CHAPTER THREE

GROWTH OF OC-I EXPRESSING TRANSFORMED TOBACCO PLANTS UNDER ABIOTIC STRESS

3.1 Abstract

Growth characteristics of transformed tobacco (Nicotiana tabacum L. cv Samsun) plants expressing a rice cystatin (OC-I) gene and non-transformed tobacco plants in response to drought, heat and a combination of theses stresses were measured. For the experiments two water regimes, which was well watered (80 – 100% field capacity) and drought stress (25 – 35 % field capacity) and two temperature regimes were used which was a 26/18ºC day/night cycle and a 38/30ºC day/night cycle. Measurement of plant growth characteristics showed that individual stresses significantly reduced plant growth and net photosynthetic rates of both transformed and non-transformed plants as compared to non-stressed tobacco plants. The degree of reduction of both parameters was further greater in plants challenged with a combination of drought and heat stress. Although under non-stress condition non-transformed plants had slightly higher total dry mass, photosynthetic rates and plant height, no highly significant differences could be found between the two types of plants in their response to a drought or heat stress or a combination of the stresses. Over-expression of exogenous OC-I in transformed tobacco did not confer any increased tolerance to drought or heat stress.

3.2 Introduction

About one third of the world's arable land suffers from inadequate supplies of water for agriculture, and in virtually all agricultural regions, yields of rain-fed crops are periodically reduced by drought (Kramer 1980; Boyer1982). Drought and heat stress almost invariably co-occur under arid-region field conditions and limit crop productivity. Drought stress hampers productivity by reducing or modifying the plant's essential processes like photosynthesis (Chaves *et al*., 2002; Lawlor, 2002; Lawlor and Cornic, 2002), which is reflected in decreased growth and productivity at the whole plant level. Similarly, temperature extremes above the optimal requirement for a plant can limit plant growth and productivity by impairing plant function including photosynthesis (Law and Crafts-Brandner, 1999) and reproductive development (Prasad *et al*., 2002). Drought stress can also cause oxidative damage to plant molecules like enzymes, lipids, RNA as a result of imbalance between production of reactive oxygen species and their metabolism (Foyer *et al*., 1994; Noctor *et al*., 2000). Combined drought and heat stress has been further shown to limit crop productivity more than the individual stresses (Craufurd and Peacock, 1993; Jiang and Huang, 2001). At the molecular level, the plant response to combined stress was also found to be greater than for individual stresses (Rizhsky *et al*., 2004). Further varietal/cultivar differences within a species in response to these stresses have been identified (Jagtab *et al*., 1998; Xu and Huang, 2001; Solomon and Labuschagne, 2003).

The aim of this part of the study was to investigate plant growth characteristics of transformed tobacco plants expressing an exogenous OC-I gene under drought and heat

stress. In particular, the plant growth and photosynthetic activity of OC-I expressing transformed tobacco plants were determined and compared to non-transformed tobacco plants. Plant growth characteristics measured included total plant dry mass, leaf area, leaf number and plant height. Leaf gas leaf gas exchange was measured to determine photosynthetic activity. The result obtained showed only marginal differences in plant growth and photosynthetic activity between transformed and non-transformed tobacco plants in response to drought or heat stress or a combination of the stresses.

3.3 Materials and Methods

3.3.1 Glasshouse experiments

Seeds from OC-I expressing transformed and non-transformed tobacco plants that have been selfed twice were raised on germinating tray on a glasshouse maintained at 26/20ºC day/night temperatures and 12 hours light with photosynthesis photon flux density of 240 ± 20 µmolm^{-2} s^{-1} and a relative humidity of 60 ± 4%. Four weeks seedlings of transformed and non-transformed seedlings were then transplanted to 5 litre capacity plastic pots filled with sand and coconut coir (50:50 by volume) and transferred to a glasshouse and well watered for two weeks. The growth condition was in the glasshouse during the study period were a relative humidity in the range of 40 – 80%, a temperature 26 ± 4ºC and a photosynthesis photon flux density of 650 - 900 µmol m^{-2} s^{-1}.

Drought stress was induced based on a gravimetric method. For that, half of the experimental plants were left without watering until plants showed wilting symptoms (10 days) while the remaining half were maintained at 80 – 100% field capacity. The amount of water evaporated was monitored daily by weighing unplanted pots placed randomly between planted pots in both stressed and non-stress treatments in each block. Pots were watered with the amount of water equivalent to the loss of weight. This was done to bring them to the pre-determined level of moisture whenever the weight of pots fell below the lower limit established for the treatment (25 – 35% for drought and 80 – 100% for non-drought stress treatments) until the end of the experiment. Plants received Hoagland nutrient solution 3-times a week. Two weeks after drought stress, the rate of photosynthesis was measured using a portable photosynthesis system (CIRAS-1, 1998, UK) on 10

randomly selected plants from each treatment combination. The rate of photosynthesis was measured on the 3rd or 4th fully expanded younger leaf counted from the shoot apex. The photosynthetic photon flux density incident at the level of the leaf was in the range of 770 - 850 µmol m^{-2} s^{-1}. Plants from 32 replicates per treatment were harvested three weeks after drought stress treatment. At harvest, plants were separated into leaves, stems and roots. Roots were washed off any soil debris. The leaf area was measured using a leaf area meter (Li-3000A, LI-COR, Inc. Lincoln, USA). The dry weight of roots and shoots was determined after drying the plant material at 70°C to a constant weight.

3.3.2 Growth cabinet experiments

Seedlings were raised as outlined above and eight weeks old seedlings were used for this experiment. The experiment with transformed and non-transformed plants included treatment with two growth temperatures, which were 26/20 ± 2°C and 38/30 ± 2°C, and two water regimes which were 25 – 35% and 80 – 100% field capacity. This resulted in a 2 x 2 x 2 factorial treatment combination in a randomized complete block design, where each treatment set is replicated 10-times. Drought stress treatment was induced based on a gravimetric method and watering was done as outlined above. 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 growth cabinet during the study period was 60 ± 4%. Plants received Hoagland nutrient solution 3-times a week. Net photosynthesis was measured (as in experiment outlined above) four weeks after stress treatment. For measurements, plants were moved out of the growth cabinet. Plants were harvested six weeks after stress treatment

induction for growth characteristic measurements and data were collected as outlined in glasshouse experiment above.

3.3.3 Statistical analysis

Plant total dry mass, leaf area, leaf number, plant height, root/shoot ratios and net photosynthesis were analyzed as two factor experiment (genotypes, drought treatment) in the glasshouse experiment and as three factor (genotypes, drought and heat treatments) in growth cabinet experiment. ANOVA model of Minitab release 11.12 software package Minitab Inc (1996) was used for statistical analysis. Differences were considered significant at $P \leq 0.05$.

3.4 Results

3.4.1 Effect of drought stress on plant growth and net photosynthetic rate in the glasshouse

A significant difference ($P<0.01$) in total dry mass, leaf area, leaf number, plant height and shoot/root ratio was found when drought stressed and non-stressed plants were compared. Drought stress reduced total dry mass by 58%, leaf area by 58%, plant height by 50% and leaf number by 37% in both transformed and non-transformed plants (Figure 1A-D). In contrast, drought stress increased the root/shoot ratio by 40% in plants when compared to non-stressed plants (Figure 1E). Although non-transformed plants were slightly higher and had greater total dry mass and net photosynthetic rates (10-15%) when compared to transformed plants once these differences were not highly significant ($P>0.05$).

When leaf gas exchange was measured, a significant difference ($P<0.01$) was found between drought stressed and non-stressed plants (Tab. 1). Net photosynthetic rate was reduced by 75%, stomatal conductance by 84% and transpiration rate by 77% in both transformed and non-transformed plants. However, no significant difference was found between transformed and non-transformed plants in net photosynthetic rate, stomatal conductance and transpiration rates.

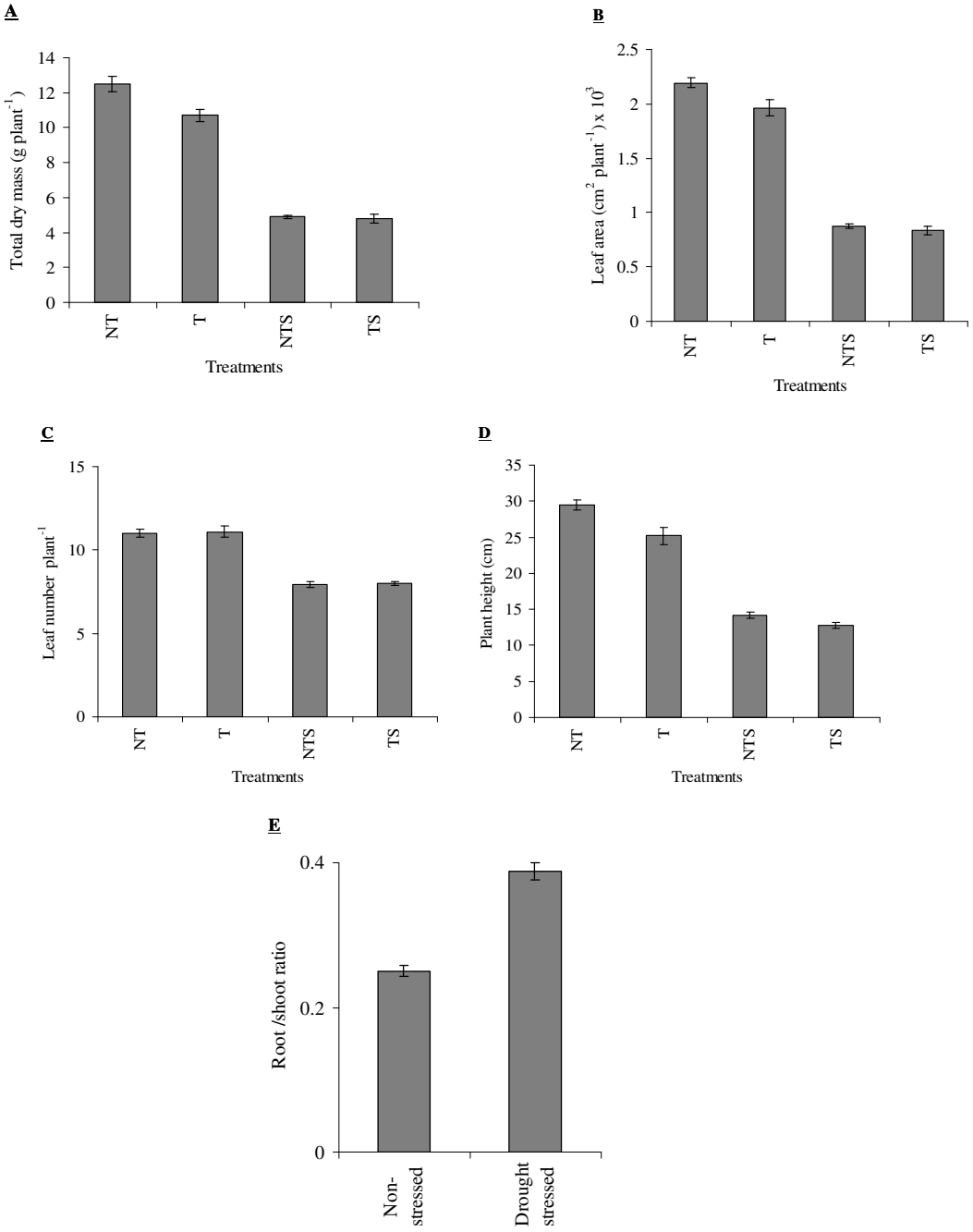

Figure 3.1 Growth characteristics of non-stressed, transformed (T) and non-stressed, non-transformed (NT) tobacco plants and drought-stressed transformed (TS) and drought-stressed non-transformed (NTS) tobacco plants. (A) Total plant dry mass, (B) leaf area, (C) leaf number, (D) plant height and (E) root/ shoot ratios of the different types of plants. Bars represent the mean of 32 plants ± standard error (S.E.).

Table 3.1 Effect of drought stress on photosynthesis, stomatal conductance and transpiration rates of non-transformed and transformed tobacco plants (data are mean ± S.E. of 10 individual plants)

Treatments	Photosynthetic rate (P_n, µmol m^{-2} s^{-1})	Stomatal conductance g_s, mmol m^{-2} s^{-1}	Transpiration rate (E, mmol m^{-2} s^{-1})
Non-stressed			
Non-transformed	18.0±0.90	1.19±0.22	11.5±1.07
Transformed	16.1±0.82	0.81±0.26	9.5±0.77
Drought-stressed			
Non-transformed	4.1±0.55	0.027±0.005	0.94±0.17
Transformed	4.2±0.29	0.016±0.004	0.62±0.16

3.4.2 Effect of drought and heat stresses on plant growth and net photosynthetic rate (growth cabinet)

Growth performance of transformed and non-transformed tobacco plants was evaluated either under drought or heat stress or under a combination of both stresses. Drought and heat stress significantly ($P<0.05$) decreased total dry mass, leaf area, plant height, leaf number and leaf net photosynthetic activity. Plant total dry mass was reduced following drought stress by 68%, following heat stress by 44% and following a combination of both stresses by 79% (Figure 2A). Similarly, the leaf area was reduced by 67% (drought stress), 38% (heat stress) and by 82% (combination of both stresses) when compared to non-stressed plants (Figure 2B). Also, plant height was reduced following drought stress 48%, following heat stress by 48% and following a combination of both stresses by 75% (Figure 2C). Further, leaf number per plant was reduced by 12%, 50% and 60% by heat, drought and combination of both stresses, respectively, and net photosynthetic rate was reduced by 35% (heat), 64% (drought) and by 75% (combination of both heat and

drought stress) when compared to non-stressed plants. However, both growth characteristics and photosynthetic activity were not significantly different ($P>0.05$) between transformed and non-transformed tobacco plants when either drought, heat or a combination of both stresses was applied.

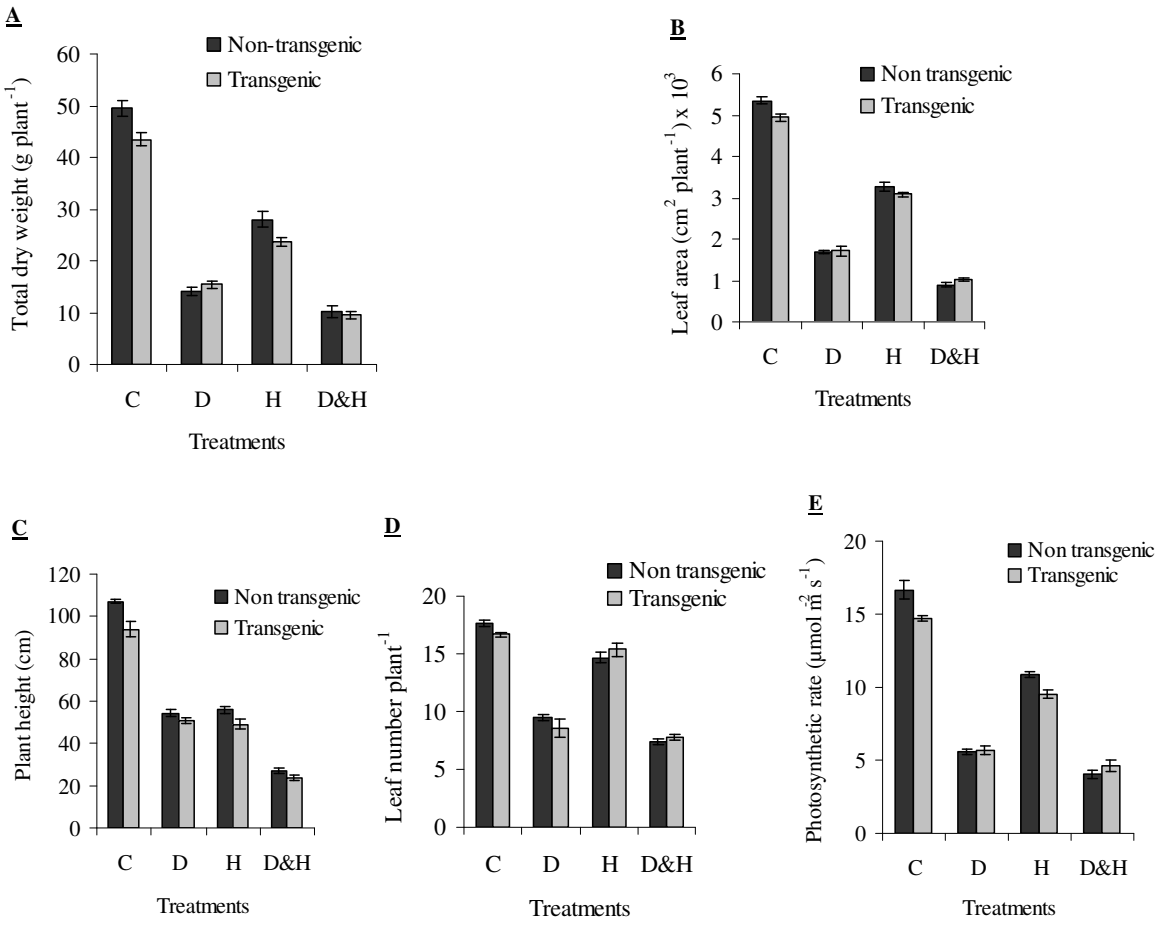

Figure 3.2 Plant growth and net photosynthetic rates of non-transformed and transformed plants under drought (D), heat (H) or a combination of both stresses (D&H). (A) Plant total dry mass, (B) leaf area (C), stem height (D), leaf number and (E) photosynthetic rate of transformed and non-transformed tobacco plants under non-stress (control), drought, heat and a combination of drought and heat stress. Bars represent mean of 8 plants ± standard error (SE).

3.5 Discussion

In this part of the study it was found that plant total dry mass, leaf number and area, stem height and photosynthetic rate were significantly reduced by drought, heat or a combination of these stresses. The effects of high temperature and drought stress on plant growth and productivity have been well documented in plants including tobacco, which has been also used in this study (Craufurd and Peacock, 1993; Savin and Nicolas, 1996; Jiang and Huang, 2001; Rizhsky *et al*., 2002). Biochemical and physiological alterations that occur during a combination of heat and drought stress on plants include enhancement of respiration and leaf temperature, reduction in photosynthesis, reduction/changes in type of antioxidant enzymes, which would lead to an increase in membrane lipid peroxidation (Jagtap *et al*., 1998; Jiang and Huang, 2001; Rizhsky *et al*., 2002). In tobacco, drought stress resulted in the suppression of both respiration and photosynthesis, whereas heat treatment resulted in enhanced respiration and stomatal conductance to lower leaf temperature by transpiration, but did not significantly alter photosynthesis (Rizhsky *et al*., 2002). In contrast, drought or a combination of drought and heat suppressed stomatal conductance raising the temperature of leaves (Rizhsky *et al*., 2002).

The present study also showed that net photosynthesis was significantly reduced by drought and combined drought and heat stress but less affected by heat stress. This also confirms the results reported by Jagtap *et al*. (1998) about the photosynthetic rate in sorghum where drought stress significantly reduced photosynthetic rates of different sorghum varieties when compared to heat or light stress. Further, in the present study it was also found that reduction in plant total biomass, leaf area and leaf number was more

severe than under heat stress. Reduction in growth is one of the most known effects of drought stress. It is mainly caused by inhibition of leaf and stem elongation when water the potential decreases below threshold. This differs among species and genotypes or cultivars within a species (Pelleschi *et al*., 1997; Younis *et al*., 2000). Also, the various mechanism by which drought stress reduces CO_2 assimilation and activity of photosynthetic enzymes includes, stomatal closure, the differences in the activation state of enzymes, decrease in the total protein content per leaf area or regulation at transcription, and translation of specific protein synthesis (Maroco, *et al*., 1999; Lawlor, 2002; Chaves *et al.*, 2002). Further, root growth is less sensitive to drought stress than stem growth (Creelman *et al*., 1990). This leads to an increase in root/shoot ratio that is commonly observed in plants exposed to drought stress and could also be confirmed in the present study. A decrease in available soil water decreases water uptake per unit root mass and may also reduce nutrient uptake, as delivery of nutrients by mass flow is hampered in dry soil (Poorter and Nagal, 2000; Marschner, 1995) resulting in overall growth reduction under drought stress.

However, in the present study it was found that, whatever growth parameter was measured following stress treatment, there was no significant difference in the response to stress between transformed and non-transformed plants. This was also true when the photosynthetic rate was measured after stress treatment. Under non-stress conditions in the greenhouse and growth cabinet, non-transformed plants even showed slightly higher total dry mass yield, stem height, leaf area and net photosynthetic rates than transformed plants. Therefore, expression of exogenous OC-I in transformed plants has no beneficial effect under drought or heat stress conditions when total dry mass, leaf area, leaf gas

exchange, leaf number and stem height were measured. Such an absence of difference in response between transformed and non-transformed tobacco plants might indicate an absence of interaction between exogenous OC-I and endogenous plant cysteine proteinases expressed following drought or heat stress. Such an absence of interaction could also emanate from differences in the localization of plant proteinases, the majority of which residing in the vacuole, and OC-I presumably expressed in the cytosol.

Since no highly significant differences in plant performance under drought or heat stress was found between the two types of plants, the influence of OCI expression on gene expression has been investigated to find out if both types of plants differ at all in gene expression. For that, cDNAs from heat-stressed transformed OC-I expressing and non-transformed plants were subtracted and identified cDNAs differently expressed between the two types of plant were used to study their transcription under heat stress.