Chapter 3: Detection of genome modifications in genetically modified tobacco plants using representational difference analysis.

Abstract

Representational Difference Analysis was applied to identify and characterize genome differences in genetically modified tobacco plants. The plant material used was wild-type tobacco of the variety '*Samsun/UK*' and genetically modified '*Samsun/UK*' in which the rice cystatin gene had been introduced by *Agrobacterium tumefaciens*-mediated transformation. RDA was performed using the combined DNA from two independent transformants as the tester and wild-type, non-regenerated 'Samsun' as the driver. Three different DNA sequences were isolated from the two genetically modified plants representing possible DNA sequence differences between the genomes of genetically modified and a non-modified wild-type tobacco plant. Two subtraction products from a subtraction using the methylation-sensitive restriction enzyme *Hpa*II were characterized. One had 96% homology to part of the tobacco chloroplast 23S and 4.5S rRNA genes and the other a 99% homology to the 18S rRNA gene of tobacco. A third DNA subtraction sequence derived from *Hind*III-digested genomic DNA had no significant homology to any already reported DNA sequences. Results indicated that the genetic modification process both alters genome methylation and also causes minor sequence alterations.

Introduction

Introducing foreign DNA molecules into the plant genome using plant tissue culture is a stressful event and carries the risk of genomic variation (Lee and Phillips, 1987; Brown et al., 1990; Phillips et al., 1994; Cullis, 1999). Phenotypic changes in genetically modified plants are well documented

(Lynch et al., 1995; Bao et al., 1996) including altered growth characteristics as found for genetically modified tobacco expressing the *gus* gene selfed over several generations (Caligari et al., 1993). In addition, a high frequency of cytogenetic aberrations in transgenic oat, has also been reported (Choi et al., 2000). Molecular tools have further recently verified the occurrence of transformation-specific genomic changes in several genetically modified plants (Sala et al., 2000; Labra et al., 2001). AFLP (amplified fragment length polymorphisms) and RAPD (random amplified polymorphic DNAs) analysis of genetically modified and non-modified rice plants using the *Agrobacterium tumefaciens* system for plant transformation has demonstrated genomic homogeneity among non-modified rice plants and verified genomic changes within the genetically modified plants (Labra et al., 2001). Genome variation is in addition to any changes arising from T-DNA integration and has been shown to cause complex rearrangements during T-DNA/transgene integration. Modifications found around T-DNA/plant DNA junctions include base substitutions, duplication of border and genomic sequences and small nucleotide deletions (Ohba et al., 1995; Windels et al., 2001; Zheng et al., 2001; Stahl et al., 2002). Strategies to eliminate such variation include the selection of the most "normal" plants, which will be used in subsequent crossings to obtain a plant, which is morphologically, but not necessarily genetically, identical to the mother plant (Labra et al., 2001).

In order to assess possible genetic changes in the genome of genetically modified tobacco plants other than around junction sites, the technique of representational difference analysis (RDA) was used in this study. RDA is a DNA subtractive technology and can be applied to derive probes for genomic losses, rearrangements, amplifications, point mutations and pathogenic organisms found within any of two genomes to be compared (Lisitsyn et al., 1993; Ushijima et al., 1997; Michiels et al., 1998). Although the RDA technique was first reported in 1993 and has been widely applied with cDNAs (Hubank and Schatz, 2000), the technique has been applied only on a limited scale with genomic DNAs. In RDA, target DNA fragments are sequentially enriched by favorable hybridization kinetics and subsequently amplified by PCR. The technique is based on altering the ends of the DNA sequences and

amplifying certain combinations, which results in a steady depletion of common sequences. Depletion is carried out by hybridization with increasingly large amounts of driver DNA (the source of the sequences to be eliminated) and with reducing amounts of the tester DNA (the source of the sequences of interest to be isolated). After each round of hybridization and amplification, only those sequences, which have formed a double stranded DNA fragment, with both strands arising from the tester DNA, will be amplified by PCR.

Results

A) Characterization of genetically modified tobacco with RDA.

Non-modified wild-type and genetically modified tobacco plants (*Nicotiana tabacum* L., var 'Samsun') expressing a rice cystatin as described in Annex (A) under "Materials and method" for Section I: "Plant transformation and selection" were used for execution of RDA. Genome subtractions between genetically modified and wild-type tobacco plants were performed with pooled DNA of two genetically modified plants mixed in a 1:1 ratio used as tester and wild-type plant genomic DNA as driver (experiment 1 and 3) and *vice versa* (experiment 2 and 4). Figures 3.1A and 3.2A shows DNA profiles after electrophoretic separation on an agarose gel of genomic DNA from modified and wild-type tobacco DNA digested with the restriction enzymes *Hpa*II and *Hind*III, respectively. Several subtraction products were produced for experiments 1 and 3 (modified DNA as tester and wild-type DNA as driver) after applying three rounds of subtractive hybridization and amplification using driver to tester ratios for first, second and third round subtractions of 75:1, 300:1 and 15000:1 for *Hpa*II-digested DNA (Figure 3.1B, C and D) and 3:1, 100:1 and 4500:1 for *Hind*III-digested DNA (Figure 3.2B, C and D). In experiments 2 and 4 (non-modified DNA tester/modified DNA driver) subtraction products were only found for *Hpa*II amplicons (experiment 2).

University of Pretoria etd – Van der Vyver C 2002

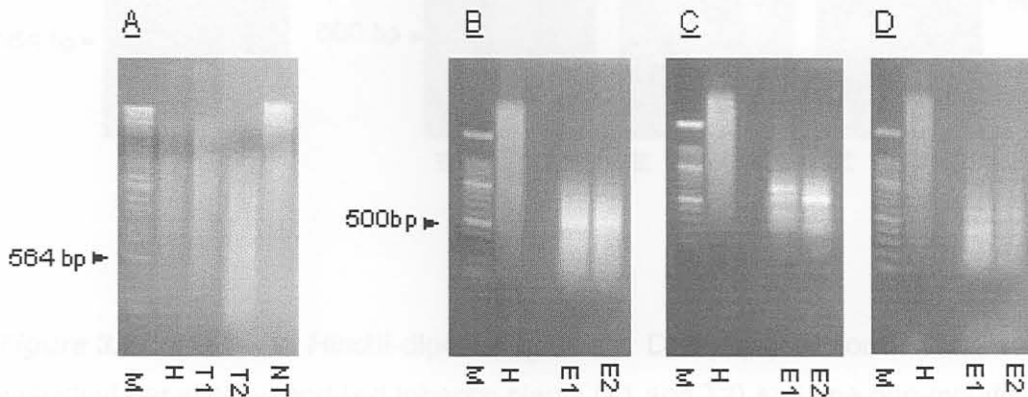

Figure 3.1: RDA of genetically modified and non-modified wild-type tobacco plant DNA. (A) Genomic *Hpa*II-digested DNA after separation on an agarose gel and stained with ethidium bromide from two individual genetically modified tobacco plants (T1 and T2) and from one wild-type tobacco plant (NT). Lane M represents molecular size marker (*Eco*RI/*Hind*III-digested λ DNA) and lane H represents 500ng herring sperm DNA. (B), (C) and (D) PCR amplified subtraction products from experiment 1 and 2 (E1 and E2) generated from *Hpa*II-digested DNA after the first (B), second (C) and third round (D) of subtractions. Lane M represents a 100 bp size marker and lane H represents 1µg of herring sperm DNA.

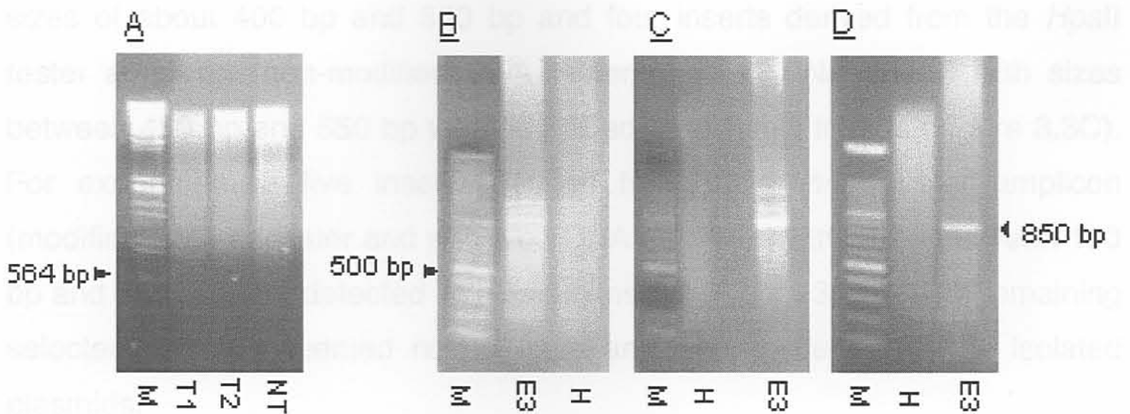

Figure 3.2: RDA on *Hind*III-digested genomic DNA. (A) Genomic DNA from two individual genetically modified tobacco plants (T1 and T2) and one non-modified wild-type plant (NT), digested with the restriction enzyme *Hind*III after separation on an agarose gel, stained with ethidium bromide. Lane M represents molecular size marker (*Eco*RI/*Hind*III-digested λ DNA). (B), (C) and (D) PCR amplified subtraction products from experiment 3 (E3) generated from *Hind*III-digested genomic DNA after the first (B), second (C) and third round (D) of subtractions. Lane M represents a 100 bp DNA marker and lane H represents 1μg of herring sperm DNA.

All pooled amplified third-round *Hpa*II and *Hind*III subtractions were cloned into the cloning vector p*MOSBlue* (Amersham Pharmacia Biotech, UK) and *E. coli* competent cells (Amersham Pharmacia Biotech, UK) were transformed with the plasmid allowing blue/white selection on an ampicillin-containing medium. Sixty white *E. coli* colonies derived from the three transformations were hybridized separately with either labeled *Hpa*II or *Hind*III driver amplicons. From each hybridization, ten colonies with the lowest hybridization signal were selected using the *Gene Images* random prime labeling module (Amersham Pharmacia Biotech, UK) (Figures 3.3 and 3.4). From these colonies plasmids were isolated, which possibly contained putative specific tester DNA subtraction products. Low level hybridization could also be due to hybridization to vector sequences or sections of probes hybridizing to parts of clone inserts. For experiments 1 and 2, two inserts derived from the *Hpa*II tester amplicon (modified DNA as tester and wild-type DNA as driver) with

sizes of about 400 bp and 550 bp and four inserts derived from the *Hpa*II tester amplicon (non-modified DNA tester/modified DNA driver) with sizes between 450 bp and 550 bp were identified as plasmid inserts (Figure 3.3C). For experiment 3 five inserts derived from the *Hind*III tester amplicon (modified DNA as tester and wild-type DNA as driver) with sizes between 150 bp and 400 bp were detected as plasmid inserts (Figure 3.4B). The remaining selected colonies seemed not to have any inserts present in the isolated plasmids.

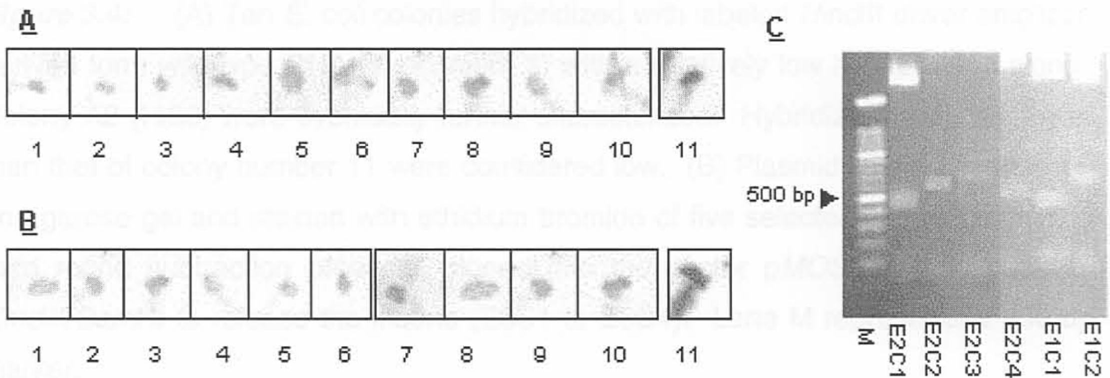

Figure 3.3: (A and B) Ten *E. coli* colonies each for the two transformations hybridized separately with labeled *Hpa*II driver amplicon derived either from wild-type DNA (A; experiment 1) or genetically modified DNA (B; experiment 2) with a relatively low hybridization signal. Hybridization signals lower than that of colony number 11 were considered low. Colonies A7 and B5 (Hp12 and Hp14) were the final clones, which were eventually further characterized. (C) Third round subtraction products cloned into the cloning vector P*MOSBlue* and cut with *Hind*III/*Bam*HI to release the cloned inserts E1C1 and E1C2 derived from experiment 1 and E2C1, E2C2, E2C3 and E2C4 derived from experiment 2. Cloned inserts were separated on an agarose gel stained with ethidium bromide. Lane M represents a 100 bp DNA marker.

University of Pretoria etd – Van der Vyver C 2002

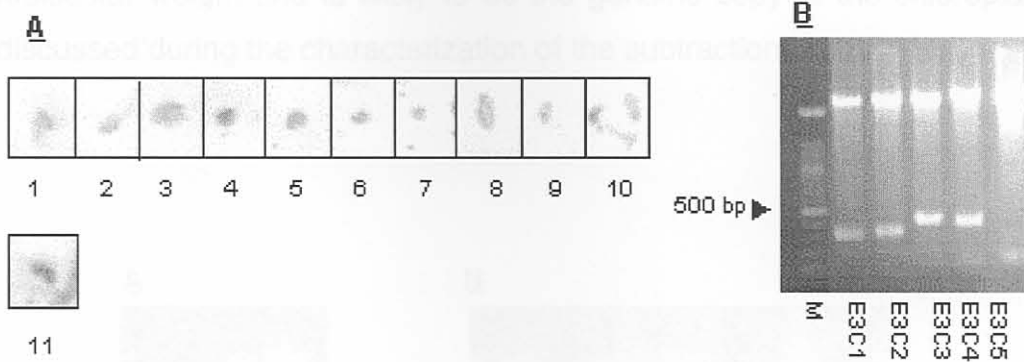

Figure 3.4: (A) Ten *E. coli* colonies hybridized with labeled *Hind*III driver amplicon derived form wild-type DNA (experiment 3) with a relatively low hybridization signal. Colony A2 (Hi30) were eventually further characterized. Hybridization signals lower than that of colony number 11 were considered low. (B) Plasmid DNA separated on an agarose gel and stained with ethidium bromide of five selected clones containing third round subtraction products, cloned into the vector p*MOSBlue* and cut with *Hind*III/*Bam*HI to release the inserts (E3C1 to E3C4). Lane M represents a 100 bp marker.

DNAs of the different rounds of subtraction were probed against all cloned and labeled final amplified subtraction products where pooled genetically modified DNA acted as tester DNA and wild-type tobacco DNA as driver. Three final subtraction products, Hp12, Hp14 and Hi30, did not hybridize with the original driver DNA amplicons derived from amplification of *Hpa*II or *Hind*III-digested genomic DNAs (Figures 3.5 and 3.6). Southern blot hybridization with other labeled isolated clones showed that these clones were not unique to the tester amplicon but were also present in the driver amplicon, indicating possible incomplete subtraction allowing background sequences through or possible size differences resulted in the enrichment of certain DNA fragments. All three final subtraction products hybridized with amplified DNAs of first, second and third round subtractions and very weakly with tester DNA amplicons (Figures 3.5 and 3.6). However, subtraction product Hp12 also

seemed to be related to the driver amplicon. The driver band is at a different molecular weight and is likely to be the genuine copy of the chloroplast (as discussed during the characterization of the subtraction product).

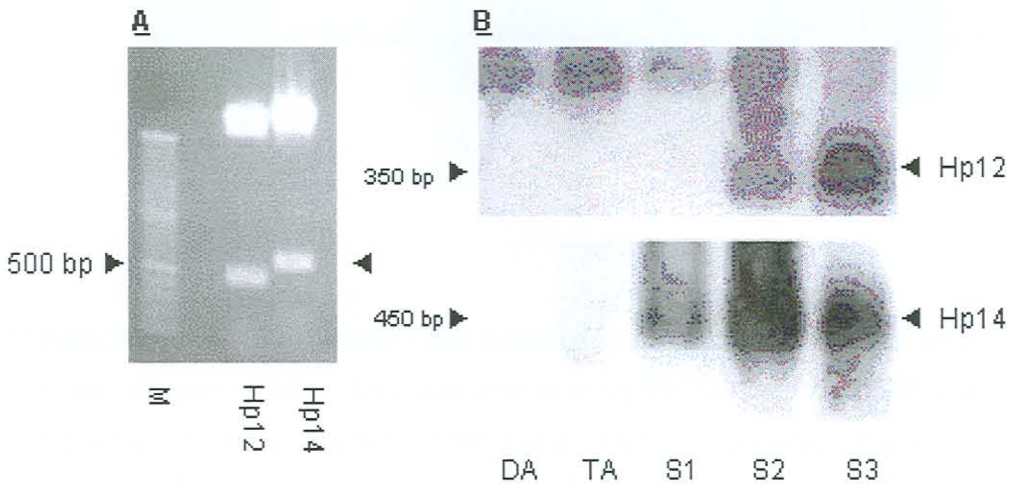

Figure 3.5: (A) Third round amplified Hp12 and Hp14 subtraction products separated on an agarose gel and stained with ethidium bromide after cloning into plasmid p*MOSBlue* and release of cloned product by a *BamH*I/*EcoR*I digest of isolated plasmid. Digestion of plasmid with *BamH*I/*EcoR*I added an additional 50 bp of cloning vector to the insert size. Arrow indicates position of Hp12 and Hp14 on the gel. Lane M represents a 100 bp marker. (B) Hybridization of subtraction products Hp12 (upper part) and Hp14 (lower part) to a filter containing *Hpa*II-digested and amplified DNAs derived from a wild-type tobacco plant (DA/driver amplicon), two pooled DNAs of genetically modified tobacco plants (TA/tester amplicon) and amplified subtraction products after first (S1), second (S2), and third round (S3) of subtraction.

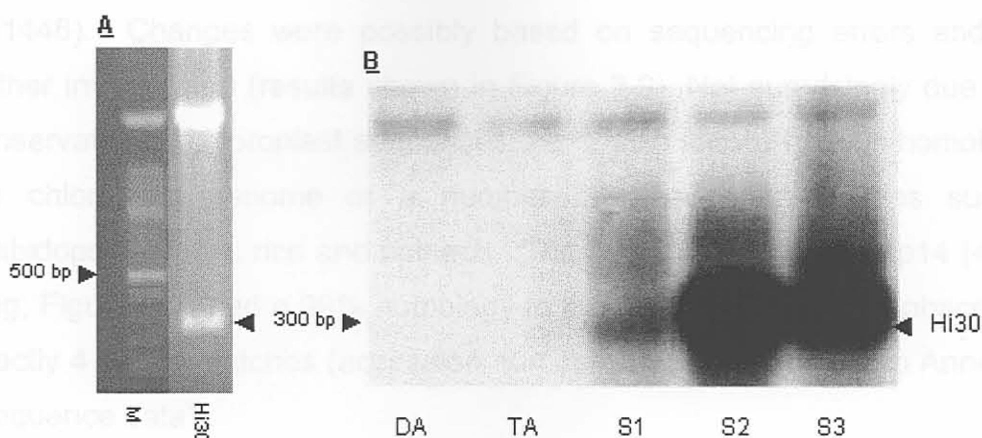

Figure 3.6: (A) Third round Hi30 subtraction product separated on an agarose gel and stained with ethidium bromide after cloning into plasmid p*MOSBlue* and release of cloned product by a *BamHI*/*EcoRI* digest of isolated plasmid. Digestion of plasmid with *BamHI*/*EcoRI* added an additional 50 bp of cloning vector to the insert size. Arrow indicates position of Hi30 on the gel. Lane M represents a 100 bp marker. (B) Hybridization of Hi30 to a filter containing *Hind*III-digested and amplified DNAs derived from a wild-type tobacco plant (DA/driver amplicon), two pooled DNAs of genetically modified tobacco plants (TA/tester amplicon) and amplified subtraction products after first (S1), second (S2), and third round (S3) of subtraction.

B) Characterization of methylation sensitive and non-sensitive RDA subtraction products.

Hp12, Hp14 and Hi30 were sequenced and obtained sequence was analyzed using the DNA analysis tools Blast, FastA and the Smith-Waterman algorithm. Remaining undigested adaptor sequences present on the subtraction products were not included in the sequence analysis. The subtraction product Hi30 was 214 bp long (Figure 3.6) and had no significant homology to any already reported DNA sequence. In contrast, product Hp12 (331 bp long; Figure 3.5) had a 97% homology to part of the tobacco chloroplast genome DNA, specifically to part of the tobacco chloroplast 23S and 4.5S rRNA genes

University of Pretoria etd – Van der Vyver C 2002

with 9 bp changes at different locations (accession numbers Z00044 and J0I1446). Changes were possibly based on sequencing errors and were further investigated (results shown in Figure 3.9). Not surprisingly due to the conservation of chloroplast sequences, Hp12 also showed a high homology to the chloroplast genome of a number of other plant species such as Arabidopsis, maize, rice and spinach. The subtraction product Hp14 (444 bp long; Figure 3.5) had a 99% homology to the 18S rRNA gene of tobacco with exactly 4 bp mismatches (accession number AJ236016) (see also Annex (B): "Sequence data").

The subtraction products Hp12 and 14 were used to determine the methylation status of these regions in the genetically modified and wild-type genomic DNAs. The two labeled subtraction products were hybridized to *HpaII*-digested genomic DNA derived from either the two original genetically modified tobacco plants or the one wild-type plant used in the experiments. Target detection was optimized regarding signal background ration, to two hours of filter exposure to a hyperfilm. After exposure, two additional larger size hybridization products were identified in wild-type DNA when the subtraction product Hp12 was the probe (Figure 3.7; Hp12/NT). The lower molecular weight band most likely represented the non-methylated plastidic DNA. The hybridization pattern observed with Hp14 gave a relatively larger amount at the higher molecular weight regions of the DNAs from the genetically modified plants (T1 and T2), and a missing band in these same DNAs at the lowest molecular weight site (Figure 3.7).

University of Pretoria etd – Van der Vyver C 2002

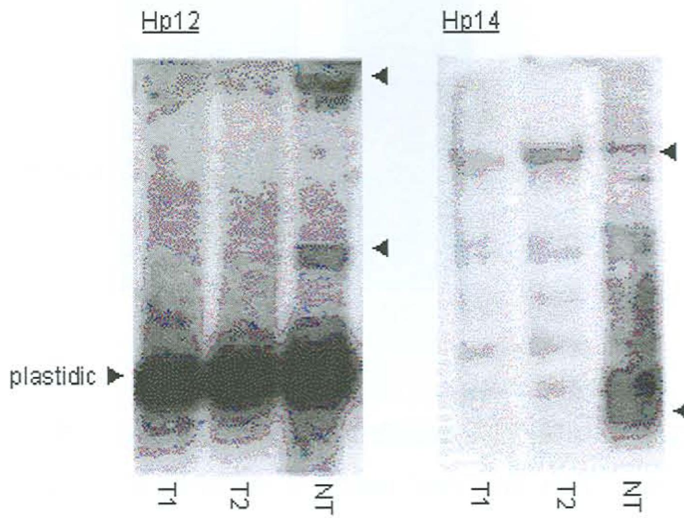

Figure 3.7: Hybridization of final amplified Hp12 and Hp14 subtraction products to a filter containing *Hpa*II-digested genomic DNA derived from two individual genetically modified tobacco plants used in the experiments for pooling DNAs (T1 and T2) and from the non-modified wild-type tobacco plant (NT). Detected band of plastidic DNA with Hp12 is indicated.

In a second experiment, wild-type genomic tobacco plant DNA was digested with *Msp*I to determine possible loss of methylation between modified and non-modified tobacco plants. After exposing the filter for three hours to hyperfilm, a single hybridization product was observed in wild-type plant DNA possibly representing the non-methylated plastidic DNA indicating an decrease in methylation in genetically modified plants when compared to wild-type plants (Figure 3.8).

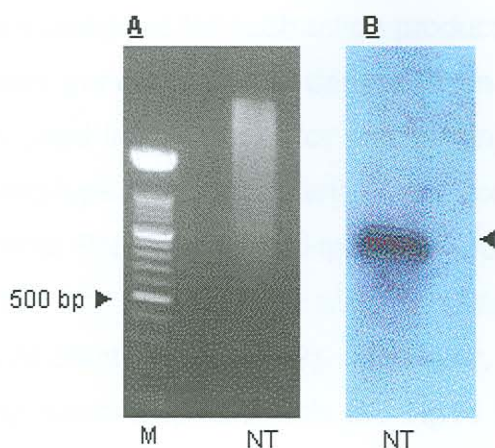

Figure 3.8: (A) Genomic *Msp*I-digested DNA after separation on an agarose gel and stained with ethidium bromide from one non-modified wild-type tobacco plant (NT). Lane M represents a 100 bp marker. (B) Hybridization of final amplified Hp12 subtraction products to a filter containing *Msp*I-digested genomic DNA derived from a non-modified wild-type tobacco plant (NT). Detected band of plastidic DNA with Hp12 is indicated.

The sequence information obtained for Hi30 and Hp12 and 14 was used to design pairs of primers to test for genomic variation in a PCR-based assay. Pairs of primers were designed using a standard design program (Expasy, Switzerland). Primers used to amplify subtraction product Hp12 were Hp12R and Hp12L, Hp14R and Hp14L for product Hp14 and Hi30R and Hi30L for subtraction product Hi30 (Table 3.1).

Table 3.1: Sequences of primers used for PCR amplification.

Name	Sequence
Hp12L	5' CGA GCC AAT GTT CGA ATA CC 3'
Hp12R	5' CCG AAG TTA CGG GGC TAT TT 3'
Hp14L	5' TGT CGG CCA AGG CTA TAA AC 3'
Hp14R	5' TTC CGT TAA CGA ACG AGA CC 3'
Hi30L	5' GGA ATG ATT TCC CAA AAC TCC 3'
Hi30R	5' CGA CGT CGA CTA TCC ATG AAC 3'

University of Pretoria etd – Van der Vyver C 2002

Primers designed for subtraction products Hp12 and Hp14 did not differentiate between genomic DNAs derived from either genetically modified tobacco plants used in this study for production of amplicons and DNA derived from the wild-type tobacco plant. An expected band from chloroplast and ribosomal RNA genes for Hp12 and Hp14, with the predicted size of 190 bp and 196 bp, respectively, were amplified out of the genomic DNA of both types of plants (Figure 3.9). However, a second bigger band with a size of 390 bp were amplified with the Hp14 primers from both the wild-type and genetically modified genomic DNAs. Subsequent cloning of amplification products into the vector p*MOSBlue* and sequencing of the amplified products showed that amplification products were identical for genetically modified and wild-type DNA with no base pair changes. The bigger amplification product amplified with the Hp14 primers were a direct repeat of the smaller amplification product found with the same primers. When the sequences of the amplification products were aligned with the two DNA sequences of the subtraction products Hp12 and Hp14, 1 bp and 4 bp mismatches were observed, respectively. However, these sequences aligned 100% with the known sequences of the tobacco chloroplast genome and the tobacco 18S rRNA gene. Although primers designed for subtraction product Hi30 amplified a predicted 199 bp product from genomic DNA derived from the two genetically modified plants, these primers failed to amplify, after 42 cycles of PCR, a fragment from genomic DNA from the wild-type plant (Figure 3.9). This result was confirmed when labeled Hi30 subtraction product was hybridized to a filter containing *Hind*III-digested genomic DNA from the non-modified wild-type tobacco plant. No hybridization products were detected on this filter, indicating the absence of the Hi30 subtraction product in the wild-type plant DNA used for the production of the RDA amplicons (data not showed).

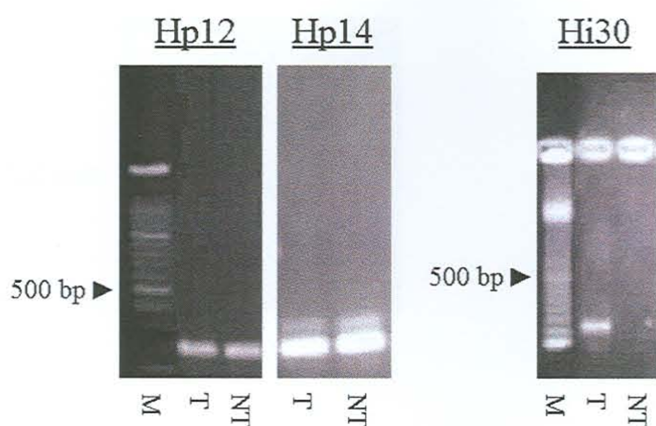

Figure 3.9: PCR amplification of genomic target DNAs with primers designed for Hi30, Hp12 and Hp14 with genomic template DNA derived from pooling DNAs of two genetically modified tobacco plants (T) and genomic DNA derived from the non-modified wild-type tobacco plant (NT). Lane M represents a 100 bp marker.

The Hp12 subtraction product was further characterized by designing the primer pair Hp12A and Hp12B (Table 3.2). Both primers had a single base pair change between the difference product and the known tobacco chloroplast sequence. DNA amplification using PCR with this primer pair resulted in an amplification product from genetically modified genomic DNA (Figure 3.10). In contrast, amplification of the identical product was much weaker in wild-type genomic DNA, which might suggest a base pair change in the primer site in the genetically modified tobacco DNA.

Table 3.2: Sequences of primers used for the characterization of subtraction product Hp12.

Name	Sequence
Hp12A	5' TTGTCTCGCGCCCCTAGGTAC '3
Hp12B	5' TACCAGGCGCTACGGCGCTGG '3
Hp12C	5' CGCAAACATTGGTGAGAATC '3

University of Pretoria etd – Van der Vyver C 2002

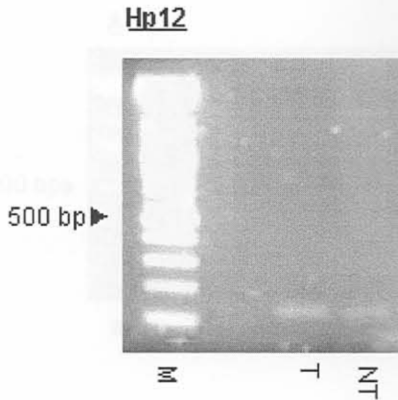

Figure 3.10: PCR amplification of genomic DNA with primer pair Hp12A and Hp12B with genomic template DNA derived from pooling DNAs of two genetically modified tobacco plants (T) and genomic DNA derived from the wild-type tobacco plant (NT). Lane M represents a 100 bp marker.

A third primer, Hp12C, was also designed outside the sequence of the Hp12 difference product. Design of Hp12C was based on available sequence information obtained from known tobacco chloroplast DNA. PCR analysis with primers Hp12C and Hp12R included the region containing the *Hpa*II cutting site present at the end of the Hp12 subtraction product, which are not present in the known tobacco chloroplast sequence. Amplification of both types of genomic DNAs by PCR, with primers Hp12C and Hp12R, followed by digestion of amplified products with the restriction enzyme *Hpa*II, showed almost identical patterns in both genetically modified and wild-type DNA (Figure 3.11A). When compared to undigested amplified DNA, a slight shift of the size of the amplified and *Hpa*II-digested DNA was found after separation on an agarose gel (Figure 3.11A). When amplifications products were transferred to a filter and probed with the Hp12 subtraction product all amplified products hybridized to Hp12 and no difference was detected between modified and wild-type tobacco DNAs (Figure 3.11B). This pattern was the same when the genomic DNAs of both genetically modified and wild-type tobacco plants were first digested with *Hpa*II or *Msp*I followed by amplification with primers Hp12C and Hp12R (Figure 3.12).

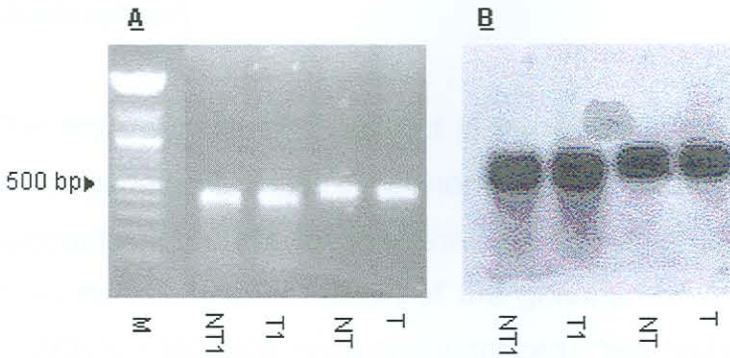

Figure 3.11: (A) PCR amplification of genomic target DNAs with primers Hp12R and Hp12C with genomic template DNA derived from pooling DNAs of two genetically modified tobacco plants (T) and genomic DNA derived from wild-type tobacco plant (NT). Amplified products from modified and wild-type plants were either undigested (NT and T) or digested with restriction enzyme *Hpa*II (NT1 and T1). Lane M represents a 100 bp marker. (B) Hybridization of Hp12 to a filter containing amplified products from A.

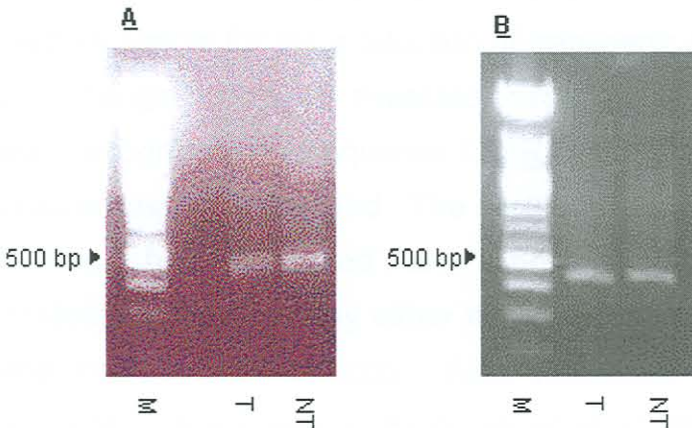

Figure 3.12: PCR amplification of genomic target DNAs with primers Hp12R and Hp12C with genomic template DNA derived from pooling (A) *Msp*I-digested (B) *Hpa*II-digested DNAs of two genetically modified tobacco plants (T) and digested genomic DNA derived from wild-type tobacco plant (NT). Lane M represents a 100 bp marker.

Discussion

The RDA experiments resulted in the isolation of DNA sequences limited to either genetically modified or non-modified wild-type tobacco plants. These subtraction products could be the result of either DNA changes in the genome or in the methylation status of the genomic DNA or both. Three of these subtraction products were characterized. The first subtraction product, Hp14, was isolated from *Hpa*II digestion and was identical to part of the 18S rRNA gene. It is therefore likely that the isolation of Hp14 was due to changes in the methylation patterns between genetically modified and wild-type tobacco plants. Changes in genome methylation due to stressful events including foreign gene insertion into a plant has been previously reported and examination of regenerated plants with methylation-sensitive restriction enzymes has revealed both hyper- and hypo-methylation (Phillips et al., 1994). Since plant tissue culture, as part of the transformation/gene insertion process, can change DNA methylation and also occurs as a consequence of treatment with an antibiotic commonly used in a tissue culture process, as selective agents for the production of transgenic plants (Schmitt et al., 1997), such changes could be expected. The methylation-sensitive endonulease *Hpa*II recognizes the sequence CCGG and does not cleave if the internal cytosine base is methylated. The section of the plant genome detected might therefore have acquired an altered methylation status during the transformation, caused by either the tissue culture process or by the foreign gene insertion into tobacco. Also, ribosomal RNA genes can be highly methylated. For example, Fulnecek et al. (1998) found that the density of methylation along the 5S rRNA genes exceeds the average methylation density in the tobacco genome. In the past is has also been proposed that methylation patterns change in correlation with gene activity. This was found to be true especially in rRNAs where methylation controlled the transcription potential of rRNA genes, which served as a mechanism to direct the number of active rRNAs (Flavell and O'Dell, 1988). When methylation-sensitive RDA was applied to different date palm varieties, some of the isolated difference products showed high homologies to the 18S, 25S and 4.5S rDNA of tobacco

indicating potential changes in methylation patterns of the rDNA in different varieties (J. Vorster, personal communication). Altered rRNA gene methylation can have a dramatic effect on protein expression, subsequently associated with a plant phenotype such as dwarfism (Neves et al., 1995). Such an association might also be present in the genetically modified tobacco characterized in this study, where the modified plants expressed a conditional dwarf phenotype.

In contrast to Hp14, subtraction product Hp12 clearly hybridized to two larger DNA fragments derived from wild-type genomic DNA. These fragments were not found in the genomic DNA derived from genetically modified plants. Since tobacco chloroplast DNA (cpDNA) is among a few well-characterized chloroplast genomes in which sequence of the complete cpDNA has been reported (Shinozaki et al., 1986), Hp12 could be identified being of plastidic origin when searching DNA sequence databases. From the hybridization experiment it was concluded that this subtraction product also seems to be present in the nuclear genome, and that the nuclear DNA copy derived from a genetically modified plant is either less methylated than in the wild-type plant nuclear DNA or is absent from the genetically modified plant.

In plants, methylation is mainly restricted to the nuclear genome, where methyl-cytosine is especially concentrated in repeated sequences (Finnegan et al., 1998). Since Hp12 has a high level of similarity to chloroplast DNA of tobacco with differences only in a few base pairs, Hp12 is possibly a nuclear localized copy (Ayliffe et al., 1998). Chloroplast and nuclear DNA are in separate cellular compartments and DNA generally does not pass in either direction through the membranes of organelles (Lewin, 1994). However, that does not exclude that such exchanges of DNA between organelles and the nucleus have occurred during evolution in endo-symbiotic events with prokaryotic organisms (Leon et al., 1998). Sequence analysis of the Hp12 difference product also showed a small number of base pair changes including changes of C to G and T to A when compared to the known tobacco chloroplast sequence. According to Phillips et al. (1994), such base pair changes represent the most commonly observed point mutations in plants,

University of Pretoria etd – Van der Vyver C 2002

which are also a consequence of plant tissue culture procedures using plant growth regulators. Such changes can occur either as a result of deamination of methylated cytosine or loss of precision in the DNA replication repair mechanism. Genomic DNA amplification by PCR using the primer pair Hp12A and Hp12B, which were specifically designed to detect single base pair mutations, resulted indeed in better amplification of genomic DNA of genetically modified plants than of DNA from a wild-type plant. It is therefore likely, that these single mutations have occurred in DNA of genetically modified plants, which consequently resulted in less efficient amplification from the wild-type DNA due to base pair changes in the primer site. When genomic DNA from both genetically modified and non-modified plants were first digested with *Msp*I or *Hpa*II followed by PCR amplification using the primers Hp12C and Hp12R, the same amplification pattern were observed in both types of plants. *Msp*I, is an isoschizomer of *Hpa*II and when the external C in the sequence CCGG is methylated, *Msp*I and *Hpa*II cannot cleave DNA. However, unlike *Hpa*II, *Msp*I can cleave the DNA sequence when the internal C residue is methylated. Since methylation is considered to be restricted to the plant nuclear genome, the non-methylated chloroplast DNA would have been digested with both methylation- sensitive restriction enzymes, *Hpa*II and *Msp*I, preventing amplification of a product due to digestion of DNA. No amplification would also have occurred when nuclear DNA would have been non-methylated at the *Hpa*II site allowing digestion with the enzymes. In contrast, amplification would have occurred when either the *Hpa*II site in the nuclear DNA is absent or the *Hpa*II / *Msp*I site is methylated at the internal and external C preventing digestion with the enzymes. Since amplification of a fragment was found despite digestion of DNA with *Hpa*II and *Msp*I, the *Hpa*II site is seemingly missing in the genomic plant DNA. Since the *Hpa*II site was found in the Hp12 subtraction product but not in the sequence data base of the known tobacco chloroplast genome, the *Hpa*II site present in the subtraction product very likely originates from a mutation in the DNA sequence by a A to C exchange.

Beside two methylation-sensitive products, one non-methylation sensitive subtraction product from genomic DNA of genetically modified tobacco plants

University of Pretoria etd – Van der Vyver C 2002

(Hi30) was identified in the study. This subtraction product was absent in genomic DNA of the wild-type tobacco plant used for RDA amplicon production. Hi30 might represent a variable DNA region, which has easily changed under plant transformation conditions. However, a primer pair designed for a subtraction product with a mutated sequence derived from DNA of a genetically modified plant seems not have allowed the amplification of a fragment with DNA of a wild-type plant. Minor DNA variations between individual wild-type plants can not be completely excluded although the original '*Samsun/UK*' cultivar used for this study is both an inbred and very likely a true-breeding cultivar. However, before any generalization about the occurrence and nature of Hi30 in the DNA of wild-type tobacco could be made a more detailed study with a greater number of genetically modified and wild-type plants was carried out, which is outlined in Chapter 5 of this thesis.

References

Ayliffe M.A., Scott N.S. and Timmis J.N. 1998. Analysis of plastid DNA-like sequences within the nuclear genomes of higher plants. Molecular Biology and Evolution 15: 738-745.

Bao P.H., Granata S., Castiglione S., Wang G.J., Giordani C., Cuzzoni E., Damiani G., Bandi C., Datta S.K., Datta K., Potrykus I., Callegarin A. and Sala F. 1996. Evidence for genomic changes in transgenic rice (*Oryza sativa* L.) recovered from protoplasts. Transgenic Research 5: 97-103.

Brown P.T.H., Kyozla J., Suleklyo V., Kimura Y., Shimamoto K., and Lörz H. 1990. Molecular changes in protoplast-derived rice plants. Molecular and General Genetics 223: 324-328.

Caligari P.D.S., Yapabandara Y.M.H.B., Paul E.M., Perret J., Roger P. and Dunwell J.M. 1993. Field performance of derived generations of transgenic tobacco. Theoretical and Applied Genetics 86: 875-879.

Choi H.W., Lemaux P.G. and Cho M.J. 2000. High frequency of cytogenetic aberration in transgenic oat (*Avena sativa* L.) plants. Plant Science 156: 85-94.

Cullis C.A. 1999. Environmental Stress - a generator of adaptive variation? In: Lerner H.R. (ed.), Plant Adaptations to Stress Environments. Marcel Dekker, New York, p. 149-160.

Cullis C.A. and Kunert K. 2000. Isolation of tissue culture-induced polymorphisms in bananas by representational difference analysis. Acta Horticulturae 530: 421-428.

Finnegan E.J., Genger R.K., Peacock W.J. and Dennis E.S. 1998. DNA methylation in plants. Annual Review of Plant Physiology and Plant Molecular Biology 49: 223-247.

Flavell R.B. and O'Dell M. 1988. Regulation of cytosine methylation in ribosomal DNA and nucleolus organizer expression in wheat. Journal of Molecular Biology 204: 523-534.

Fulnecek J., Matyasek R., Kovarik A. and Bezdek M. 1998. Mapping of 5-methylcytosine residues in *Nicotiana tabacum* 5S rRNA genes by genomic sequencing. Molecular and General Genetics 259: 133-141.

Gawel N.J. and Jarret R.L. 1991. A modified CTAB DNA extraction procedure for *Musa* and *Ipomoea*. Plant Molecular Biology Reporter 9: 262-266.

Hubank M. and Schatz D.D. 2000. Representational difference analysis of cDNA. In: Hunt S.P. and Livesey F.J. (eds.), Functional Genomics. Oxford University Press, Oxford New York, p. 45-112.

University of Pretoria etd – Van der Vyver C 2002

Labra M., Savini C., Bracale M., Pelucchi N., Colombo L., Bardini M. and Sala F. 2001. Genomic changes in transgenic rice (*Oryza sativa* L.) plants produced by infecting calli with *Agrobacterium tumefaciens*. Plant Cell Reports 20: 325-330.

Lee M. and Phillips R.L. 1987. Genomic rearrangements in maize are induced by tissue culture. Genome 29: 122-128.

Leon P., Arroyo A. and Mackenzie S. 1998. Nuclear control of plastid and mitochondrial development in higher plants. Annual Review of Plant Physiology and Molecular Biology 49: 453-480.

Lewin B. 1994. Genes. Oxford University Press, Oxford, UK.

Lisitsyn N., Lisitsyn N. and Wigler M. 1993. Cloning the differences between two complex genomes. Science 259: 946-951.

Lisitsyn N.A., Segre J.A., Jusumi K., Lisitsyn N.M., Nadeau J.H., Frankel W.N., Wigler M.H. and Lander E.S. 1994. Direst isolation of polymorphic markers linked to a trait by genetically directed representational difference analysis. Nature Genetics 6: 57-63.

Lynch P.T., Jones J., Blackhall N.W., Davey M.R., Power J.B., Cocking E.C., Nelson M.R., Bigelow D.M., Orum T.V., Orth C.E. and Schuh W. 1995. The phenotypic characterisation of F(2) generation transgenic rice plants under field and glasshouse conditions. Euphytica 85: 395-401.

Michiels L., Van Leuven F., van den Oord J.J., De Wolf-Peeters C. and Delabie J. 1998. Representational difference analysis using minute quantities of DNA. Nucleic Acids Research 26: 3608-3610.

University of Pretoria etd – Van der Vyver C 2002

Neves N., Heslop-Harrison J.S. and Viegas W. 1995. rRNA gene activity and control of expression mediated by methylation and imprinting during embryo development in wheat x rye hybrids. Theoretically and Applied Genetics 91: 529-533.

Ohba T., Yoshioka Y., Machida C. and Machida Y. 1995. DNA rearrangement associated with the integration of T-DNA in tobacco - an example for multiple duplications of DNA around the integration target. Plant Journal 7: 157-164.

Phillips R.L., Kaeppler S.M. and Olhoff P. 1994. Genetic instability of plant tissue cultures: Breakdown of normal controls. Proceedings of the National Academy of Science, U.S.A. 91: 5222-5226.

Sala F., Arencibia A., Castiglione S., Yifan H., Labra M., Savini C., Bracale M. and Pelucchi N. 2000. Somaclonal variation in transgenic plants. Proceedings of the International Symposium On Methods and Markers for Quality Assurance in Micropropagation (eds). A.C. Cassells, B.M. Doyle, R.F. Curry Acta Horticulturae 530, ISHS.

Sambrook J., Fritsch E.F. and Maniatis T. 1989. Molecular cloning: a laboratory manual, 2nd edition, Cold Spring Harbor Laboratory Press, New York.

Schmitt F., Oakeley E.J. and Jost J.P. 1997. Antibiotics induce genome-wide hypermethylation in cultured *Nicotiana tabacum* plants. Journal of Biological Chemistry 272: 1534-1540.

Shinozaki K., Ohme M., Tanaka M., Wakasugi T., Hayashida N., Matsubayashi T., Zaita N., Chunwongse J., Obokata J., Yamaguchi-Shinozaki K., Ohto C., Torazawa K., Meng B.Y., Sugita M., Deno H., Kamogashira T., Yamada K., Kusuda J., Takaiwa F., Kato A., Tohdoh N., Shimada H. and Suguira M. 1986. The complete nucleotide sequence of tobacco chloroplast genome: its gene organization and expression. EMBO Journal 5: 2043-2049.

University of Pretoria etd – Van der Vyver C 2002

Stahl R., Horvath H., Van Fleet J., Voetz M., von Wettstein D. and Wolf N. 2002. T-DNA integration into the barley genome from single and double cassette vectors. Proceedings of the National Academy of Science, U.S.A. 99: 2146-2151.

Ushijima T., Morimura K., Hosoya Y., Okonogi H., Tatematsu M., Sugimura T. and Nagao M. 1997. Establishment of methylation-sensitive representational difference analysis and isolation of hypo- and hypermethylated genomic fragments in mouse liver tumors. Proceedings of the National Academy of Science, U.S.A. 94: 2284-2289.

Windels P., Taverniers I., Depicker A., Van Bockstaele E. and De Loose M. 2001. Characterisation of the Roundup Ready soybean insert. European Food Research Technology 213: 107-112.

Zheng S.J., Henken B., Sofiari E., Jacobsen E., Krens F.A. and Kik C. 2001. Molecular characterization of transgenic shallots (*Allium cepa* L.) by adaptor ligation PCR (AL-PCR) and sequencing of genomic DNA flanking T-DNA borders. Transgenic Research 10: 237-245.

This work has been submitted to Molecular Breeding entitled “Representational difference analysis as a tool to detect possible genome changes in genetically modified tobacco plants” by C Van der Vyver, KJ Kunert and CA Cullis.