotine that would be present in said plant in the absence of said exogenous nucleic acid.

24. A tobacco plant according to claim 23, wherein said *Nic* gene product responsive element is selected from the group consisting of :

(a) an isolated nucleic acid consisting essentially of the nucleotide sequence of SEQ ID NO: 1 or a fragment thereof, wherein said fragment is between about 20-455 consecutive nucleotides;

(b) an isolated nucleic acid that hybridizes to the complement of SEQ ID NO:1 or to the complement of a fragment thereof of (a); and

(c) an isolated nucleic acid that is at least 90% identical to the nucleic acid of (a),

wherein said nucleic acid is capable of binding to or is otherwise responsive to a *Nic* gene product .

25. A tobacco plant according to claim 23, wherein said *Nic* gene product responsive element consists essentially of a nucleic acid consisting of the nucleotide sequence of SEQ ID NO :1.

26. A tobacco plant according to claim 23, wherein said exogenous nucleic acid is contained within a recombinant nucleic acid construct, wherein said isolated nucleic acid is joined to said recombinant nucleic acid construct, said recombinant nucleic acid construct does not contain *NtQPT1* coding sequence, and said recombinant construct is linear.

27.The tobacco plant according to claim 26, wherein said recombinant nucleic acid construct is circular.

28.The tobacco plant according to claim 23, wherein said exogenous nucleic acid is DNA.

29. Tobacco leaf collected from the tobacco plant of claim 23.

30. Tobacco seed that germinate into the tobacco plant of claim 23.

31. Tobacco seed collected from the tobacco plant of claim 23.

32. A method of making a plant having an altered content of a protein of interest therein, wherein said protein of interest is regulated by a *cis*-acting element, said method comprising the steps of :

introducing an exogenous nucleic acid comprising said *cis*-acting element into at least one plant cell to produce at least one transformed plant cell,

said at least one transformed plant cell containing said exogenous nucleic acid in a copy number sufficient to increase or reduce the level of said protein of interest in a plant regenerated from said cells as compared to the amount of said protein of interest that would be present in the absence of said exogenous nucleic acid, subject to the proviso that said *cis*-acting element is not operably linked to a coding sequence or complement thereof for said protein of interest.

33. The method according to claim 32, wherein said *cis* acting element is a *cis*-acting activating element that binds an activator compound, which activator compound increases expression of said protein of interest in said plant, and with said at least one transformed plant cell containing said exogenous nucleic acid in a copy number sufficient to reduce the level of said protein of interest in a plant regenerated from said cells as compared to the amount of said protein of interest that would be present in the absence of said exogenous nucleic acid.

34. The method according to claim 32, wherein said *cis*-acting element is a *cis*-acting repressor element that binds a repressor compound, which repressor compound decreases expression of said protein of interest in said plant, and with said at least one transformed plant cell containing said exogenous nucleic acid in a copy number sufficient to increase the level of said protein of interest in a plant regenerated from said cells as compared to the amount of said protein of interest that would be present in the absence of said exogenous nucleic acid.

35. The method according to claim 32, further comprising generating a plant from said transformed plant cells.

36. The method according to claim 35, further comprising:

collecting seed from said plant, said seed containing said exogenous nucleic acid in a copy number sufficient to reduce the level of said protein of interest in a plant regenerated from said seed as compared to the level of said protein of interest that would be present in the absence of said exogenous nucleic acid.

37. The method according to claim 32, wherein said *cis*-acting element is selected from the group consisting of UAS-1, the vicilin box, site B, and the tobacco RB7 promoter root-specific *cis*-acting element.

38. The method according to claim 32, wherein said exogenous nucleic acid is linear.

39. The method according to claim 32, wherein said exogenous nucleic acid is circular.

40. The method according to claim 32, wherein said exogenous nucleic acid is DNA.

41. The method according to claim 32, wherein said introducing step comprises ballistic transformation.

42. The method according to claim 32, wherein said introducing step comprises *Agrobacterium* transformation.

43. A plant produced by the method of claim 32.

44. Leaves, fruit, flowers, roots or tubers collected from the plant of claim 43.

45. Seed collected from the plant of claim 43.

46. A plant having altered levels of a protein of interest therein, said plant comprising cells containing an exogenous nucleic acid,

which exogenous nucleic acid comprises a *cis*-acting element that regulates the level of said protein of interest in said plant,

said cells containing said exogenous nucleic acid in a copy number sufficient to increase or reduce the level of said protein of interest in said plant as compared to the amount of said protein of interest that would be present in the absence of said exogenous nucleic acid, subject to the proviso that said *cis*-acting element is not operably linked to a coding sequence or complement thereof for said protein of interest.

47. The plant according to claim 46, wherein said *cis* acting element is a *cis* acting activating element that binds an activator compound, which activator compound increases expression of said protein of interest in said plant, and with said at least one transformed plant cell containing said exogenous nucleic acid in a copy number sufficient to reduce the level of said protein of interest in said plant as compared to the amount of said protein of interest that would be present in the absence of said exogenous nucleic acid.

48. The plant according to claim 46, wherein said *cis*-acting element is a *cis* acting repressor element that binds a repressor compound, which repressor compound decreases expression of said protein of interest in said plant, and with said at least one transformed plant cell containing said exogenous nucleic acid in a copy number sufficient to increase the level of said protein of interest in said plant as compared to the amount of said protein of interest that would be present in the absence of said exogenous nucleic acid.

49. The plant according to claim 46, wherein said exogenous nucleic acid is linear.

50. The plant according to claim 46, wherein said exogenous nucleic acid is circular.

51. The plant according to claim 46, wherein said exogenous nucleic acid is DNA.

52. Leaf, fruit, flowers, roots or tubers collected from the plant of claim 46.

53. Seed that germinates into the plant of claim 46.

54. Seed collected from the plant of claim 46.

55. A method of decreasing expression of a protein of interest in a host cell, wherein transcription of said protein of interest is enhanced by a *cis*-acting activating element that binds an activator compound, which activator compound increases expression of said protein of interest in said host cell, said method comprising the steps of :

(a) providing a decoy recombinant nucleic acid construct comprising said *cis* acting activating element; and

(b) introducing said decoy construct into said host cell in an amount sufficient to bind said activator compound and reduce expression of said protein of interest, subject to the proviso that said *cis*-acting element is not operably linked to a coding sequence or complement thereof for said protein of interest..

56. The method according to claim 55, wherein said host cell is a prokaryotic or eukaryotic cell.

57. The method according to claim 55, wherein said host cell is a bacterial cell.

58. The method according to claim 55, wherein said host cell is a fungal cell.

59. The method according to claim 55, wherein said host cell is an animal cell.

60. The method according to claim 55, wherein said host cell is a mammalian cell.

61. The method according to claim 55, wherein said host cell is a vascular plant cell.

62. The method according to claim 55, wherein said host cell is a monocot or dicot plant cell.

63. The method according to claim 55, wherein said decoy recombinant nucleic acid construct is a plasmid.

64. A method of increasing expression of a protein of interest in a host cell, wherein transcription of said protein of interest is reduced by a *cis*-acting repressor element that binds a repressor compound, which repressor compound reduces expression of said protein of interest in said host cell, said method comprising the steps of :

(a) providing a decoy recombinant nucleic acid construct comprising said *cis* acting repressor element ; and

(b) introducing said decoy construct into said host cell in an amount sufficient to bind said repressor compound and increase expression of said protein of interest, subject to the proviso that said *cis*-acting element is not operably linked to a coding sequence or complement thereof for said protein of interest..

65. The method according to claim 64, wherein said host cell is a prokaryotic or eukaryotic cell.

66. The method according to claim 64, wherein said host cell is a bacterial cell.

67. The method according to claim 64, wherein said host cell is a fungal cell.

68. The method according to claim 64, wherein said host cell is an animal cell.

69. The method according to claim 64, wherein said host cell is a mammalian cell.

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70. The method according to claim 64, wherein said host cell is a vascular plant cell.

71. The method according to claim 64, wherein said host cell is a monocot or dicot plant cell.

72. The method according to claim 64, wherein said decoy recombinant nucleic acid construct is a plasmid.

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SEQUENCE LISTING

<110> Conkling, Mark A.
Li, Yan

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