Chapter 2: Preliminary study to physiologically and biochemically analyse oryzacystatin I expressing tobacco.

In collaboration with the group of Prof. C. Foyer at Rothamsted Research, (UK), possible improved tolerance to abiotic stress has been evaluated in genetically modified tobacco expressing the cysteine proteinase inhibitor oryzacystatin I (*OC-I*) in the cytosol. This study carried out in benefit to the PhD thesis, proceeded the study on the analysis of the tobacco genome and was mainly conducted in the first year of the PhD thesis.

Abstract

The primary goal of this study was to assess whether expression of exogenous *OC-I* in genetically modified tobacco plants provides improved tolerance to chilling stress. The plants had a conditional phenotype, possible not directly connected to the expression of the transgene, affecting plant morphology and general plant growth characteristics. A marked effect on stem elongation was observed in plants grown under low light intensities. After 7 weeks of growth at low light, the plants expressing *OC-I* were smaller with fewer expanded leaves and a slightly lower total biomass than wild-type plants. Maximal rates of photosynthesis (A_{max}) were also decreased, the inhibitory effect being greatest in the plants wth highest *OC-I* expression. After 12 weeks of growth at low light, however, the plants expressing *OC-I* performed better in terms of shoot biomass production, which was nearly double that of wild-type controls. All plants showed similar responses to drought, however photosynthesis was better protected against chilling injury in plants constitutively expressing *OC-I*. Photosynthesis CO_2 assimilation was decreased in all plants following exposure to 5°C, but the inhibition was significantly less in the *OC-I* expressing plants than in controls. The results of this study are reported below and are important to bring in line with the genetic analysis of the plants used in the study for my PhD thesis.

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Introduction

Cystatins bind tightly and reversibly to the papain-like group of cysteine proteinases. Cystatins have been used in attempts to engineer better pest control in plants by targeting the digestive system of Coleopteran insects and nematodes (Leplé et al., 1995; Urwin et al., 2001). The best characterized of these is the oryzacystatin-I (*OC-I*) from rice (Abe et al., 1987) which has been successfully expressed in tobacco without any deleterious effect on the plant (Masoud et al., 1993).

Several cystatins have been isolated from plants and two observations have led to the conclusion that cystatins are involved in the regulation of protein turnover during seed development (Abe et al., 1987; Abe et al., 1992). Other recent studies have also shown that endogenous cystatins are specifically induced during cold or salt stress (Pernas et al., 2000), wounding and/or following treatment with methyl jasmonate (Botella et al., 1996), or by prosystemin overexpression (Jacinto et al., 1998). All of these observations support the hypothesis that cystatins play a crucial role in general plant defence mechanisms. However, additional benefits (or disadvantages) arising from constitutive overexpression of cystatins controlling the action of cysteine proteinases in transgenic plants have been largely ignored. Such cysteine proteinases have acidic pH optima *in vitro*, suggesting that they are localized to the vacuole *in vivo* (Callis, 1995). They are expressed mainly in young and senescent leaves and flowers (Buchanan-Wollastan and Ainsworth, 1997; Guerrero et al., 1998; Xu and Chye, 1999) and accumulate in response to oxidative stress (Schaffer and Fischer, 1988). Recently, a role of cysteine proteinases has also been proposed in programmed cell death (Solomon et al., 1999; Xu and Chye, 1999) and an involvement in developmentally regulated programmed suicide pathways has been found (Hadfield and Bennett 1997; Penell and Lamb, 1997). Linthorst et al. (1993) further reported that expression of the tobacco cysteine proteinase, CYP-8, is regulated by a circadian rhythm and that the proteinase is involved in the wound response in tobacco. However, the interaction between endogenous

cysteine proteinases and endogenous or exogenous cystatins, as their natural inhibitors, during abiotic stress remains to be elucidated.

Results

A) OC-I expression

Plants of 3 selected genetically modified lines T4/5, T4/3-1 and T4/3-2 expressed the *OC-I* coding sequence (Van der Vyver et al., 2002; in press). After immunoblotting, a band at the predicted size of about 12 kDa equivalent to the 102 amino acid *OC-I* protein was observed following SDS-PAGE and using an antiserum raised against *OC-I* (Leplé et al., 1995) (Figure 2.1).

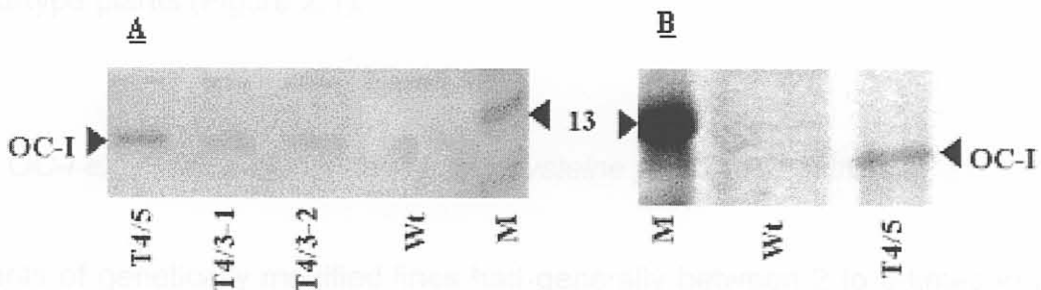

Figure 2.1: Immunoblotting of leaf extracts from plants of genetically modified lines T4/5, T4/3-1 and T4/3-2 and of the wild-type line (Wt). For blotting a polyclonal antibody raised against *OC-I* (A) was used. Separation of plant extracts on SDS PAGE and Coomassie Blue staining of separated plant extracts after heat treatment and concentration of extracts (B). For each line, 10 µg of protein from an extract of a fully expanded leaf (A) or from a concentrated extract (B) was loaded onto the gel. Size marker (M) is shown in kDa.

An accurate direct measurement of the inhibitor level in plant extracts is difficult due to possible binding of the inhibitor to endogenous cysteine proteinases. In the future this problem might be solved under strong

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denaturing conditions, breaking the binding between *OC-I* and putative cysteine proteinases. The strongest expression after immunoblotting was detected in modified plants of line T4/5. Recently, it was suggested that *OC-I* is poorly expressed in transgenic plants (Womack et al., 2000). This conclusion perhaps arose because the detection of *OC-I* transgene expression (based solely on immunoblotting) can be problematic. For this reason, we have included two selectable marker genes (*nptII* and *gus*), in addition to *OC-I*, in the transgenic lines. This allowed us to avoid selection of false positives for kanamycin resistance and to increase selection efficiency by using *gus* expression together with *OC-I*, because it is unlikely that the *gus* gene would segregate away from *OC-I* in the progeny. Expression of *OC-I* was also detected after separating a plant extract on a SDS PAGE following heat treatment of the plant extract to remove the bulk of heat-labile proteins and concentrating the expressed heat-stabile *OC-I* by freeze drying (Figure 2.1). No reaction with the antiserum or a protein band for *OC-I* was found in wild-type plants (Figure 2.1).

B) OC-I expressing plants have lower cysteine proteinase activity.

Plants of genetically modified lines had generally between 2 to 5-times lower endogenous cysteine proteinase activity than wild-type control plants when grown under *in vitro* conditions at 25°C on a half-strength MS medium (Figure 2.2). However, a great variability was found in endogenous cysteine proteinase activity between individual plants and batches of plants. One possible explanation is that different levels or forms of cysteine proteinases are present at different stages of plant development, which might be differentially inhibited by exogenous *OC-I* in the different lines (Figure 2.2).

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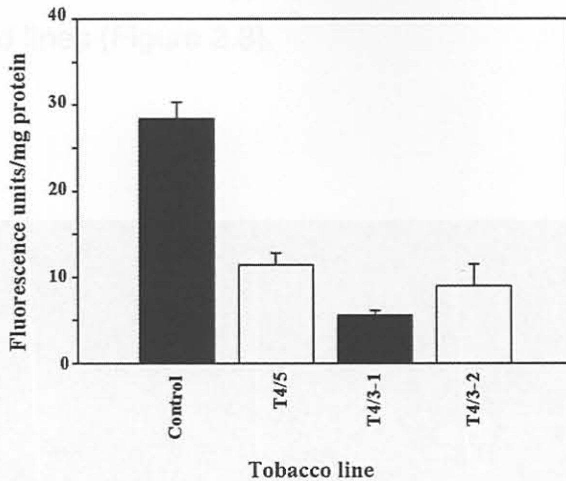

Figure 2.2: Cysteine proteinase activity present in leaves of the lines T4/5, T4/3-1 and T4/3-2 and controls (control). Plants were grown in culture at and irradiance of 50 µmol m^{-2} s^{-1} light intensity. After 4 weeks at 10ºC (A) the plants were allowed to recover 2 weeks at 25ºC (B). In each case values represent the mean ± standard error of 5 different plants.

C) Conditional phenotype and growth characteristics – Rothamsted Research.

When *OC-I* expressing plants were grown for 7 weeks at a relatively low light intensity (300-350 µmol m^{-2} s^{-1}) in a greenhouse at 20°C or during fall/winter in South Africa without extra light supplementation, stem elongation was substantially decreased compared to controls (Figure 2.3). This phenotype was observed in all the *OC*-I expressing (*OC-I/nptII/gus*) lines but not in the Tgus (*nptII/gus*) control line, which showed a similar phenotype to the non-transformed wild-type controls. This indicates that decreased stem elongation is linked to *OC-I* expression and was not due to somaclonal variation consecutive to the transformation or tissue culture processes. Under low light conditions, flower development was further delayed in plants of the genetically modified lines when compared to wild-type plants and flowering was initiated

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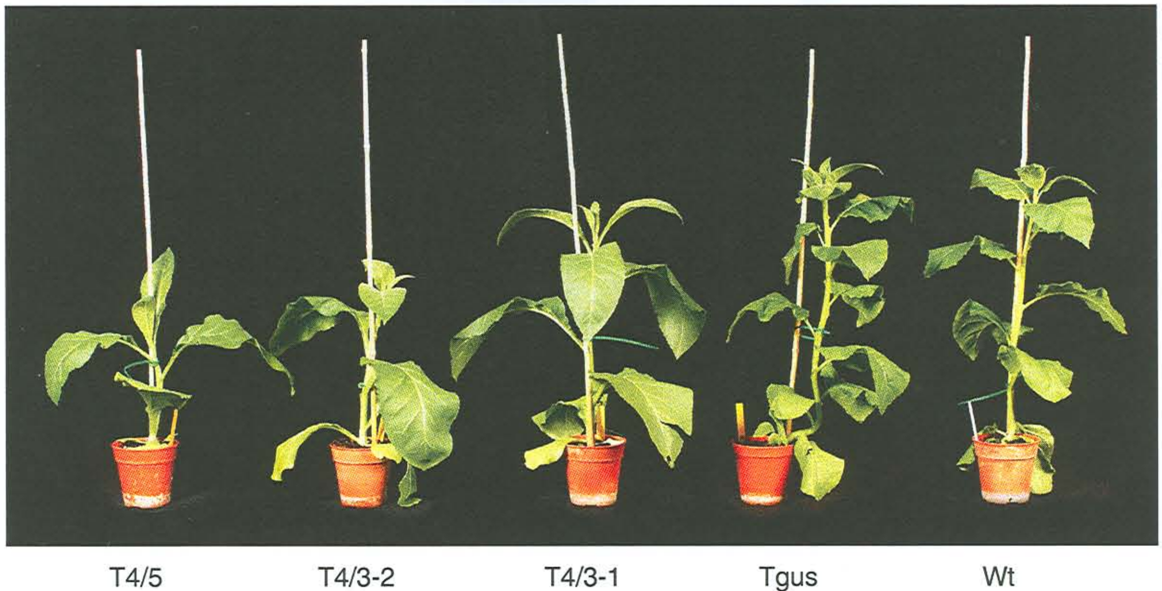
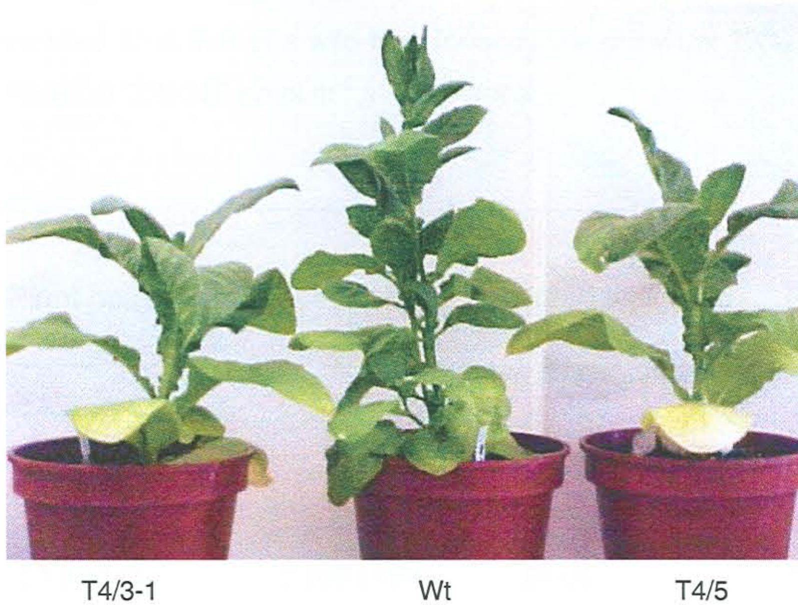
after 7 weeks in wild-type plants compared to 10 weeks in the genetically modified lines (Figure 2.3).



T4/5 T4/3-2 T4/3-1 Tgus Wt

Figure 2.3: Growth of plants of genetically engineered lines T4/5, T4/3-2, T4/3-1 and Tgus compared to that of a plant from a wild-type tobacco line (Wt) grown for 7 weeks under a low light intensity (300-350 μmol m^{-2} s^{-1}).

These phenotypic differences between 7 weeks old wild-type and *OC-I* expressing plants were, however, much less apparent at higher growth light intensities, for example 900 μmol m^{-2} s^{-1} when higher light intensity suppresses rapid stem elongation (Figure 2.4).



T4/3-1 Wt T4/5

Figure 2.4: Growth of plants of genetically modified lines T4/3-1 and T4/5 and of a wild-type plant (Wt) grown for 7 weeks under a high light intensity (900 $\mu mol\ m^{-2}\ s^{-1}$) (Van der Vyver et al., 2000).

The leaves of the wild-type expanded more rapidly under low light conditions than those of the *OC-I* expressing plants after 7 weeks (Table 2.1). Total leaf area was thus slightly less in *OC-I* expressing lines than in the wild-type at 7 weeks. At 7 weeks the *OC-I* expressing plants had also lower total biomass (fresh and dry weight) than the wild-type (Table 2.1). After 12 weeks growth at a lower light intensity (300-350 $\mu mol\ m^{-2}\ s^{-1}$) the stems of the *OC-I* expressing plants were still much shorter (55% - 70%) and thicker than the stems of the wild-type plants although all lines had similar leaf numbers.

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Table 2.1: Height, leaf area, fresh and dry weights of plants of individual genetically modified lines and of a wild-type tobacco line grown at 20°C for 7 weeks in the greenhouse at 300-350 μmol m^{-2} s^{-1} irradiance.

Line	Plant height (cm)	Leaf area (cm^2)	Fresh weight (g)	Dry weight (g)
Wild-type	51.2±4.8	2400±166	110±3	16.8±1.2
T4/5	12.0±0.8	2100±83	96±7	13.0±1.1
T4/3-1	11.6±1.2	2166±167	95±5	12.1±1.1
T4/3-2	15.6±0.4	2116±103	103±4	12.4±1.2

In each case values represent the mean ± standard error of 3 different plants.

D) Photosynthesis and respiration – Rothamsted Research

Photosynthesis was lower ($p \geq 0.05$) in *OC-I*-expressing tobacco plants grown at 20°C with an irradiance of 300-350 μmol m^{-2} s^{-1} for 7 weeks, than in the wild-type (Table 2.2). Maximal rates of photosynthesis (A_{max}; 18.1±0.2μmol CO_2 m^{-2} s^{-1}) were decreased as a result of *OC-I* expression (Table 2.2). All *OC-I*-expressing plants had significantly lower ($p \leq 0.05$) rates of CO_2 assimilation than wild-type plants. The inhibitory effect was greatest (11.0±0.6 μmol CO_2 $m^{-2}s^{-1}$) in transformed plants of line T4/5, which had also the highest level of *OC-I* protein. However, the apparent quantum efficiencies of photosynthesis (AQE) were similar in all lines. This observation indicates that while the absolute amount of photosynthetic machinery is decreased in the *OC-I*-expressing plants there is no photoinhibition (Table 2.2).

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Dark respiration rates varied between the plants of the different lines (Table 2.2). Leaves from line T4/3-1 had similar rates of dark respiration to those of wild-type plants. Respiration rates in T4/5 plants with highest *OC-I* expression were half ($p \leq 0.05$) of those of the wild-type. Moreover, respiration was significantly higher in transformed plants of line T4/3-2 ($p \leq 0.05$; Table 2.2).

Table 2.2: Apparent quantum efficiencies (AQE), photosynthetic CO_2 assimilation rates (A_{max}) and dark respiration rates (R) in leaves from genetically modified and wild-type plants grown at 20°C in the greenhouse at 300-350 μmol m^{-2} s^{-1} irradiance.

Line	AQE (mol [CO_2] mol^{-1}[light]) x 10^{-2}	A_{max} (μmol [CO_2] $m^{-2}s^{-1}$)	R (μmol [CO_2] $m^{-2}s^{-1}$)
Wild-type	3.56±0.36	18.1±0.2	0.82±0.10
T4/5	2.92±0.24	11.0±0.6	0.44±0.21
T4/3-1	2.88±0.16	15.6±1.4	0.89±0.19
T4/3-2	3.12±0.24	15.8±1.8	1.34±0.09

In each case values represent the mean ± standard error of samples of 6 different plants. The statistical significance of the difference between the mean values was determined by the Student's two-tailed *t* test and P values ≤ 0.05 were considered significant.

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E) OC-I expression protects against chilling stress – Rothamsted Research

Photosynthetic CO_2 assimilation was decreased following exposure to 5°C for two days, with all plants showing lower A_{max} (65-80% lower; Figure 2.6) and AQE (20-75% lower; Figure 2.5) values. However, the decline in AQE in two *OC-I*-expressing lines (T4/3-1 and T4/3-2) was significantly less (20% and 31% respectively; $p \leq 0.05$) than that measured in wild-type plants in which AQE declined by 75% (Figure 2.5A). While the low-temperature-induced decline in AQE (65%) was also less in T4/5 plants (with highest *OC-I* expression), this was not significantly different ($p \geq 0.05$) to that observed in the wild-type. In all cases, low-temperature-induced changes in measured values for photochemical and non-photochemical quenching of chlorophyll *a* fluorescence were in agreement with the changes in AQE (data not shown). Following the 2 days exposure to low growth temperatures, plants were allowed to recover at 20°C for 2 days (Figure 2.5B). After 2 days recovery, AQE had returned to values measured in non-chilled plants in all lines (Figure 2.5B). There were no significant differences in AQE between plants of the different lines ($p \geq 0.05$) in the recovery phase (Figure 2.5B). A_{max} was reduced by 84% in wild-type plants after two days at 5°C (Figure 2.6A). The chilling-induced decrease in A_{max} was, however, less in the *OC-I*-expressing lines. Compared to values measured at 20°C, A_{max} was decreased by 72% in plants of lines T4/3-1 and T4/5 and by 65% in line T4/3-2 (Figure 2.6A). Two days after return to 20°C, A_{max} had recovered to 70% of the original values in line T4/3-1 and in wild-type plants. A trend to higher recoveries was observed in plants of lines T4/3-2 (78%) and T4/5 (85%), but A_{max} being significantly higher in line T4/5 with the highest *OC-I*-expression ($p \leq 0.05$; Figure 2.6B).

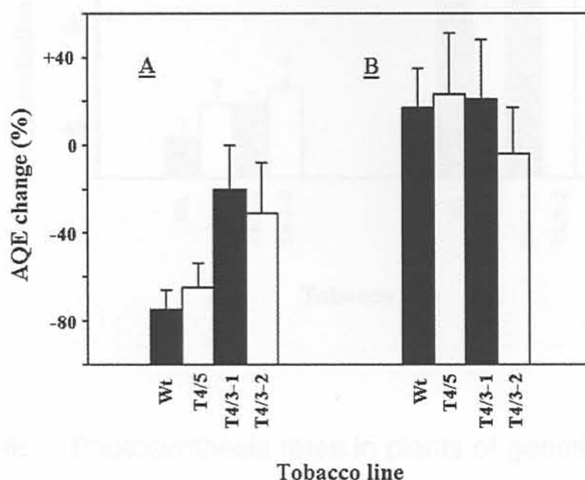

Figure 2.5: Effect of chilling on photosynthesis AQE in plants of genetically modified lines T4/5, T4/3-1 and T4/3-2 and a non-modified wild-type line (Wt). All lines were grown at low light (300-350 µmol m^{-2} s^{-1}). Measurements were made after 2 days at 5ºC (A) and after a subsequent 2 days recovery at 20ºC (B). Values are expressed as percentages of those measured before cold treatment. These were 3.56±0.36 mol [CO_2] mol^{-1}[light] x 10^{-2} for control plants, 2.92±0.24 mol [CO_2] mol^{-1}[light] x 10^{-2} for T4/5, 2.88±0.16 mol [CO_2] mol^{-1}[light] x 10^{-2} for T4/3-1 and 3.12±0.24 mol [CO_2] mol^{-1}[light] x 10^{-2} for T4/3-2. In each case values represent the mean ± standard error of leaves of 6 different plants.

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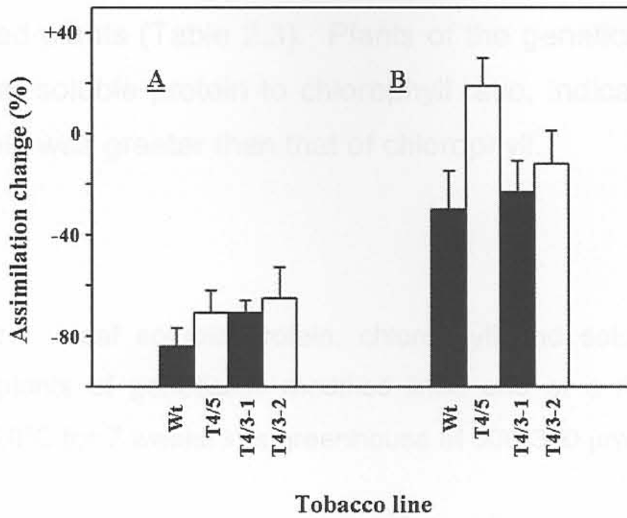

Figure 2.6: Photosynthesis rates in plants of genetically modified lines T4/5, T4/3-1 and T4/3-2 and in a non-modified wild-type line (Wt). In all cases plants were grown under low light (300-350 μmol m^{-2} s^{-1}). Measurements were made after 2 days at 5ºC (A) and after a subsequent 2 days recovery at 20ºC (B). Values are expressed as percentages of those measured before cold treatment. These were 18.1±0.2 μmol [CO_2] $m^{-2}s^{-1}$ for controls, 11.0±0.6 μmol [CO_2] $m^{-2}s^{-1}$ for T4/5, 15.6±1.4 μmol [CO_2] $m^{-2}s^{-1}$ for T4/3-1 and 15.8±1.8 μmol [CO_2] $m^{-2}s^{-1}$ for T4/3-2. In each case values represent the mean ± standard error of leaves of 6 different plants.

F) Protein and chlorophyll content – Rothamsted Research

The study carried out at Rothamsted also showed that leaves of plants of all genetically modified lines grown under low light intensity had a significantly higher soluble protein content ($p \leq 0.05$) than leaves of wild-type plants under the same growth conditions. Plants from all genetically modified lines T4/5, T4/3-1 and T4/3-2 had between 1.37 and 1.48 times as much soluble protein in their leaves as non-engineered plants and there was no significant difference between the engineered lines ($p \geq 0.05$) (Table 2.3). Plants of the engineered lines also contained significantly higher leaf chlorophyll contents ($p \leq 0.05$) than wild-type plants. Leaves from lines T4/5, T4/3-1 and T4/3-2 had

between 1.17 and 1.32-times as much chlorophyll as the leaves of non-engineered plants (Table 2.3). Plants of the genetically modified lines had a higher leaf soluble protein to chlorophyll ratio, indicating that the increase in leaf protein was greater than that of chlorophyll.

Table 2.3: Leaf soluble protein, chlorophyll and soluble protein to chlorophyll ratios of plants of genetically modified lines and of a non-modified wild-type line grown at 20°C for 7 weeks in a greenhouse at 300-350 μmol m^{-2} s^{-1} irradiance.

Line	*Protein* (μg cm^{-2})	*Chlorophyll* (μg cm^{-2})	*Ratio*
Wild-type	348±24	46±4	7.6
T4/5	*480±56*	*57±5*	*8.4*
T4/3-1	492±60	61±3	8.1
T4/3-2	516±20	54±2	9.6

In each case values for protein and chlorophyll represent the mean ± standard error of samples from 6 different plants. The statistical significance of the difference between the mean values was determined by the Student's two-tailed *t* test and P values ≤ 0.05 were considered significant.

G) Growth of OC-I expressing and wild-type tobacco under low temperature.

When wild-type control plants and plants of genetically modified lines were grown for extended time periods *in vitro* on MS medium and exposed for 4 weeks to 10°C at a light intensity of 50 μmol m^{-2} s^{-1}, the fresh weight doubled.

But no significant differences were observed between the lines ($p>0.05$; Figure 2.7A). After 4 weeks at low temperature, however, the wild-type plants developed necrosis on the expanding leaves (Figure 2.8). This was not observed in plants of the genetically modified lines expressing *OC-I*. In these lines the leaves did not expand but stayed green and compact.

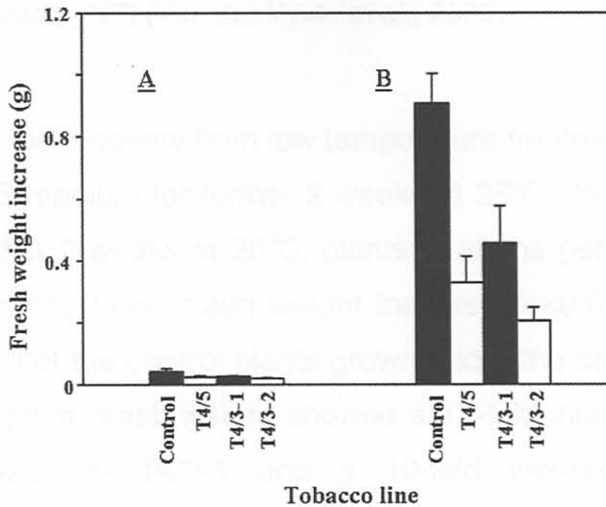

Figure 2.7: Influence of constitutive *OC*-I expression on plant growth. The increase in growth (g fresh weight) in plants of genetically modified lines T4/5, T4/3-1 and T4/3-2 and in wild-type control plants was measured in plants grown in culture at 50 μmol m^{-2} s^{-1} light intensity. They were grown for 4 weeks at 10ºC (A) and then for 2 weeks at 25ºC (B). At the beginning of the experiment the average fresh weight of individual plants from each line was about 12 mg. At this stage each plant was 2 cm in height. In each case values represent the mean ± standard error of 5 different plants.

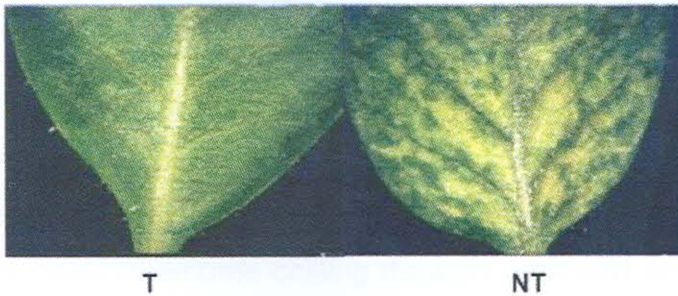

Figure 2.8: Chilling-induced chlorosis in leaves of genetically modified plants (T) and wild-type plants (NT) (Van der Vyver et al., 2000).

To determine the recovery from low temperature treatment, plants were grown *in vitro* on MS medium for further 2 weeks at 25°C after the low temperature treatment. After 2 weeks at 25°C, plants of all the genetically modified lines had a significantly lower fresh weight increase ($p \leq 0.05$) and remained only half of the size of the control plants grown under the same conditions (Figure 2.9). At this point, fresh weight showed a 12-fold increase in T4/5 plants, a 17-fold increase in T4/3-1 and a 10-fold increase in T4/3-2 plants, respectively. By contrast the fresh weight had increased 22-fold in wild-type plants (Figure 2.7B).

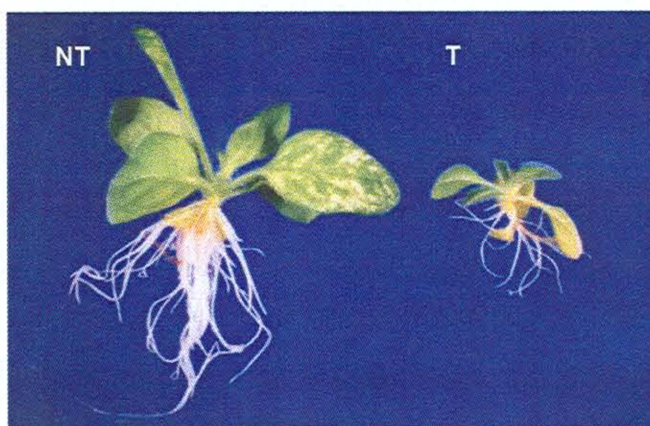

Figure 2.9: Growth of wild-type plants (NT) and genetically modified tobacco plants (T) at 10°C for 4 weeks followed by a further 2 weeks at 25°C (Van der Vyver et al., 2000).

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Discussion

Several genetically modified plants expressing the proteinase inhibitor *OC-I* in the cytosol had phenotypic changes when compared with wild-type tobacco plants. Results provided evidence that under sub-optimal growth conditions modified plants could overcome chilling through improved stress acclimation. Photosynthesis was better protected against chilling-induced photoinhibition (determined by AQE) in these plants when compared to wild-type plants.

Constitutive *OC-I* expression also facilitated better post-chilling recovery of CO_2 assimilation and rates of photosynthesis were higher in the recovery phase than before cold treatment. In addition, modified plants had also less chlorophyll degradation after long-term chilling conditions than wild-type plants. This suggests that cystatin expression has the potential to increase abiotic stress tolerance by better recovery of the photosynthetic apparatus and limiting cell degradation after chilling in genetically modified plants.

A fine line seems, however, to exist between possible disadvantages and benefits attained from constitutive *OC-I* expression. Although highest recovery from chilling was observed in modified plants of line T4/5 with the highest *OC-I* expression, this line had both the lowest CO_2 assimilation rate and the lowest dark respiration rate under greenhouse conditions. Under these conditions constitutive *OC*-I expression tend to have negative rather than positive effects on photosynthesis. One possible major source of negative interference by *OC-I* could be the modification of the protein turnover rates in the cytosol. This could have major consequences for proteins whose rapid turnover is essential for the maintenance of the photosynthetic apparatus. These effects could be an advantage at sub-optimal low temperatures but not at an optimal growth temperature and such features would, therefore, have severe implications if a high cystatin expression were, for example, to be exploited for optimal pest control in crop species.

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Unclear is why *OC-I* expression in the cytosol has such a profound effect on photosynthesis in the chloroplast. However, most chloroplast proteins are synthesised in the cytosol and then transported across the chloroplast envelope. This includes, for example, the small subunit of Rubisco and also chaperons required for Rubisco assembly. Also there is evidence that cysteine proteinases are expressed during chilling (Schaffer and Fischer, 1988). While the chloroplast itself has its own complement of proteinases that degrade proteins such as D1, degradation of other chloroplast proteins may be shared between different compartments of the photosynthetic cell. To date, cysteine proteinases have been found in the vacuole and recently evidence was provided that ER bodies appear to be a novel proteinase-storing system that assists in cell death under stressed conditions (Hayashi et al., 2001). Pompe-Novak et al. (2002) also found the potato leaf cysteine proteinase, PLCP-2, in protein bodies in the vacuoles, cytoplasm and in cell walls of shoots tips, leaves, stems and root tips presumably involved in organogenesis. But their location in other parts of the cell, such as the chloroplast, remains to be elucidated.

Growth characteristics were also significantly changed in genetically modified plants carrying *OC-I* when compared to wild-type plants. When plants were grown in the greenhouse with supplemented lighting of relative low intensity, which promotes rapid stem elongation in plants, a significant inhibition of plant elongation was observed in the modified plants irrespectively of the expression level of *OC-I*. However, while elongation was significantly affected, plants of the modified lines and the wild-type line had no significant differences in total leaf areas and biomass (dry weight), leaf number, and fresh weight. Leaf chlorophyll contents increased in modified plants but this did not modify photosynthesis, expressed on a surface area basis, grown under artificial low light in the greenhouse at 20°C, which is similar to growth in shaded environments or within crowded plant communities (Ballaré et al., 1994).

The "dwarf" phenotype, which has not been reported by other investigators studying cystatin expression in plants (Masoud et al., 1993; Leple et al., 1995)

was, however, dependent on the light environment in which the modified plants were grown. Differences in stem elongation were much less obvious when genetically modified plants were grown at a much higher light intensity or under natural light conditions during the summer at 25°C, which does not induce a rapid stem elongation. From this study the possibility could not be excluded that the transformation process itself contributed to altered elongation characteristics and not the transgene expression as changes in growth characteristics were inherent in all genetically modified plants tested irrespectively of the expression level of the transgene. For example, reduced growth and delayed flowering has also been reported in the tobacco cultivar *Samsun* expressing *gus* (Caligari et al., 1993). The occurrence of such genomic changes in transgenic plants produced by *Agrobacterium tumefaciens* infection has also been recently verified in rice by molecular tools (Labra et al., 2001). To identify possible genome differences between modified and wild-type plants, a detailed genetic analysis on genetically modified plants was therefore conducted after this initial physiological and biochemical characterization. This characterization study is outlined in the following chapters.

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