CHAPTER TWO

EXPRESSION OF ORYZACYSTATIN-I IN DROUGHT AND HEAT-STRESSED TRANSFORMED TOBACCO PLANTS

2.1   Abstract

Expression of a rice cysteine proteinase inhibitor transgene, oryzacystatin-I (OC-I), was studied under drought, heat and a combination of both stresses in transformed tobacco plants (*Nicotiana tabacum* L. cv. Samsun). Transformed plants either exposed to individual or combined stresses had higher OC-I transcript and protein levels than non-stressed transformed plants and expressed OC-I was active against the plant cysteine proteinase papain. No OC-I degradation products could be detected in transformed plants by immuno-blotting following application of drought, heat or a combination of stresses. This indicates that OC-I transgene expression and the stability of the encoded protein in plants was not affected by drought or heat stress.

2.2   Introduction

The success of studying the function of an exogenous gene in a given plant background strongly depends on the expression of the gene and the stability of the expressed protein. Sub-optimal growth conditions might severely affect transgene expression and/or accumulation of encoded protein and activity. A study by Neumann *et al.* (1997) showed that heat treatment of transformed tobacco plants resulted in reversible reduction or complete loss of exogenous luciferase and neomycin phosphotransferase activity in 40% of transformed tobacco plants. Similarly, Sousa-Majer *et al*. (2004) found that the level of α-amylase inhibitor 1 (α-AI-1) in transgenic peas (*Pisum sativum* L.) was reduced both in the amount and protective ability against a seed weevil as a result of exposure to high temperature (32/27°C day/night). In addition, plant endogenous proteinases (Outchkourov *et al*., 2003), developmental stages of plants as well as environmental abiotic stresses that enhance abundance of certain plant endogenous proteinases can be a limiting factor in the accumulation of foreign proteins in plants (Dolja *et al*., 1998; Stevens *et al*., 2000; Down *et al*., 2001). However, the stability and expression of an exogenous cystatin expressed in a transformed plant during stress has so far not been studied in detail.

The aim of this part of the study was to investigate the expression and the stability of the OC-I transgene under drought and heat stress and a combination of these stresses. For that, different molecular and biochemical methods were used to study (i) the integration of the transgene into the plant genome, such as PCR and Southern blotting, (ii) to analyse the expression level of the gene by using northern blotting for transcript detection, and

(iii) to detect protein expression by either immuno-blotting or enzymatic assays to monitor protein activity.

2.3 Materials and Methods

2.3.1 Plant material

Transformed plants (T4/5) of the cultivar 'Samsun' (Tobacco and Cotton Research Institute at Rustenburg/South Africa) were produced following the procedure outlined by Horsch *et al*. (1985). They carried a *gus* marker gene coding for β-glucuronidase (GUS) and the gene coding for OCI under the control of a constitutive cauliflower mosaic virus (CaMV) 35S promoter (Van der Vyver *et al*., 2003). The non-transformed plants used this experiment and in other subsequent experiments refer to plants that passed through an identical transformation process and that have been selected from segregating population of primary transformation event. These plants lacked the insertion of the genes coding for GUS and OCI.

2.3.2 Detection of OC-I sequence in transformed plants

Genomic DNA was isolated from putative young leaves of transformed and control tobacco plants using a commercial DNA isolation kit (Amersham Phyto Pure DNA isolation kit, Amersham, UK). The quantity and quality of genomic DNA was determined by the NanoDrop® reading technique. A standard Polymerase Chain Reaction (PCR) using 100 ng genomic DNA as template was applied to amplify a portion of the OC-I coding sequence from transformed tobacco plants with a OC-I forward (5'-TCACCGAGCACAACAAGA-3') and reverse (5'– CATCGACAGGCTTGAACT - 3') primer. Plant DNAs, from which DNA bands of the expected size of 200 base pairs (bp) could be amplified and visualized on a 1.5% agarose gel, were considered as transformed with OC-I. These plants were labelled and transplanted to 5 l capacity pots containing 1:1

river sand/coconut coir potting media along with a corresponding non-transformed control plant and used for induction of drought, heat and combination of drought and heat stress two weeks after transplanting.

2.3.3   Stress treatment of plants

The experiment was conducted at growth cabinate facility situated on experimental farm of the University of Pretoria, South Africa. The two types of tobacco (Samsun) plants, transformed and non-transformed (non-transformed plants used in all experiments described in this thesis are obtained from segregating population of primary transformants after twice selfing) were treated with two growth temperatures consisting of treatments at: 26/20 ± 2°C (normal temperature) and 38/30 ± 2°C day/night (heat treatment) at 12 hours light photoperiod, and also two water regimes consisting of treatments with 25 – 35% (drought stress) and 80 – 100% field capacity (non-drought stress). This experimental design resulted in a 2 x 2 x 2 factorial treatment combination in a randomised complete block design, where each treatment set was replicated ten-times. Drought stress treatment was induced based on a gravimetric method. Watering was done on a daily basis by weighing individual pots based on the field capacity determination and the treatments. Light in the growth cabinet was provided by a combination of incandescent and fluorescent lamps generating a photosynthetic photon flux density of 240 ± 10 µmol m^{-2} s^{-1}. The relative humidity in the individual cabinet during the study period was $60 \pm 4\%$. Plants received Hoagland nutrient solution three-times a week. Four weeks after treatment induction, leaf samples were collected from fully expanded 3rd or 4th leaf position from shoot tip, flash frozen in liquid nitrogen and either immediately

used or stored at -80°C until needed. The entire experiment was repeated twice. Leaf samples from both experiments were used as DNA and RNA sources in the analysis.

2.3.4   Preparation of leaf protein extract

Frozen leaf samples were homogenized in liquid nitrogen in the presence of an extraction buffer. The buffer contained 50 mM Tris-HCl, (pH 8), and to block proteinase activity, 1 mM PMSF (phenylmethylsulphonyl fluoride), 1 mM EDTA (Ethylenediaminetetraacetic acid), 10 µM *trans*-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64) and 10 µM pepstatin A. The homogenate was centrifuged at 13000 rpm at 4°C for ten minutes in an Eppendorf centrifuge (Eppendorf, Gemany) and the resulting supernatant was used for further analysis after determination of the protein concentration according to Bradford (1976) using BSA as a standard (Bio-Rad, Hercules, USA).

2.3.5   Immuno-blotting

Protein containing supernatants from leaf homogenates were added to an equal volume of a 2X sample-loading buffer (90 mM Tris-HCl, pH 6.8; 20% glycerol; 2% SDS, 5% (v/v) β-mercaptoethanol and 0.2% bromophenol blue) and boiled at 93°C for 4 minutes. Boiled protein extracts were subjected to 12% (w/v) SDS-PAGE according to Laemmli (1970). Separated proteins on gels were then transferred to a Hybond™ P membrane (Amersham, UK), and blocked overnight at room temperature with gently shaking in a solution of 5% (w/v) low fat milk powder in Tris-buffered saline (TBS-T) containing 0.1% Tween-20. Blots were incubated for 1 hour under gentle shaking in primary OC-I antiserum (for detection of OC-I) or alternatively in primary Rubisco antiserum (for detection of the Rubisco small and large subunits). Antisera were diluted 1:5000 in TBS-T for 1 hour and

blots were washed three-times each in TBS-T containing 0.5% low fat milk. This was followed by incubation in horseradish peroxidase-conjugated anti-rabbit IgG (Amersham, UK) allowing fluorescence detection. Anti-rabbit IgG was used as the secondary antibody (1:10000 dilution). Treatment was done under gentle shaking for 1 hour at room temperature followed by three washes of blots for 10 minutes each in TBS-T containing 0.5% low fat milk. Detection of labelled proteins was done by chemiluminescence using an ECL™-Plus kit (Amersham, UK) according to the instructions by manufacturer.

2.3.6   Cysteine proteinase inhibition by tobacco leaf protein extract

A protein extract was prepared as outlined under immuno-blotting, but without addition of any proteinase inhibitors. The supernatant was used after centrifugation for an papain inhibition assay after protein quantification. A stock solution of papain dissolved in 0.1 M citrate phosphate buffer (pH 6.0) was diluted to 2 µg ml $^{-1}$ protein in proteolysis buffer (0.1 M citrate phosphate buffer, pH 6.0, containing 10 mM L-cysteine). The papain solution (100 µl) was pre-incubated with or without 100 µg leaf extract from either transformed or non-transformed tobacco plants in a total reaction volume of 300 µl. After 10 minutes of pre-incubation at 37°C, 200 µl of the cysteine proteinase substrate benzyloxycarbonyl-phenylalanine-arginie aminomythylcoumarin (20 µM Z-phe-arg-AMC), prepared by diluting in proteolysis buffer of a 1.0 mM in dimethylsulfoxide stock) was added to the reaction mixture and the resulting mixture was incubated again for another 10 minutes at 37°C. The reaction was then stopped by addition of 1.0 ml stopping reagent to the reaction mixture. The stopping reagent contained 10 mM sodium monochloroacetate, 30 mM sodium acetate and 70 mM acetic acid, pH 4.3. The fluorescence of the released AMC was determined by using a fluorescence

spectrophotometer (Hitachi Model F-2000) at an excitation and emission wavelengths set at 370 nm and 460 nm, respectively.

2.3.7   Southern blot analysis

Genomic DNA (20 μg) from transformed and non-transformed tobacco plants was digested overnight with restriction enzymes *Bam*HI, *Eco*RI, *Kpn*I, *Xba*I. Digested DNA was then run on 1% agarose gel and then transferred to a Hybond™-N+ membrane (Amersham, UK) according to the protocol described by Sambrook *et al*. (1989). A *Pst*I/ *Eco*RI cut of the OC-I coding sequences from plasmid pBluescript SKII and labelled by Random-Prime Labelling (Amersham, UK) was used as a probe. Hybridisation of blotted DNA with the probe was performed overnight after pre-hybridisation for 1 hour at 60°C in a hybridisation buffer (5X SSC, 0.1% SDS, 5% w/v dextran sulphate and 20-times dilution of liquid block, supplied with CDP- Star™ detection kit). Subsequent stringency washes and the detection with Gene Images™ CDP- Star™ and exposure to Hyper™ film were performed according to the manufacturer's instruction (Amersham, UK).

2.3.8   Northern blot analysis

Total RNA from transformed and non-transformed tobacco plants was extracted using the TriPure total RNA isolation kit (Roche, Germany). Quality of RNA was tested after running the RNA on a denaturing agarose gel and staining the RNA with ethidium bromide. RNA was quantified using the NanoDrop® technique. Northern blot analysis was carried out essentially as described by Sambrook and Russell (2001) with minor changes. Total RNA (20 μg) was first size-separated on a 1.2% agarose gel containing 2.2

M formaldehyde and then transferred to a Hybond-N+ membrane (Amersham, UK) and the DNA was UV cross-linked. For detection of an OC-I transcript, a 200 bp PCR amplified product representing the OC-I coding region was used as a probe after labelling with Random-Prime (Amersham, UK).

For the PCR reaction, the plasmid pBluescript SKII containing the cloned OC-I insert was used as a DNA template. For obtaining a probe for the Rubisco small subunit (*rcbS*), a portion of its coding sequence (359 bp) was amplified by PCR using designed primers from the tobacco *rbcS* cDNA sequence available on public database (GenBank accession AY220079). The primers used were forward 5-GCTGCCTCATTCCCTGTTTTC-3' and reverse 5'-TATGCCTTCTTCGCCTCTCC-3'. Both OC-I and *rbcS* probes were labelled by a Random-Prime labelling kit according to the manufacturer's instruction (Amersham, UK). Pre-hybridization for 2 hours and hybridisation of the probe with a membrane bound RNA were performed overnight at 65°C in a hybridisation buffer containing 0.5 M Na_2HPO_4, pH 7.2, 7% (w/v) SDS and 1mM EDTA. Subsequent stringency washes were performed at 65°C for 15 minutes each. The first washing solution contained 0.1% SDS (w/v), 2X SSC, the second solution 0.1% SDS (w/v), 1X SSC and the third washing solution 0.5X SSC and 0.1% SDS. Detection with the Gene Images™ CDP- Star™ and exposure to the Hyper™ film were performed according to the manufacturer's instruction (Amersham, UK).

2.4   Results

2.4.1   OC-I gene and protein detection

When different putative transformed tobacco plants expressing GUS were tested for the OC-I gene insertion by PCR analysis, all putative transformed plants showed an amplified PCR fragment with the predicted size of about 200 bp (Figure 1A; lanes T1-T4). An identical fragment was not amplified from genomic DNA obtained from a non-transformed plant (Figure 1A; lane NT). To test for the presence of an OC-I transgene encoded protein, an immuno-blot analysis was carried out using a polyclonal antibody raised against OC-I. Figure 1B shows the predicted OC-I protein band with an approximate size of 11.5 kDa detected from four putative transformed plants (Figure 1B; lanes T1-T4). A similar protein band was not detected in an extract from a non-transformed plant (Figure 1B; lane NT).

After restriction enzyme digestion of genomic DNA obtained from putative transformed plants, several hybridization products were detected when probed with an OC-I coding sequence. When *Bam*HI was used, three bands were detected (Figure 1C) and two bands were detected after digestion with either restriction enzymes *Eco*RI, *Kpn*I or *Xba*I. Such hybridization profile, however, was not detected in *Bam*HI digested genomic DNA obtained from a non-transformed tobacco plant (Fig 1C; lane NT). For all restriction enzymes used, only one major band beside less intense bands could be detected. This possibly indicates a single gene integration of exogenous OC-I into the tobacco genome and any detected additional band might have originated from cross-hybridization with endogenous tobacco cystatin sequences.
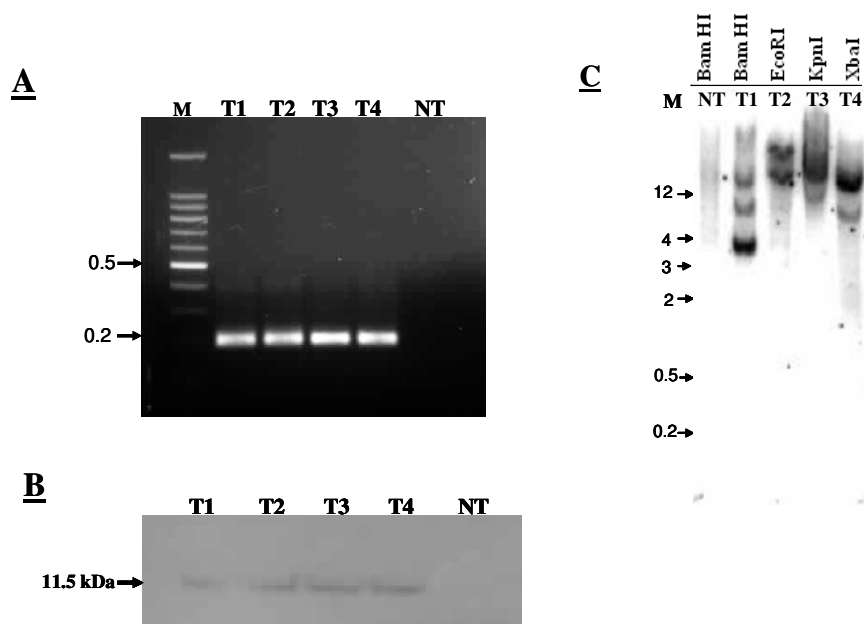
Figure 2.1 Characterization of putative transformed plants. (A) PCR amplified genomic DNA extracted from different putative transformed plants (lanes T1 –T4) and a non-transformed tobacco plant without OC-I gene insert (NT) as a control. M represents a 0.1 kbp DNA size marker (Roche, Germany). (B) Immuno-blotting of leaf protein extracts from different transformed plants (lanes T1-T4) and a non-transformed plant (lane NT) detected with an antibody raised against OC-I. (C) Genomic Southern blot analysis of genomic DNA derived from different transformed tobacco plants (lanes T1 – T4) and from a non-transformed tobacco plant (NT) probed with the complete OC-I sequence (598 bp) after digestion of genomic DNA with different restriction enzymes and transfer of the DNA onto a Hybond-N+ membrane. M represents a 1 kbp DNA size marker (Invitrogene, USA).

2.4.2   Expression of the small and large subunit of Rubisco

Transcription level of the small subunit (SSU) of the Rubisco gene (*rbcS* gene) and expression of the Rubisco large subunit (LSU) and SSU were used as indicators for the action of different stresses on transformed and non-transformed tobacco plants. On the protein level, LSU and SSU content were reduced in both transformed and non-transformed plants under drought stress when compared to non-stressed controls (Figure A). This was in comparison to heat-stressed or plants stressed with a combination of both stresses. On the transcription level, only transcription of SSU for non-transformed plants was greatly reduced under drought stress (Figure B). With all other stress treatments no obvious reduction in transcription was found when compared to transcription of SSU in a control plant.

A

	Transformed				Non-transformed			
	C	D	H	D&H	C	D	H	D&H

LSU →

SSU →

B

	C		D		H		D&H	
	T	NT	T	NT	T	NT	T	NT
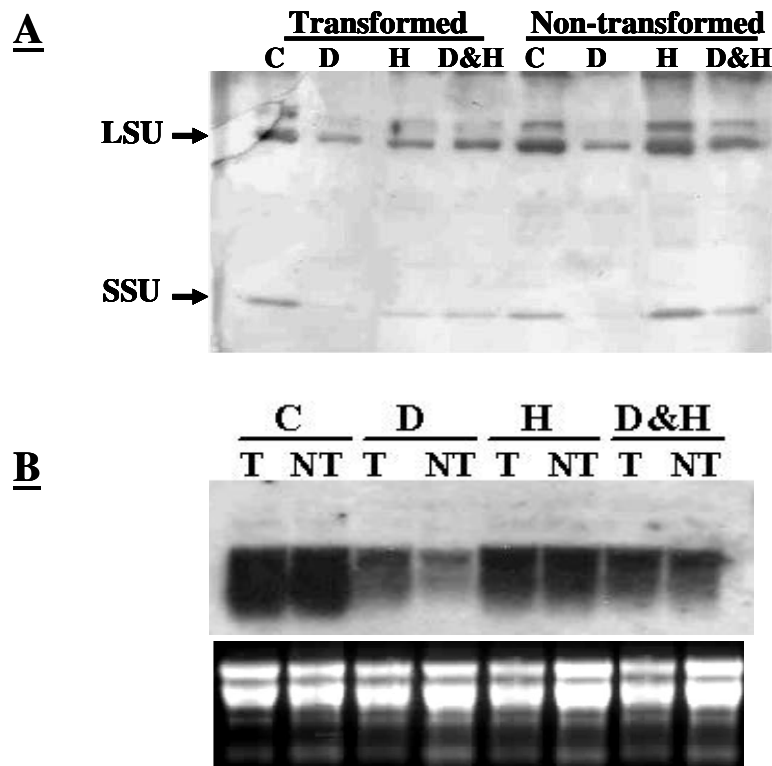
Figure 2.2 (A) Immuno-blot analysis of the a large subunit (LSU) and small subunit (SSU) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in transformed (T) and non-transformed (NT) tobacco plants under non-stress (C), drought (D), heat (H) and combined drought and heat stress (D&H). Polyclonal antibody for Rubisco subunit detection was raised against barley (*Hordieum vulgarae*) Rubisco (kindly provided by Dr Kepova K. Bulgarain Academy of Sceinces). (B; upper part) Northern blot analysis of the transcript of the small subunit of Rubisco (*rbcS* gene) and ethidium bromide stained total RNA (lower part) to ensure equal loading of RNA.

2.4.3   OC-I expression under stress

When protein extracts from transformed plants exposed to drought, heat and a combination of both stresses were analyzed by immuno-blotting using an OC-I antiserum, expression of exogenous OC-I could be detected in both non-stressed and stressed plants. A protein band of approximate 11.5 kDa was found (Figure 3A). The level of expressed OC-I increased by about 65% under both drought and heat stress and by 100% under a combination of drought and heat stress when compared to non-stressed transformed plants. Figure 4B shows the increase of the transcript level of OC-I following exposure of plants to the different types of stresses when compared to non-stressed transformed plants. All stress treatments increased transcription of OC-I in transformed plants.

A

B
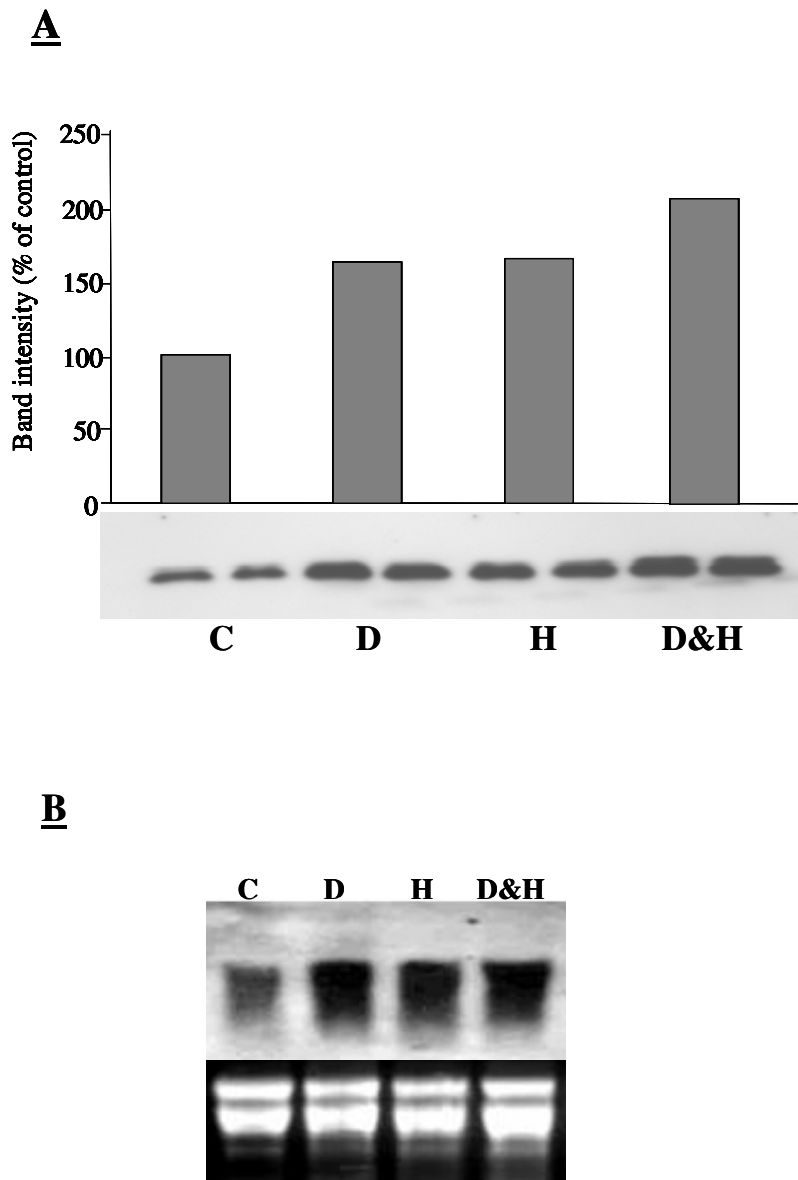
Figure 2.3 (A) Immuno-blot analysis to detect expression of OC-I in transformed non-stressed tobacco (C) and in transformed tobacco plants exposed to drought (D), heat (H) and a combination of drought and heat stress (D&H). Bars indicate average protein band intensity relative to the band from a non-stressed transformed plant. (B; upper part) Northern blot analysis for detection of an OC-I transcript in stressed and non-stressed

tobacco plants and B lower part ethidium bromide stained total RNA to ensure equal loading of RNA.

2.4.4   Activity of expressed OC-I

In order to test the activity of expressed exogenous OC-I, an *in vitro* cysteine proteinase activity assay was applied using a plant cysteine proteinase, papain, and the synthetic cysteine proteinase substrate Z-Phe-Arg-AMC. This substrate releases fluorescent AMC upon the action of a cysteine proteinase which can be quantified using fluorescence spectrophotometer. Figure 4A shows the residual activity of papain after incubation with a plant protein extract obtained from either non-stressed or stressed transformed or non-transformed plants. Inhibition of papain by a leaf protein extract from stressed transformed plants was greater than for an extract from non-transformed plants. Inhibition from extracts of non-transformed plants was 17% under drought, 13% under heat and 20% under both drought and heat stress when compared to 5% obtained from a non-transformed, non-stressed plant extract. In contrast, inhibition of papain from a non-stressed transformed plant extract was 24% and the level of inhibition was 45% for an extract derived from drought treated plants, 41% for heat and 54% for a combination of both stresses (Fig 4A). Inhibition was therefore always greater (P<0.05) for extracts from transformed plants than for extracts from non-transformed plants. E-64, a known inhibitor of cysteine proteinases, almost totally inhibited activity of papain (98%) under this experimental condition.
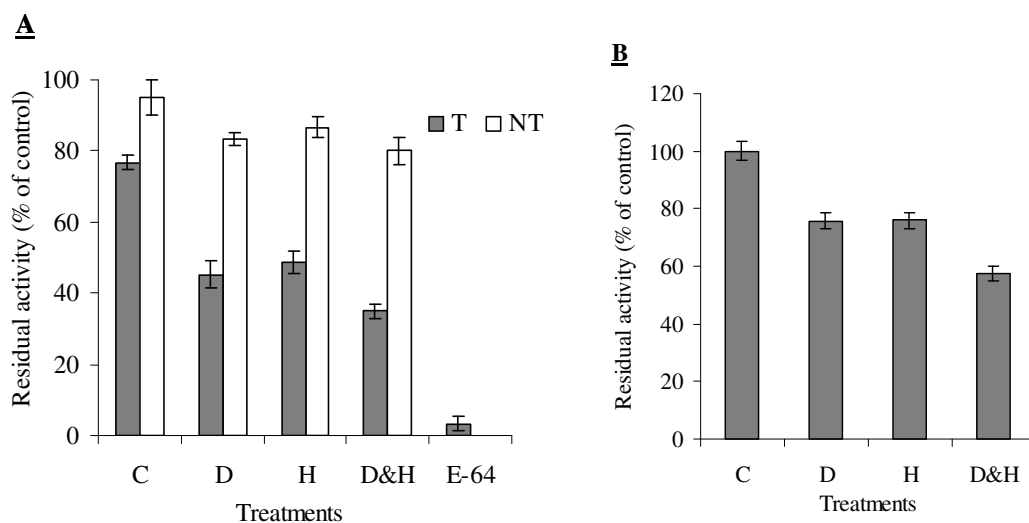
Figure 2.4 Residual papain activity (%) in the presence of 100 μg of plant soluble proteins. (A) Proteins derived from non-transformed (NT) and from OC-I expressing transformed (T) tobacco plants either un-stressed (C) or exposed to drought (D), heat (H) or a combination of both stresses (D&H). (B) Residual papain activity (%) after addition of a leaf protein extract derived from non-stressed transformed plants (C) and transformed plants stressed with drought (D), heat (H) and a combination of drought and heat (D&H). Values shown in A and B are relative to a papain activity without addition of a plant extract. Bars represent the mean of 4 different experiments ±SE.

2.5   Discussion

This part of the study showed that, the expression of the OC-I transgene in tobacco measured on the transcript and translational level was not reduced by drought, heat or a combination of both stresses when compared to non-stressed tobacco plants. Even further, an increase in the amount of both OC-I transcript and protein of OC-I was found. So far there is no evidence that the 35S CaMV promoter sequence, which is controlled in its activity by tissue and plant developmental stages (Benfey *et al.*, 1989), provides higher expression activity under drought or heat stress. Therefore, since equal amounts of transcripts and proteins were analyzed in the experiments from stressed and non-stressed plants, the increased transcript and protein level could be due to maintenance of an unchanged mRNA and protein level, while the majority of other transcripts decreased following stress treatment. In contrast, when an equal amount of RNA or protein was used for comparison to detect the Rubisco SSU and LSU protein or a SSU transcript, drought stress significantly reduced the amount of both protein and RNA transcripts.

Degradation of an exogenous protein can occur naturally or under stress. Expressed corn cystatin in transgenic rice showed a slight natural degradation in leaves (Irie *et al.*, 1996). Further, Outchkourov *et al.* (2003) also found stepwise degradation of potato cystatin (PhyCys), equistatin from Sea Anemone and a chicken egg white cystatin in transgenic potato plants. This was very likely caused as a result of action of certain sub-groups of plant endogenous cysteine proteinases. Such degradation following heat stress has also been found with other expressed exogenous proteins. Further, heat stress has shown to promote degradation of exogenous proteins in transgenic plants. Peas expressing a seed

specific bean α -amylase inhibitor 1 resulted in reduction of both the quantity and activity of the transgene encoded protein (Sousa-Majer *et al*., 2004). Similarly, loss of transgene-encoded activity that directly correlated with a stressful heat treatment was observed in a single-cell-suspension culture of alfalfa *(Medicago sativa)* carrying a single copy of an introduced synthetic phosphinothricin-resistance gene (Eckes *et al*., 1989 and Walter *et al*., 1992). Neumann *et al*. (1997) further showed that heat treatment of transformed tobacco lines can result in reversible reduction or complete loss of exogenous luciferase and neomycin phosphotransferase activity in transformed tobacco plants. Any degradation of OC-I was not evident in this study despite exposing plants to drought or heat stress because none of the immuno-blotting experiments showed any OC-I degradation products.

In the following chapter the consequences of a stable expressed OC-I under stressful conditions in a transgenic plant have been investigated in greater detail. A major focus was on the investigation of any benefit for the plant under drought and heat stress by measuring general growth parameters and photosynthetic activity.