

## **CHAPTER FOUR**

**APPLICATION OF cDNA REPRESENTATIONAL DIFFERENCE ANALYSIS  
(cDNA RDA) FOR DETECTION OF DIFFERENTIALLY EXPRESSED GENES  
IN OC-I EXPRESSING TOBACCO**

#### **4.1 Abstract**

Enhanced expression of proteinase inhibitor in transformed plant might confer multiple stress tolerance. The objective of this part of the study was to investigate differentially expressed gene(s) between oryzacystatin (OC-I) expressing transformed tobacco (*Nicotiana tabacum* L. cv. Samsun) and non-transformed tobacco plants by using cDNA Representational Difference Analysis (cDNA RDA). Three putative differentially expressed sequences were isolated from two weeks heat-treated transformed and non-transformed plants. Sequences included a fragment coding for a putative light harvesting chlorophyll *a/b* binding protein of photosystem II (LHC II) isolated from tester non-transformed plant DNA and a 60S ribosomal L12 like protein isolated from tester transformed plant DNA. A temporal expression study of the putative light harvesting chlorophyll *a/b* gene under heat treatment showed a difference in expression between transformed and non-transformed plants under non-stress conditions where the gene was down-regulated in transformed plants. Measurement of chlorophyll content and soluble proteins using two-dimensional gel electrophoresis also showed differences between the two plant types both under non-stress and heat stress conditions. This suggests that constitutive overexpression of OC-I transgene affects gene expression as a plant response to heat stress in tobacco.

## 4.2 Introduction

Representational Difference Analysis (RDA) was first described by Lisitsyn *et al.* (1993) for the identification of difference between two complex genomes. RDA belongs to the general class of DNA subtractive methodologies, in which one DNA population, known as the “driver”, is hybridized in excess against a second population, which is the “tester”, to remove common (hybridizing) sequences. Thereby “target” sequences are enriched that are unique to the “tester” population. cDNA RDA is a modification of the original RDA technique, in which the starting material is derived from mRNA rather than genomic DNA. Accordingly, targets are only genes which are expressed at the time total RNA is isolated (Hubank and Schatz, 1994 and 1999). The method is flexible, sensitive, and relatively inexpensive to perform. The method has further the major advantage that sequences common to both groups of cells are eliminated. This greatly simplifies the interpretation of results and identification of the differentially expressed genes. In addition, the exponential degree of enrichment achieved by the use of PCR in cDNA RDA enables the detection of very rare transcripts. Examination of differential gene expression using cDNA RDA requires the sampling of a population (of cells) grown under the condition(s) of interest and a population grown under conditions which differ only by those of interest. mRNA is extracted from both populations and used as a template for cDNA synthesis. The cDNA RDA technique has been successfully applied to isolate differentially expressed genes in rejuvenated soybean cotyledons (Ling *et al.*, 2003), iron deficiency up-regulated genes in the bacteria *Neisseria meningitides* (Bowler *et al.*, 1999) and garlic up-regulated genes in human gastric cancer cells (Li and Lu, 2002).

The goal of this part of the study was to investigate, by using cDNA RDA, possible effects of expression of exogenous OC-I on gene expression in transformed tobacco. In particular cDNA RDA was applied to isolate and characterize differentially expressed genes between transformed OC-I expressing and non-transformed tobacco plants after exposure of plants to stress and then to characterize these isolated gene sequences under stress conditions. Applying the RDA technique, a sequence coding for a chlorophyll *a/b* binding protein (LHC II) and an unknown sequences were isolated from heat-stressed non-transformed plant DNA, while a 60S ribosomal L12-like protein gene was isolated from heat-stressed tester transformed plant DNA.

### 4.3 Materials and Methods

#### 4.3.1 mRNA isolation and cDNA synthesis

Total RNA was isolated from leaf samples collected from plants after two weeks of heat treatment. The fourth leaf from four individual plants, when leaves were counted from the top of the plant, was harvested and harvested leaves were mixed for total RNA isolation. Total RNA was extracted in TriPure total RNA isolation kit and any contaminant genomic DNA was digested by RNase free DNase (Roche, Germany). Total RNA was quantified using a NanoDrop® spectrophotometer and the quality of RNA was determined by running isolated RNA on a denaturing agarose gel containing ethidium bromide. About 250 µg of total RNA was used for mRNA purification using the Oligotex® mRNA isolation kit (QIAGEN GmbH, Hilden, Germany). Poly(A) RNA was then primed with Oligo(dT)<sub>15</sub> and reverse transcribed with AMV reverse transcriptase according to the manufacturer's recommendation (Roche, Germany) which was followed by second strand cDNA synthesis. The synthesised cDNA was visualized by running 4 µl of the reaction product on a 1% agarose gel containing ethidium bromide.

#### 4.3.2 cDNA RDA

Preparation of amplicons and subsequent hybridisation and amplifications were done following the basic RDA procedure outlined by Lisitsyn *et al.* (1993) for genomic DNA and the modified version for differentially expressed genes (Hubank and Schatz, 1994).

#### 4.3.2.1 Amplicon production

In the first step, cDNA (1  $\mu\text{g}$ ) derived from two types of tobacco, where one type served as tester cDNA and the other type as driver cDNA, was digested in a 20  $\mu\text{l}$  restriction enzyme buffer at 37°C for 120 minutes with 10 units of the restriction enzyme *Mbo*I (Amersham, UK). After digestion, digested cDNA was analyzed for effective digestion on an ethidium bromide containing 1% agarose gel in TAE (Tris-Acetate-EDTA) buffer.

A pair of single-stranded oligonucleotide adaptors of different length was used to alter the ends of digested cDNA fragments to enable cDNA amplification. The longest adaptor was used as the primer for cDNA amplification after ligation. For adaptor ligation, digested tester and driver cDNA (between 0.5 and 1  $\mu\text{g}$ ) were mixed in a total volume of 60  $\mu\text{l}$  with 7.5  $\mu\text{l}$  of a 12-mer and a 24-mer adaptor with a concentration 58  $\text{pmol } \mu\text{l}^{-1}$  (R-12 and R-24). Adaptor DNA was diluted from a 62  $\text{pmol } \mu\text{l}^{-1}$  adaptor stock solution and the adaptor ligation reaction was carried out in a ligase buffer consisting of 66 mM Tris-HCl (pH 7.6); 6.6 mM  $\text{MgCl}_2$ ; 10 mM DDT and 66  $\mu\text{M}$  ATP. To anneal the adaptors, the ligation mixture containing cDNA fragments and adaptors was incubated in Eppendorf reaction tubes at 55°C for 1 minute in a heating block. After heating, the block was immediately placed into a cold room (4°C) for approximately 2 hours until the temperature dropped in the ligation mixture between 15°C and 10°C. The reaction tubes were then incubated on ice for 3 minutes. After incubation, 1  $\mu\text{l}$  (5U  $\mu\text{l}^{-1}$ ) of T4 DNA ligase (Amersham Life Science, UK) was added to the mixture and the ligation mixture was then incubated overnight at 14°C to ligate the adaptors.

**Table 4.1** Oligonucleotide adapters and primers used for cDNA RDA

<b>Adapter/Primer Name</b>	<b>Sequence</b>
R-12	5'-GATCTGCGGTGA-3'
R-24	5'-AGCACTCTCCAGCCTCTCACCGCA-3'
J-12	5'-GATCTGTTCATG-3'
J-24	5'-ACCGACGTCGACTATCCATGAACA-3'
N-12	5'-GATCTTCCCTCG-3'
N-24	5'-AGGCAACTGTGCTATCCGAGGGAA-3'
P-12	5'-GATCCAGATGTA-3'
P-24	5'-ATACGTGCAGGCTGGTTACATCTG-3'

For preparation of tester and driver amplicons by PCR, ligated DNA was diluted up to 200  $\mu$ l with 140  $\mu$ l of dsH<sub>2</sub>O. For cDNA amplification, a PCR tube containing a PCR amplification mixture (100  $\mu$ l), which contained 40 ng of ligated cDNA; 372 pmol of the 24-mer adaptor (R-24 Table 1); 10 mM dNTPs (4  $\mu$ l); 25 mM MgCl<sub>2</sub> (6  $\mu$ l) and PCR buffer consisting of 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 1.5 mM MgCl<sub>2</sub> and 0.001% w/v gelatine, was placed into a pre-warmed (72°C) thermocycler for 3 minutes (Gene amp PCR System, Perkin Elmer, USA). During this incubation, the 12-mer (R-12) dissociates, freeing the 3'ends for subsequent fill-in. To fill in the ends complimentary to the 24-mer adapters (R-24) the PCR machine was paused and 5 units of Taq DNA polymerase (5U  $\mu$ l<sup>-1</sup>) (Amersham, Life Science, UK) and left for another 5 minutes. cDNA amplification by PCR was followed using 20 cycles of (60 seconds at 95°C; 3 minutes at 72°C) with the last cycle for DNA extension for 10 minutes at 72°C. Approximate total amount of cDNA of amplified tester and driver amplicons was determined on a 1.5% agarose gel in TAE buffer with sheared herring sperm DNA as a standard and NanoDrop (ND-1000) spectrophotometer to determine the total amount of amplified cDNA produced. Amplified cDNA was phenol/chloroform purified and after

ethanol precipitation (Sambrook *et al.*, 1989) amplicon cDNA was dissolved in TE (10 mM Tris-HCl, pH 8; 0.1 mM EDTA) buffer to obtain a cDNA concentration of about 0.5  $\mu\text{g } \mu\text{l}^{-1}$ .

#### 4.3.2.2 First round subtraction and amplification

To cleave adaptors from amplified cDNA, driver cDNA and tester cDNA (90  $\mu\text{g}$ ) were digested for 1 hour at 37°C with 20 units of *Mbo*I  $\mu\text{g}^{-1}$  cDNA. Yeast glycogen carrier (10  $\mu\text{g}$ ) was added to digested cDNA, which was then purified using QIAquick (Qiagen, Germany) PCR purification kit to clean digested R-adapters and eluted with 70  $\mu\text{l}$  of TE buffer to give a concentration of 0.5  $\mu\text{g } \mu\text{l}^{-1}$ . The tester amplicon cDNA (1  $\mu\text{g}$ ) from which adaptors were cleaved was then ligated to a second adaptor pair (J-12 and J-24; Table 1) following the procedure outlined above for adaptor ligation. Ligated tester DNA was diluted to 10 ng/ $\mu\text{l}$  in a total volume of 70  $\mu\text{l}$  with TE buffer. For hybridization, diluted tester cDNA 10  $\mu\text{l}$  (100 ng) was mixed with 20  $\mu\text{l}$  driver amplicon cDNA (10  $\mu\text{g}$ ) with a ratio of driver to tester of 100 to 1. Then 10 M ammonium acetate (12  $\mu\text{l}$ ) solution and 96% ethanol (144  $\mu\text{l}$ ) were added to the two cDNAs and mixed by sucking and blowing using an Eppendorf pipette. The mixture was incubated at -70°C for 10 minutes which was followed by an incubation period of 1 minute at 37°C. DNA was then precipitated by centrifugation for 10 minutes at 13000 rpm. and the cDNA containing pellet was washed twice with 70% ethanol and air-dried. The DNA pellet was re-suspended in 4  $\mu\text{l}$  EE buffer containing 30 mM EPPS (N- (2-hydroxyethyl piperazine) - N-(3-propene sulfonic acid) (pH 8) and 3 mM Na<sub>2</sub> EDTA 2H<sub>2</sub>O. The cDNA was overlaid with 20  $\mu\text{l}$  of sterile mineral oil and the sample was incubated at 98°C for 5 minutes to



denature the cDNA. The mix was cooled to 67°C and immediately 1 µl of 5 M sodium chloride solution was directly injected into the DNA drop and the mixture was incubated at 67°C overnight.

The mineral oil was removed and the sample was diluted by adding 200 µl TE buffer to the mixture. For initial amplification, diluted hybridized cDNA (40 µl) was added to 360 µl standard PCR reaction mixtures as outlined above for cDNA amplification. The solution was divided into 4 separate PCR tubes and 1µl of Taq DNA polymerase was added to each tube. The solution was incubated at 72°C for 5 minutes after which 4 µl of a 24-mer primer (J-24; Table. 1) were added to the solution. Eleven cycles of PCR (60 seconds at 94°C and 3 minutes at 70°C) were performed using an extension at 72°C for 10 minutes after the last cycle. To evaluate the effectiveness of the hybridization step, 20 µl of the hybridization mixture was amplified for an additional 32 cycles of amplification and any amplification products were visualized on an ethidium bromide containing 1.5% agarose gel in TAE buffer. If the amplification products were visible, 20 µl of the hybridization were digested with 20 units of mung bean nuclease at 30°C for 30 minutes to remove single-stranded DNA. The reaction was stopped by the addition of TE buffer (80 µl). The digested product was amplified in a standard PCR reaction mixture containing 4 µl of the 24-mer primer (J-24; Table 1). Amplified DNA subtraction products were purified with phenol/chloroform and precipitated with ethanol and finally dissolved in 200 µl of dsH<sub>2</sub>O.

#### 4.3.2.3 Second round subtraction and amplification

For the second round cDNA subtraction and kinetic enrichment by PCR, the first round cDNA subtraction products (5  $\mu\text{g}$ ) were digested with 50 units of *Mbo*I in a total volume of 100  $\mu\text{l}$ . The digested DNA was purified with phenol/chloroform after addition of glycogen carrier (10  $\mu\text{g}$ ), ethanol precipitated and re-suspended in dsH<sub>2</sub>O to obtain a cDNA concentration of 100 ng  $\mu\text{l}^{-1}$ . cDNA (200 ng) was ligated to a third set of adaptors (N-12 and N-24; Table1) in a total volume of 30  $\mu\text{l}$  as described above for first round subtraction and amplification. The ligated cDNA was diluted to 1 ng  $\mu\text{l}^{-1}$  with addition of 130  $\mu\text{l}$  of dsH<sub>2</sub>O. cDNA hybridization and kinetic enrichment by a PCR reaction was carried out with 5 ng of ligated cDNA (5  $\mu\text{l}$ ) and an appropriate amount of driver amplicon cDNA (20  $\mu\text{l}$ ) as described above, in tester and driver ratio of 1: 2000.

#### 4.3.2.4 Third and fourth round subtraction and amplification

For the third round cDNA subtraction and kinetic enrichment by PCR, 100 ng of the second round subtraction products were ligated to a set of adaptors (P-12 and P-24, Pastorian *et al.*, 2000, Table 1). Tester DNA (500 pg) was mixed with 10  $\mu\text{g}$  of driver amplicon cDNA (20  $\mu\text{l}$ ) in a ratio of 1 to 20000, hybridized and amplified as described above. For the fourth round subtraction adaptors of the third round subtraction product were changed to a new adaptor set (J-12 and J-24; Table 1). The concentration of the ligated cDNA was adjusted to 10 pg  $\mu\text{l}^{-1}$  using consecutive dilutions with TE buffer. Before cDNA hybridization and kinetic enrichment of cDNA by PCR, hybridization and PCR amplification was carried out with 50 pg J-adaptors ligated cDNA and 10  $\mu\text{g}$  of

driver amplicon in 20 µl low TE buffer as described above in a tester and driver ratio of 1:200000.

#### 4.3.2.5 Cloning and sequence analysis of difference products

Final RDA subtraction products were treated with appropriate restriction enzyme to remove ligated adaptors, separated on a 1.5 % ethidium bromide containing agarose gel in TAE buffer and visualized on a UV transilluminator. cDNA fragments were eluted from the agarose gel and purified using a QIAquick gel purification kit (Qiagen). Purified DNA fragments were cloned into pGEM-T Easy vector system II (Promega, USA). Blue/white bacterial colony selection was made and isolated plasmids containing inserts were sequenced. Sequencing of the inserts were performed by using the BigDye<sup>®</sup> Terminator Cycle Sequencing FS Ready Reaction Kit, v 3.1 on ABI PRISM<sup>®</sup> 3100 automatic DNA-Sequencer (Applied Biosystems, Foster City, CA, USA). The BLASTN and BLASTX programs (Altschul *et al.*, 1997) were used for homology search. Amino acid sequence alignment was made using Clustal W multiple alignment software (Thompson *et al.*, 1994).

#### 4.3.4 Gene expression under heat stress

Eight weeks old tobacco plants raised from transformed and non-transformed plants were used for exposure to a high temperature. The heat treatment was achieved by moving 4 plants per treatment, as indicated in Figure 4.1, from a growth chamber maintained at 26/18°C (control) to a growth chamber maintained at a temperature of 38/30°C day/night (heat stress). The light photoperiod was set at 12 hours in both growth cabinets. Light in

growth cabinets was provided by a combination of incandescent and fluorescent light generating a photosynthetic photon flux density of  $240 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$ . The relative humidity in the growth cabinets during the study period was  $70 \pm 4\%$ . Plants grown under  $26^\circ\text{C}$  day temperature and  $20^\circ\text{C}$  night temperature were considered to have been exposed to a standard control growth temperature. Watering was done on a daily basis and plants received a Hoagland nutrient solution three times per week. To minimize water deficit in the pots maintained at  $38^\circ\text{C}$ , plants were watered twice a day if required. Harvesting of leaves for analysis was done simultaneously for all four treated plants that have been exposed to heat for a varying time period. Leaf harvest was carried during the day photoperiod. Fully expanded leaves, (fourth leaf counted from the shoot apex) were harvested for all treatments and the experiments were repeated once. Harvested leaves were flash frozen in liquid nitrogen and either used immediately or stored at  $-80^\circ\text{C}$  until required. Leaf samples from both experiments were used for chlorophyll determination and as a source for RNA and protein.

#### 4.3.5 Chlorophyll determination

The chlorophyll content of leaves was measured from three different plants per treatment. For determination of chlorophyll *a* and *b* content, the absorption of a 80% acetone extract containing the chlorophyll was measured at 663 and 645 nm in a spectrophotometer (Pharmacia LKB, Ultrospec III, UK) and the chlorophyll content was determined using absorption coefficients according to MacKinney (1941).

#### 4.3.6 Two-dimensional gel electrophoresis (2-DE)

Leaf protein extraction and preparation for 2-DGE was performed as described by Salekdeh *et al.* (2002) with minor changes. A leaf protein extract from a leaf (0.3 g of fresh weight) was crashed in liquid nitrogen and rapidly homogenized in 1 ml of extraction buffer (20 mM Tris-HCl pH 8.0 containing 5 mM EDTA and 1 mM PMSF). The homogenate was transferred to 1.5 ml Eppendorf tube and centrifuged at 4°C for 10 minutes at full speed in an Eppendorf bench-top centrifuge. The supernatant containing the soluble protein was transferred to a new tube and centrifuged again as outlined above. The clear supernatant was precipitated with an equal volume of 10 % (w/v) trichloroacetic acid containing 0.07%  $\beta$ -mercaptoethanol and cold acetone (-20°C) for 2 hours at -20°C and centrifuged for 15 minutes in an Eppendorf centrifuge at full speed. The resulting pellet was treated twice with cold acetone containing 0.07%  $\beta$ -mercaptoethanol following incubation for 1 hour at -20°C and centrifugation at 4°C. The pellet was finally freeze-dried. The protein pellet was then dissolved in lysis buffer (9.5 urea, 2% (w/v) a detergent CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate), 0.8% (w/v) Pharmalyte pH 3-10 (Amersham), 1% (w/v) DTT). Approximately 200  $\mu$ g protein was loaded onto a single immobilized pH gradient (IPG) gel strip (70 mm, pH 3-10, Amersham Pharmacia Biotech, UK). Iso-electric focusing (IEF) was conducted using the Pharmacia Multiphore system and the DryStrip kit (Amersham). IPG strips were rehydrated in buffer (8 M urea, 0.5% (w/v) CHAPS, 20 mM DTT, 0.5% (v/v) IPG buffers) overnight before carrying out electrophoresis. IEF was performed at 500 V for 1 hour, followed by 1000 V for 1 hour, and 3000 V for 16 hour. The IPG gel strips were placed for 15 minutes in an equilibration buffer (6 M urea, 15 mM DTT, 30% glycerol and 2% (w/v) SDS in 0.05 M Tris-HCl buffer, pH 8.8). A second equilibration was performed

with 2.5% iodoacetamide replacing DTT. The second dimension was run in vertical slabs (MINI PROTEAN II, Bio-Rad, USA) of 12% SDS-polyacrylamide gels. Duplicate gels were produced using same procedures from heat stressed and non-stressed transformed and non-transformed plants and gels were stained with Coomassie R250 or silver nitrate for detection of protein bands and compared visually.

## 4.4 Results

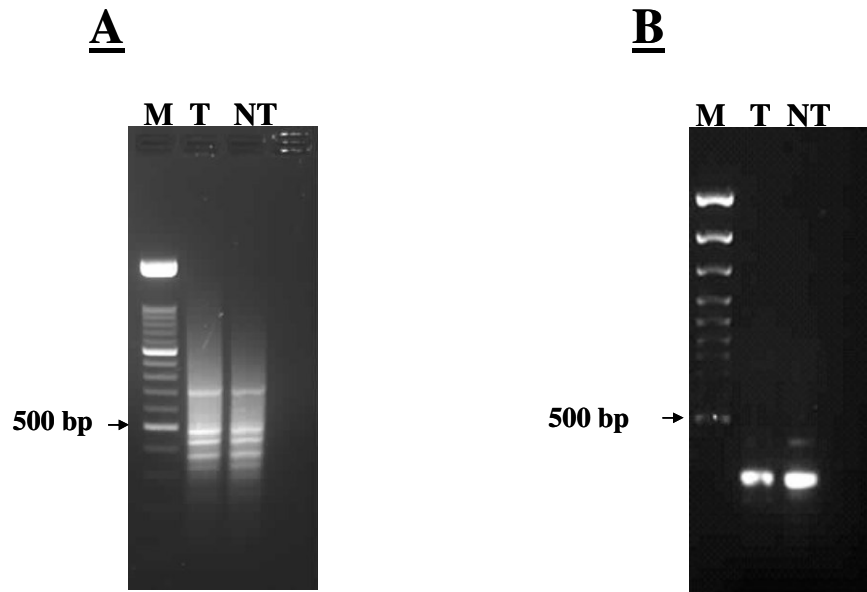
### 4.4.1 DNA RDA

A reciprocal cDNA RDA was used to identify gene sequences differentially expressed between transformed and non-transformed tobacco plants under stress conditions from cDNA population synthesized from mRNA obtained after two weeks of heat treatment. Figure 4.1 shows the amplicons (A) and difference products (DP) after the fourth round of subtraction (B) of heat-treated transformed and non-transformed plants. Sequential hybridisation and PCR amplification following the cDNA RDA procedure for differentially expressed sequences, as outlined by Hubank and Schatz (1994), allowed enrichment of difference products visible as distinct bands on an agarose gel (Figure 4.1 B).

Difference products were cloned and 70 cloned products were sequenced. BLAST search showed that the majority of the clones contained tobacco ribosomal RNA (28S, 26S, 23S, 18S and 16S) genes. Three products were identified as nuclear-encoded, two having a matching sequence on the database, and the third product with no matching sequence on the database. The two products (GBDP4-5d-12 and GBDP5-5d-11) were identified when cDNA from heat-stressed non-transformed plants were used as tester and cDNA from heat-stressed transformed plants as driver. The third difference product (clone GBDP4-3d-7) was isolated when cDNA from heat-treated transformed plant was used as a tester using cDNA from non-transformed plants as a driver. These three sequences were deposited on the Expressed Sequence Tag (EST) database with GenBank accession numbers CF931536, CF931537 and CF931538, respectively, for clones GBDP4-3d-7,

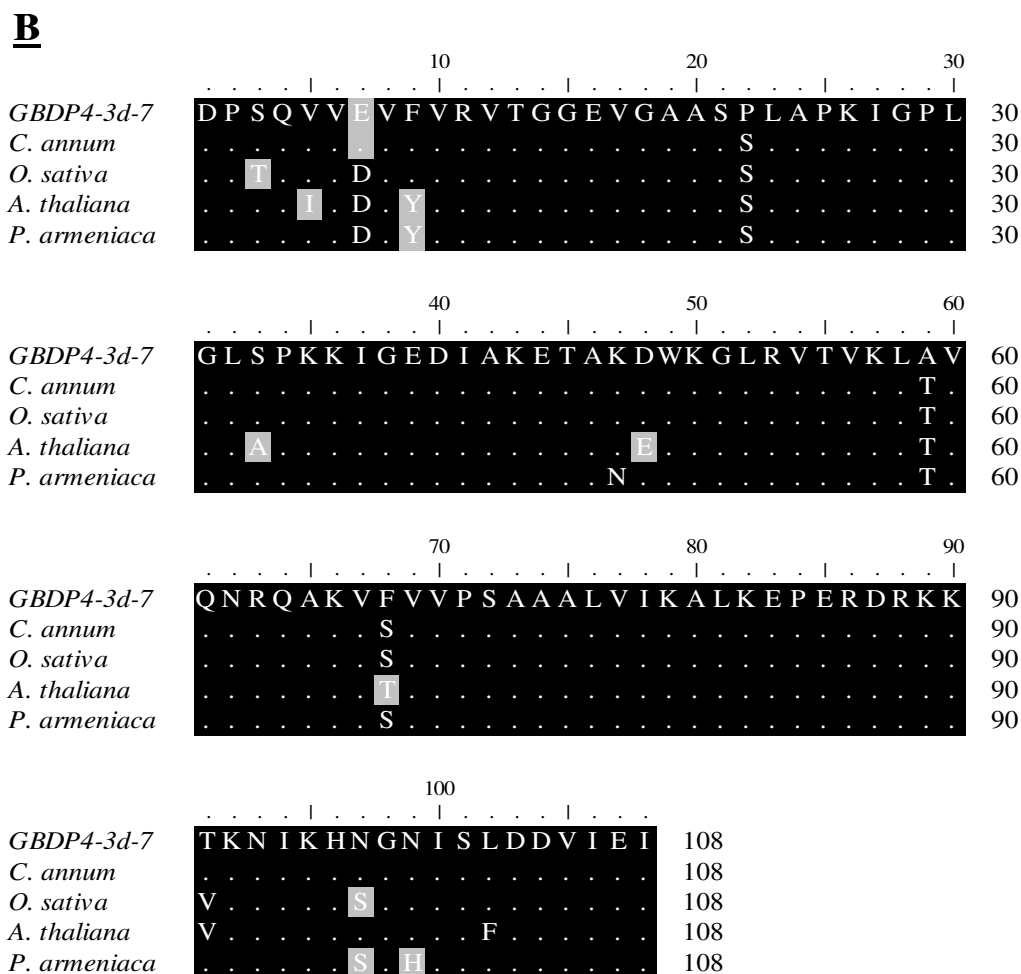
GBDP4-5d-12 and GBDP5-5d-11. Of the two cDNAs difference products from tester (non-transformed plant), the deduced amino acid sequence of one of the sequences (GBDP4-5d-12) with GenBank accession number CF931537 had a 98 – 100% identity with tobacco and other *Solanaceae* chlorophyll *a/b* binding protein sequences of photosystem II (Figure 4.2A), while the second 211 bp difference product (GBDP5-5d-11) with GenBank accession number CF931538 had no matching plant sequence on any public database. The third difference product (GBDP4-3d-7) with GenBank accession number CF931536, isolated from heat-stressed tester transformed plant was 324 bp long. By using a BLAST search, a matching plant sequence (90-97%, identity) was found, which is the 60S ribosomal L12 protein sequence of different plant species (Figure 4.2 B). However, this sequence had a very low (12%) similarity with known tobacco 50S ribosomal L12 sequences (EMBL accession number X62339, Elhag *et al.*, 1992). Thus, this product might represent a novel nuclear-encoded tobacco chloroplast ribosomal protein sequence.





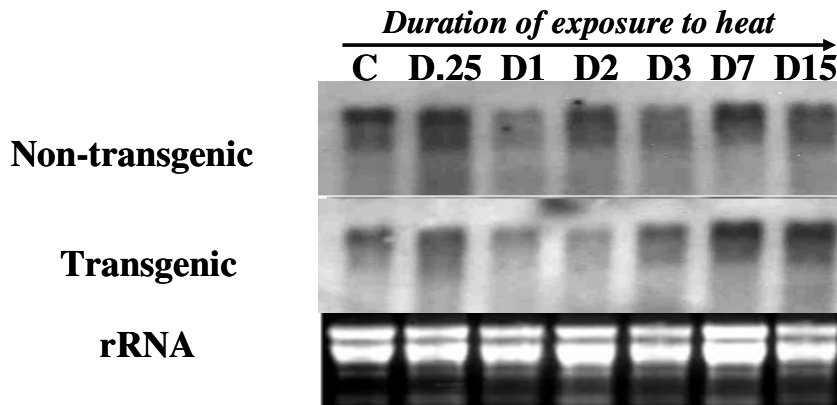
**Figure 4.1** (A) Amplicons of heat-stressed transformed (T) and non-transformed (NT) plant for cDNA RDA. (B) Difference products after the fourth subtraction when cDNA of transformed plants were used as driver and non-transformed plant cDNA used as tester (lane T) and when cDNA of non-transformed plants were used as driver and cDNA of transformed plants was used as tester (lane NT). Ten  $\mu$ l of a PCR product was run on 1.5% agarose gel containing ethidium bromide for DNA visualization. M represents a 100 bp molecular weight marker purchased from Roche, Switzerland (A) and Fermentas, Litavia (B).





**Figure 4.2** Alignment of deduced amino acid sequences. (A) GBDP4-5d-12 amino acid sequence alignment with the amino acid sequence of the plant light harvesting chlorophyll *a/b* binding protein derived from different plant species. (B) GBDP4-3d-7 amino acid sequence alignment with the L12 ribosomal protein amino acid sequences derived from different plant species. Identical amino acids are shown in dots, while similar amino acids (amino acids that have similar function) are light-shaded. Amino acid sequences in (A) represent *Nicotiana tabacum* cab 16 (P27492), *Nicotiana tabacum* cab 50 (P27496), *Petunia hybrida* cab 91R (P04783), *Nicotiana sylvestris* Lhcb1-7 (AB012639), *Solanum tuberosum* Lhcb1-2 (AAA80589) and *Lycopersicon esculentum* cab 1B (P07370). Sequences in (B) represent 60S ribosomal protein L12 from *Capsicum annum* (AAR83868), *Oryza sativa* (XP\_467310), *Prunus armeniaca* (AAB97143) and *Arabidopsis thaliana* (AAM65708).

4.4.2 Expression of GBDP4-5d-12 Expression pattern of GBDP4-5d-12, which had a high homology to chlorophyll *a/b* binding protein of photosystem II, was further studied under non-stress and heat stress condition. A northern analysis was carried to determine the transcription of *GBDP4-5d-12* (putative chlorophyll *a/b* binding protein gene, *lhcb1*) in transformed and non-transformed tobacco plants either under un-stressed conditions (C, control) or exposed to heat stress for a varying length of time. Transcription of the chlorophyll *a/b* binding protein gene (Figure 4.3) was lower in non-treated transformed plants expressing OC-I and also in OC-I expressing material until three days after heat treatment. Both types of plants showed, however, a decline in transcript level reaching a minimum 24 h after heat treatment. This was followed by an increase in accumulation at seven and fifteen days after heat treatment where transcript levels in both types of plants were almost identical. Increase in transcript levels might reflect an adaptive response to heat treatment not affected by expression of OC-I.

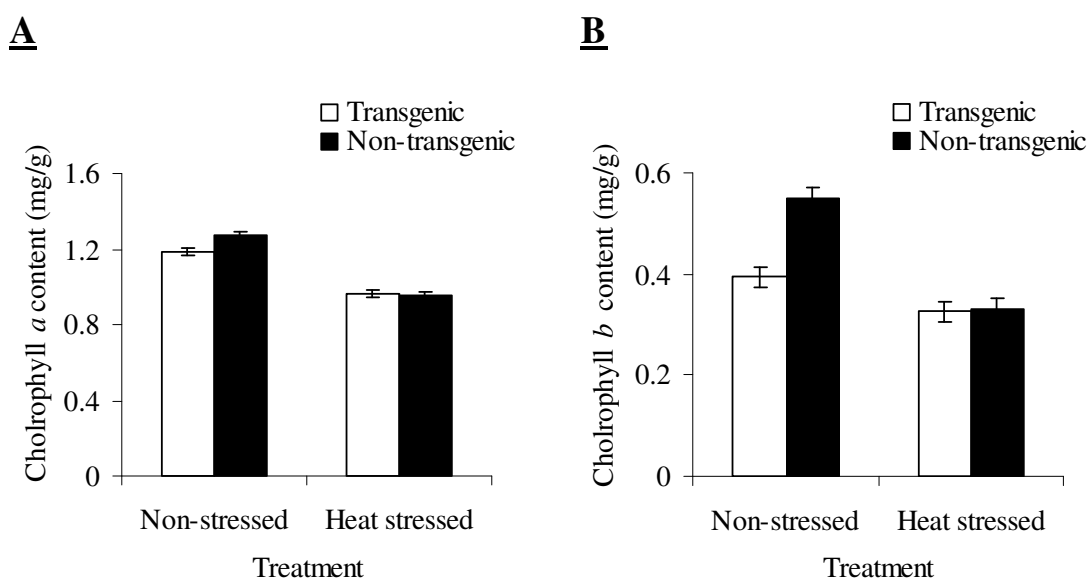


**Figure 4.3** Temporal expression of a GBDP4-5d-12 under non-stress and during heat stress exposure. Total RNA was isolated from transformed and non-transformed tobacco plants that have been either non-stressed (C, control) or heat-stressed for various time periods. RNA was separated on a 1.2% agarose gel containing 2.2 M formaldehyde transferred to Hybond™-N+ membrane and probed with *GBDP4-5d-12*. For heat exposure, plants were grown at 38°C/30°C (day/night) and control (non-stressed) plants were grown at 26°C/18°C (day/night) temperatures. Ethidium bromide stained rRNA was used to show equal loading of RNA and a degree of RNA degradation was evident in all samples.

#### 4.4.3 Chlorophyll content

Leaf chlorophyll content was measured to demonstrate the extent of treatment effect. Measurements of leaf chlorophyll *a* and *b* contents were made under non-stress (control) and after 15 days of heat treatment. In both transformed and non-transformed plants,

chlorophyll *a* and *b* contents were significantly ( $P < 0.05$ ) higher under non-stress condition when compared to heat-treated plants (Figure 4.4 A and B). Non-transformed plants had significantly ( $P < 0.05$ ) higher chlorophyll *a* and *b* content than transformed plants under non-stress condition, but there was no difference in the chlorophyll content between the two plant types after 15 days of heat treatment.

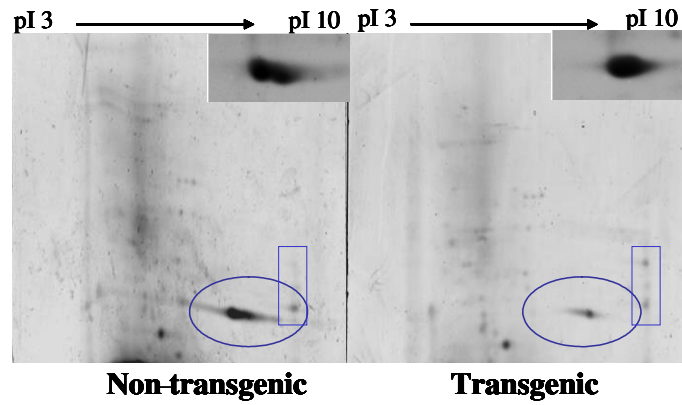


**Figure 4.4** (A) Chlorophyll *a* and (B) chlorophyll *b* content ( $\text{mg g}^{-1}$  on fresh weight basis) of transformed and non-transformed tobacco leaves under non-stress and after heat stress exposure. Heat stress treatment was made at  $38/30^{\circ}\text{C}$  (day/night) temperature for 15 days, while non-stress treatment was at  $26/18^{\circ}\text{C}$  (day/night) temperature. Each data point represents the mean  $\pm$  standard error (SE) of three independent observations.

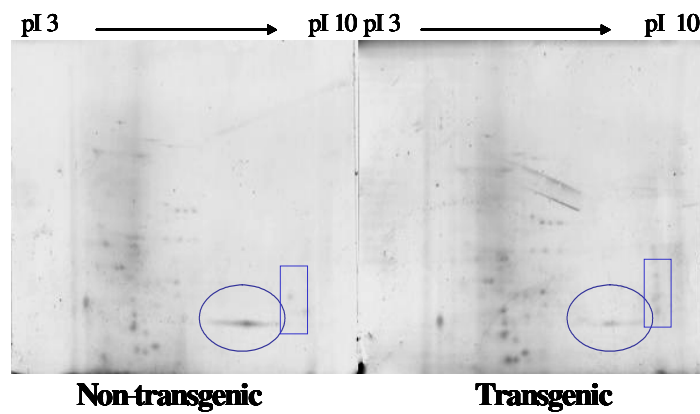
#### 4.4.4 Protein expression detected by 2-DE

To study the differential expression of leaf protein, protein extract from transformed and non-transformed plants were also compared by two-dimensional gel electrophoresis (2-DE) both under non-stress and after heat exposure for 15 days. Comparison of the spots on the gels from protein samples of transformed and non-transformed plants showed the presence of additional spots in transformed plant material which were absent or very weak in non-transformed plant material (boxed spots). Further, a single, visually distinguishable major band, appeared different between non-transformed and transformed plants (Figure 4.5 A and B; circled spots). The position of this single differentially expressed spot on the gel was compared with available data from the tobacco 2-DE database (<http://tby2-www.uia.ac.be/tby2/>). Comparison with other 2-DE from tobacco and other plant leaf proteins indicated that the abundant spot very likely contains the small subunit of Rubisco among possible other proteins. Proteins at this location were more abundant in non-transformed plants when compared to transformed plants under both non-stressed and heat-stressed conditions. The intensity of protein spots decreased under heat stress in both types of plants. However, the decrease is also somehow greater for non-transformed plants when compared to transformed plants (Figure 4.5 A and B). This possibly indicates a higher degree of protein degradation in non-OC-I expressing plants due to heat treatment.

**A**



**B**



**Figure 4.5** Two-dimensional gel electrophoresis of a plant leaf protein extract derived from an OC-I expressing (transformed) and a non-transformed tobacco plant. Coomassie blue-stained protein spots from (A) non heat-stressed and (B) heat-stressed non-transformed and transformed plants. Encircled spots represent major protein differences, and inserts in (A) are enlarged bigger spots showing possibly degradation in non-transformed plants.



#### 4.5 Discussion

In general, cDNA RDA has been used for the isolation of differentially expressed genes between two populations of cDNAs. In principle the technique offers the advantage of isolating non-abundant, rare transcripts by using PCR amplification of differences. RDA has been further shown to be a reliable procedure for the discovery of target genes whose abundance differs in a magnitude of at least ten-fold. Nonetheless, the detection difference in abundance lower than that magnitude, although possible, is unpredictable (Hubank and Schatz, 2000).

cDNA RDA was applied in this study to isolate differentially expressed sequences from leaf cDNA preparations obtained from heat-stressed OC-I expressing and non-transformed tobacco plants. The technique was further applied for drought-stressed transformed and non-transformed plants and compared with non-stressed transformed and non-transformed plants. Cloning of the difference products after two rounds of hybridization and amplification in these experiments resulted in the isolation of 16S, 18S, 23S, 25S and 26S ribosomal RNAs. Primary attention to prove the usefulness of the technique in isolating unique difference products was then given to a single treatment, which was heat stress, and the rounds of hybridization and amplifications were increased to four following a standard procedure of cDNA RDA. At the same time the stringency in terms of driver/tester ratio was also increased. The increase in rounds of hybridization and amplification steps limited the appearance of rRNAs in the cloned difference products and resulted in the isolation of three sequences, which were two from non-transformed tester cDNA and one from transformed tester cDNA. Among them were a

nuclear-encoded chloroplast light-harvesting chlorophyll *a/b* binding protein gene of photosystem II that was isolated from non-transformed tester plants and a 60S ribosomal L12 like protein isolated from a tester transformed plant cDNA. Both of these sequences represent abundant transcripts encoded by multigene families in higher plants and are not rare transcripts. The third sequence isolated from tester non-transformed plant cDNA did not have a matching sequence on the database.

Of the three sequences expression pattern of one of the sequences (clone GBDP4-5d-12) that had higher identity with tobacco nuclear-encoded, the chloroplast light-harvesting chlorophyll *a/b* binding protein gene of photosystem II was studied in more detail. Light-harvesting chlorophyll *a/b* binding proteins are major components of the antenna complexes that collect and deliver light energy to the photosynthetic reaction centre in chloroplasts. The major complex (LHC II), which is associated with photosystem II, consists in higher plants of six types (Lhcb1–6) (Jansson, 1994). LHC II type I proteins (Lhcb1s) are encoded by the *lhcb1* multigene family, which is composed of eight members in *Nicotiana tabacum* L., with 95 – 99% protein identity and are expressed in the green part of the plant (Hasegawa *et al.*, 2002). This fragment was initially isolated from two weeks heat-treated tester non-transformed plants in this study, and it was expected to have been more abundant in non-transformed plants after two weeks of heat treatment by northern analysis. By using northern blotting for detection, it was found in this study that the expression pattern of this gene differed between non-transformed and transformed plants until seven days after exposure to heat treatment and its transcript was more abundant in non-transformed plants than in transformed plants. This possibly might

indicate a protective effect of OC-I expressing plants on protein degradation processes whereby down-regulating transcription of this gene. However, it is rather unclear why this sequence was detected as a unique difference product using the RDA technique although the gene sequence was expressed in the two types of plants. There is the possibility that this abundant gene sequence was not completely removed during the hybridization process. The stringency at the first round of hybridization with a 100:1 driver: tester ratio has a typical 10% probability for appearance of false positive differences (Hubank and Schatz, 2000). By using then an amplification process all remaining un-hybridized DNA will be amplified resulting ultimately in the appearance of a false unique DNA subtraction product. In addition, the isolated *lhcb1* fragment also corresponds to the most conserved region within *lhcb1* gene family of tobacco and other plant species. Differential expression of the members of the family has been reported in tobacco under non-stress condition (Hasegawa *et al.*, 2002). The possibility cannot be excluded that the gene sequence might have hybridized in the Northern blot procedure to all family members masking the actual differential expression of one or more members within the gene family.

In this study, under heat stress both chlorophyll content and intensity of protein spots decreased in the two types of plants. Reduction of chlorophyll content under heat stress has also been reported by Tewari and Tripathy (1998), which was caused by impaired biosynthesis of certain enzymes such as 5-aminolevulinic acid. The chlorophyll *a* and *b* contents of non-transformed plants were however higher than in transformed plants under non-stress conditions. This relates to the higher transcript level of the *lhcb1* found in non-

transformed plants. Both might have also contributed to the observed slightly higher photosynthetic activity and growth in non-transformed plants under non-stress conditions. In contrast, transformed plants had a lower transcript level for the chlorophyll *a/b* binding protein gene and also a lower chlorophyll content under non-stress condition. In addition, the intensity of certain protein spots on a 2D-gel was significantly lower in transformed plants when compared to non-transformed plants and also some proteins were protected additionally expressed under non-stress and stress conditions in OC-I expressing plants. Therefore, OC-I expression might have lowered protein turnover due to prevention of proteinase action resulting in a lower protein spot intensity and might also have protected a variety of expressed proteins against degradation.

The results obtained necessitated to design a strategy to identify tobacco proteinases that might be involved in the degradation of proteins and serve as a possible target for exogenous OC-I in a transformed plant. Therefore, in the following chapter cloning and characterization of two unique papain-like cysteine proteinases is presented as a first step to investigate possible OC-I cysteine proteinase targets in the tobacco.