

APPENDIX I

INTERNET DATABASES ON GMO FIELD TRIALS

Given the frequent updates in the publicly available databases, a comprehensive list of internet addresses of public GMO field trials databases has been included:

Argentina

<http://siiap.sagyp.mecon.ar/http-hsi/english/conabia/liuk.HTM>

Australia

<http://www.health.gov.au/tga/gene/gmac/gmac.htm>

Brazil

<http://www.fiocruz.br/cict/oquee/estrut/dect/bis/lib.htm>

Canada

<http://www.cfia-acia.agr.ca/english/ppc/biotech/field.html>

Germany

http://www.rki.de/GENTEC/GENENG/GENTEC_E.HTM

EU

<http://biotech.jrc.it/gmo.htm>

Japan

<http://ss.s.affrc.go.jp/docs/sentan/index.htm>

USA

<http://aphisweb.aphis.usda.gov/oa/new/ab.html>

Worldwide

<http://www.nbiap.vt.edu/>

<http://www.olis.oecd.org/biotrack.nsf/>

<http://www.gibip.org/database/index.html>

<http://binas.unido.or.at/binas/binas.html>

<http://www.nbiap.vt.edu/cfdocs/globalfieldtests.cfm>

APPENDIX II

TOBACCO DNA EXTRACTION FOR PCR ANALYSIS

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Introduction

This Section describes a practical approach for extraction of DNA from raw tobacco, or cigarette tobaccos for PCR analysis. It addresses the questions “How do varieties of tobacco (e.g. Bright, Orient, Burley etc.) differ from each other in respect to quality and quantity of DNA, that can be extracted, and how does curing and manufacturing of these tobaccos (e.g. air-cured, sun-cured, expanded, reconstituted tobaccos) influence DNA quantity and quality?”

Methods

DNA Extraction and Purification

Initially, a modified CTAB-Method was used for DNA-Extraction.

Depending on the quality of DNA in respect to the PCR the following sequence of additional methods for purification of the DNA were used on the same sample:

- | | | |
|---------------------------|---------------------|------------------------------|
| 1. Wizard [®] | DNA Clean-Up System | (Promega) |
| 2. MicroSpin [™] | S-300HR column | (Amersham Pharmacia Biotech) |
| 3. Wizard [®] | DNA Clean-Up System | |
| 4. MicroSpin [™] | S-300HR column | |

After each step the DNA was again tested for quantity and quality in respect to PCR.

DNA Quantity Test

For testing the quantity of DNA, an aliquot of the extracted sample was separated on a 1% Agarose gel stained with Ethidium bromide. Quantity was calculated by comparison of fluorescence of the sample to calf-thymus DNA standards also loaded onto the same gel in a different slot.

DNA Quality Test

Quality of DNA was checked mainly by its capability to inhibit PCR. The samples were spiked with about 20 copies of 35S-DNA.

Primer:	35S-af1	CAC CTA CAA ATG CCA TCA TTG C
	35S-ar1	GGG TCT TGC GAA GGA TAG TG
50 cycles:	94°C	25sec
	62°C	30sec
	72°C	45sec
Fragment:	207 bp	

Results

Usually each purification step resulted in loss of DNA, and especially fragments smaller than 175bp were lost. As PCR screening methods for GMOs usually amplify fragments of about 200bp in size, the loss of small fragments should not interfere with e.g. 35S-screening.

Results are summarised in Table 1.

An additional observation is that contamination of raw material with fungi can result in very strong inhibition of PCR.

Table 1. Summary of results.

	Type of curing / manufacture	DNA after CTAB (ng per g sample ^a)	DNA after last purification step (ng per g sample)	PCR	Purification Sequence			
					Wizard [®] 1	MicroSpin [™] 2	Wizard [®] 3	MicroSpin [™] 4
Bright	Flue-cured	1000-3000	<2000	Yes	100% ^b	20% ^c		
Dark-fired	Fire-cured	<500	n.d.	No	100%	100%	100% ^d	100% ^e
Burley	Air-cured	500-1500	<1500	Yes	100%	50%		
Orient	Sun-cured	500-1500	<1000	Yes	100%	100%		
Stems		1000	<1000	Yes	100%			
Reconstituted		<1000	<500	Yes	100%	100%		
20-40% Burley 60-80% Bright	CO ₂ expanded	500-1000	<1000	Yes	100%	20%		
Cigarettes		500-3000	<3000	Yes	100%	100%	5%	
Cigars		1500-3000	<2500	Yes	100%	100%	20%	

- a) DNA was usually extracted from 2g material.
- b) 100% of all templates had to be purified at least once with Wizard system not to inhibit PCR.
- c) 20% of all templates had to be purified at least once with Wizard and once with Microspin not to inhibit PCR.
- d) 100% of all templates had to be purified at least twice with Wizard and once with Microspin not to inhibit PCR.
- e) 100% of all templates had to be purified at least twice with Wizard and twice with Microspin. In the case of dark fired tobacco PCR was still inhibited.

Summary

- In general, too much DNA-matrix inhibits PCR. However, in the case of tobacco it is not yet clear, whether this depends only on the amount of DNA-matrix used or also on some compounds from tobacco, which could not be separated from DNA during extraction and purification and which inhibit PCR.
- The more tobacco is processed, either by curing or by manufacturing, the less DNA can be extracted.
- Quality of extracted DNA depends firstly on the variety of tobacco and secondly on the manufacturing process.
- Dark-fired tobaccos could not be analyzed by PCR; even when only very low amounts of samples were applied, PCR was inhibited.

APPENDIX III

CHINESE METHOD FOR SCREENING GENETICALLY MODIFIED TOBACCO

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PURPOSE

Worldwide agreement on the use of genetically modified (GM) plants including tobacco has not yet been achieved. In order to confirm that the product from tobacco fields in China, especially for export, is GM-free, we carry out screening for GM tobacco.

The methods described here aim to detect genetic modification in tobacco material, such as seeds, seedlings, fresh leaf and cured tobacco leaf. They provide a means of identifying a limited number of genes that have been reported to be inserted in certain tobacco varieties.

The methods have been developed in our laboratory based on published work and many detection methods for GM tobacco from other laboratories, especially the screening methods for cured tobacco leaf. These have provided acceptable results in our laboratory with positive control samples of genetically modified (GM) tobacco.

Introduction

Three genes (*nptII*, 35S, *nos*) are commonly used as markers in plant transformation research. These are used for early screening of transgenic plants.

Kanamycin Resistance and *nptII* PCR testing

As a selection gene, neomycin phosphotransferase (which encodes resistance to kanamycin) is popularly used in genetically modified tobacco. According to the results of experiments in our laboratory, cells of GM tobacco can tolerate more than 100 ppm kanamycin, but the normal tobacco cell will become yellow and die after more than 7 days in this condition. Thus kanamycin resistance can be used to screen GM tobacco seeds.

Since the *nptII* gene was transferred to the tobacco genomic DNA from a bacterium, it cannot be found in the non-GM plant DNA. Using the nucleotide sequence of *nptII*, primers were designed to test extracted DNA of tobacco seed samples. This is not used to test cured tobacco leaf since there are bacteria which may contain the *nptII* gene on the surface of the cured leaf which can result in false positive results.

35S and *nos* PCR Testing

As 35S and *nos* are the most frequently used plant promoter and terminator, they can be used in a PCR test. This is capable of detecting modification in a majority of currently-available GM plant materials. Also the screening result cannot be affected by bacteria on the fresh or cured leaf.

Confirmation Tests

After PCR screening *nptII*, 35S or *nos*, some samples may give a positive result. Confirmation tests aim to avoid the risk of false positives. The tests, which include restriction analysis and nested- or semi-nested PCR, seek to confirm the presence of the 35S and *nos* sequences in these samples, especially in cases where the initial sample result appears less intense than the positive control.

Detection of Specific Transgenes

Reported work on GM tobacco has introduced resistance genes to virus or insects, including the following:

- Tobacco Mosaic Virus coat protein gene (TMV-CP),
- Cucumber Mosaic Virus coat protein gene (CMV-CP),
- Potato Virus Y coat protein gene (PVY CP),
- Bt insect resistance crystal protein gene.

GENERAL REQUIRMENTS

Duplication

In order to obtain a reliable result, each sample is analysed in duplicate, i.e. extraction is performed on two separate test samples and two aliquots of extract are submitted to PCR analysis.

Control Samples

Positive Control

The positive control samples are previously-tested GM tobacco samples obtained from the Institute of Microbiology Academia Sinica and other institutes, and have been extensively tested in our laboratory according to the present procedure.

Negative Control

The negative control samples were previously-tested non-GM tobacco (Seed origin from Henan Agriculture Academic Tobacco Institute).

Reagent Blank

No tobacco material is included in the sample. It is prepared using the same reagents and analytical procedures as for the test samples to control the quality of the extraction buffer.

Extraction Blanks

During the grinding of the samples, two microtubes are positioned next to the work place and subsequently submitted to PCR in order to control sample cross-contamination.

Accuracy Check

After every PCR procedure, the results are accepted only if a single band in the right position relative to the DNA size marker is obtained from the positive control, and at the same time there are no bands from the negative controls, reagent blank, or extraction blanks. Otherwise, the procedure is repeated. When samples yield weak bands on gel-electrophoresis (not as bright as the positive control), or more than one band is found on the gel, the DNA extraction and PCR should be repeated.

Contamination

One of the major problems in PCR-based methods is the accidental contamination of samples and reagents with DNA from previous experiments or from non-target sources especially from the positive control. Even minute cross-contamination is very likely to lead to false-positive results. To minimize such risks, sample preparation, DNA extraction, PCR and gel electrophoresis are carried out in different laboratories and the working surfaces are decontaminated with UV light (254 nm) overnight.

All samples are handled using sterile DNA- and DNAase-free equipment, wearing gloves at all times and changing the tips of the micro-pipette after each operation.

Airborne dust and aerosols may be formed during grinding or handling of tobacco samples, so laboratories should be well ventilated and samples are handled under a chemical fume hood area provided with strong exhaust ventilation.

Safety Precautions

A laboratory coat, safety glasses and gloves are worn throughout all steps of the procedure. Special care and chemical-resistant gloves, safety glasses and a mask must be used when working with ethidium bromide (EB), which should be used only in the chemical fume hood.

Screening by Kanamycin Resistance

Method 1.

1. Put seeds of each sample in a 1.5 ml micro tube.
2. Add 1.0 ml of 70% ethanol, then pipette out immediately.
3. Add 1.0 ml of 20% Clorox and soak for 30 min. Invert vials regularly.
4. Wash 4 times with sterile distilled water.
5. Distribute seeds evenly on plates containing ½ MS medium using a 1 ml pipette with a tip widened by cutting.
6. Transfer the germinating seeds to ½ MS medium plates containing kanamycin at 0, 50, and 100ppm. Observe growth on every treatment after 14 days.

Method 2.

Steps (1)~(4) are as Method 1.

5. Steam sterilise some 9cm filter paper, and filter sterilise the kanamycin solution.
6. Place a double layer of filter paper in a petridish and add 4 ml sterile water with 0, 50, 100ppm kanamycin solution. Distribute the sterilized seeds evenly and incubate at 26.5° in continuous light. Observe growth after 10 days.

Screening PCR for *nptII*, 35S and *nos* genes

Sample preparation

Seeds and Fresh Leaf

Sterilise seeds as described above. Cultivate (without adding kanamycin) for more than 5 days in the incubator.

Cured Leaf

See the protocol for DNA isolation from tobacco cured leaf (Below).

DNA extraction

Materials and Reagents

High speed freezer centrifuge
Micropipettes and tips
Gel electrophoresis system and Transilluminator
Analytical balance accurate to 0.1 mg
Heating bath (65°C)
Spectrophotometer
Microwave oven and heating plate
Steam sterilizer
Ultra-purity water system
Eppendorf tubes
CTAB
Ethanol
Agarose
10xTBE buffer
EB (Ethidium bromide)
Sample loading buffer
Dneasy plant mini kit (Qiagen)
RNase
DNA ladder

DNA Isolation from Seeds and Fresh Leaf

1. Germinate seeds on filter paper. Then grind *c*200 mg seedlings into powder in liquid nitrogen in a mortar and pestle (pre-chilled at -85°).
2. Transfer the powder to a microfuge tube, and add 2x CTAB extraction buffer (2% CTAB (w/v); 100mM Tris (pH8.0); 20 mM EDTA (pH8.0); 1.4M NaCl; 1% PVP; pre-warmed to 65°) according to the proportion of $1\mu\text{l}/\text{mg}$. Heat the mixture in a 65° water bath for 1-3 mins.
3. Add an equal volume of chloroform /isoamyl alcohol (24:1, v/v). Mix gently but thoroughly and centrifuge at 11000g for 4 mins.
4. Transfer the top phase to a new microtube and add 1/10 volume 10% CTAB(w/v); 0.7 M NaCl (pre-warmed to 65°). Add a second equal volume of chloroform / isoamyl alcohol (24:1, v/v) and centrifuge.
5. Transfer the top phase to a new microtube and add one volume of CTAB precipitation buffer (1% CTAB; 50mM Tris (pH 8.0); 10mM EDTA (pH8.0)) followed by gentle mixing. Centrifuge for 8min at 15000g.
6. Rehydrate the pellet in high salt TE buffer (10mM Tris (pH8.0); 1mM EDTA (pH8.0); 1M NaCl), in a 65° water bath for 10 min.
7. Reprecipitate the nucleic acid with two volumes of ethanol, and 8 min of centrifugation .
8. Wash the pellet with 80% ethanol and dry in a desiccator.
9. Rehydrate the nucleic acid pellet in $50\mu\text{l}$ 0.1xTE buffer (1mM Tris (pH8.0); 0.1mM EDTA).

DNA Isolation from Cured Leaf

(A) Modified CTAB Method

1. Add $700\mu\text{l}$ 1xCTAB extraction buffer, mix and heat for 10 min at 65°C .
2. Add $700\mu\text{l}$ chloroform/isoamyl alcohol and centrifuge at 12000g for 4 min.
3. Take the top phase and add 1/10 volume of 10% CTAB extraction buffer. Re-extract with chloroform/isoamyl alcohol and centrifuge.
4. Repeat Step 3. Then add one volume of CTAB precipitation buffer and centrifuge at 15000g for 8 min.
5. Rehydrate the pellet in high salt TE buffer at 65°C for 5-10 min.
6. Add two volumes of ethanol and hold at 70°C for 30 min.
7. Centrifuge for 8 min. at 15000g and rehydrate the pellet in $70\mu\text{l}$ TE buffer.

(B) Modified Protocol for DNA Isolation from Plant Tissue with the DNEASY Plant Mini Kit (Quiagen Inc.)

1. Place half of the sample (max. 20g) in a plastic bag, immerse in liquid nitrogen and grind with a cell disrupter (Bioreba) to obtain a homogenous powder. Transfer to a labeled 50ml sterile centrifuge tube. Weigh out 20-25mg of ground dry tobacco power into a 1.5ml microtube.

CORESTA Task Force Genetically Modified Tobacco – Detection Methods

2. Prepare the following Stock solution: 400µl AP1 + 5µl Rnase (100µg/µl) per sample to be extracted. Add 400µl Stock solution to the 20-25mg tobacco powder. Vortex to obtain a homogenous mixture.
3. Incubate the mixture 10 minutes at 65°C.
4. Add 130µl AP2 to the lysate, and incubate for 5 minutes on ice.
5. Remove from ice and pour the lysate onto the QIAshedder spin column, sitting on a 2ml collection tube. Centrifuge for 2 minutes at 14,000rpm.
6. Transfer the flow-through fraction to a new tube without disturbing the pellet.
7. Add 225µl AP3 buffer and 450µl ethanol (room temperature) to the 450µl lysate.
8. Apply half of the solution obtained in Step 7 onto the Dneasy mini spin column sitting in a 2ml collection tube. Centrifuge for 2 minutes at 10000rpm.
9. Repeat Step 8 with the remaining solution from Step 7.
10. Place Dneasy column in a new 2ml collection tube and add 500µl AW buffer onto the Dneasy column and centrifuge for 2 minutes at 10000rpm.
11. Add 500µl AW buffer onto the Dneasy column and centrifuge for 2 minutes at 14 000rpm.
12. Transfer the Dneasy column to a new 1.5ml microtube and add 130µl preheated (65°C) AE buffer onto the center of the Dneasy column and centrifuge for 2 minutes at 10000rpm .

Determination of DNA Yield and Purity

Electrophoresis Observation

Nucleic acid extracted from each sample is subjected to electrophoresis on an 0.8% agarose gel. DNA concentration and length is estimated by comparison of the position and brightness of the bands on the gel with a dilution series of lambda DNA (0, 10, 25, 50, 100, 200 ng/μl).

Measurement by UV spectrophotometer

The yield and purity of the nucleic acid can be estimated by the measurement of OD at 260nm and 280nm using a UV-spectrophotometer.

1. Transfer 5μl of the DNA extraction sample into a microtube. Add deionized water to 1ml and mix completely. Then fill into the cup of the spectrophotometer .
2. Correct the zero of the spectrophotometer with distilled water.
3. The Optical Density is measured at 260nm and 280nm alternately. Calculate the DNA concentration by $OD_{260nm} \times \text{dilution fold} \times 50/1000$ (μg/μl).
4. If the value of OD_{260}/OD_{280} is more than 1.8, this indicates the presence of RNA in the extract. If the value is less than 1.6 this indicates protein or phenol in the DNA extraction sample.

Testing Tobacco Chloroplast Non-coding DNA

The quality of DNA extraction can also be checked by detection of a tobacco chloroplast non-coding DNA region sequence using PCR amplification methods.

Chloroplast Universal Primer:

Plant -1 5' CGA AAT CGG TAG ACG CTA CG -3'
Plant -2 5' GGG GAT AGA GGG ACT TGA AC -3'

PCR of tobacco chloroplast DNA with these primers yields a product of 550 bp, which can be used as a quality marker for DNA preparation. As the PCR assay is very sensitive, this method will determine if the DNA extract can be used as a template for the GM screening assays. This is particularly useful following the DNA extraction of cured tobacco leaf sample.

PCR Process

Material and Reagents

Thermocyclers: Thermolyyn Gene Amp PCR 9600

Micropipette (brand and Reinin) and tips

Sterile deionized water (Millipore, Ultrafiltered)

Taq enzyme; 10xbuffer; Mg²⁺; dNTP (Promega)

Synthesized Oligonucleotide Primers (COL purity, storage in -40°C freezer)

Primers for Screening

For GM tobacco screening the following DNA sequences or genes are tested:

- CaMV 35S-promoter sequence
- *A. tumefaciens nos*-terminator sequence
- *nptII* sequence

Primers were custom synthesized and HPLC-purified by the commercial supplier, and dissolved in water to give a 400µM stock solution .

A: 35S-promoter

A 195bp PCR product is used as a marker to detect the presence of the promoter sequence from Cauliflower Mosaic Virus

35S-1 5' GCT CCT ACA AAT GCC ATC A -3
35S-2 5' GAT AGT GGG ATT GTG CGT CA -3

B: *nos*-terminator

A 180bp PCR product is used as a marker to detect the presence of the *nos*-terminator sequence from *Agrobacterium tumefaciens*.

NOS -1 5'- GAA TCC TGT TGC CGG TCT TG -3
NOS -2 5'- TTA TCC TAG TTT GCG CGC TA -3

C: *nptII*, Neomycin phosphotransferase II

A 411bp PCR product can be used as a marker to detect the *nptII* gene sequence using the following primers.

NPTII -1 5' GCC CTG AAT GAA CTG CAG GAC GAG GC -3'
NPTII -2 5' GCA GGC ATC GCC ATG GGT CAC GAC GA -3'

A 785bp PCR product can be used as a marker to detect the *nptII* gene sequence using the following primers.

NPTII-3 5' GAA CAA GAT GGA TTG CAC GC -3'
NPTII-4 5' GAA GAA CTC GTC AAG AAG GC -3'

A 699bp PCR product can be used as a marker to detect the *nptII* gene sequence using the following primers.

NPTII-3 5' - AGA GGC TAT TCG GCT ATG ACT GGG C -3'
NPTII-4 5' - CGG GAG CGG CGA TAC CGT AAA GC -3'

PCR Reaction

The reaction is performed in a 0.25ml microtube. The following components are added sequentially to a pre-chilled sterile microfuge tube and the tube was finger-vortexed after each addition.

Reagent	Loading Order	Volume	Final Concentration
10xBuffer	1	5.0µl	1xBuffer
dNTP	2	1.5 µl	200µM
Primer 1	3	2.5 µl	25pmol/rxn
Primer 2	4	2.5 µl	25pmol/rxn
DNA Template	5	1.0 µl	10ng/rxn
Water	6	18.5 µl	
Taq DNA Polymerase	7	3 units	2.5-3u/rxn
Mineral Oil	8	40 µl	

After reagents 1-6 are added the reaction solution is mixed thoroughly and centrifuged at 6500rpm for 30 sec. The tube is placed into the Air thermocycler to perform the pre-denaturation at 94°C for 7 min. Then Taq DNA Polymerase and mineral oil are added. The capillary tube is centrifuges at 6500rpm for 30sec. Finally the loaded tube is placed into the reaction chamber of the Air thermocycler and the program is run. After the run the PCR tube is stored in a –40°C freezer.

PCR Conditions

For the 35S test, the following cycle program is used:

- Pre-cycle: Hold temperature at 95°C for 7 minutes,
- Cycle: Cycle 35 times as follows
 - Denaturation at 94°C for 30 sec.,
 - Annealing at 62°C for 45 sec.,
 - Extension at 72°C for 90 sec.,
- Post-cycle: Hold at 72°C for 7 minutes.

In all PCR reactions a positive (DNA extracted from GM tobacco), a negative (DNA extracted from non-GM tobacco), a reagent blank (no DNA), and an extraction blank are tested with the same primer set.

Analysis of the PCR Products

Material and Reagents

Gel electrophoresis system

Microwave oven

Transilluminator (UV light, 312nm)

Micropipette and tips

5xTBE buffer

DNA ladder (100bp) length standard

Sample loading buffer (1.5g Ficoll type 400, 25mg Bromophenol Blue in 10ml water).

Gel Electrophoresis and Documentation

Agarose (1.5% for amplicons of 250-600bp) is completely dissolved in boiling 0.5xTBE buffer. After cooling to about 45°C, the agarose solution is poured into the gel mould, fitted with a comb. The gel is cooled and the comb removed. The gel is placed in the electrophoresis chamber and covered with about 5mm of TBE buffer. 2µl of loading buffer is added to 10µl of the amplified PCR solution, and the mixture is loaded onto the gel in 0.5xTBE buffer. On each gel plate at least 3 lanes must be left at each side of the plate for positive control, negative control, and DNA ladder.

After loading, the gel is run for 2 hours at 100V in 0.5xTBE buffer. Finally the gel is stained in 0.5µg/ml ethidium bromide for about 40 minutes and the DNA bands visualised using a UV transilluminator.

Confirmation test

If a sample is PCR-positive either the promoter or the terminator sequence, a restriction enzyme and sequence analysis of the amplified DNA fragment can be performed to confirm the results. To increase the sensitivity of the screening, nested- or semi-nested PCR can be applied. When the PCR product bands of the samples are less intense than the positive control, the confirmation test must be done.

Restriction Analysis of 35S and *nos* Amplified Fragments

Material and Reagents

Asp700 (*XmnI*) restriction enzyme

NsiI restriction enzyme

Gel electrophoresis system

Microwave oven

Transilluminator (UV light, 312nm) and documentation device

Micro-pipette and tips

Agarose

5xTBE buffer

Sample loading buffer

DNA ladder

Ethidium bromide

Restriction Enzyme Reaction Preparation

Besides the produced DNA fragment, PCR reactions contain reagents such as Taq DNA Polymerase, dNTP, DNA template and other materials which can inhibit the activity of the restriction enzyme. These can be removed by DNA purification as follows;

Method 1

Remove the top phase (mineral oil) with a micro-pipette. Add 2 volumes of chloroform and vortex. Centrifuge for 5 min. at 12000g, and collect the top phase to a new tube.

Method 2.

Transfer the PCR product to another sterile microtube, and add 100µl ethanol to precipitate the DNA fragments. The pellet is rehydrated in 50µl high salt TE buffer.

Method 3.

Purify the amplified DNA fragment using a commercial kit (Promega).

Restriction Enzyme Reaction

1. Transfer 10µl of the purified PCR product to a new tube. Add 10x restriction enzyme buffer and 1µl restriction enzyme (4-10units). Vortex and incubate at 37°C for 3 hours.
2. Mix 2µl restriction enzyme cut product with 1µl loading buffer and examine by 1.5% agarose gel electrophoresis.

35S Promoter

If the sample is 35S promoter positive, an *Asp700* (*XmnI*) restriction enzyme analysis of the amplified DNA fragment is performed. The 195bp amplified fragment can be cut into two pieces of 115bp and 80bp length with the restriction enzyme *Asp700*. This reaction can be performed by adding 5units of *Asp700* enzyme to 10µl PCR reaction solution and incubating the solution for 3 hours at 37°C. After that, the products are analyzed by electrophoresis through a 1.5 % agarose gel in 0.5xTBE buffer followed by staining in ethidium bromide and visualization of the DNA bands using a UV transilluminator.

nos terminator

If the sample is *nos*-terminator positive, a *NsiI* restriction enzyme analysis of the amplified DNA fragment is performed. The 180 bp amplified fragment can be cut into two pieces of 96bp and 84bp length with the restriction enzyme *NsiI*. This can be performed by adding 5 units of *NsiI* enzyme to 10µl PCR reaction solution and incubating the solution for 2 hours at 37°C. The products are analysed by electrophoresis through a 2.5% agarose gel in 0.5xTBE buffer followed by staining in ethidium bromide and visualization of the DNA bands using a UV transilluminator.

Nested or Semi-nested PCR

If the amplified PCR fragment is very weak or not visible (the presence of the transgene is uncertain), it is recommended to perform a nested or semi-nested PCR for detection of 35S or *nos*- sequences.

There are two pairs of primers in the nested PCR assays, one being the same as for the normal PCR, and is added into the reaction solution to perform the first PCR. Another pair of primers called specific primers is added into the the second PCR reaction. The sequence of the specific primers is designed according to the sequence of the target DNA such that they fall within the first amplified fragment. Thus the fragment can be used as the template of the second PCR process. It is necessary that the fragment amplified by the second primers is shorter than that of the first one. PCR products are analysed by electrophoresis through a 1.5% agarose gel followed by staining in ethidium bromide and visualization of DNA bands using a UV transilluminator. By using nested PCR, the sensitivity of PCR screening is increased.

Nested PCR Assay of 35S

Primer:

35 S -nest -1 5' CGT TCA CCT AAC TAC ACT AT 3'
35 S -nest -2 5' GTG ACT GCC GTC TCC GTA GA 3'

The product of the 35S nested PCR is 110 bp in length.

Nested PCR Assay of nos-terminator

The product of *nos* nested PCR is 100 bp in length.

Nested PCR Assay of *nptII*

The products obtained are very different when different primers are used in the initial PCR process. In the screening methods of tobacco cured leaf described above, primers NPTII-1 and NPTII-2 are usually used. The second pair of primers (Below) produce a product of the nested PCR of 209bp.

NPTII-nest-1 5' CTG GCT GCT ATT GGG CGA AG 3'
NPTII-nest-2 5' TCG TCC AGA TCA TCC TGA TC 3'

Determination of specific transgenes

TMV - CP: Tobacco Mosaic Virus Coat Protein

A 327bp PCR product is used as a marker to detect the presence of the TMVcp gene sequence with the following primers:

TMV1 5' - GTG TTC TTG TCA TCA GCG TGG GC -3'
TMV2 5' - CAC CGT TGC GTC GTC TAC TCT AC G -3'

The following cycle program is used:

- Pre-cycle: Hold temperature at 95°C for 5 minutes,
- Cycle: Cycle 30 times as follows
 - Denaturation at 94°C for 1 minute,
 - Annealing at 55°C for 1 minute,
 - Extension at 72°C for 2 minutes,
- Post-cycle: Hold at 72°C for 7 minutes.

CMV – CP: Cucumber Mosaic Virus Coat Protein

A 202 bp PCR product is used as a marker to detect the presence of the CMVcp gene sequence with the following primers:

CMV1 5' - AAG ACG TTG GCA GCT GGT CG -3'
CMV2 5' - CTC GAA TTT GAA TGC GCG AA -3'

A 263 bp PCR product can be used as a marker to detect the presence of the CMV cp gene sequences with primers CMV3 and CMV4.

CMV3 5' - ACC CAA CCT TTG TAG GGA GTG AGC G -3'
CMV4 5' - ACA TAG CAG AGA TGG CGG CAA CG -3'

The PCR cycle used is as for TMVcp, with the exception that the Pre-cycle phase is:

- Pre-cycle: Hold temperature at 94°C for 2 minutes

PVY CP: Potato Virus Y Coat Protein

A 362 bp PCR product can be used as a marker to detect the presence of the PVYcp gene sequence using the primers PVY1 and PVY2:

PVY1 5' - GAT ATT TCA AAT ACT CGG GCA -3'
PVY2 5' - GCA TAA CGC GCT AAA CCC AC -3'

A 272 bp PCR product can be used as a marker to detect the presence of the PVYcp gene sequence using the primers PVY3 and PVY4:

PVY3 5' - CTC AGA TGT TGC AGA AGC GT -3'
PVY4 5' - CCT CGG TGG TGT GCC TCT CT -3'

The PCR cycle program used is the same as for the CMVcp gene.

TMV 54 kD Gene

A 295 bp PCR product can be used as a marker to detect the presence of the TMV 54 kD gene sequence with following primers:

TMV54 1 5' - GAG TTG TCT GGC ATC ATT GA -3'
TMV54 2 5' - ACA ATG GTC AAA GCC GGG TA -3'

The following cycle program is used:

- Pre-cycle: Hold temperature at 95°C for 5 minutes,
- Cycle: Cycle 35 times as follows
 - Denaturation at 94°C for 1 minute,
 - Annealing at 60°C for 1 minute,
 - Extension at 72°C for 2 minutes,
- Post-cycle: Hold at 72°C for 7 minutes.

Bt gene

A 299bp PCR product can be used as a marker to detect the presence of the Bt gene sequence using the following primers:

BT -1 5' - CCC ACT AGT TAA CAA TTT GAT TGG A -3'
BT -2 5' - CCG GAA GCT TTA GGA CTG TAG GT -3'

The following cycle program is used:

- Pre-cycle: Hold temperature at 94°C for 5 minutes,
- Cycle: Cycle 30 times as follows
 - Denaturation at 94°C for 45 sec.,
 - Annealing at 55°C for 1 minute,
 - Extension at 72°C for 2 minutes,
- Post-cycle: Hold at 72°C for 7 minutes.

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APPENDIX IV

GLOSSARY OF TERMS

35S	<i>Promoter</i> from Cauliflower Mosaic Virus, commonly used in <i>transgene constructs</i>
AFLP	Amplified Fragment Length Polymorphism, a method of DNA fingerprinting
agarose gel	A medium for <i>DNA electrophoresis</i>
<i>Agrobacterium tumefaciens</i>	Bacterium used as a vector for plant <i>transformation</i>
amplicon	<i>DNA fragment</i> produced by <i>PCR amplification</i> of a specific <i>target</i> region
amplification	Increasing the concentration of a specific <i>DNA fragment</i> to detectable levels by <i>PCR</i>
annealing, anneal	Interaction between two <i>DNA</i> molecules to form a double strand (see also <i>hybridisation</i>)
base pair (bp)	Paired <i>nucleotides</i> in a <i>DNA</i> molecule. Used as a unit of length of a <i>DNA</i> strand.
Bt	<i>Bacillus thuringensis</i> , or the insecticide produced by this bacterium
CaMV	Cauliflower Mosaic Virus
construct	<i>Spliced DNA</i> molecule prepared for <i>transformation</i>
<i>cryIA</i>	One <i>gene</i> encoding a <i>Bt</i> insecticidal protein
cycle	The repetitive step of a <i>PCR</i> reaction
denaturation (of DNA)	Separation of double-stranded <i>DNA</i> into single strands. Necessary for replication and <i>PCR amplification</i>

DNA, Deoxyribonucleic acid	Linear molecule composed of two strands of <i>nucleotides</i> . Carrier of genetic information.
electrophoresis	Process used to separate and identify <i>DNA fragments</i> , by applying an electric field.
ELISA	Enzyme Linked ImmunoSorbent Assay. Protein detection method
endogenous	Occurring naturally within the organism
EPSPS, EPSP	Enol puruvyl shikimate-3-phosphate synthase. Enzyme inhibited by <i>glyphosate</i>
fragment	A <i>DNA</i> molecule of defined length
gene	Unit of genetic information, comprising <i>DNA</i> and usually encoding a protein
genetic engineering, genetic modification	Producing a novel organism with desirable characteristics by <i>transformation</i> using <i>DNA constructs</i> in the laboratory.
glyphosate	Herbicide marketed as RoundUp.
GM	Genetically modified
GMO	Genetically modified organism
homologous / homology	<i>DNA</i> molecules having identical or highly similar sequence
hybridisation	<i>Annealing</i> of <i>homologous DNA</i> molecules
junction sequence	Region between two <i>spliced</i> sections of a <i>transgene</i>
multiplex PCR	<i>PCR</i> of multiple <i>target genes</i> in a single reaction tube.
neomycin phosphotransferase	<i>Gene</i> conferring Kanamycin resistance

nested PCR	Sequential <i>PCR</i> focussing on a specific <i>target DNA</i> region
nopaline synthase	Enzyme from <i>Agrobacterium tumefaciens</i>
<i>nos</i>	<i>Nopaline synthase gene</i> used as a source of <i>promoter</i> or <i>terminator sequences</i>
NPTII, <i>nptII</i>, <i>neo</i>	<i>Neomycin phosphotransferase gene</i>
nucleotide	Basic repeat unit of a <i>DNA</i> molecule
PCR	Polymerase Chain Reaction. Method of detecting <i>DNA sequences</i> , comprising multiple <i>cycles</i> of <i>amplification</i>
primer	Short single-stranded <i>DNA</i> molecule used in <i>PCR</i> to specify the <i>target sequence</i>
probe	A labelled <i>DNA</i> molecule used in <i>hybridisation</i> assays.
promoter	<i>DNA sequence</i> regulating <i>gene</i> expression
restriction analysis	<i>DNA</i> analysis by enzymatic cleavage at specific <i>sequences</i>
selectable marker	<i>Gene</i> allowing selection of <i>GM</i> from non- <i>GM</i> plants (e.g. by conferring antibiotic resistance)
sequence	<i>DNA</i> molecule whose identity can be described by the order of <i>nucleotides</i> along it
sequencing	Determining the order of <i>nucleotides</i> along a <i>DNA</i> strand.
Southern blotting	Transfer of <i>DNA</i> onto a membrane for a <i>hybridisation</i> assay.
spliced	Conjoined, as of different <i>DNA sequences</i> in a <i>transgene construct</i>
Taq DNA polymerase	Enzyme used in <i>PCR</i>

target	Region of <i>DNA</i> under detection in an assay
template	Sample <i>DNA</i> used in a <i>PCR</i> to which a <i>primer anneals</i>
terminator	<i>DNA sequence</i> regulating <i>gene</i> expression
threshold	Maximum acceptable level, or minimum detectable level.
transformation	Transfer of <i>DNA</i> during <i>genetic modification</i>
transgene	Novel <i>gene</i> introduced by <i>transformation</i>
Western blotting	Transfer of protein onto a membrane for an immunological assay