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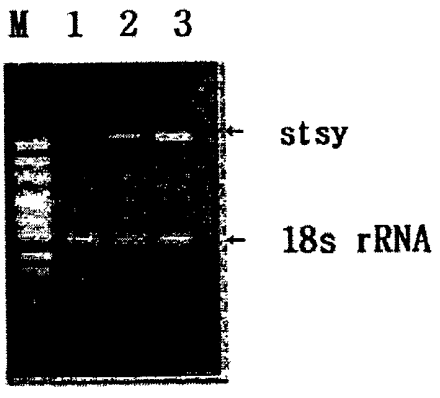
(54) Title: NOVEL TRANSGENIC TOBACCO RESTRAINING ACTIVITY OF HARMFUL MATERIALS THEREOF

(57) Abstract: The present invention relates to novel transgenic tobacco which restrains activity of harmful materials of it by using resveratrol biosynthesis gene of *Vitis vinifera* X *Vitis labrusca* L. cv. Kyoho.



[Example]
Lane M : Molecular marker
Lane 1 : Wild type tobacco leaves
Lane 2 : Leaves of transgenic plants(3X1)
Lane 3 : Leaves of transgenic plants(3W1)

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NOVEL TRANSGENIC TABACCO RESTRAINING ACTIVITY OF HARMFUL MATERIALS THEREOF

Technical Field

The present invention relates to a product of novel transgenic tobacco, which restrains negative activities of harmful materials of tobacco by using resveratrol biosynthesis gene of *Vistis vinifera* X *Vistis labrusca* L. cv. Kyoho. More specifically the present invention relates to a new product of novel transgenic tobacco which is actively able to neutralize harmful components of tobacco, This product is produced by inserting the resveratrol biosynthesis gene of *Vistis vinifera* X *Vistis labrusca* L. cv. Kyoho. to pBI 121 vector, .that has been known to have anti-stress and anti-cancer effects , thus constructing a recombinant vector(pBIstsyv1), which is again introduced into Agrobacterium-LBA4404 to transfer Agarobacterium strain(pBIstsyv1/ LBA4404) thereafter inoculating with leaf explant

Background Art

Resveratrol and it's Polymer Viniferin have been known as phytoalexin in plants, which is a blocking agent that inhibits the growth of penetrated molds from outside. Resveratrol(or pseudostilbene) is produced from a very few plants and so far *Polygonum cuspidatum*, Eucalyptus, Spruce, Scottish pine, grapes, *Arachis hypogaea*, lilly ect are known to belong in these plants.

It has been reported that in these plants, resveratrol is synthesized from

malony-CoA and p-coumaroyl-CoA by stilbene synthesis and induced by external stresses including molds, UV and ozone(schoepper and Kindil H, 1979; Fliegmann et al., 1992; Fritzemeier et al., 1983; Vornam et al., 1988).

Recently many researches have been directed to the biosynthesis pathways and regulations of this substance in molecular and genetic levels. As a result, a gene which relates to the biosynthesis of this substance has been isolated from pine trees, grapes and *Arachis hypogaea* ect (Sparvoli et al., 1994; Preising-Muller et al., 1999; Schubert et al., 1997). This accumulated information on gene expression and regulations provided a stepping stone to use the gene.

On the other hand, research on resveratrol of grapes has been started a long time ago and the anti-stress effect of this chemical compound has been well known(Langcake et al., 1979).

Earlier studies have mainly confined for a few specific crops in the development of transformed crops that have strong disease tolerance including anti-biotic, anti-bacterial, anti-virus and anti-insect by transforming a synthesis gene constructed by using anti-stress and stress resistance of resveratrol(Kindle et al., 1999; Hain et al., 1998; Schroder et al., 2000). However, the recent discovery of trans-resveratrol in wine and the dynamic report on the induction of the death rate by the coronary heart disease (CHD) by drinking an appropriate amount of wine have boosted many experimental results in the medical effects of resveratrol (Siemann and Creasy, 1922). In particular, one of the remarkable results was that the red wine increases anti-oxidation activity in the blood, thereby restraining the production of oxidative LDL (Maxwell et al. 1994 ; Fuhrman et al, 1995) Most recently many research results on the anti-mutation and anti-cancer activities of the

resveratrol have been reported. (Carbo et al., 1999: Clement et al , 1998; Huang et al, 1999).

Tobacco contains several thousands of chemical substances. More than 200 substances of them are harmful to the human body and cause diseases or worsen the disease including lung cancer, angina pectoris, cerebral infarction, pulmonary emphysema, bronchitis, asthma, stomach ulcer, arteriosclerosis, myocardinal infarction and optical defect. Moreover, the harmful activities of tobacco are due to toxic substances in the tobacco smoke like carcinogens such as nicotine, nitrosamine and benzypyrene. Nicotine affects the lipid metabolism through autonomic nerve system and promotes deposition of cholesterol in arterial wall by decreasing HDL in blood to cause arteriosclerosis. Nicotine also increases the ability of coagulation of blood platelets when blood coagulates, thus causing blood coagulation more easy. Consequently, it is easy for smokers to have blood clot in their blood stream and to cause pulmonary emphysema. The mechanism of carcinogen by the harmful substances of tobacco is not known well, however it is generally hypothesized that smoking reduces vitamin C and beta-carotenes in the body, thereby causing the accumulation of harmful substances such as free-radical oxygen. The free-radical oxygen is produced when lipid is oxidized in the body and in particular when the cell membrane composed of unsaturated fatty acid is oxidized. The free radical causes DNA damage in cells, thereby leading cancer cells. Recently, there has been reports that vitamin A,C,E and β-carotene ect. could decrease the cancer generation rate through their oxidation activities. Actually there was a report that the incident rate of lung cancer by nicotine was reduced by the green tea extract which has an anti-oxidation effect. In addition, there have been attempts to eliminate harmful

substrate of tobacco using physical and chemical methods. The first transformed tobacco by using gene conversion is the TMV, PVY resistant tobacco developed by the Korean Ginseng Tobacco Research Institute. However, novel transgenic tobacco repressing and decreasing harmful substances by the gene conversion has not been developed yet.

The present invention was performed based on the above facts to produce a novel transgenic tobacco which can neutralize harmful substances of tobacco and decrease it's effect remarkably by transforming *Vistis vinifera* X *Vistis labrusca* L. cv. Kyoho resveratrol biosynthesis gene extracted as above which has anti-mutation and anti-cancer effects in tobacco. The present product is the novel transgenic tobacco produced on the basis of new techniques of gene cloning and transformed plants, that have been developed for last five years and has an excellent effect on anti-cancer and anti-oxidation.

The present invention uses the plant breeding gene that has the functions of the original anti-biotic and anti-virus effect so that damages of insects occurring in cultivating tobacco can be reduced and also applied to harvest better quality of tobacco in a large amount. Consequently, the object of the present invention is to provide a constructed recombinant vector by inserting the resveratrol biosynthesis gene of *Vistis vinifera* X *Vistis labrusca* L. cv. Kyoho resveratrol in the vector. Another object of the present invention is to provide a transformed Agrobacterium strain (pBIstsyv/LBA4404) obtained by the above recombinant vector introducing into Agrobacterium-LBA4404. Still another object of the present invention is to provide a novel transgenic tobacco that has an effect on neutralizing tobacco toxin substance effectively by inoculating the above strain into tobacco.

Disclosure of invention

The object of the present invention is to construct a recombinant vector (pBIstsyvl) by inserting the *stsy* cDNA whose open reading frame and partially untranslated region was digested with a restriction enzyme into the downstream of the CaMV promoter of the pB1121 vector which the GUS gene had been eliminated. The *stsy* cDNA was amplified by PCR using the first strand cDNA as a primer that was synthesized from the total RNA, which was extracted from each steps of developing tissues and organs of *Vistis vinifera* X *Vistis labrusca* L. cv. Kyoho. After producing a transgenic strain by introducing the above recombinant vector into Agrobacterium-LBA4404, the leaf explant of the tobacco was transformed by inoculating on above transformed Agrobacterium strain suspension and was cultured on antibiotic added medium. The grown shoots from the medium was rooted and graft-taken to produce a novel transgenic tobacco which restrains toxic substance activity.

Brief Description of the drawings

FIG 1. is showing the nucleotide sequence of stilbene synthase cDNA of *Vistis vinifera* X *Vistis labrusca* L. cv. Kyoho.

FIG 2. is a graph showing the present invention of the recombinant vector(pBIstsyv1) that carries the gene which synthesize the de-toxicating substance and which is used for transformation.

FIG 3. is showing the wild type species of the tobacco and the novel transgenic tobacco which was inserted by FIG 1 gene.

FIG 4. is showing the RT-PCR results that shows the expression of detoxicating substance gene of the novel transgenic tobacco.

FIG 5. is showing the analyzed results of the detoxicating substance production yield of novel transgenic tobacco by HPLC.

Best Mode for carrying out the invention

The present invention is executed, comprising the steps of: constructing the recombinant vector by inserting the PCR amplified resveratrol biosynthesis gene in pBI 121 vector; selecting cultured Agrobacterium-LBA4404 as a strain for transformation and introducing the recombinant vector which has resveratrol biosynthesis gene into the strain; inoculating the leaf explant with the transformed Agrobacterium; producing a novel transgenic tobacco restraining toxic activity of tobacco by growing shoots and rooting of the transformed leaf explant in the antibiotic added medium; confirming the resveratrol gene introduction and expression by RT-PCR after isolating total RNA from the leaf of transgenic novel tobacco and isolating genomic DNA followed by genomic PCR; and analyzing the resveratrol content of the transgenic tobacco by HPLC.

Hereinafter, the present invention will be described by way of the following examples, which are not limit the scope of the present invention.

Example 1: A recombinant vector inserted by the resveratrol gene of *Vistis vinifera* X *Vistis labrusca* L. cv. Kyoho and the transformed Agrobacterium introduced by the above recombinant vector.

Step 1: RNA and genomic DNA isolation of each organ and tissue *of Vistis vinifera* X *Vistis labrusca* L. cv. Kyoho for the production of novel transgenic tobacco.

Vistis vinifera X *Vistis labrusca* L. cv. Kyoho was harvested at every week for 15 weeks after blooming. 5 grapes from the harvested ones were chosen randomly to investigate the fruit development stage with checking the length, width and weight of the fruit.

The harvested grapes were divided into pericarp and pulp stored at –80℃ after freezing rapidly with the liquid nitrogen. RNA was isolated from each organs and tissues of *Vistis vinifera* X *Vistis labrusca* L. cv. Kyoho that were in diverse developing stages.

Parts of tissues and each organ obtained from the grape fruits that aged 2, 3, 8, 10, 13 and 15 weeks after blooming were frozen and grinded by a coffee grinder to obtain powder. The powder was mixed with the RNA extraction buffer and was centrifuged for 15 minutes at 200 X g in a syringe, which was filled with glass wool. The extract was harvested and precipitated with 2.5 volumes of ethanol. The pellet was dissolved in 10mM Tris-HCl, 1mM EDTA, 0.2% β -mercaptoethanol and then extracted with phenol-chloroform. The supernatant was collected and the RNA was precipitated with 0.1 volume of 3M sodium acetate and 2.5 volume of ethanol.

After centrifuging again, the pellet was dissolved in DEPC-treated distilled water. If necessary poly A-Tract mRNA isolation system was used to

isolate mRNA and the amount of the total RNA ect. RNA was measured by UV spectrophotometer and RNA gel. Next, for isolating genomic DNA, 2g of young leaf tissue was grinded by using liquid nitrogen and resuspended with 25ml of buffer A followed by centrifugation at 5000 rpm for 10 minutes. 5ml of extraction buffer was added to the pellet and was incubated at 37 for 30 minutes at a rotary shaker. After adding equal volume of chloroform: isoamylalcohol it was mixed and centrifuged at 12000 rpm for 10 minutes and then the upper supernatant was precipitated with 0.54 volume of isopropanol. The pellet was dissolved in distilled water, which contained RNase and incubated at 37℃ for 30 minutes. Proteins including RNase were removed by precipitating with 0.5 volumes of 7.5M ammonium acetate and DNA was collected from the supernatant by adding 0.54 volume of isopropanol..

Step 2: Amplifying cDNA by RT-PCR using primers.

Isolated total RNA from each organs and tissues were used for synthesizing the first strand cDNA by using first-strand cDNA synthesis kit (Pharmacia) and the produced first strand cDNA was used as PCR templates. After analyzing the nucleotide sequence of stilbene synthase cDNA of Shiraz grape, that has already been known, a primer was designed on the basis of each starting and ending regions of the open reading frame and was used for RT-PCR primers.

The primer which is to amplify the first strand cDNA by PCR.

HPPN : 5'ATGGCTTCAGTCGAGGAAATT 3' (21mer)

HPPC ; 5' TTAATTTGTCACCATAGGAATG 3' (22mer)

PCR conditions were decided according to the procedure of Sambrook et al.

Conditions were as follow: 94℃ for 30 sec. 55℃ for 1min, 72℃ for 1min. and 30 cycles. The PCR product was checked by gel electrophoresis.

Step3: Sequencing and analyzing the above cDNA

Referring to FIG.1, the above RT-PCR amplified cDNA product was serial deleted or subcloned into pCR2.1-TOPO cloning vector several times and the sequence were determined by dideoxy termination method using sequenase kit. After translating the nucleotide into amino acids, the homology between cDNA and the well-known stilbene synthase gene, was analyzed. As a result, it had over 99% homology with the Shiraz stilbene synthase cDNA sequence. In addition, this cDNA was used as a probe for genomic southern hybridization. For analysis, DNASIS/PROSI program was used.

Step 4; Culturing Agrobacterium as a strain for Transformation

Selecting Agrobacterium-LBA4404 for transformation, it was incubated at 28℃ for 2-3 days after streaking on a YEP solid medium(An, 1987) added rifampicin 100mg/L, kanamycin(Km) 100mg/L, agar 15g/L. After picking a colony and inoculating 3ml of YEP broth containing Km 100mg/L, it was incubated at 28 for 8 hours in a shaking incubator. 1ml of shaking incubated Agrobacterium suspension was transferred to 50 ml YEP broth containing Km 100mg/L and incubated for 16 hours as the above condition.

The O.D (optical density) of the cultured Agrobacterium suspension

was adjusted to 0.8- 1.0 and was used in transforming the tobacco.

Step 5: Construction of *stsy* cDNA inserted recombinant vector, pBIstsyv1, which is used for transformation.

To construct a recombinant vector pBIstsyvl, *stsy* cDNA clone was obtained by RT-PCR and the open reading frame region and some untranslated region was digested by restriction enzyme. Subsequently, it was inserted into the downstream of the CaMV 35S promoter of the pBI 121 vector, which the GUS gene was eliminated(FIG. 2).

This recombinant vector was introduced into step4 Agrobacterium producing transformed strain, pBIstsyv1/LBA44040

The transformed Agrobacterium pBIstsyv1/LBA44040 was deposited to the Korean inoculum society on June 18, 2001(KCCM Accession NO. 10284)

Example 2: Inoculation of transformed Agrobacterium and plant redifferentiation by tissue culture of tobacco

Step1: inoculation of transformed Agrobacterium

Grown *Nicotiana tabacum* L. Wisconsin 38 or *Nicotiana tabacum* L. Xanthi was selected as a material and the leaf was cross-sectioned. To avoid oxidating and drying, it was stored at a petri-dish that contained 20 ml of broth, made of the MS media added with BA 2.0mg/L, IBA 0.01mg/L, sucrose 30g/L, agar 8g/L and adjusted to pH 5.8.

Put 20ml of Agrobacterium suspension and the prepared leaf explant cultured on the YEP in a petri-dish to dip in the suspension fully and shake

the petri- dish carefully for 5 minutes at 25 ± 2℃.

After finishing inoculation, the explant was placed on the sterilized Whatman No.2 paper and the excessive Agrobacterium was removed. A small amount of explant taken out of the petri-dish not to dry the explant was placed to the antibiotic added callus inducing media. The explant was incubated until the shoot was induced from callus and subcultured every 2 weeks at 25 ± 2℃ under continuous lightening. 2 weeks after inoculating on a antibiotic added callus inducing media, callus was starting to form from the transformed Agrobacterium and the leaf explant. After 3 weeks, callus was generating shoots and getting thicker.

Step 2; Rooting the regenerated transformed shoot.

The selected regenerated shoot taller than 2 cm was transferred to the shoot inducing media for subculturing and was induced to root by the processes of elongation and cultivation. Incubation has been done for 2-4 weeks at a rooting inducing media which Kanamycin 50-100mg/L, carbenicillin 150-300mg/L, α -naphthaleneacetic acid 0.5g/L, sucrose 30g/L, agar 8g/L was added to MS medium. After taking out rooted small plants from the incubating plate, agar was washed with flowing water and immerged for a moment in a 700 diluted sterilizer(Iminoctadine Tris:Demethomorph=10: 23%) and then dried. After that it was planted in the autoclaved vermiculite. When incubating in the 1000 diluted hyponex solution under the condition of bottom watering, the plate was sealed to maintain the humidity and was opened slowly to induce rooting. And then the rooted plants were moved to the greenhouse.

Example 3: genomic PCR, RT-PCR of genomic DNA and total RNA isolated from transformed tobacco and reverse phase HPLC of resveratrol synthetic substance

Using the method as described in example 2, a genomic DNA was isolated from the transformed novel tobacco leaf and the genomic PCR was performed.

To find if the *stsy* gene has introduced into the transformed *Vistis vinifera* X *Vistis labrusca* L. cv. Kyoho, the genomic PCR has been performed. The result indicates that the transformed novel tobacco had resveratrol synthesis gene of *Vistis vinifera* X *Vistis labrusca* L. cv. Kyoho. Also total RNA was isolated from the wild type tobacco and transformed new tobacco leaf as described in example 2. RT-PCR was accomplished to analyze the gene expression pattern in tissue

As a result transformed tobacco expressed the *Vistis vinifera* X *Vistis labrusca* L. cv. Kyoho gene successfully, knowing their individual expression level was different.

Resveratrol was extracted using the Siemann and Cress method. 1~5g of tobacco leaf was add in a 3ml ethyl acetate test tube and vortexed for 15 seconds. After that the test tube was stored at 4 for several seconds and then at –20, the organic solvent layer was separated with the pasteur pipette while the aqueous solution was extracted in 2ml twice with ethyl acetate to separate the organic solvent layer. Hydrating with anhydrous sodium sulfate, the organic solvent phase was concentrated using Rotavopor in the low-pressure conditions.

The extract was dissolved in methanol and was analyzed with HPLC by the Celotti method. Partisphere C18, 5 ㎛(125 X 4.6 X mm I.D., Whatman) was used for a column and water/acetic acid / acetonitrile(75:5:20) for mobile phase. Also the flow rate was adjusted experimentally. For standard, trans-resveratrol(Sigma R5010), catechin(Sigma C1251), epicatechin(Sigma E1753), rutin(Sigma R5143), quercetin(Sigma Q0125) was used.

Extracting a substance which is soluble in ethyl acetate from the wild type tobacco and the transformant leaf, it was separated by thin layer chromatography and its active region was re-extracted by methanol. After using C18 column, resveratrol was analyzed by HPLC and as a result 0.5ug/g of anti-cancer substance was produced (Fig. 5).

Industrial applicability

As mentioned above, the present invention provides a novel transgenic tobacco produced by introducing the grape resveratrol biosynthesis gene that has strong anti-mutation, anti-oxidation and anti-cancer effects to tobacco. Also genes that have been used for the breed development have the original anti-biotic and anti-virus effects so that this invented product can reduce damages of disease and insect pest in cultivating tobacco and produce mass production of a good quality of tobacco. Further more, this also promotes people's health by alleviating the harmful toxic components of tobacco so that inhibits lung cancer caused by smoking and can be contributed usefully in the seed industry.

Applicant's or agent's file reference	International application No.
YL01014PCT	PCT/KR01/01113

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 10, line 12-14.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

Korean Culture Center of Microorganisms(KCCM)

Address of depositary institution *(including postal code and country)*

Korean Culture Center of Microorganisms(KCCM)
#361-221 Yourim Bldg., Hongjae-1-dong, Seodaemoon-gu
Seoul 120-091, Republic of Korea
Korean Federation of Culture Collections

Date of deposit	Accession Number
June 18, 2001	KCCM-10284

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

What is claimed is:

1. A resveratrol synthesis gene having anti-cancer effect that includes No.1 nucleotide sequence which was isolated from *Vistis vinifera* X *Vistis labrusca* L. cv. Kyoho.

2. A recombinant vector pBIstsyv1, including anti-cancer resveratrol synthesis gene according to claim 1, which was originated from *Vistis vinifera* X *Vistis labrusca* L. cv. Kyoho.

3. A transformed *Agrobacterium tumofeciens* LBA4404/pBIstsyv1(KCCM-10284) by recombinant vector according to claim 2.

4. A transformed tobacco which redifferentiates through tissue culture and synthesize the anti-cancer recombinant resveratrol by transforming the tobacco explant with Agrobacterium strain according to claim 3.

FIG. 1

atggcttcag tcgaggaaat tagaaacgct caacgtgcca agggtccggc caccatccta 60
gccattggca cagctacccc cgaccactgt gtctaccagt ctgattatgc tgatttctat 120
ttcagggtca ctaagagcga gcacatgact gcgttgaaga agaagttcaa tcgcatatgt 180
gacaaatcca tgatcaagaa gcgttacatt catttgaccg aagaaatgct tgaggagcac 240
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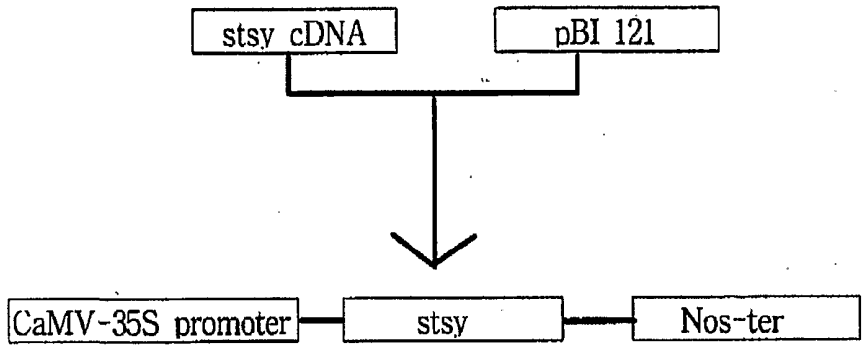
FIG. 2



[Example]
stsy cDNA, stilbene synthase cDNA isolated from Vitis vinifera X Vitis labrusca L. cv. Kyoho
CaMV, Cauliflower Mosaic Virus
Nos, Nopaline synthase gene

FIG. 3

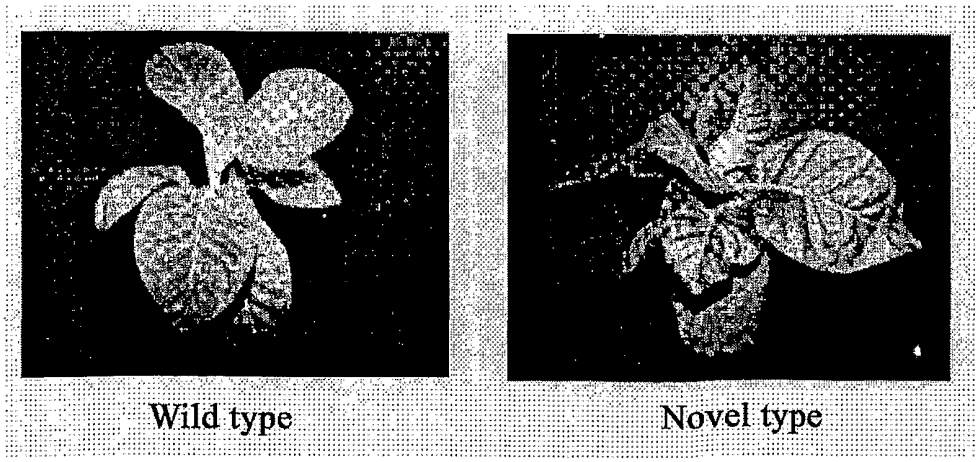
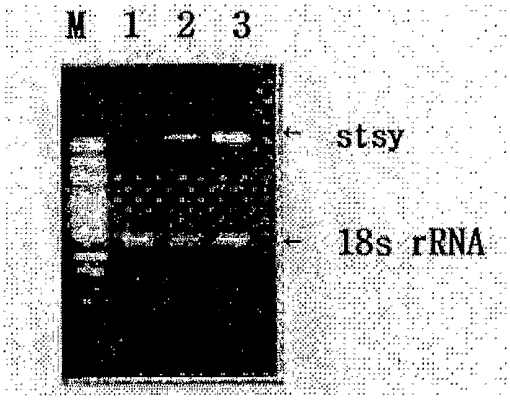
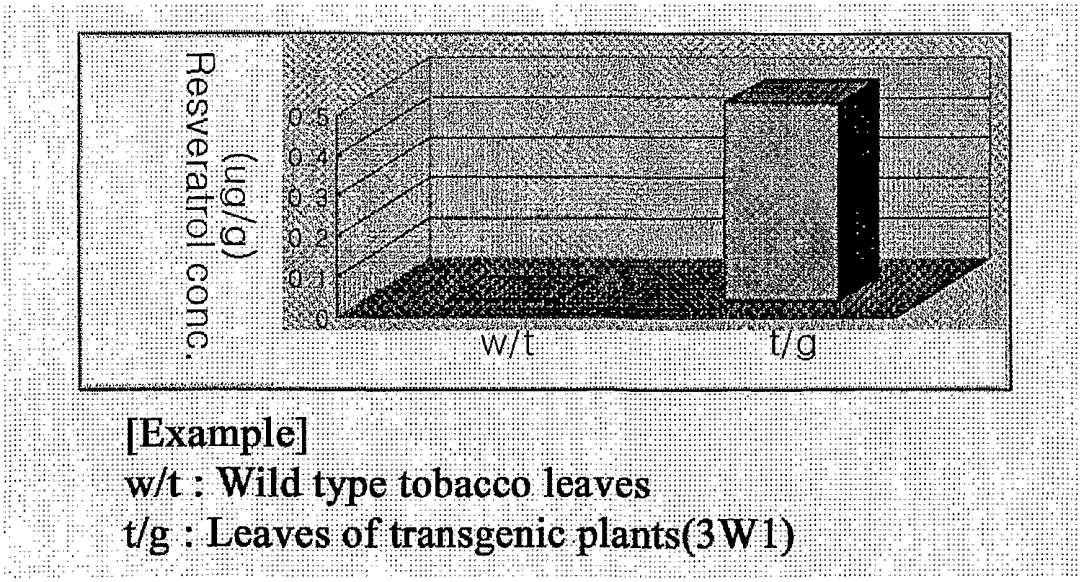


FIG. 4



[Example]
Lane M : Molecular marker
Lane 1 : Wild type tobacco leaves
Lane 2 : Leaves of transgenic plants(3X1)
Lane 3 : Leaves of transgenic plants(3W1)

FIG. 5



[Example]
w/t : Wild type tobacco leaves
t/g : Leaves of transgenic plants(3W1)

Sequence Listing

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<151> 2000-06-29

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<213> Vitis vinifera L.x Vitis labrusca cv. Kyoho

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ttcagggtca ctaagagcga gcacatgact gcgttgaaga agaagttcaa tcgcatatgt 180

gacaaatcca tgatcaagaa gcgttacatt catttgaccg aagaaatgct tgaggagcac 240

ccaaacattg gtgcttatac ggctccatct cttaacatac gccaagagat tatcactgcc 300

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Sequence Listing

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actgttgtgt tgcatagcat tcctacggtg acaaattaa 1179