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The protective role of Oryzacystatin-I under abiotic stress

By

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DECLARATION

I, the undersigned, hereby declare that the thesis submitted herewith for the degree Magister Scientiae to the University of Pretoria, contains my own independent work and has not been submitted for any degree at any other university.

Anneke Prins
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The protective role of Oryzacystatin-I under abiotic stress

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ABSTRACT

One of the most important photosynthetic enzymes in a plant is ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which plays a key role in carbon fixation. Degradation of this enzyme leads to decreased carbon fixation and poor photosynthetic performance by the plant. It is therefore of interest to investigate possible ways of protecting this enzyme during stress conditions in order to generate plants that would perform better under extreme climates. In this study the effect of an expressed, exogenous rice cysteine proteinase inhibitor (OCI) in transformed tobacco plants on Rubisco stability/content under chilling and senescence was investigated. Results showed that there is no significant protective role for exogenous OCI on the degradation/content of Rubisco when tobacco plants were exposed to chilling. This result was found using native gel-based quantification procedures, as well as immuno-blotting, spot densitometric analysis, and a radioactive quantification assay as analysis techniques. The study, however, provided evidence for protection of Rubisco against degradation by expression of OCI under a more severe stress condition, such as senescence using native gel-

based quantification procedures as detection techniques. Tobacco plants were also transformed with a newly designed vector allowing expressed OCI to be transported to the chloroplast. Failure to detect so far any OCI-expressing transformed plants and the idea that delay of senescence could prove beneficial to farmers by providing a more nutrient-dense crop with higher tolerance against stress-induced cell death are discussed.

SAMEVATTING

Een van die belangrikste ensieme tydens fotosintese in 'n plant is ribulose-1,5-bisfosfaat karboksilase/oksigenase (Rubisco) wat 'n sleutelrol in koolstoffiksering speel. Afbraak van hierdie ensiem lei tot 'n afname in koolstoffiksering en verlaagde fotosintese deur die plant. Dit is dus van belang om moontlike maniere te ondersoek wat hierdie ensiem tydens streskondisies kan beskerm, ten doel om plante te genereer wat beter sal funksioneer in uiterste klimate. In hierdie studie is die uitwerking van 'n uitgedrukte, eksogene rys sisteïen proteïenase inhibitor (OCI) op Rubisco stabiliteit/inhoud in getransformeerde tabakplante onder koue en veroudering, bestudeer. Resultate het getoon dat daar geen betekenisvolle beskermende uitwerking van eksogene OCI op Rubisco afbraak/-inhoud is wanneer tabakplante blootgestel is aan koue. Resultate is verkry deur van nie-denaturerende gel-gebaseerde kwantifisering, sowel as immuno-blottering, kol densitometriese analise, en 'n radioaktiewe kwantifiseringstoets as analitiese tegnieke gebruik te maak. Die studie het wel bewys gelewer vir die beskerming van Rubisco teen afbraak met die uitdrukking van OCI onder 'n erger stressituasie, soos veroudering, met die gebruik van non-denaturerende gel-gebaseerde kwantifisering as analitiese tegniek. Tabakplante is ook getransformeer met 'n nuut-ontwerpte vektor wat oordrag van uitgedrukte OCI na die chloroplas toelaat. Mislukking om tot dusver enige getransformeerde plante wat OCI uitdruk te vind, en die idee dat vertraging van veroudering voordelig kan blyk vir boere, deur 'n meer voedingsdigte gewas met hoër weerstand teen seldood as gevolg van stres daar te stel, word bespreek.

RESEARCH AIM AND OBJECTIVES

The aim of this study was to prove the hypothesis that an exogenous cystatin, Oryzacystatin I (OCI), has a protective effect on the degradation of Rubisco under cold stress. For that the interaction between plant cysteine proteinases and a cystatin, and protection of a plant key enzyme, Rubisco, under stress was investigated. Specifically it was asked in this study whether endogenous cysteine proteinases could be inhibited by an exogenous OCI expressed in a genetically modified (GM) plant, and how this might affect plants growing under chilling.

The specific objectives of this study were therefore to:

1. Physiologically and biochemically characterise transformed tobacco plants expressing exogenous OCI. In particular, protection of Rubisco by exogenous OCI against chilling damage in tobacco plants was determined.

2. Investigate the effect of senescence on Rubisco stability in transformed plants expressing OCI.

3. Undertake an attempt to direct OCI expression to the chloroplast of tobacco. This was done by designing a suitable vector containing the coding sequence of OCI and a transit peptide sequence which would direct the preprotein towards the chloroplast, as well as transforming tobacco (*Nicotiana tabacum* L. cv. Samsun) with the vector using the *Agrobacterium* system.

PREFACE

Chapter 1 of this thesis presents an introduction into stress and plants, how plants respond to abiotic stress, and the role of protein degradation during stress. It also explains the role of cystatins in plants, and describes the current applications of cystatins in genetically modified crop plants. **Chapter 2** explains the experimental procedures that were applied in this study. **Chapter 3** describes how tobacco plants expressing OCI in the cytosol were selected and characterised with regard to cold-tolerance and resistance to senescence. In particular, the degradation pattern of Rubisco was studied. This chapter also details how a vector was produced and used to modify tobacco plants to express chloroplast-targeted OCI. **Section 3.1** describes the process by which transgenic seedlings were grown from seed and selected for the expression of antibiotic resistance, the *GUS* marker gene, and the *OCI* gene. **Section 3.2** details the results of the study on protein degradation in unstressed tobacco plants (both transformed and non-transformed) in two different buffer systems. It also outlines the degradation sensitivity of Rubisco to cysteine proteinases. **Section 3.3** focuses on the effect of exogenous OCI expression on the sensitivity of Rubisco to cysteine proteinase degradation in plants under cold stress. **Section 3.4** focuses on the effect of exogenous OCI expression on the sensitivity of Rubisco to cysteine proteinase degradation in plants during senescence. **Section 3.5** explains how a plant transformation vector was constructed, which would allow expression of OCI that would be transported into the chloroplast. It also describes the results of initial transformation attempts with the newly designed plant transformation vector. **Chapter 4** is a general discussion chapter in which the results obtained in this study are discussed. In particular, phenotypic characterisation, general protein degradation profiles due to cysteine proteinase action, the effect of cold stress and senescence on Rubisco stability, as well as the first attempts to express OCI in the chloroplast is discussed. Achievements of this study and future perspectives are also discussed. The **Annexure** details the composition of buffers, solutions, and other chemicals used in this study

ABBREVIATIONS AND SYMBOLS

A_{585}	-	Absorbancy at wavelength 585nm
β	-	Beta
BAP	-	6-benzylamino purine
°C	-	Degrees Celsius
CABP	-	2-carboxyarabinitol -1,5- bisphosphate
CAM	-	Crassulacean acid metabolism
CaMV	-	Cauliflower mosaic virus
cm	-	Centimeter
CO_2	-	Carbon dioxide
CP	-	Chloroplast
dATP	-	Deoxyadenosine triphosphate
dCTP	-	Deoxycytosine triphosphate
dGTP	-	Deoxyguanosine triphosphate
dH_2O	-	Distilled water
DNA	-	Deoxyribonucleic acid
dsDNA	-	Double stranded deoxyribonucleic acid
DTT	-	Dithiotreitol
dTTP	-	Deoxythymine triphosphate
E. coli	-	*Escherichia coli*
EDTA	-	Ethylenediamine tetraacetic acid
FBPase	-	Fructose 1,6-bisphosphatase
F units	-	Fluorescence units
g	-	Grams
GUS	-	β-glucoronidase
HCl	-	Hydrochloric acid
H_2O_2	-	Hydrogen peroxide
HR	-	Hypersensitive response
HRP	-	Horseradish peroxidase
IDV	-	Integrated density value
L	-	Litres
LB	-	Luria Bertani
LSU	-	Large subunit

M	-	Molarity
mg	-	Milligrams
$MgCl_2$	-	Magnesium chloride
mL	-	Millilitres
mM	-	Millimolar
MS	-	Murashige and Skoog
NaAc	-	Sodium acetate
NaCl	-	Sodium chloride
$NADP^+$	-	Nicotinamide adenine dinucleotide phosphate (oxidised)
$NaHCO_3$	-	Sodium bicarbonate
NaH_2PO_4	-	Sodium hypophosphate
NaOH	-	Sodium hydroxide
Na_2SO_4	-	Sodium sulphate
ng	-	Nanograms
nm	-	Nanometer
NPT II	-	Neomycin phosphotransferase II
O_2	-	Dioxygen
OCI	-	Oryzacystatin I
OH^-	-	Hydroxyl radical
PA	-	Polyacrylamide
PAGE	-	Polyacrylamide gel electrophoresis
PCD	-	Programmed cell death
PEG	-	Polyethylene glycol
pH	-	log hydrogen ion concentration
P_i	-	Inorganic phosphate
PSI	-	Photosystem I
PSII	-	Photosystem II
PVDF	-	Polyvinylidene difluoride
RbcS	-	Rubisco small subunit
RNA	-	Ribonucleic acid
ROS	-	Reactive oxygen species
Rubisco	-	Ribulose -1,5- bisphosphate carboxylase/oxygenase
RuBP	-	Ribulose –1,5- bisphosphate
SBPase	-	Seduheptulose 1,7-bisphosphatase

SDS	-	Sodium dodecyl sulphate
SPS	-	Sucrose phosphate synthase
ssDNA	-	Single stranded deoxyribonucleic acid
SSU	-	Small subunit
TBS	-	Tris-buffered saline
TEMED	-	N, N, N', N' –tetramethyl ethylenediamine
Triose-P	-	Triose phosphate
Tris-HCl	-	2-Amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride
μg	-	Micrograms
μL	-	Microliters
μM	-	Micromolar
UV	-	Ultra violet
Z-Phe-Arg-NMec	-	benzyloxycarbonyl-phenylanine-arginine-aminomethyl coumarin
%	-	Percentage

CHAPTER 1

1. INTRODUCTION

1.1 Stress and Plants

Plants are the main source of energy for all animals on earth. They serve to incorporate atmospheric carbon into a form accessible for life, giving humans and animals nutrition, as well as providing them with essential micronutrients. Within any environment, however, conditions are not static, and plants are exposed to changes in climate to which they must adapt, or to which they must be tolerant. Any change in an environmental condition that results in a response of an organism might be considered stressful (Levitt, 1972). Koehn and Bayne (1989) defined stress as the reduction of the fitness of an organism by a change in an environmental condition that might reduce or adversely change growth and development of an organism. The study of plant response to environmental stress can improve any strategy aimed at improving crop productivity, since it elucidates the reasons why some plants are more tolerant than others. This information can then be used in breeding programmes and other strategies employing the tools of biotechnology to improve crop production.

The study of stress on plants, and the way plants respond to these stresses, has become increasingly complex. With the tools available to researchers today it is possible to investigate the response of plants to environmental stress at a molecular level. The amount of information available has reached almost unmanageable proportions with the numerous genome-sequencing programmes currently investigating the genetic layout of a great variety of organisms, including plants, animals, and microbes. The task of modern day plant biologists is to employ the available technologies in an effective way, in order to get meaningful answers to simple questions. Where plant stress response is concerned, an integrative approach is essential, where the route of research should include the study of the environment, plant physiology,

patterns in gene expression, and metabolic change. Stress factors may be biotic (living) or abiotic (non-living). Biotic stresses include a variety of pathogenic microorganisms, such as viruses, fungi and bacteria, as well as the effects of herbivorous insects that feed off the plant material. Abiotic stresses include water logging, drought, extreme temperature, intense light, excessive soil salinity, inadequate mineral nutrients in the soil and also treatment with plant growth regulators and antibiotics (Smirnoff, 1998).

Since plants are not mobile and cannot remove themselves from an unfavourable environment, they need to adapt to stress in order to survive. Environmental stress has played a significant role in the evolution of plants, to allow them to adapt to, and survive in different climates. This is obvious when comparing plants that have adapted to grow in very dry climates such as cacti and other succulents, and plants that have adapted to grow in very wet environments such as water lilies and other macrophytes. There may even be differences in stress tolerance within plants of the same species but different cultivars (Tomashow, 1990; Komatsu and Katu, 1997; Gale and Devos, 1998). Humans have used natural variation in stress-resistance in plants for centuries in order to breed hardier, more productive crops. A plant's response to stress can be influenced by its genes (whether the plant possesses "stress-resistant" genes), by the level of development it has reached, the duration and severity of the stress, and whether there are more than one stress factor present. If adaptation and repair mechanisms are not sufficient and stress factors are ultimately not removed, the outcome of stress will be death of the organism (Figure 1.1).

The strategy a plant employs could be either to avoid the stress, or to tolerate it. Plants avoid stress by, for example, extending their roots through dry soil down towards the water table, or by employing a different form of photosynthesis, such as CAM-type photosynthesis. Both of these are examples of adaptation mechanisms plants have evolved to counteract severe water deficit. Plants might also tolerate stress by altering their metabolism in response to it. Much research has been done on the expression of temporary phenotypes and the regulation of genes by

environmental cues (Smirnoff, 1998). Work has been done on gene regulation by low temperatures (Guo *et al.*, 2002, Nemeth *et al.*, 2002), pathogens and senescence (Robatzek and Somssich, 2002), water stress (Tambussi *et al.*, 2002), heat stress (Kleinhenz and Palta, 2002), and salt stress (Zhu, 2001), as well as the way that these stresses affect a plant's metabolism and physiology. Protective systems that are up-regulated as a result of stress include the anti-oxidative system, which protects plants against the harmful effect of reactive oxygen species, and the production of pathogenesis-related proteins during pathogenic attack (Foyer and Noctor, 2000; Kitajima and Sato, 1999). A general stress response in all kingdoms is the accumulation of ions (potassium, sodium, and calcium) and increased amounts of metabolites which are a part of normal metabolism and which are considered compatible solutes. Examples are sugars, sugar alcohols, low-complexity carbohydrates, tertiary amines, sulfonium compounds and amino acids (Pilon-Smits *et al.*, 1995; Bohnert and Jensen, 1996; Holmström *et al.*, 1996; Nelson *et al.*, 1998). During sudden increases in temperature, heat shock proteins are induced as a completely new set of proteins possibly playing a role in protecting essential enzymes and nucleic acids from denaturing (Sabehat *et al.*, 1998). The defence response can also be elicited far from the initial site of invasion, and sometimes even in neighbouring plants. The chewing action of insects activates proteinase inhibitors initially involving the release of a signal molecule, such as systemin, leading to a signalling cascade and eventually resulting in transcriptional activation of proteinase inhibitors far from invasion (Ryan, 2000).

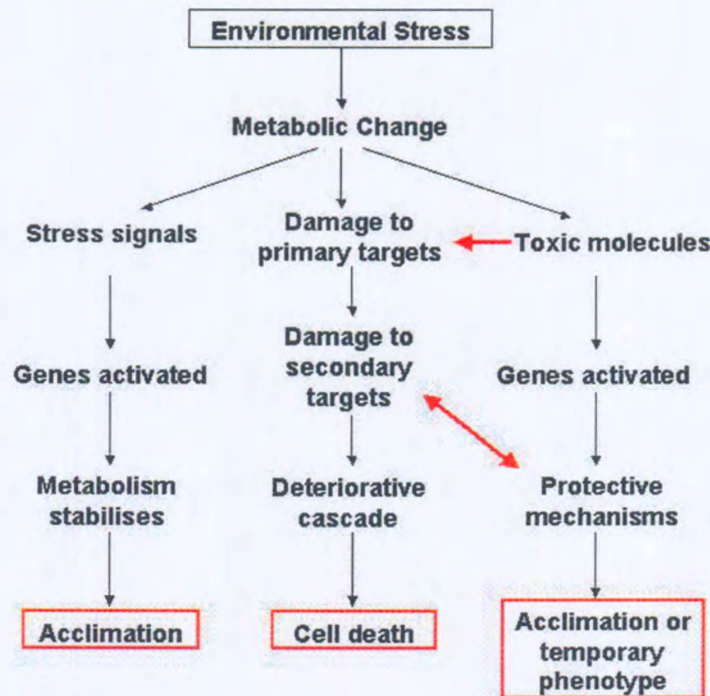

Fig. 1.1. A schematic representation of some of the possible ways in which plants can respond to environmental stress. Stress causes a change in the plant's normal metabolism. This causes a number of stress signals to be perceived by the plant, as well as the production of possible toxic molecules. Primary targets are damaged directly. A number of stress-responsive genes are activated in the plant, causing metabolic changes and triggering protective mechanisms. The eventual effect of the stress is acclimation, the expression of a temporary phenotype, or cell death (Asada, 1994).

1.2 Oxidative Stress and Plants

In photosynthetic organisms, the inevitable production of reactive oxygen species (ROS) leads to singlet oxygen, superoxide, hydrogen peroxide and hydroxyl radicals and consequently oxidative stress. ROS are produced under the conditions of senescence (Pastori and Del Rio, 1997), drought and heat (Price and Hendry, 1991), cold stress (Koukalová *et al.*, 1997), UV radiation (Green and Fluhr, 1995), and during the hypersensitive response (HR) to pathogen attack (Levine *et al.*, 1994) (Figure 1.2). Plants primarily protect

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themselves from the harmful effects of oxidative stress by regulating electron transport and photosynthesis. Furthermore a number of enzymes act as scavengers that find and inactivate harmful ROS. These enzymes include superoxide dismutase (Bannister *et al.,* 1987), ascorbate peroxidase, glutathione reductase (Foyer and Halliwell, 1976), and catalase (Willekens *et al.*, 1995). Non-enzymatic antioxidants, such as the vitamins C and E and the carotenoids, also exist. Vitamin C is water-soluble and reacts with ROS, thereby inactivating them (Yu, 1994), whereas vitamin E is lipid-soluble playing a protective role in plant membranes.

The effect of chilling temperatures on plants has been especially well investigated. At low temperatures there is a decrease in membrane fluidity, diffusion rates of molecules, and chemical and enzyme reaction rates. Besides this there is an inhibition of photosystem I (PSI) caused by the production of ROS. These ROS include singlet oxygen and hydroxyl radicals (Sonoike *et al.*, 1995). Terashima and co-workers (1998) observed an increase in the amount of hydrogen peroxide in cucumber leaves illuminated at 5°C because of decreased ascorbate peroxidase activity at that temperature. The investigators concluded that photoinhibition of PSI was due to the suppression of ascorbate peroxidase activity. It is still unclear, however, whether the Calvin cycle enzymes in chilling-sensitive plants are more sensitive to low temperatures than those in tolerant plants. The production of ROS at low temperatures does not always associate with light, which might also imply alternate sources for the damaging molecules.

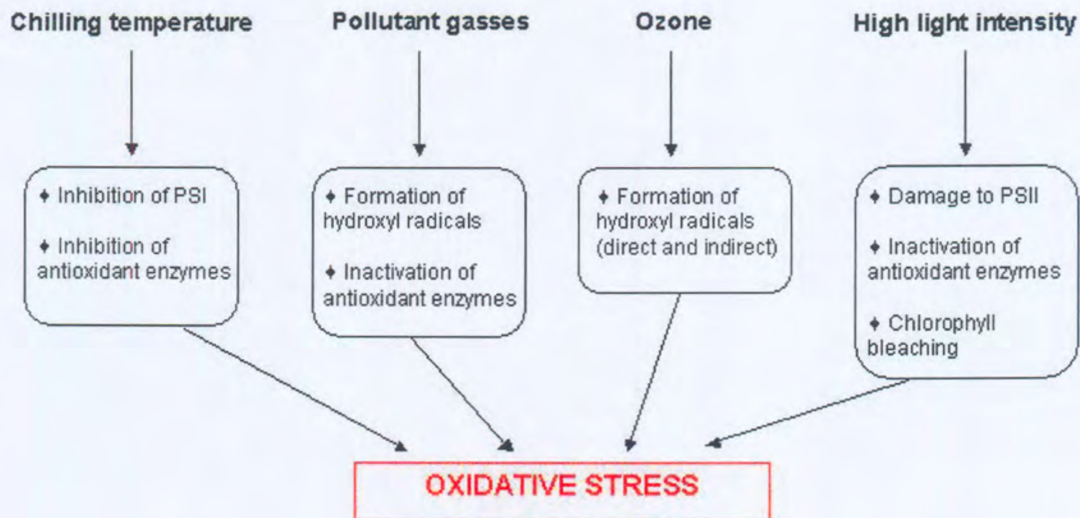

Fig. 1.2. Some of the environmental factors that lead to oxidative stress in plants, as well as their primary results. PSI – Photosystem I; PSII – Photosystem II (Asada, 1994).

1.3 Cold Stress and Photosynthesis

Low temperature is one of the most important factors affecting plant performance and distribution, and also causes significant crop losses (Boyer, 1982). Many crops cultivated in temperate climates come from tropical and subtropical evolutionary backgrounds. These species apparently lack the genetic information to adjust to low temperatures, and provide the perfect model organisms for study of the effects of chilling on plants (Allen and Ort, 2001). Chilling refers to non-freezing temperatures of 0-12ºC. Occasional short chilling episodes in an area that generally has a mild, constant temperature is typical of what occurs in many temperate regions where thermophilic crops are grown. Reports on the effects of such a short chill confirm the disruption of essentially all major components of photosynthesis, including thylakoid electron transport, the carbon reduction cycle, and control of stomatal conductance (Kingston-Smith *et al.*, 1997; Ribas-Carbo *et al.*, 2000; Wilkinson *et al.,* 2001). One of the most important challenges to research in this field is identifying the primary effects within this complex and highly regulated system that are the actual reasons for *in vivo* dysfunction.

A number of reports state that PSI has a greater sensitivity to chilling damage than photosystem II (PSII) (Kingston-Smith *et al.*, 1999). Evidence that PSI is the primary target of chilling has not been conclusive, since in research investigating PSI response to cold stress downstream chill-susceptible processes were not studied (Terashima *et al.*, 1998). These processes (e.g. carbon metabolism and stomatal conductance) could be the primary target, with PSI and/or PSII activities a secondary effect. The most extreme sub-freezing temperature causes ice formation in plant structures, leading to widespread cellular damage. Ice crystallises in extracellular compartments, causing water loss from cells by osmosis, and eventually leading to dehydration (Shinozaki and Yamaguchi-Shinozaki, 2000).

When chilling is accompanied by light, chronic photoinhibition of PSII can be the result (Martin *et al.*, 1981; Melis, 1999). This is thought to be the result of extra excitation energy being absorbed into a system that has slowed down, which increases the potential for oxidative damage to PSII. Low temperatures also interfere with the normal replacement rate of the protein D1 of PSII in the turnover-repair cycle. Low temperature reduces membrane fluidity and this might reduce the rate of D1 protein turnover by slowing the diffusion of photo-damaged D1 proteins destined for degradation to regions of the thylakoid (Moon *et al.*, 1995). It has been speculated that the change in membrane fluidity associated with low temperatures is the plant's biological "thermometer" (Örvar *et al.*, 2000), being the initial signal that controls cold-induced genes. Damage to PSII is, however, not the only factor causing inhibition of photosynthesis in thermophilic plants.

The downregulation of PSII upon chilling stress occurs very rapidly, and is reversible. It has a photo-protective role in leaves, ensuring that any excess light energy absorbed does not damage the photosynthetic machinery, but is given off as heat (Kingston-Smith, 1997). However, in warm climate plants, such as tomato, dynamic photoinhibition does not seem to be the primary cause of the reduction in photosynthesis following a chill (Martin *et al.*, 1981). Analysis of the relative rate of PSII electron transport with the relative rate of CO_2 assimilation in grapevine leaves seemed to imply that chilling leads to an

increase in alternative electron sinks (Flexas, 1999). Oxygen can be used as a terminal electron acceptor, which could protect plants from photo-damage in bright light. This would unfortunately lead to the formation of additional reactive oxygen species. In order to prevent oxidative damage to essential proteins and lipids, the antioxidant system of the plant would have to be activated. Antioxidants would then scavenge for ROS, and act as important electron sinks to prevent oxidative damage. In maize, antioxidant enzymes become more active when grown under cool conditions in the field (Fryer *et al.*, 1998) but decline following a short chill under controlled conditions (Jahnke *et al.*, 1991). Antioxidants therefore don't seem to be regenerated, and might cause the observed inhibition of photosynthesis. In addition to the direct effects of this oxidative potential, the oxidative stress also affects the redox state of the stroma, which interferes with the normal activation of several enzymes involved in CO_2 assimilation.

It has also been reported that low temperatures affect carbohydrate metabolism to a much greater extent than other components of photosynthesis, for example in the studies done by Paul and co-workers (1992), and Jones and co-workers (1998) (Figure 1.3). The accumulation of soluble carbohydrates might lead to end-product inhibition of photosynthesis. Photosynthesis might also be limited by the inability of a chilled plant to regenerate RuBP. Two stromsl bisphosphatases that play an important role in RuBP regeneration is seduheptulose 1,7-bisphosphatase (SBPase) and chloroplast fructose 1,6-bisphosphatase (FBPase). These enzymes are activated by the ferredoxin-thioredoxin system and their activity is tightly coupled to the redox state of the chloroplast. In tomato, the primary restriction on photosynthesis is caused by a decrease in activity of both these enzymes as a result of impairment in their reductive activation function (Sassenrath *et al.*, 1990).

Declines in photosynthesis after a chill, both under light and dark conditions, have been attributed to a loss in Rubisco activity. It has been suggested that chilling damages Rubisco itself (Kingston-Smith *et al.*, 1997), or that Rubisco activation is disrupted by the chill (Allen *et al.*, 2000). During chilling

temperatures, both the air around the leaf and the leaf itself also cool down. Therefore, under this condition plants need not transpire so much to cool down and they close the stomata (Guye and Wilson, 1987). It might be that this stomatal closure during chilling temperatures decreases the amount of CO_2 that enters the leaf, and therefore decreases the rate of photosynthesis.

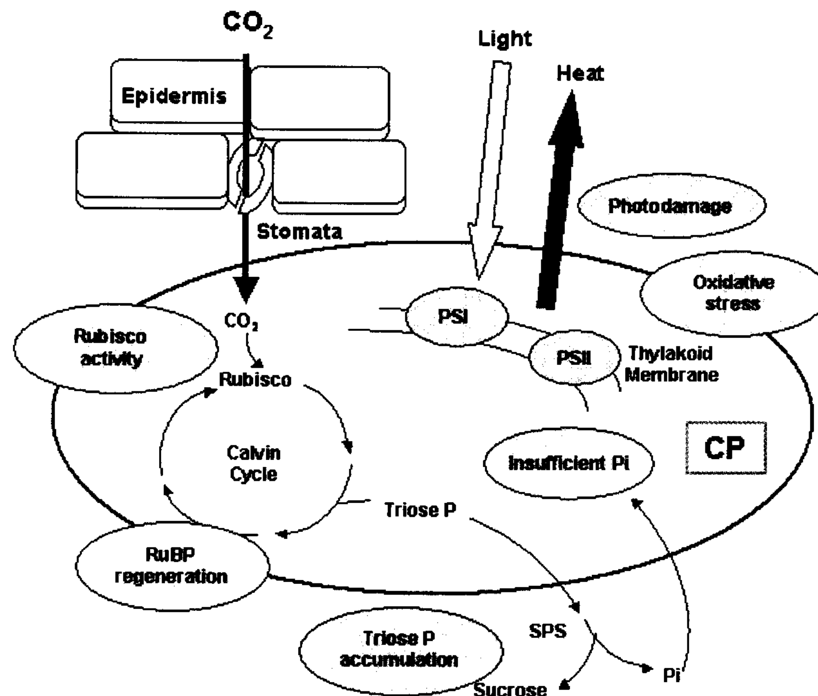

Fig. 1.3. The effect of chilling on various elements of the photosynthetic machinery. By stomata closure less carbon dioxide (CO_2) enters the plant cell and Rubisco activity can be negatively affected. RuBP regeneration may be hampered due to changes in the redox state of the plant. Triose accumulation leads to end-product inhibition. Insufficient inorganic orthophosphate limits photosynthesis. Oxidative stress caused by low temperatures may damage the photosystems (PSI and PSII). Excess light accompanied by cold temperatures causes photo-damage. SPS - Sucrose phosphate synthase. CP – Chloroplast. (Karpinski *et al.*, 2002)

Many plants increase their freezing tolerance upon exposure to low non-freezing temperatures, a phenomenon known as cold acclimation (Xin and Browse, 2000). The trigger for acclimation is exposure to a low temperature.

This means that plants that would be killed by freezing can be "hardened-off" when kept at low temperatures for a while, and then placed at freezing temperatures. This is in contrast with brief chilling spells, such as those that might occur at night, interspersed with warmer temperatures during the day. Guy and co-workers (1985) established that changes in gene expression occur with cold acclimation. Down-regulation of metabolism, as well as synthesis of new proteins and cryo-protectants occurs as a response to the cold (Paul *et al.* 1992). A large number of genes induced during cold acclimation encode proteins with known enzyme activities that potentially contribute to freezing tolerance. In a study done by Kingston-Smith and co-workers (1999) to investigate photosynthetic acclimation in maize, plants were grown at 14, 18, and 20°C until the fourth leaf had emerged. Growth rate and chlorophyll content were much lower in plants grown at temperatures below 20°C. Total foliar Rubisco content was decreased by about 50% at 18°C and by 70% at 14°C. Conversely, the activation state of Rubisco was increased in plants grown at 14 and 18°C relative to those grown at 20°C. There was an increase in the abundance of Rubisco breakdown products in plants grown at 14°C, which might reflect an increase in proteolysis. Savitch and co-workers (2001) have also shown that short-term cold stress inhibits light-saturated rates of CO_2 assimilation and O_2 evolution by approximately 75% in *Arabidopsis thaliana.* Long-term cold acclimation resulted in incomplete recovery of photosynthetic capacity, associated with an increased reduction of the chloroplast stroma. A study conducted by Komatsu and Kato (1997), indicated a possible increase in proteinase activity in response to cold stress, which accompanied the degradation of the Rubisco LSU. In a similar study performed by Byrd and co-workers (1995) it was shown that photosynthesis rate, Rubisco activation state, and ribulose -1,5- bisphosphate (RuBP) concentration were all reduced after exposing tomato plants to light at 4°C for 6 hours. Experiments were performed in the light, since it has now been demonstrated that stimulation of Rubisco activation is light-dependent. It has been widely reported that Rubisco activity is impaired during chilling in the light, in short-term (Sassenrath *et al.,* 1990) and long-term (Brüggeman *et al.*, 1992) experiments. The inhibition of photosynthesis after exposure to low temperatures may be partially due to the inhibition of sucrose synthesis, which

leads to the accumulation of phosphorylated intermediates causing Pi (inorganic orthophosphate)-limitation of photosynthesis. During acclimation, this process is reversed, and photosynthesis recovers to better levels. It is also known that transcript levels of the SSU of Rubisco decreases after transfer of plants to low temperatures (Strand *et al.*, 1997), which would mean that the level of photosynthesis is limited by the decrease in SSUs, which concurrently decreases the amount of holo-enzyme able to form.

1.4 Protein Degradation and Stress

The life span of most cellular proteins is significantly shorter than the life span of the organism. It follows, therefore, that most proteins are degraded by cellular proteinases of one sort or another. Some proteins are degraded when they become damaged. Other proteins are degraded when their constituents, carbon and nitrogen, are required to support the life of the organism (Vierstra, 1993 and 1996). Still others are degraded in response to specific environmental or cellular signals (Ellis *et al.*, 1991; Callis, 1995). In each case, proteolysis is a specific and highly regulated process. A great diversity of cellular processes depends upon regulated protein degradation, including photoinhibition in the chloroplast (Aro *et al.*, 1990; Mano, 2002), programmed cell death (Huffaker, 1990; Solomon *et al.*, 1999), and photo-morphogenesis in the developing seedling (Staswick, 1994).

In chloroplasts, the degradation of proteins has been an important area of research, especially since this is the location of the photosynthetic apparatus (Adam, 2000). All of the chloroplast proteinases described to date are related to bacterial enzymes (Estelle, 2001). Degradation of various photo-synthetically important enzymes has been investigated. For example, when plants are exposed to intense light, reactive oxygen species are formed that cause irreversible damage to the D1 protein, thus arresting electron transport. This is called photoinhibition. To recover from photoinhibition, the D1 protein must be removed from the reaction center and degraded (Aro *et al.*, 1990; Lindahl *et al.*, 2000).

Programmed cell death (PCD) is a physiological process that affects single cells or small groups of cells during plant development or under pathological conditions. The destruction of old cells is necessary during reproductive and vegetative stages of development such as sex determination, gamete development, embryogenesis, formation of fluid conducting channels called vessels and tracheids, leaf abscission, and during the hypersensitive response (HR) to pathogen infection (Jones and Dangl, 1996). Cell death during the HR could prevent the spread of infection by removing the source of nutrition (the plant cells) from the pathogen.

PCD is characterised by chromatin aggregation (eventually leading to chromatin fragmentation), cytoplasmic and nuclear condensation, and the partitioning of cytoplasm and nucleus into membrane-bound vesicles (called apoptotic bodies), as well as membrane blebbing (Martins and Earnshaw, 1997). There are three distinct phases in PCD, namely induction, effector phase, and degradation (Greenberg, 1996). During the induction phase, plant cells receive the signal that triggers PCD. This might include the binding of certain molecules generated by the HR, heat shock, UV, or oxidative stress. The effector phase is mostly a regulatory phase which sends the cells that have received any of a number of diverse signals down the path of PCD. Degradation then takes place in the nucleus and the other compartments of the cell due to the action of degradative enzymes, including proteinases and nucleases (Jones and Dangl, 1996).

Chloroplasts contain up to 50% of the total cellular protein, and therefore provide most of the substrate during cellular proteolysis. The matter of chloroplastic protein degradation is an issue of contention, with a number of theories existing as to how it actually occurs. One theory speculates that chloroplast proteins are degraded by vacuolar proteinases (Wittenbach *et al.*, 1982; Moriyasu, 1995). Later on it was suspected that the ubiquitin pathway may also be involved (Vierstra, 1996), and more recently it has been found that chloroplasts have a variety of internal proteinases, some of which require ATP (Shanklin *et al.*, 1995). Besides these proteinases, the chloroplast has a number of neutral proteinases (Liu and Jagendorf, 1986), a prolyl

endopeptidase (Kuwabara, 1992), a stroma-located metalloproteinase EP1 (possibly involved in Rubisco degradation) (Bushnell *et al.*, 1993), and two proteinases required for the removal of transit peptides (Oblong and Lamppa, 1992). None of the proteinases that have been cloned seem to be encoded by the chloroplast genome. This would mean that proteinases are imported from the cytosol into the chloroplast, degradation of proteins occurs inside the chloroplast, and the degradation products are then exported to the cytosol.

1.5 Rubisco Degradation

The biggest source of amino acids in growing plants is the degradation products of Rubisco located in the chloroplast. Rubisco present in plant leaves can count for 40-60% of the total soluble protein and the enzyme is possibly the most important and most abundant protein on earth, catalysing the first step of the Calvin cycle. It ultimately fixes atmospheric carbon dioxide into a form accessible for use by animals. Rubisco itself is a hexa-decameric enzyme consisting of 8 large subunits (LSU) and 8 small subunits (SSU) (Schneider *et al.*, 1992). In higher plant Rubisco, the LSUs (55kDa) are arranged in an octameric core, and the SSUs (about 14kDa) occur in layers of four on opposite sides of the molecule (Chapman *et al.*, 1988). It seems as if the SSU stabilizes the LSU_8 core, since it does not contribute directly to the structure of the active site, which is located on the LSU (Chapman *et al.*, 1988). The LSUs are encoded by a single gene in the chloroplast genome, under the control of a strong promoter, which is light-inducible. The SSUs are encoded by a family of nuclear *rbcS* genes, synthesized in a precursor form on cytosolic ribosomes, and imported into the chloroplast stroma where the N-terminal transit sequence is cleaved to yield the mature polypeptide. The expression of these genes is regulated by the presence or absence of light. Rubisco is synthesised predominantly during leaf expansion or during the greening of etiolated leaf tissue (Kleinkopf *et al.*, 1970), after which the cellular concentration remains nearly constant for several days with little or no turnover.

The degradation of total protein during senescence (which is a form of PCD) happens very rapidly, with Rubisco being one of the main substrates of proteolysis (Friedrich and Huffaker, 1980). In general, proteolysis is an important function in the chloroplast to regulate the amount of Rubisco, especially to correct the quantity of subunits when they're not available in the same amounts. It has been determined that unassembled SSU is rapidly degraded upon import into the chloroplast, when there is no LSU available to bind with (Schmidt and Mishkind, 1983). Rubisco degradation plays an important regulatory role in at least two physiological processes:

- During foliar senescence, when nutrients are redistributed through the plant from the leaves to the reproductive structures. Since Rubisco is the most abundant protein in leaves, it could be considered the greatest source of such nutrients.

- Environmental stress factors can cause reversible and irreversible inactivation of Rubisco. Irreversibly inactivated Rubisco has to be degraded and replaced by newly synthesised copies, in order to have full recovery of the photosynthetic system.

These two processes share a common result, namely the development of oxidative processes caused by ROS. In chloroplasts, oxidative stress causes inhibition of the enzymes of the Calvin cycle, and modifications of Rubisco structure, amongst other things. It has been shown that the oxidized form of Rubisco is more sensitive than the reduced form to general proteinases, such as papain and trypsin (Peñarrubia and Moreno, 1990) and that oxidative stress induces partial degradation of the LSU (Desimone *et al.*, 1996). The oxidised form of Rubisco is irreversibly inactivated, which therefore necessitates breakdown.

1.6 Proteinases and Stress

Proteinases are enzymes that break down proteins. They are present in the digestive system of many plant pests and catalyse the hydrolysis of various

polypeptide substrates, such as plant proteins, being most active under reducing and mildly acidic conditions. Proteolysis is an essential metabolic process required for protein processing and turnover in plants. During germination, proteinases catalyse the degradation of storage proteins in order to provide nitrogen for assimilation into biosynthetic pathways (Toyooka, *et al.*, 2000). It has also been implicated that these enzymes play a role during developmental processes, such as PCD (of which senescence is a sub-category), as well as being important components in the interaction between plants and other organisms. In general, protein degradation functions as a means of cellular housekeeping and ensures the correct cellular concentration of enzymes. Protein degradation is also necessary for the removal of signal or targeting peptides, is responsible for the generation of peptides that act as hormones, and has a role in homeostasis (Vierstra, 1996).

The role of vacuoles in protein degradation has been a matter of intense investigation. Originally it was hypothesised that vacuoles might act like animal lysosomes, being mostly responsible for the degradation of cellular proteins, including cytosolic and chloroplastic proteins proposed to enter the vacuole by autophagy (Wittenbach *et al.*, 1982; Matile *et al.*, 1988). Subsequent identification of plant proteolytic pathways outside of the vacuole (Callis, 1995) and the ability of plants to degrade intracellular proteins even when most vacuolar proteinase activities are inhibited (Moriyasu, 1995) suggest that vacuolar proteinases have little, if any role to play in total protein breakdown. However, the method by which vacuoles may be involved in protein breakdown might be more complex than simple autophagy. Recent data indicates that plants contain two types of vacuoles: one with an acidic pH (like the animal lysosome), and another type called protein bodies, which is a specialised type of vacuole responsible for the storage and mobilisation of protein reserves during seed germination (Paris *et al.*, 1996). Some of the storage protein-degrading proteinases are related to the cathepsin class of cysteine proteinases, found in mammalian lysosomes (Bethke *et al.*, 1996). This type of storage and mobilisation is not restricted to seeds, but is also evident in leaves, seedpods, and seedling hypocotyls. Here storage proteins are synthesised and sequestered in vacuoles during periods of high nitrogen

availability, and subsequently degraded when nitrogen becomes limited, when the plant tissue becomes senescent, or when stored amino acids are needed by sink tissues (Staswick, 1994). Other functions of the vacuolar proteinases might include plant defence mechanisms against pathogens, parasites, and herbivores, where the proteinases may attack the invader once the cell is lysed. Vacuolar proteinases might also act during the last stages of senescence by degrading any remaining cytoplasmic and organellar substrates after rupture of the tonoplast. Vacuolar proteinases might lastly also assist in supplying free amino acids during times of rapid growth, starvation, or stress (Staswick, 1994). The targets for these proteinases could be storage proteins, but also other vacuolar or cytosolic proteins. If cytosolic proteins are involved, stress-enhanced vacuolar degradation would require the active transport of proteins into the organelle. Such a system has not yet been demonstrated in plants, but has been described in animal cells (Dice, 1987).

In recent years, one of the best-studied examples of cellular regulation by proteinases is that occurring during apoptosis in animal cells. Research on this phenomenon in animal cells lead to the identification of the caspases, which contain a cysteine in their active site, and cleave at specific aspartic acid residues (Grutter, 2000). Plants exhibit a process similar to apoptosis, namely PCD. PCD in plant cells occurs most notably during the hypersensitive response to pathogen attack, tracheary-element differentiation, and senescence (Lam *et al.*, 1999). Cysteine proteinases are the key enzymes regulating apoptosis in animal cells (Martin and Green, 1995; Solomon *et al.*, 1999; Xu and Chye, 1999), and therefore it is speculated that these proteinases, as well as their inhibitors (cystatins), would be the regulating factors in plant PCD. In soybean, it has been determined that PCD-activating oxidative stress induces a set of cysteine proteinases. Expression of an ectopic cystatin in these plants prevented PCD (Solomon *et al.*, 1999). Research to date has further shown that cysteine proteinases are expressed mainly in young and senescent leaves and flowers (Buchanan-Wollastan *et al.*, 1997; Guerrero *et al.*, 1998; Xu and Chye, 1999) and accumulate in

response to oxidative stress which has been shown to be caused by exposure to low temperatures (Schaffer and Fischer, 1988).

1.7 Proteinase Inhibitors and Stress

Throughout the animal and plant kingdom, proteinases are controlled by peptidal proteinase inhibitors, in order to regulate proteolytic activity, as well as protecting tissues from degradation of unwanted or foreign proteolytic activities. In plants, these inhibitors are encoded by small gene families that are expressed either developmentally, or in response to general environmental stresses and insect or pathogen attack (Table 1.1). The serine and cysteine proteinase inhibitors (cystatins) have been studied more intensely than metallo- and aspartyl proteinase inhibitors, since the latter two families have only rarely been found in plants (Ryan, 1990).

Table 1.1. Proteinase inhibitor families in plant tissues.

1.	Soybean trypsin inhibitor family
2.	Bowman-Birk inhibitor family
3.	Barley trypsin inhibitor
4.	Potato Inhibitor I family
5.	Potato Inhibitor II family
6.	Squash Inhibitor family
7.	Ragi I-2/ Maize bifunctional inhibitor family
8.	Carboxypeptidase A, B inhibitor family
9.	Cysteine proteinase inhibitor family (cystatins)
10.	Aspartyl proteinase inhibitor family

Cystatins are one class of proteinase inhibitor (Brown and Dziegielewska, 1997) that bind tightly and reversibly to the group of papain-like cysteine proteinases. This group includes several animal catheptic enzymes and a number of plant enzymes, including bromelain, ficin, actinidin, and papain (Turk and Bode, 1991). Cystatins have an important role in plant development, especially where seed development (Abe *et al.,* 1987; Abe *et*

al., 1992), maturation, and plant defence is concerned. They are involved in regulation of protein turnover during these stages, and also seem to play a role in plant stress responses.

In plants, several cystatins have been characterized. The first research into plant cystatins was undertaken by Abe and co-workers (1985 and 1987) using rice. The rice cystatin isolated, oryzacystatin I (OCI), has endogenous target enzymes (oryzains) and is assumed to play a defensive role in maturing and mature seeds, protecting seeds from herbivorous insects or some other invaders (Kondo *et al.,* 1989). After the initial discovery of OCI, a second cystatin (Oryzacystatin II) was discovered. Both cystatins contain a conserved central pentapeptide motif Gln-X-Val-X-Gly, which is believed to be the target enzyme-binding site, and is similar to the conserved motif in animal cystatins (Abe *et al.,* 1991). Meanwhile, cystatins have been identified in many other plants, such as corn cystatin I and II (Abe *et al.*, 1992), soyacystatin (Misaka *et al.*, 1996), and cystatins from potato (Waldron *et al.*, 1993), ragweed (Rogers *et al.*, 1993), cowpea (Fernandes *et al.,* 1993), avocado (Kimura *et al.,* 1995), and papaya (Song *et al.*, 1995). Not all of these cystatins are completely homologous in expression, as corn and wheat cystatins are synthesised as pre-proteins, whereas soyacystatin is characterised by a very large N-terminal extension. Recently it has been shown that expression of the *OCI* gene in a transformed plant improved resistance against different plant pests (Table 1.2), including insects and nematodes (Michaud *et al.*, 1993; Leplè *et al.*, 1995; Michaud *et al.*, 1995; Urwin *et al.*, 1995). The OCI protein has certain useful qualities, such as being very heat-stable, even under cooking conditions (Abe *et al.*, 1987).

Table 1.2 Plants that have been transformed to express proteinase inhibitors (drawn from Michaud and Vrain, 1998).

Plant	Inhibitor	Class
Rapeseed	Oryzacystatin I	Cysteine
Poplar	Oryzacystatin I	Cysteine
Potato	Oryzacystatin I	Cysteine
Tobacco	Cowpea trypsin inhibitor	Serine
	Tomato Proteinase inhibitor I	Serine
	Oryzacystatin I	Cysteine
Tomato	Oryzacystatin I	Cysteine

Other recent studies have 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 over-expression (Jacinto *et al.*, 1997). All of these observations support the hypothesis that cystatins play a crucial and central role in general plant defence mechanisms. However, a possible interaction between an exogenous cystatin and endogenous cysteine proteinases has only been studied in few cases (Michaud *et al.*, 1995). There is a need for a continuous investigation into possible additional benefits (or disadvantages) of expression of an exogenous cystatin, specifically for abiotic stress tolerance. Plant cysteine proteinases have acidic pH optima *in vitro*, suggesting that they are localised to the vacuole *in vivo* (Callis, 1995). So far no significant cysteine proteinase activity has been measured in other cell compartments and also no detailed information is available about the cellular localisation of cystatins.

1.8 Cystatins and Metabolic Change

As technology develops, humans have a much greater capacity for impacting and changing their environment. This includes the molecular manipulation of plants aimed at increasing crop yield, and giving plants a greater resistance to pests and pathogens. The more we influence and change our environment, the greater our responsibility to investigate the way the environment has been

impacted, whether it be positive or negative. In this capacity, plant physiologists have begun to characterise a new generation of developed plants that might be genetically enhanced by foreign gene transfer to have certain additional character traits. Research into the effect of such genetic modification on a plant's normal metabolic state is therefore of utmost importance, to ensure healthy, nutritious and safe crops, as well as securing the continuing existence of diverse plant species.

In recent years a lot of work has been done in understanding metabolic changes in plants due to abiotic and biotic stress (Aro *et al.*, 1990; Bohnert and Jensen, 1996; Holmström *et al.*, 1996; Smirnoff, 1998; Grover *et al.*, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2001). In particular, the effect of abiotic stress on plants has been under intensive investigation due to its importance for crop production (Guy *et al.*, 1985; Holmström *et al.*, 1996; Grover *et al.*, 1999; Karpinski *et al.*, 2002). The impact of extreme or unstable environments on plants can be severe and leads to great losses in crop yield, as well as a limitation in the amount of available arable land (Smirnoff, 1998). Research aimed at improvement of plant resistance to abiotic stress will ultimately mean better yields and a potentially larger area of land available for crop production.

In the last 15 years specific focus has been placed on genetically modified (GM) plants aimed at producing hardier and more resistant or tolerant crops. Introduction of foreign genes (called transgenes) that give plants a greater resistance or tolerance to biotic pests, such as insects, has been done successfully (Ryan, 1990; Michaud *et al.*, 1993; Urwin *et al.*, 1995; Irie *et al.*, 1996). Furthermore, the identification and isolation of "stress-resistance" genes has been enhanced by large-scale mutational analysis studies, as well as breeding trials. In this respect, the model organism for plant research, *Arabidopsis thaliana,* has played a major role. Whereas many researchers focus on crop improvement alone, only recently others have started to focus on the impact of such improvements on the general metabolism and physiology of the plant. In particular, this research aims to characterise GM plants on various levels, including changes in photosynthesis and metabolic

pathways. In order to fully understand the impact of genetic manipulation on the plant as a whole, such work is vitally important.

In a recent pioneering study undertaken by Van der Vyver *et al.* (2003) the effects of constitutive OCI expression on whole plant physiology was investigated, with the focus on photosynthesis, respiration, and growth characteristics. The effect of expression of OCI in transformed tobacco was studied under different environmental conditions, including low light intensity, drought, and low temperatures. The transformed plants all showed a conditional phenotype, where stem elongation was markedly decreased when grown under low light conditions. Transformed plants also had lower maximal rates of photosynthesis and a slightly lower total biomass after seven weeks of growth at low light intensities. After prolonged (12 weeks) growth at low light intensity, however, transformed plants surpassed wild type plants in shoot biomass production. OCI-expressing tobacco plants grown at low light intensities had significantly higher leaf chlorophyll and total soluble protein content than wild type plants grown under the same conditions. In comparison, when plants were grown in full sunlight, the differences between transformed and wild type tobacco was much less apparent. Another characteristic of transformed plants was their better recovery of photosynthesis after chilling, compared to wild type plants. When grown at 20°C, however, photosynthesis was lower in OCI-expressing tobacco. The higher the level of OCI expression, the greater the inhibition of CO_2 assimilation rate. The apparent quantum efficiencies of photosynthesis were similar in all lines, which meant that even though the absolute amount of photosynthetic machinery is decreased in transformed tobacco, there is no photoinhibition. Photosynthetic CO_2 assimilation was decreased in all plants upon exposure to 5°C, but transformed plants showed less inhibition than wild type plants. Transformed and wild type plants recovered equally well after two days recovery at 20°C.

The differences in protein content between transformed and wild type tobacco plants, as well as the other observations made by Van der Vyver *et al.* (2003) suggests that an exogenous cystatin influences protein turnover rates in the

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cytosol by interacting with endogenous cysteine proteinases. It also seems to protect the photosynthetic machinery from cold-induced damage. The questions arising from these results are: how would a cytosolically expressed inhibitor inhibit a proteinase suspected to be resident in the vacuole, and how could this same inhibitor protect the photosynthetic machinery resident in the chloroplast (Figure 1.4)?

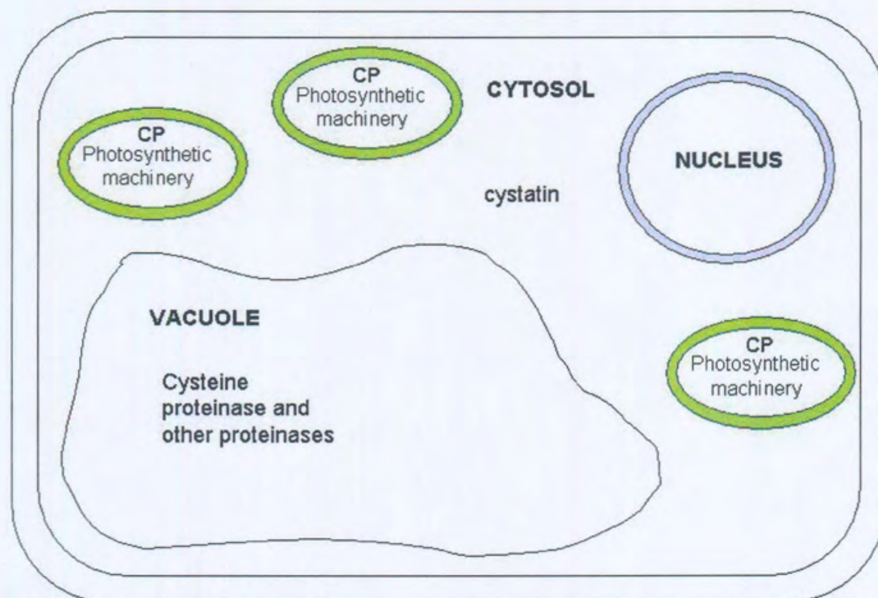

Fig. 1.4 Cellular location of various cellular components studied by Van der Vyver *et al.* (2003). A cytosolically expressed cysteine proteinase inhibitor (cystatin) seemingly protects photosynthetic machinery from degradation by cysteine proteinases under cold stress. CP – Chloroplast.

CHAPTER 2

2. EXPERIMENTAL PROCEDURES

2.1 Materials and Methods

2.1.1 Plant material and chemicals

Non-transformed tobacco seeds (*Nicotiana tabacum* L. cv. Samsun) as well as transformed tobacco seeds carrying the oryzacystatin I (OCI) gene from rice were obtained from Prof. Karl Kunert at the Forestry and Agricultural Biotechnology Institute, University of Pretoria. The transformed seedlings contain the gene for OCI under the control of a double 35S promoter from cauliflower mosaic virus, an Ω leader sequence for gene expression enhancement, the *nptII* gene under control of a 35S promoter for kanamycin resistance, and an intron-containing *gus* gene encoding β-glucuronidase under the control of a 35S promoter. Kanamycin resistance was used as a selectable marker, and GUS expression was used as a specific and easily detectable reporter for plant transformation.

Chemicals used were purchased either from Sigma (St. Louis, Mo), Boehringer Mannheim (Germany), Life Technologies (Scotland) or BioRad (Hercules, California). All products used were of analytical or molecular biology grade.

2.1.2 Aseptic techniques and apparatus

All possible preventative measures were taken to work aseptically and all experiments were carried out in a laminar flow cabinet where necessary. Ethanol (70%) was used for flaming and cleaning of utensils and all solutions were sterilised by autoclaving for 20 min at 120°C.

2.2 Plant Protein Analysis

2.2.1 Protein extraction

Tobacco leaf material was ground in extraction buffer to which a small amount of sea sand was added. The extraction buffer consisted of 50mM Tris-HCl (pH 8.9) (buffer A) or 50mM NaAc (pH 5.4) (buffer B) containing 10mM β-mercaptoethanol (Yoshida and Minamikawa, 1996). Buffer A, which has a basic pH, was used in experiments where plant protein degradation was not desired in the extraction buffer itself. Buffer B, which has an acidic pH, was specifically used to study plant protein degradation in an acidic extraction buffer.

2.2.2 Protein determination

The protein concentration of extracts was determined according to the method described by Bradford (1976). Bradford colour reagent (200µL) was diluted with 800µL water. Crude plant extract (2µL) was added to this reaction mixture, and left at room temperature for 20 min. The absorbancy of the reaction mixture was determined on a spectrophotometer at a wavelength of 595 nm. The protein concentration was determined by comparing absorbancy values to those on a standard protein curve. The standard curve was drawn by using known quantities of bovine serum albumin, and measuring its absorbancy at 595nm.

2.2.3 Protein degradation

Total soluble protein (30µg) from either transformed or non-transformed tobacco leaves was incubated in 50mM NaAc extraction buffer at 37°C for 0 - 4 hours. This procedure was done in duplicate, with 0.5nmol E64 (a synthetic inhibitor of cysteine proteinases) added to the duplicate samples.

In order to test the type of extraction buffer best suited to studying protein degradation in plants, parallel extractions of plant proteins were done in either 50mM Tris-HCl or 50mM NaAc extraction buffer, and 30μg of total soluble protein was immediately loaded on a native 6% polyacrylamide (PA) gel. The ratio of bisacrylamide:acrylamide in the 30% stock solution was 1:29 (w/w). The amount of Rubisco on these native gels was then quantified using the native gel quantification method.

2.2.4 Rubisco quantification

a) *Labelling of Rubisco with radioactive inhibitor (^{14}CABP)*

Leaf material (200mg) was ground in a chilled mortar and pestle, over ice. Extraction buffer consisted of 50mM bicine, 20mM $MgCl_2$, 1mM EDTA, and 50mM β-mercaptoethanol (pH 8.2). A protease inhibitor cocktail (Sigma) was added to the extraction buffer just before grinding, at a concentration of 1% (v/v). Crude extract was then centrifuged at 14,000rpm for 2 min at 4°C. Supernatant was transferred to clean microcentrifuge tubes and kept on ice. Extract (200μL) was added to 200μL activation buffer. Activation buffer consisted of 100mM bicine, 20mM $MgCl_2$, 10mM $NaHCO_3$, 0.6M Na_2SO_4, ^{14}CABP, and 50mM β-mercaptoethanol (pH 8.0). This mixture was left at room temperature for 16 min after which 290μL 60% polyethylene glycol (PEG) was added, mixed well, and kept on ice. The solution was centrifuged at 12,000rpm for 10 min at 4°C. Supernatant was aspirated, and the pellet resuspended in 500μL 25% PEG in activation buffer. This wash step was repeated once. After the solution was again centrifuged at 12,000rpm for 10 min, the pellet was resuspended in 500μL 1% (v/v) Triton X-100. This solution (400μL) was pipetted into a scintillation vial to which 3.6mL scintillation cocktail was added. The radioactivity was then counted in a scintillation counter (Beckman, USA). The radioactivity level still present in the solution indicates the amount of Rubisco present, since the radioactively labelled CABP binds in a quantitative way to the enzyme (one molecule inhibitor to one active site).

b) Native polyacrylamide gel method

The amount of Rubisco holoprotein was determined using the procedure employed by Rintamäki *et al.* (1988). Proteins of crude extracts were separated in a native 6% PA gel. The gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in a water:propan-2-ol:glacial acetic acid (65:25:10, v/v/v) mixture, being gently shaken in the dark for 16 hours, at room temperature. Destaining was done first in staining solution (with no dye) for 8 hours, and then in 7% acetic acid in water. Destaining fixes the proteins in the gel, and extracts the excess stain from the gel. Bands of Rubisco holoprotein were cut out and placed in capped 2mL vials containing a 1% (w/v) aqueous SDS solution. This was done to elute the proteins, as well as the dye bound to the protein, from the gel. After a 24-hour incubation period at 4 °C, during which the dye bound to the proteins were extracted, the absorbancy of the 1% (w/v) SDS solution was determined at 585nm. The amount of dye bound to the protein is equivalent to the amount of protein present in the gel. Therefore, the amount of dye present in the elution mixture would give an indication of the relative amount of protein present in the gel. These relative values were used to compare Rubisco quantity in different plants.

c) Spot densitometric analysis of SDS-PAGE gel

The AlphaEase™ software (Alpha Innotech Ltd, UK) was used to compare the intensity of Rubisco LSU bands in SDS-PAGE gels, after proteins had been extracted from tobacco leaves, and separated by SDS-PAGE. The Spot Denso tool of the software was used to measure the density of specific bands that corresponded to Rubisco LSU in the SDS-PAGE gel. Each band specified was assigned a number and its associated numerical data was displayed in a data table. The Integrated Density Value (IDV), which is the sum of all the pixel values detected by the software after background correction, was used directly to compare protein quantity between plants.

2.2.5 SDS-PAGE gel electrophoresis

SDS-PAGE gel electrophoresis was carried out in gradient gels (5-7.5%, 5-10% or 5-12%) according to the method described by Sambrook *et al.* (1989), employing the discontinuous electrophoresis buffer system of Laemlli (1970). Generally, 30µg of total soluble protein of crude leaf extracts was separated on gels to be used in studies on Rubisco degradation, whereas 50µg were separated on gels to be used in studies on OCI expression. Samples were first incubated at 95°C for 5 min in loading buffer containing 62.5mM Tris-HCl, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.001% (w/v) bromophenol blue (pH 6.8), and then loaded onto the SDS-PAGE gel. Electrophoresis was performed at constant current and varying voltage. Samples were run at 80V through the stacking gel and 100V through the resolving gel untill the blue front of the loading dye reached the bottom of the gel.

2.2.6 Western blotting

After extraction of soluble protein from leaves, samples were run on SDS-PAGE gels. The method according to Sambrook *et al.* (1989) was used for SDS-PAGE and the method as described in the technical booklet for the ECL Plus Western Blotting system (Amersham, UK) was used for Western Blotting. After electrophoresis, the PAGE gel was equilibrated for 20 min in transfer buffer containing 25mM Tris and 192mM glycine in 15% methanol, pH 8.2. A polyvinylidene difluoride (PVDF) membrane (PVDF-Plus, Micron Separations Inc., USA) was cut to size and pre-wet in 100% methanol for 5 sec, after which the membrane was washed in distilled water for 5 min, and equilibrated in transfer buffer (500mL) for 15 min. Transfer of proteins was done in a BioRad Mini protein II transfer apparatus filled with transfer buffer (Sambrook *et al.*, 1989), at 4°C and 60V for 1 h. After transfer, the membrane was washed once for 5 min in Tris buffered saline (TBS) pH 7.6. Thereafter the membrane was incubated in a 5% fat free milk powder/TBS buffer solution containing 0.1% (v/v) Tween 20 for one hour. This blocks non-specific binding

sites. The membrane was then washed three times for 10 min each in the same milk powder/TBS/Tween solution, and once for 10 min in TBS containing 0.1% (v/v) Tween 20 only (TTBS).

The membrane was incubated in primary antibody in TTBS containing 5% (w/v) fat free milk powder at room temperature, on an orbital shaker, overnight. Primary antibodies directed against Rubisco and OCI were raised in rabbit, and obtained from Prof. A.-M. Oberholster at the Forestry and Agricultural Biotechnology Institute at the University of Pretoria. To remove all unbound antibodies, the membrane was washed four times for 10 min each in TTBS. Detection was done using the ECLPlus Western blotting detection system (Amersham Pharmacia Biotech, UK), as outlined by the supplier. This detection system detects immobilised specific antigens conjugated to horseradish peroxidase (HRP) labelled antibodies. The membrane was incubated in secondary antibody (HRP labelled goat anti-rat antibody) diluted in TTBS containing 5% (w/v) fat free milk powder for 2 hours, at a dilution of 1:5,000. Afterwards, the membrane was washed four times for 10 min each in TTBS. Detection reagent (2mL) containing a Lumigen PS-3 acridan substrate was pipetted onto the membrane, and incubated for 5 min at room temperature. The substrate reacts with the HRP, and generates acridinium ester intermediates. These intermediates react with peroxide (present in the detection reagent) under slight alkaline conditions and produce a sustained, high intensity chemiluminescence. Excess detection reagent was drained off by touching the edge of the membrane against a tissue. The membrane was wrapped in clean Saran Wrap, and placed in an X-ray film cassette. A sheet of autoradiography film (Hyperfilm ECL, Amersham Pharmacia Biotech, UK) was placed on top of the membrane in a dark room under red safe light conditions. After exposure, the film was developed, rinsed in water, and fixed.

2.2.7 Proteinase assay

A fluorometric assay was applied to compare the amount of cysteine proteinase activity between transformed and non-transformed tobacco plants. For that, the specific cysteine proteinase substrate Z-Phe-Arg-Nmec

(Novabiochem Ltd., UK) was used as described by Barret and Kirschke (1981). The substrate, Z-Phe-Arg-Nmec, which is barely fluorescent, is hydrolysed to liberate 7-amino-4-methylcoumarin, and this is quantified by its intense fluorescence after the reaction has been stopped with monochloroacetate.

Total soluble protein (50µg) from crude leaf extract was diluted with a 0.1% (v/v) Tween 20 solution to a total volume of 500µL. Proteinase reaction buffer (250µl) was added. Reaction buffer consisted of 340mM sodium acetate, 60mM acetic acid, 4mM disodium EDTA, and 8mM DTT. For temperature equilibration and activation of enzymes, the solution was placed at 30°C for 1 min. After equilibration, 250µL of a 20µM solution of the cysteine proteinase substrate Z-Phe-Arg-Nmec dissolved in dimethyl sulfoxide was added. After 10 min at 30°C, stopping reagent (100mM sodium monochloroacetate, 30mM sodium acetate, 70mM acetic acid, pH 4.3) (1mL) was added. The fluorescence of the free 7-amino-4-methylcoumarin was determined with excitation at 370nm and emission at 460nm in a Hitachi fluorometer (Hitachi Corp., Japan).

2.3 DNA Analysis

2.3.1 Plasmid purification from *Escherichia coli* (*E. coli*)

All plasmids were replicated in the *E. coli* cell line MOS*Blue* (Amersham Pharmacia Biotech, UK). Bacteria were plated out on solid medium consisting of Luria-Bertani (LB) broth to which 14g/L agar was added. Single colonies were picked out, and grown in LB broth containing 10g tryptone, 5g yeast extract, and 10g NaCl per liter, at a pH of 7.4. Cultures in liquid solution were incubated overnight at 37°C in an orbital shaker. Plasmid DNA was isolated from bacteria using the Qiagen Plasmid Purification kit (Qiagen Ltd., UK). The selection of pUC18 plasmids containing the *OCI* gene (pUC-OCI) was done on selection medium containing 50µg/mL ampicillin. The pUC18 plasmid contains an ampicillin resistance gene that allows bacteria that contain the

plasmid to multiply in medium containing the antibiotic. Furthermore, insertion of the OCI fragment into the multiple cloning site was monitored by adding 87.5µg/mL X-gal and 0.1mM IPTG to the solid growth medium. Bacterial colonies that have the OCI fragment inserted into the multiple cloning site will produce white colonies. This is because the *lac*I'OPZ' sequence has been disrupted, making it impossible for the bacteria to break down the X-gal substrate to a blue product. Bacterial cells containing the plasmid pJIT were selected on medium containing 50µg/mL ampicillin, since this plasmid allows bacterial growth on this selective medium.

2.3.2 Gel electrophoresis of DNA

DNA molecules were separated on a 1% agarose gel according to the method of Sambrook *et al.* (1986). Samples were loaded in a loading buffer containing 50% (v/v) glycerol, 1xTAE buffer and 1% (w/v) bromophenol blue. TAE buffer (1x) was also used to fill up electrophoresis cells. Agarose gels were run at 100V and 80mA to separate the DNA.

2.3.3 Polymerase chain reaction (PCR)

The PCR reaction mixture was made up as stated in annex 1, and contained reaction buffer, deoxyribonucleotides, primers, and polymerase. Primer annealing occurred as outlined in the text (Chapter 6). Primers were designed from the OCI sequence (Abe *et al.*, 1987) and commercially synthesised (Roche, Switzerland). The forward primer was OCI-LI-KK: 5'–TCA CCG AGC ACA ACA AGA AG-3' and the reverse primer was OCI-RI-KK: 5'–CAT CGA CAG GCT TGA ACT CC-3'. The melting temperature for primer OCI-LI-KK was 57.3°C, and for primer OCI-RI-KK it was 59.4°C.Temperature cycles were as specified in table 2.1

Table 2.1. PCR cycle temperatures and number of cycles.

Temperature	Duration	Number of Cycles	Purpose
94°C	5 min	1 (First cycle)	Complete denaturation of dsDNA to yield single stranded templates
94°C	1 min	42	Denaturation of dsDNA
60°C	1 min		Annealing of primers to ssDNA
72°C	2 min		Polymerase extends primer by incorporating dNTPs into growing strand
72°C	5 min	1 (Last cycle)	Final elongation of dsDNA products

2.6 Physiological Study

2.4.1 Cold treatment

Plants were grown on Murashige and Skoog (MS) (Murashige and Skoog, 1962) growth medium in closed tissue culture flasks, in a growth room at 25°C. When plants reached 2-3 cm in height, they were placed in a 10°C growth chamber (Labcon, Labex, Orange Grove) with enclosed light source (Gro-Lux, Sylvania, UK) (6x8W bulbs), for 10 days under constant illumination. Plant material from these plants grown on MS medium was used to quantify Rubisco using the PA native gel method. These experiments were done at the University of Pretoria.

Plants were also grown in potting soil, under normal environmental conditions in a greenhouse at 20°C (at Rothamsted, UK). Mature plants were used for *in vivo* experiments. Cold treatment of plants grown in soil consisted of a 2-day chill of the whole plant at 6°C in a growth cabinet both in the light and in the dark, followed by a two-day recovery period at 20°C. A 16-h photoperiod and light intensity of 1000 $\mu mol.m^{-2}.s^{-1}$ was used. Samples were taken from plants before cold treatment, after cold treatment, and after recovery. Plant material from these plants grown in soil was used to quantify Rubisco using the radioactive labelling method as well as the spot densitometry method.

2.4.2 Senescence

Plants grown on MS medium for 3–4 weeks were used for senescence studies. Transformed plants were first screened using the GUS histochemical assay, to test for expression of the GUS gene. Leaves of about the same size from transformed (T) and non-transformed (NT) seedlings were detached with a scalpel and placed in petri dishes on filter paper. Sterile distilled water (3-4mL) was pipetted onto filter paper. Leaves were left in constant light (Gro-Lux, Sylvania, UK) in a growth chamber (Labcon, Labex, Orange Grove) and at a temperature of 20°C. Proteins were extracted after 5-12 days, and Rubisco quantities were compared.

2.5 Plant Transformation and Selection

2.5.1 Plant growth

Seeds were germinated on half-strength MS medium (Highveld Biological LTD, South Africa), containing 2% sucrose and 8g/L agar (pH 5.8). Seedlings were grown in tissue culture flasks in a growth room at 20°C. Seeds were also germinated in soil medium, and grown under environmental conditions at 20°C in a greenhouse.

2.5.2 Plant selection

a) Antibiotic selection

Transformed tobacco seedlings were screened by growing them on selective medium containing 100mg/mL kanamycin. Tobacco seedlings possessing the kanamycin resistance gene would remain green when grown on selective medium, while seedlings that don't possess the resistance gene would be chlorotic and eventually turn white.

b) GUS assay

Mature tobacco plants were screened using the GUS histochemical assay. Plant tissue was incubated at 37°C overnight in GUS-staining solution (1mg/mL 5-bromo-4-chloro-3-indolyl glucoronide in 50mM NaH_2PO_4 buffer, pH 7 containing 0.01% (v/v) Tween 80 and 10mM Na_2EDTA). Transformed plants posess the β-glucuronidase coding sequence (*gus* gene), which produces the hydrolase GUS. This enzyme catalyses the cleavage of 5-bromo-4-chloro-3-indolyl glucoronide, leading to a blue precipitate.

2.5.3 *Agrobacterium* infection

The *Agrobacterium* binary vector system was used to transform tobacco plant material. Plasmid pBIN19 was used as a cloning vector. This vector is capable of replicating in both *E. coli* and *Agrobacterium*, and carries unique cloning sites and plant selectable markers between its disarmed T-DNA borders. This plasmid was altered as outlined in Chapter 7 to include the OCI coding sequence, a Rubisco SSU transit sequence, and a poly-A tail. This new construct, pBIN-TP-OCI, was used to transform Samsun tobacco plants. The *Agrobacterium* strain used in conjunction with the vector plasmid was LBA4404. This strain is resistant to rifampicin (Hoekema *et al.*, 1983). The plasmid pRK2013 was used to provide *in trans* the *vir* functions needed for the transfer of T-DNA into plants. Plasmid pBIN-TP-OCI was propagated in MOS*Blue E. coli* cells, and grown in LB broth containing 50µg/mL kanamycin overnight at 37°C. The *Agrobacterium* strain LBA4404 was grown for 2 days at 25°C in LB-broth containing 50µg/mL rifampicin. The *E. coli* helper strain pRK2013 was grown in LB broth containing 50µg/mL kanamycin overnight at 37°C. Three mixtures were then prepared and 0.3mL of each mixture was grown on solid LB medium containing no antibiotics at 25C° overnight (Table 2.2).

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Table 2.2. Composition of different solutions prepared for transformation of tobacco plants, as well as their respective antibiotic resistances.

Solution	S	K1	K2
Components	1mL *Agrobacterium* LBA4404	1mL *Agrobacterium* LBA4404	1mL *Agrobacterium* LBA4404
	0.5mL *E. coli* (pRK2013)	0.5mL *E. coli* (pRK2013)	-
	0.5mL *E. coli* (pBIN-TP-OCI)	-	0.5mL *E. coli* (pBIN-TP-OCI)
Antibiotic resistance	Rifampicin	Rifampicin	Rifampicin
	Kanamycin	-	-

The bacterial colonies from each mixture were scraped from the solid medium and resuspended in 10mL 10mM $MgSO_4$ separately. Dilutions of 1:100 were made from all three, with extra dilutions of mixture S being made at 1:1,000 and 1:10,000. These dilutions were then plated out on separate plates of solid LB medium containing 50µg/mL kanamycin and 50µg/mL rifampicin each. This is done to select for the *Agrobacterium* colonies that have taken up the pBIN-TP-OCI plasmid during triparental mating. Only mixture S showed colony growth. After the dilutions were incubated at 25°C overnight, a single colony was picked from the plate carrying a 1:10,000 dilution of mixture S, and once again grown at 25°C overnight, but this time in liquid LB broth. The cell density of the overnight culture was monitored the following day at hourly intervals, and centrifuged at 10,000rpm in a benchtop centrifuge for 10 min once the cell density in the medium reached an optical density of 0.4 at 600nm. Growth medium was poured off, and cells resuspended in an equal volume sterile 10mM $MgSO_4$. Fully expanded tobacco leaves were taken from mature Samsun plants and sterilised in a solution containing 1:3 bleach:sterile distilled water for 10min. Leaves were then washed three times in sterile distilled water. Leaf disks were cut out with a sterilised leaf-cutter and placed in the *Agrobacterium* solution for 5 min. The disks were blotted on sterile filter paper to remove excess *Agrobacterium*-containing liquid, and incubated for 2 days in the dark on petri dishes containing MS medium supplemented with 0.1mg/L 6-benzylamino purine (BAP) for the stimulation of shoot formation

and cell division. After incubation, disks were transferred to a medium for regeneration of transformed shoots. This consisted of MS salts supplemented with 0.1mg/L BAP, 100mg/L kanamycin for selection of transformed shoots, and 500mg/L cefotaxime to prevent further growth of *Agrobacterium*. Disks were transferred to new MS medium containing the same amounts of BAP, kanamycin and cefotaxime after 4 weeks cultivation in a growth room with a growth temperature of 25°C with a 16/8 h light/dark cycle.

After 4 weeks, a white callus appeared on the disks which developed into shoots after about 9 weeks of cultivation on MS medium supplemented with 0.1mg/L BAP, 100mg/L kanamycin and 500mg/L cefotaxime. For root formation, putative transformed shoots were placed on a medium containing half-strength MS medium, 100mg/L kanamycin and 300mg/L cefotaxime. Rooted shoots were transferred to individual containers containing half-strength MS medium.

2.6 Statistical Methods

Significant differences between comparable treatment means were determined by use of Student's t-test.

CHAPTER 3

3. PLANT CHARACTERISATION AND TRANSFORMATION

3.1 Selection of Transformed Plants

3.1.1 Introduction and objective

Transformed plants can be selected with different techniques, such as selection of plants on an antibiotic selective medium, the determination of the expression of an introduced reporter gene using an enzymatic test, and detection of the gene of interest transferred into the plant genome by an enzymatic assay or immuno-detection. The easiest procedure to select a transformed plant is by growth on a selective medium. Such a medium might contain an antibiotic that would otherwise be lethal to the plant. A variety of aminoglycoside antibiotics, such as kanamycin, neomycin, geneticin and paromomycin, have been applied in transformation experiments. These antibiotics are inactivated via phosphorylation due to expression of the neomycin phosphotransferase II (*nptII*) gene.

In general a reporter system must allow easy quantification, be highly sensitive, and the reaction of the reporter enzyme should be specific to minimise interference with normal cellular metabolism. The *E. coli* β-glucuronidase coding sequence (GUS) has been developed as a reporter gene system to study gene expression in plants by expressing GUS (Jefferson, 1987). This protein, which has a molecular weight of 68.2 kDa, is a hydrolase catalysing the cleavage of a wide variety of β-glucuronides, which are used as spectrophotometric, fluorometric or histochemical substrates. Common techniques to detect expression of an exogenous target gene on the translational level in a transformed plant include detection by an enzymatic assay or immuno-detection in a Western blot procedure using an antiserum raised against a purified protein encoded by the target gene.

The objective of this part of the study was to select transformed plants grown from seed for characterisation in physiological and biochemical studies in order to evaluate the potential of an expressed OCI in transformed plants to protect plant proteins from degradation. For that, putative transformed seedlings were selected by screening for antibiotic resistance, reporter gene expression and expression of OCI.

3.1.2 Results

3.1.2.1 *Selection on kanamycin*

To select for transformed plants used in this study, putative transformed seeds expressing the *nptII* gene for kanamycin resistance were germinated on a selective MS medium containing 100mg/L kanamycin (Figure 3.1.1). Out of a total of 52 seeds germinated on the selective medium, 38 seedlings remained dark-green (kanamycin resistant), while 14 had pale leaves that had a bleached appearance. These pale-looking seedlings eventually turned completely white when left on the kanamycin selective medium for 4 weeks. Only dark-green seedlings were further characterized in the study.

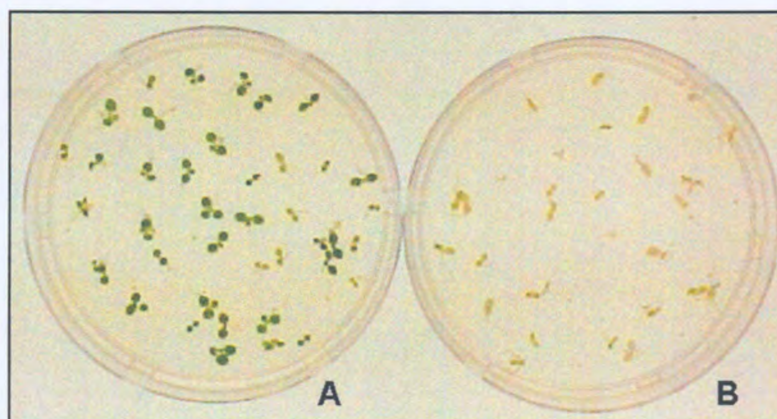


Fig. 3.1.1 Seed germination on 100mg/L kanamycin selection medium. (A) Putative transformed seedlings with the majority of seedlings dark-green indicating expression of kanamycin resistance and (B) non-transformed wild-type seedlings not expressing any kanamycin resistance.

3.1.2.2 *Selection for GUS expression*

Putative transformed seedlings expressing kanamycin resistance were also tested for GUS expression (Figure 3.1.2). Roots and leaves of putative transformed seedlings were tested for the expression of the GUS selectable marker gene (Figure 3.1.2). Since the *gus* gene is closely linked to the *OCI* gene on the T-DNA, all GUS positive plants should also carry the *OCI* gene in their genomes. Out of a total of 44 kanamycin-resistant seedlings, 35 seedlings also tested positive for expression of GUS.

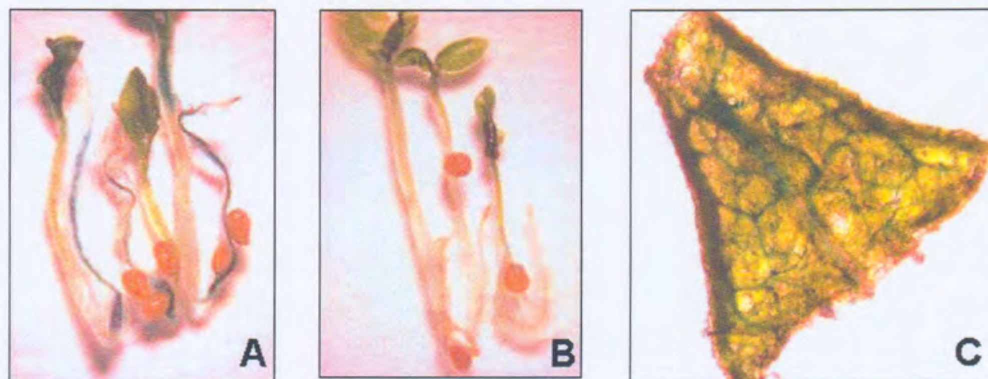


Fig. 3.1.2 GUS histochemical screening of seedlings grown from transformed seed. (A) Blue-stained roots of transformed seedlings indicating GUS expression and (B) roots of non-transformed seedlings showing no GUS expression. (C) Blue-stained leaf tissue of a transformed plant indicating GUS expression.

3.1.2.3 *Selection for OCI expression*

Putative transformed seedlings that tested positive for the expression of kanamycin-resistance and the *gus* selectable marker gene were further screened for the expression of OCI. This was done after SDS-PAGE by Western blot analysis with antiserum raised against the OCI protein (Figure 3.1.3). A single band corresponding to the OCI protein was detected in the majority of transformed plants. In contrast, no band corresponding to the OCI protein was detected when non-transformed plants were tested. Only

transformed plants that clearly expressed the *OCI* gene product detected by Western blot analysis were further characterised in the biochemical and physiological study.

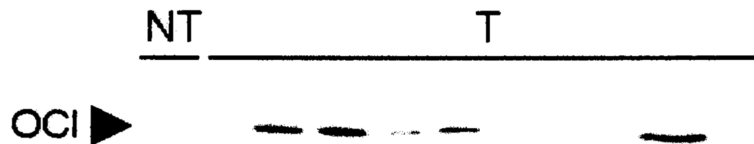


Fig. 3.1.3 Detection of OCI expression by Western blot analysis (immuno-blotting) of plant extract from non-transformed (NT) and transformed (T) plants, with antiserum directed against OCI. The position of the OCI band corresponding to a molecular weight of about 13kDa is indicated.

3.2 Protein Degradation

3.2.1 Introduction and objective

Protein degradation plays a major role in plant growth, development, and senescence, as well as protection against pathogens (Vierstra, 1996). Among the proteinases identified in the plant cell are cysteine proteinases. They have been widely studied, and are specifically expressed during a variety of stress conditions including plant senescence (Hörtensteiner and Feller, 2002). At an acidic pH, cysteine proteinases are active and these proteinases are located in the plant vacuole (Huffaker, 1990). During plant senescence, degradation of Rubisco LSU occurs (Peñarrubia and Moreno, 1990; Navarre and Wolpert, 1999; Hörtensteiner and Feller, 2002). Since there is evidence that vacuolar proteinases are involved in Rubisco degradation (Yoshida and Minamikawa, 1996), Rubisco could be a target for cysteine proteinase action.

The objective of this section was to study Rubisco stability against cysteine proteinase action in the presence/absence of a synthetic and natural cysteine proteinase inhibitor. In particular (1) the effect of different buffer systems on Rubisco stability during proteinase action and (2) Rubisco stability in non-transformed and transformed tobacco plants expressing the rice cystatin OCI

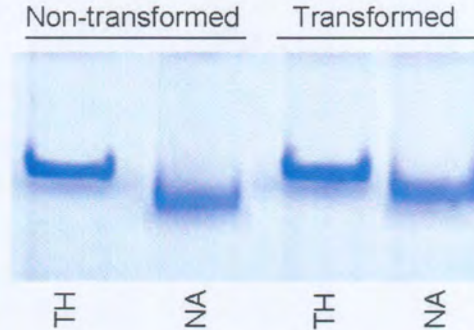
were investigated. Tobacco plants were all grown *in vitro*. Techniques applied in this study included native PA gel analysis and Western blot analysis (immuno-blotting) to determine protein profiles and Rubisco degradation, respectively. In the experiments either purified pea Rubisco (Sigma, UK) treated with purified papain (cysteine proteinase) or plant extracts containing endogenous Rubisco from non-transformed and transformed plants were used.

3.2.2 Results

3.2.2.1 *Extraction buffer optimisation for protein analysis*

When plant extracts of non-transformed and transformed tobacco plants were analysed on a native PA gel, a major protein band was detected in all samples after protein staining possibly representing mainly Rubisco, which is the most abundant protein in plant protein extracts (Figure 3.2.1A). However, a shift occurred in the position of this protein band depending on the extraction buffer system used for preparing the plant protein extract. A shift to a lower position was found for buffer B that promotes cysteine proteinase activity. With this buffer system a more diffused protein band was also observed. In addition, absorbancy of the extracted stain from the protein band decreased when buffer B was used (Figure 3.2.1B). Degradative processes possibly caused this shift although no clear degradation products were identified on the gel. To prevent any degradation by the buffer system after extraction, all experiments to determine protein stability directly in the plant were therefore carried out in buffer A. However, when degradation of a protein by a cysteine proteinase was specifically investigated in an *in vitro* enzymatic assay, buffer B was used which provides the optimal pH for cysteine proteinase activity during the assay.

A



B

Plant	Extraction buffer	A_{585}
Non-transformed	TH	0.166
	NA	0.117
Transformed	TH	0.193
	NA	0.134

Fig. 3.2.1 Stability of crude plant extracts from non-transformed (NT) and transformed (T) Samsun tobacco plants in different extraction buffers determined by native gel electrophoresis (A) and absorbancy of extracted stained proteins (B). Extraction buffers used were 50mM Tris-HCl, pH 8.9 (buffer A) and 50mM NaAc/10mM β-mercaptoethanol, pH5.8 (buffer B). Absorbancy of the blue stain was measured at 585 nm after elution of the stained protein from the PA gel presented in (A) and (B).

3.2.2.2 *Total protein degradation*

Figure 3.2.2 shows a typical incubation experiment to determine the effect of existing cysteine proteinases on proteins extracted from a non-transformed and a transformed tobacco plant, and separated on a native PA gel. After incubation of crude proteins in the NA (acidic) extraction buffer for 4 hours at 37°C the position and intensity of the protein band altered (Figure 3.2.2A and 3.2.2C). A temperature of 37°C was used as a standard temperature for testing cysteine proteinase activity (Barrett *et al.*, 1982). However, the shift of the protein band was not found when the cysteine proteinase inhibitor E64 was added (Figure 3.2.2A) or with a plant extract from a transformed plant

expressing OCI, which blocks cysteine proteinase activity (Figure 3.2.2B). This transformed plant extract also showed a higher but statistically not significant ($p>0.05$) protection against the loss of the intensity of the protein band after 4 hours incubation at 37°C (Figure 3.2.2C). No change of the pattern was obtained when E64 was further added to the transformed plant extract (Figure 3.2.2B). The result of this experiment could be confirmed in several repeats.

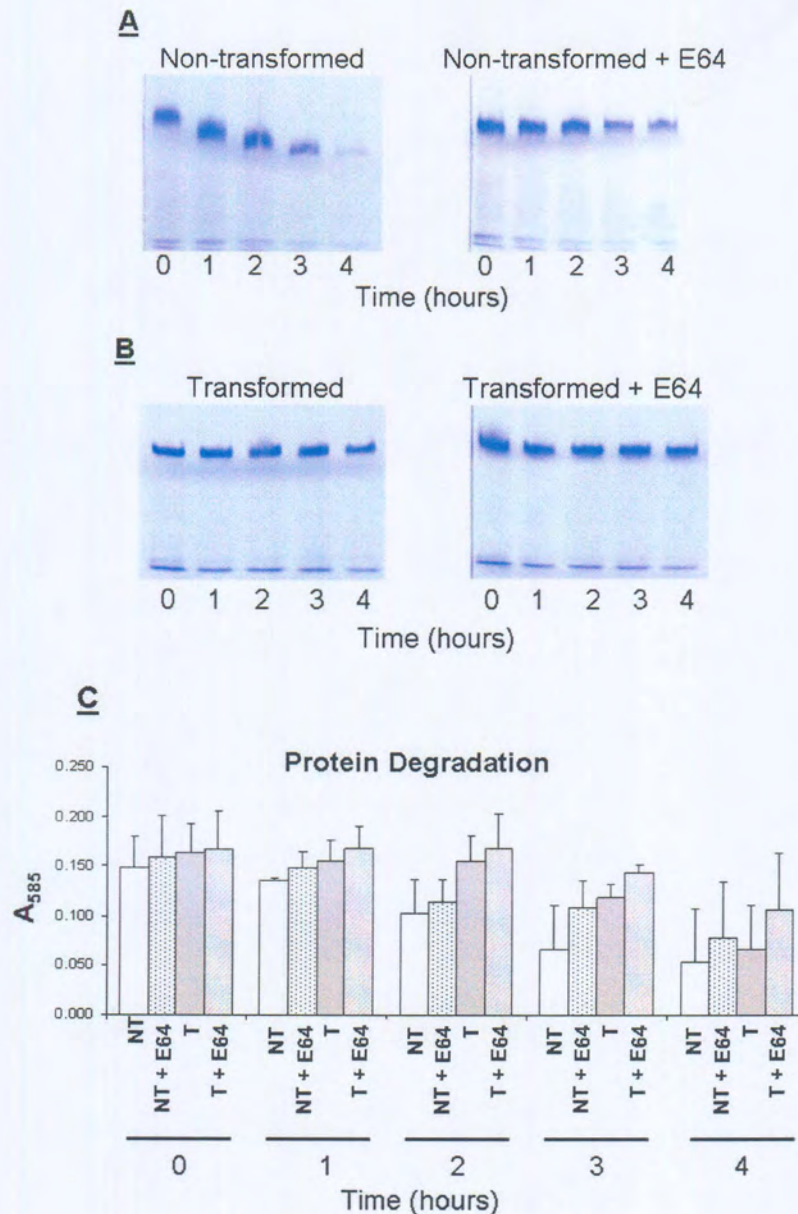


Fig. 3.2.2 Protein stability in a crude plant extract of a non-transformed (NT) and a transformed (T) tobacco plant after incubation for different time intervals at 37ºC in buffer B with and without the addition of the cysteine proteinase inhibitor E64. (A) Plant protein extract from a non-transformed plant, (B), plant protein extract from a transformed plant expressing the rice cystatin and (C) absorbancy of the blue stain measured at 585 nm after elution of the stained protein from the PA gel. Data for (C) represent the mean of 3 different experiments ± standard deviation.

3.2.2.3 *Rubisco degradation*

Purified papain (Sigma, UK), a cysteine proteinase, was used to determine the sensitivity of purified pea Rubisco (Sigma, UK) to degradation by cysteine proteinases in an acidic pH. Figure 3.2.3A shows the effect of papain on Rubisco LSU at 37°C in buffer B detected by Western blotting using an antiserum raised against LSU. Besides a major protein band (Rubisco LSU) a number of smaller bands were detected after SDS PAGE and immuno-blotting. These smaller bands very likely indicate degradation products of Rubisco LSU. When the synthetic cysteine proteinase inhibitor E64 was added to the incubation solution, only some lower molecular weight products were detected after 2 hours beside the major protein band. When the effect of papain on Rubisco SSU was investigated, a single band corresponding to the SSU was detected in all samples analysed (Figure 3.2.3B). This band did not even change after incubation of the Rubisco solution for 3 hours, and there was no appearance of smaller molecular weight products.

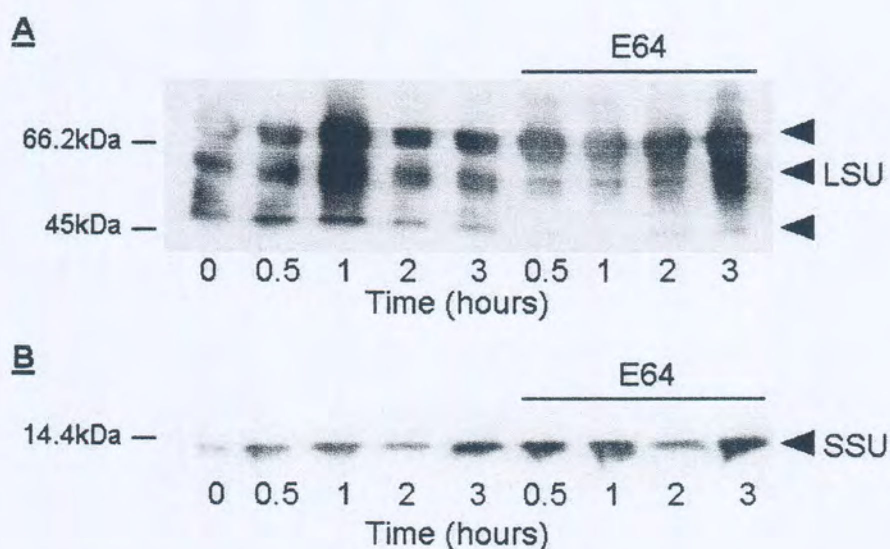


Fig. 3.2.3 Detection by Western blot analysis (immuno-blotting) of Rubisco products after SDS-PAGE from purified Rubisco (Sigma, UK) treated with the cysteine proteinase papain (0.1 µg). Products of Rubisco LSU detected by a LSU antiserum (A) and by a Rubisco SSU antiserum (B). Detection after incubation of Rubisco for different time intervals in buffer B with and without the addition of the proteinase inhibitor E64.

Plant extracts from transformed and non-transformed tobacco plants were used to determine the sensitivity of endogenous Rubisco to degradation by proteinases present in the plant. Plant proteins were extracted and incubated in buffer B at 37°C for 3 hours. The acidic pH of buffer B specifically creates an optimal condition for cysteine proteinases. Figure 3.2.4A shows the profile for Rubisco LSU obtained from crude plant extracts of transformed and non-transformed tobacco plants after 3 hours incubation. A major protein band corresponding to the LSU size is clearly visible for extracts from the transformed tobacco plant (Figure 3.2.4A) as well as a number of bigger bands possibly representing Rubisco polymers, and a smaller band possibly representing a degradation product at 51kDa. However, a similar profile was not obtained when an extract from a non-transformed plant was tested and only a single band corresponding to approximately 51kDa was found in all samples. Also, no higher molecular weight bands were found in the crude extract from a non-transformed tobacco plant even at 0h. This could be due to experimental error – tubes were kept on ice, and cysteine proteinase activity might have been present in non-transformed plant extract. In contrast to the LSU, only a single band for Rubisco SSU was detected in transformed and non-transformed plants without formation of any other product (Figure 3.2.4B).

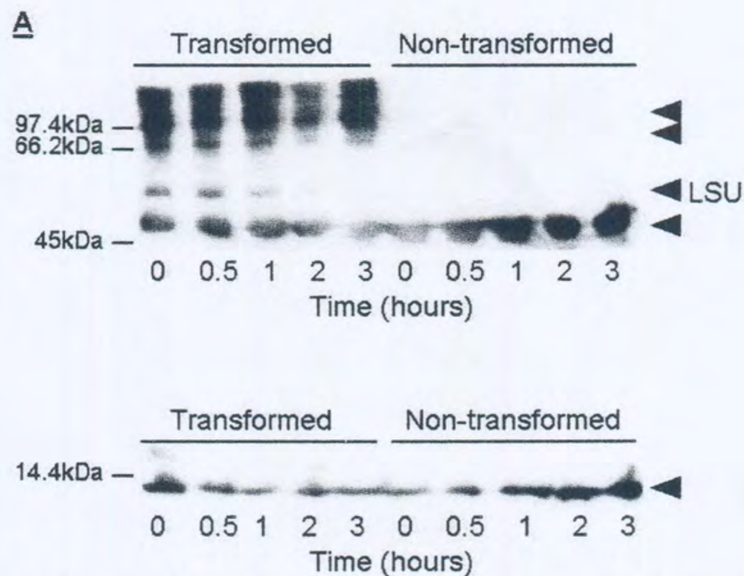


Fig. 3.2.4 Detection by Western blot analysis (immuno-blotting) of products derived from plant endogenous Rubisco after cysteine proteinase activation in an *in vitro* assay system. Products of Rubisco LSU detected by a LSU antiserum (A) and by a Rubisco SSU antiserum (B). Detection after incubation of a non-transformed and transformed tobacco plant extract for different time intervals in buffer B. Arrows indicate the position of LSU and smaller molecular weight products. The LSU size was determined by a SDS PAGE gel containing a protein size marker run in parallel.

3.3 Cold Stress And Cystatins

3.3.1 Introduction and objective

Chilling injury causes a variety of symptoms to occur in the plant (Graham and Patterson, 1982). Processes that are affected in the plant include reproductive ability and photosynthesis. The impact of chilling on transformed tobacco plants overexpressing the rice cystatin OCI was previously investigated (Van der Vyver *et al.*, 2003). It was found that photosynthesis of these transformed tobacco plants recovered much faster from a short chill than control plants and a possible protective role of cystatins against chilling damage was suggested.

The objective of this section was to investigate protection of Rubisco against chilling damage by exogenous OCI expressed in genetically engineered tobacco plants. In particular Rubisco stability under cold stress was studied in non-transformed and transformed tobacco plants expressing OCI by applying native PA electrophoresis, spot densitometric Rubisco quantification after SDS-PAGE, radioactive quantification of Rubisco, and Western blot analysis. All plants were grown *in vitro*, except for plants used in spot densitometric and radioactive quantification of Rubisco, in which case plants were grown under controlled conditions in the greenhouse.

3.3.2 Results

3.3.2.1 *Total cysteine proteinase activity*

No significant change of total cysteine proteinase activity was found when non-transformed plants were exposed to a temperature of 10°C (10.1±2.1 Funits/mg protein) in comparison to plants grown at 25°C (12.6±4.4 Funits/mg protein) ($p>0.05$) (Figure 3.3.1). Also, there was no significant difference in cysteine proteinase activity in transformed plants at 25°C and 10°C ($p>0.05$). However, transformed plants expressing the cystatin had a significant lower total cysteine proteinase activity when either treated at 25°C (2.9±0.4 Funits/mg protein) or 10°C (3.8±1.0 Funits/mg protein) when compared to non-transformed plants ($p\leq0.05$).

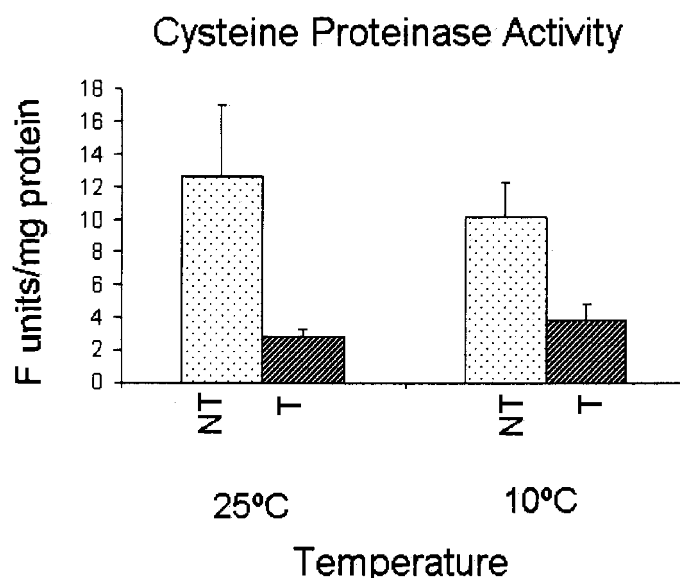


Fig. 3.3.1 Cysteine proteinase activity measured in Funits/mg of total plant protein (fluoresence units/mg protein) in non-transformed (NT) and transformed (T) tobacco plants grown under different temperatures. Data shown represent the mean of 10 different experiments ± standard deviation.

3.3.2.2 *Protein degradation*

When a crude plant protein extract was analysed by native PA gel electrophoresis a major protein band for transformed and non-transformed plants was found possibly representing several different proteins including Rubisco, which is the most abundant protein in a plant extract (Figure 3.3.2A). This protein band did not change in position or intensity when a crude extract from a transformed plant was analysed regardless the temperature used for plant treatment. However, this band shifted to a lower position when a plant extract from a non-transformed plant was analysed, which was treated at 10°C. Furthermore, transformed plants had only a slight but not significant ($p>0.05$) increase in absorbancy value when amounts of eluted dye from protein bands of transformed and non-transformed plants were compared (Figure 3.3.2B).

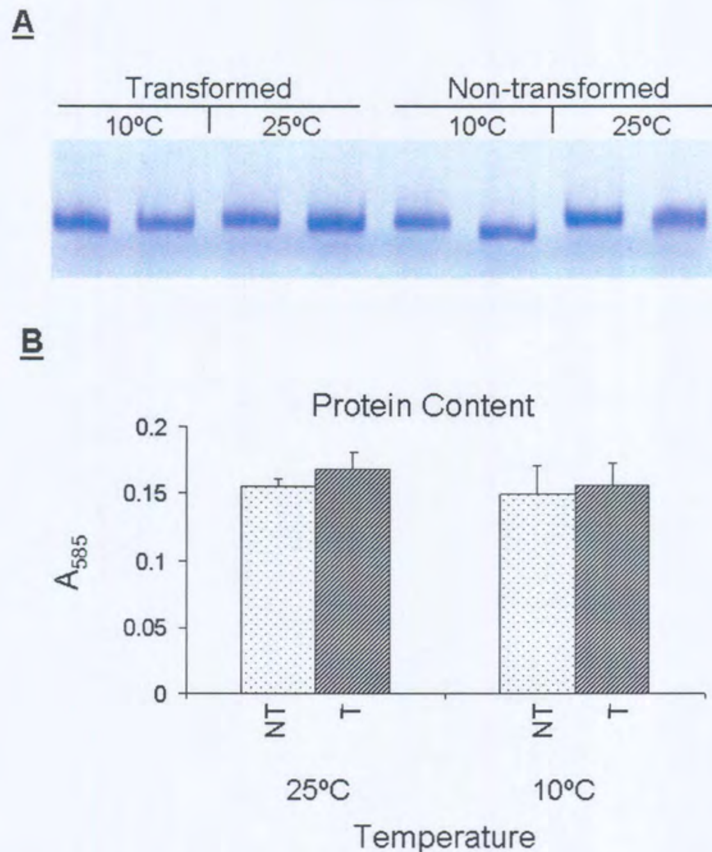


Fig. 3.3.2 Native PA gel electrophoresis (A) and absorbancy (B) of eluted dye from major protein band on native PA gel of plant extract from transformed (T) and non-transformed plants (NT) exposed to different temperatures for 5 days. Extracts from two transformed and two non-transformed plants treated at 10ºC, or left at 25ºC, were used. Proteins were extracted in buffer A. Data shown in (B) represent the mean of 15 different experiments ± standard deviation.

3.3.2.3 *Spot densitometric protein quantification*

The density of bands corresponding to the Rubisco LSU was determined with the AlphaEase software package (Alpha Innotech, UK), after proteins were extracted from tobacco leaves and separated on a 7.5% SDS-PAGE gel. Samples were taken from transformed and non-transformed plants before cold treatment, after a two-day cold treatment at 10-12ºC, and after a two-day recovery period at 20ºC. The integrated density values (IDV) for bands

corresponding to Rubisco LSU in a 7.5% SDS-PAGE gel were not different between non-transformed and transformed plants either before cold treatment, after cold treatment, or after recovery (Figure 3.3.3A and B). An IDV of 233 was observed for the LSU band from a non-transformed plant before cold treatment, compared to a value of 234 for a transformed plant. After cold treatment, the IDV value for the LSU band in a non-transformed plant decreased to 142, compared to an IDV value of 169 in a transformed plant. After recovery the IDV value was the same for a non-transformed and a transformed plant at 198. The procedure was repeated three times, and an identical result was consistently obtained in all three experiments.

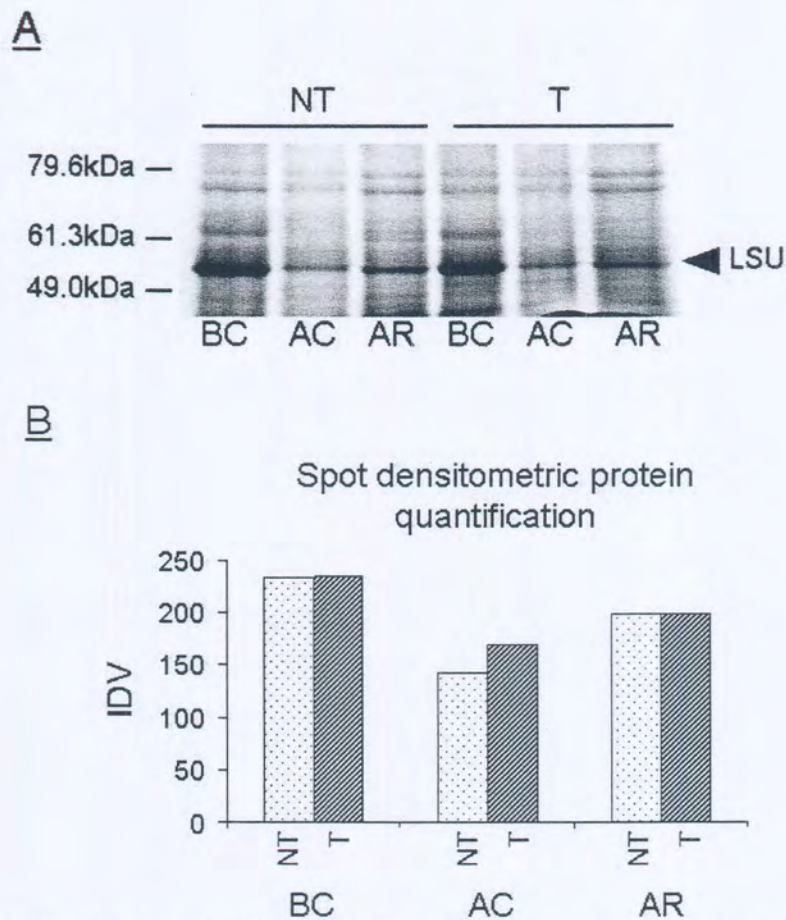


Figure 3.3.3 Spot densitometric protein quantification of Rubisco LSU after SDS-PAGE (A) in non-transformed (NT) and transformed (T) tobacco plants before cold treatment (BC), after cold treatment (AC) and after recovery (AR). The integrated density value obtained for the LSU band from non-transformed and transformed plants were compared (B) on a scale where IDV 255 was equal to black, and IDV 0 was equal to white.

3.3.2.4 *Radioactive quantification of Rubisco*

Figure 3.3.4 shows the Rubisco content, determined by a radioactive quantification technique, in plant extracts from transformed and non-transformed tobacco plants. Measurements were carried out after plants were exposed to 10-12°C for 2 days (cold treatment), and allowed to recover for 2 days at 20°C (recovery period). The Rubisco content was determined before cold treatment, after cold treatment, and after the 2-day recovery period. Transformed plants had, on average, a higher but not significant ($p>0.05$) Rubisco content than non-transformed plants regardless the treatment applied. Furthermore, in non-transformed plants the amount of Rubisco decreased by 22% after cold treatment, but increased again to 88% of its original value after a 2-day recovery period. However, decrease and recovery was not significantly different from that found in transformed plants ($p>0.05$). In these plants the Rubisco content decreased after cold treatment by 31% and increased to 74% of its original value after a 2-day recovery period.

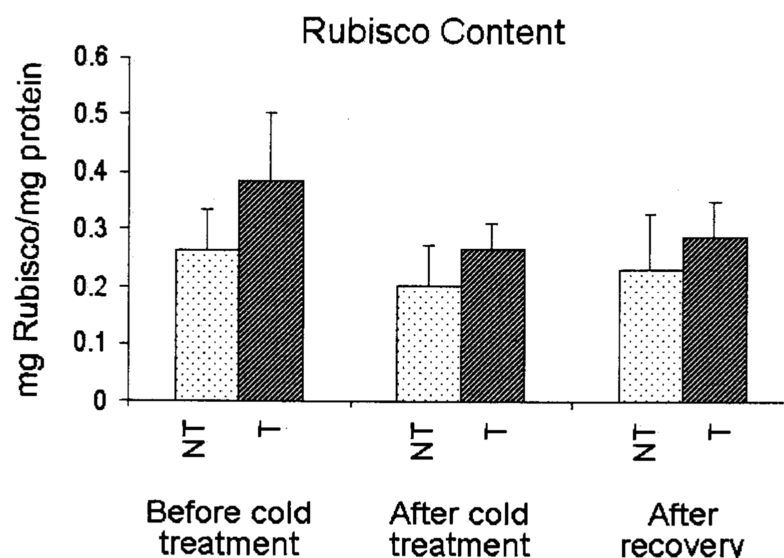


Fig. 3.3.4 Rubisco content in transformed (T) and non-transformed (NT) tobacco plants before cold treatment, after cold treatment, and after a two-day recovery period. Rubisco was quantified using radioactively labeled inhibitor (^{14}CABP) which binds to the enzyme quantitatively and measuring ^{14}C incorporation into the enzyme. Data shown represent the mean of 5 different experiments ± standard deviation.

3.3.2.5 *Immuno-blotting*

When plant proteins extracted in buffer A, which prevents cysteine proteinase activity, were separated by SDS-PAGE and analysed by immuno-blotting several protein bands were detected with an antiserum raised against Rubisco LSU (Figure 3.3.5). The higher molecular weight protein (55kDa) corresponds to the exact size of LSU, whereas the lower molecular weight band with size of approximately 51kDa very likely represents degradation products of the LSU. The high molecular weight bands most likely indicate LSU polymers, since it is known that the LSUs form dimers *in vivo*. The LSU polymer bands were detected in all tested plants (transformed and non-transformed) grown at 25°C but only in one transformed plant grown at 10°C (T3). Further, the intensity of the LSU band (55kDa) was less for the two non-transformed plants grown at 10°C (NT3 and 4) than for the two transformed plants (T3 and 4). This possibly indicates a higher amount of full-size LSU in transformed plants. Also, in one non-transformed plant (NT4) the protein band at 55kDa completely disappeared which did not occur for the two transformed plants (T3 and 4).

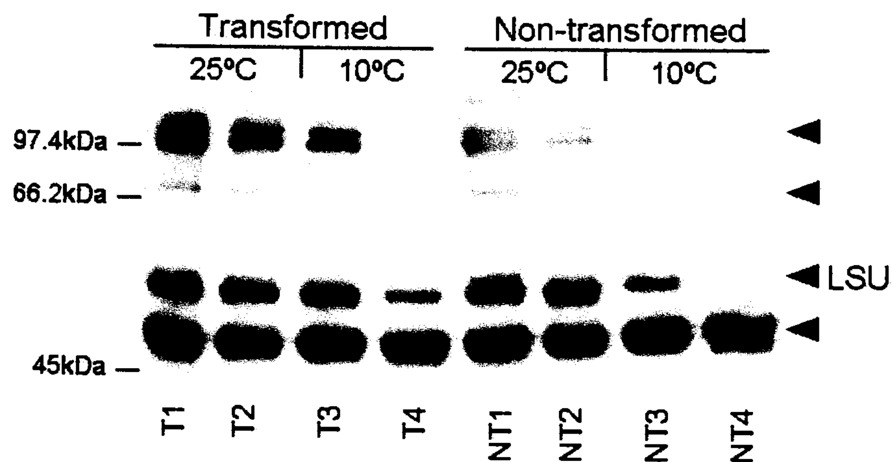


Fig. 3.3.5 Detection of Rubisco LSU by immuno-blotting (Western blot analysis) with an LSU antiserum after SDS PAGE of plant extracts derived from transformed (T1-4) and non-transformed plants (NT1-4) grown at different temperatures. Sizes of marker proteins are indicated.

3.4 Plant Senescence and Cystatins

3.4.1 Introduction and objective

Leaf senescence involves a series of degradative processes leading to the remobilisation of nutrients and eventual leaf death (Huffaker, 1990). However, the senescence process is highly regulated, involving photosynthetic decline, lipid peroxidation, protein degradation, and chlorophyll degradation (Smart, 1994). It can be genetically controlled (Friedrich and Huffaker, 1980), or environmentally induced, by factors such as nutrient deficiency, pathogenic attack, drought, light limitation, and temperature (Smart, 1994).

Rubisco seems to be the limiting factor to photosynthesis during the entire life cycle of a plant (Mae *et al.*, 1985), and its early loss, among other constituents, during senescence decreases photosynthesis. This results in crucial limitation to seed yield (Huffaker, 1990). Many studies have been done on the involvement of proteinases in senescence-associated protein degradation (Miller and Huffaker, 1985; Hensel *et al.*, 1993; Morris *et al.*, 1996). Among the proteinases investigated during plant senescence are cysteine proteinases (Granell, 1992; Hensel *et al.*, 1993; Martin and Green, 1995). However, their contribution to the general degradation of chloroplast proteins, which constitutes as much as 75% of total cellular nitrogen, remains unclear (Hörtensteiner and Feller, 2002). Previous studies have further indicated that Rubisco, and specifically the LSU, is sensitive to degradation by vacuole-localised cysteine proteinases (Mitsuhashi and Feller, 1992; Navarre and Wolpert, 1999). Although the chloroplast itself contains proteinases degrading its own protein constituents (Dalling *et al.*, 1983), vacuole-located enzymes may act on stromal proteins. This could occur after a loss of membrane intactness during the cellular senescence process (Hörtensteiner and Feller, 2002).

The objective of this part of the study was to investigate the effect of senescence on Rubisco stability in non-transformed and transformed plants that express OCI. To carry out the study the techniques of native PA gel

electrophoresis and immuno-detection of Rubisco products by Western blot analysis were applied on senescent leaf material. All tobacco plants were grown *in vitro*.

3.4.2 Results

3.4.2.1 *Leaf senescence*

Detachment of leaves from either transformed or non-transformed tobacco plants and placement of leaves in a petri dish with a wet filter paper resulted in wilting and yellowing of leaves from both transformed and non-transformed tobacco plants to the same degree (Figure 3.4.1).

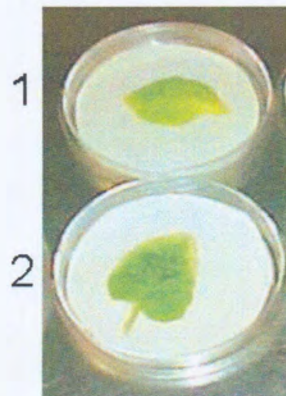


Fig. 3.4.1 Wilting and yellowing of detached transformed and non-transformed leaves in petri dishes on wet filter paper for 5 days. (1) Transformed leaf material and (2) non-transformed leaf material.

3.4.2.2 *Senescence and total cysteine proteinase activity*

A significant increase in total cysteine proteinase activity was found when non-transformed plants were senesced in petri dishes for 5 days. Total cysteine proteinase activity in non-transformed plants increased from 4.32±0.2 Funits/mg protein in non-senescent leaf material, to 7.0±2.3 Funits/mg protein in senescent leaf material (Figure 3.4.2). Under non-senescent conditions, transformed leaf material expressing the cystatin had a significantly ($p \leq 0.05$)

lower total cysteine proteinase activity (2.4±0.6 Funits/mg protein when compared to non-transformed leaf material. However, under senescent conditions the total cysteine proteinase activity in transformed leaves (7.1±1.1 Funits/mg protein) did not differ significantly from the activity found in non-transformed leaves (7.0±2.3 Funits/mg protein).

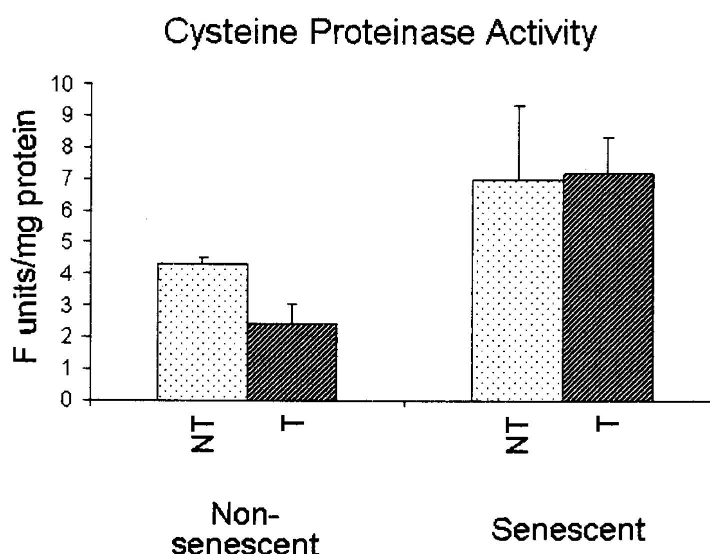


Fig. 3.4.2 Cysteine proteinase activity measured in fluorescence units (Funits)/mg of total plant protein (fluoresence units/mg protein) in non-transformed (NT) and transformed (T) tobacco plant leaf material under non-senescent and senescent conditions. Data shown represent the mean of 4 different experiments ± standard deviation.

3.4.2.3 *Senescence and protein degradation*

A major protein band was detected on native PA gel electrophoresis after separation of crude plant protein extracts from transformed and non-transformed plants (Figure 3.4.3A). In comparison to transformed leaves, this protein band decreased more rapidly in intensity due to senescence in non-transformed leaves. However, this decrease was variable and was not consistently found for all plant leaves. There was also a decrease in mean absorbancy of the eluted protein band for both the non-transformed and transformed plants with absorbancy generally higher from transformed leaves

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than from non-transformed leaves under non-senescent or senescent conditions. For non-transformed leaf material, this decrease was from A_{585} 0.15±0.01 (non-senescent) to A_{585} 0.11±0.02 (senescent) and for transformed leaf material from A_{585} 0.16±0.01 (non-senescent) to A_{585} 0.14±0.01 (senescent) (Figure 3.4.3B).

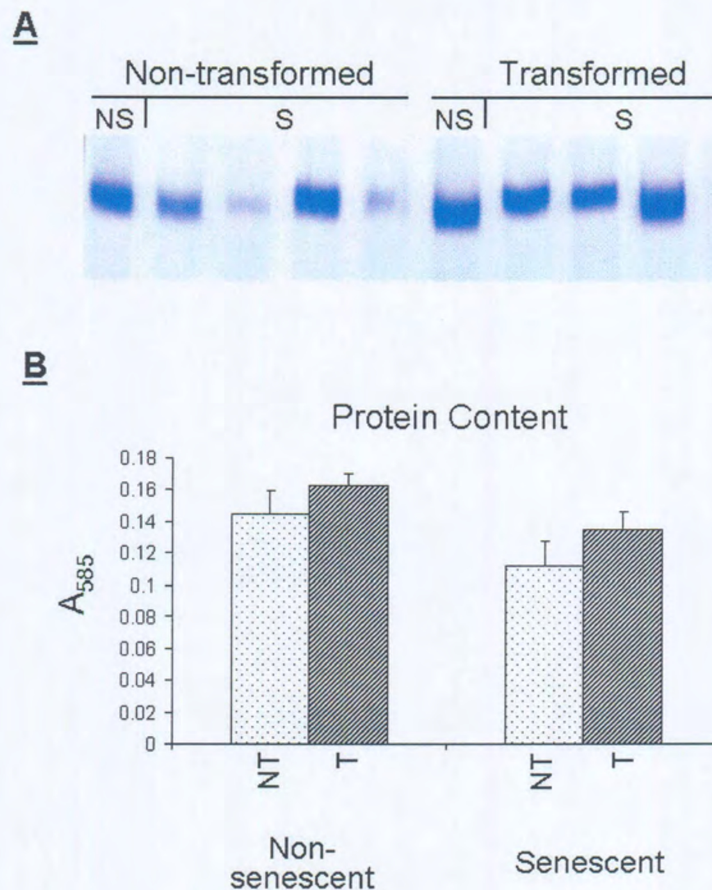

Fig. 3.4.3 Native PA gel electrophoresis (A) and absorbancy (B) of eluted dye from major protein band excised from native PA gel from transformed (T) and non-transformed leaf extracts (NT) senesced for 5 days (S) or non-senesced (NS). Proteins were extracted in buffer A. Data shown in (B) represent the mean of 4 different experiments ± standard deviation.

3.4.2.4 *Immuno-blotting*

Proteins from leaves senesced for 12 days were extracted in buffer A to prevent protein degradation in the extraction buffer, separated by SDS-PAGE and analysed by immuno-blotting with an antiserum raised against Rubisco LSU (Figure 3.4.4). A higher molecular weight protein band was found corresponding to the expected size of the LSU (55 kDa). In addition, high molecular weight bands, very likely polymers of Rubisco LSU, were detected. Although a more diffused banding pattern was found in the protein profile from non-transformed plant leaves, no major differences were detected between the protein profiles from non-transformed and transformed leaf material.

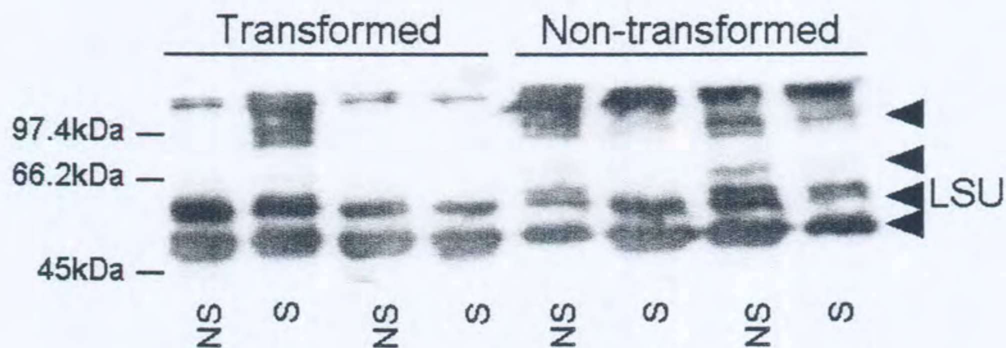

Fig. 3.4.4 Detection of Rubisco LSU by immuno-blotting (Western blot analysis) with a LSU antiserum after SDS-PAGE of plant extracts derived from transformed and non-transformed leaves that were senesced for 12 days (S) or not senesced (NS).

3.5 Construction of a Vector for OCI Targeting to the Chloroplast and Production of Transformed Tobacco

3.5.1 Introduction and objective

The plant cell is highly compartmentalised into a number of membrane-delineated compartments, called organelles. However, the majority of proteins and enzymes functional in organelles are encoded in the nucleus and synthesized in the cytosolic compartment of the cell. These proteins are

targeted towards, and imported into the organelle by specific targeting sequences, called transit sequences, situated at the amino terminal end of the protein (Chua and Schmidt, 1979). Bruce (2001) estimated that between 13% and 22% of the total gene pool are chloroplast-targeted precursors. Transit peptides direct more than 3,500 different proteins into the plastid during the life of a typical plant. Transit sequences have also been applied in plant studies, in order to target foreign gene products to specific organelles, such as the chloroplast (Van den Broeck *et al.,* 1985, Nakashita *et al.*, 2001), and the peroxisome (Nakashita *et al.*, 2001). Van den Broeck and Nakashita used the targeting sequence from Rubisco SSU, which is encoded in the nucleus and is the most used precursor with over 170 different SSU targeting sequences represented in the SWISS-PROT database.

Previous results obtained in this study showed that there was no significant protective role for exogenous OCI on the stability of Rubisco (LSU) under cold treatment and senescence, when expressed in the cytosolic compartment of a plant cell. A study was therefore initiated at the end of the thesis to determine whether targeted expression of OCI in the chloroplast might provide any protection of Rubisco against degradation by cysteine proteinases in the chloroplast itself. The objective of the first part of the study was to transform tobacco (*Nicotiana tabacum* L. cv. Samsun) with a suitable vector allowing expression of OCI as a pre-protein with a transit peptide suitable for targeting OCI towards the chloroplast. The vector used for plant transformation was based on the pJIT117 vector (Guerineau *et al.*, 1988). The vector designed from pJIT117 included the *OCI* gene inserted into the multiple cloning site with the transit sequence on the amino-terminal side of the gene, as well as the necessary regulatory sequences for OCI expression and a selectable marker gene allowing selection of transformed plants.

3.5.2 Plant transformation

3.5.2.1 *Vector construction*

The *OCI* gene was digested from the plasmid pAOCI-3 (Figure 3.5.1) using the restriction enzymes *PstI* and *EcoRI* (Roche, Switzerland), and cloned into the *PstI*/*EcoRI* sites of the plasmid pUC18 (Figure 3.5.2A) to create pUC-OCI (Figure 3.5.2B). This plasmid allowing blue/white selection for DNA insertion into its multiple cloning site was transferred into *E. coli* MOSBlue cells (Amersham, UK) and the recombinant plasmid was isolated from transformed cells. To confirm the correctness of the cloning procedure and the presence of the OCI start codon to be ultimately in frame with the transit sequence in pJIT117, the pUC-OCI construct was sequenced on an automatic sequencer. Sequencing was done by GATC Biotech AG (Germany), using the M13 forward primer. A *PstI*/*EcoRI* fragment from pUC-OCI was then cloned into identical restriction sites of the plasmid pJIT117 plasmid to create pJIT-OCI (Figure 3.5.3A and B). Plasmid pJIT-OCI contains a transit sequence from the pea Rubisco SSU, which encodes a transit peptide to direct a protein into the chloroplast, a double 35S promoter from the Cauliflower Mosaic Virus (CaMV) for constitutive expression of OCI in plants, the OCI coding sequence and the CaMV transcription termination sequence (Guerineau *et al.,* 1988).

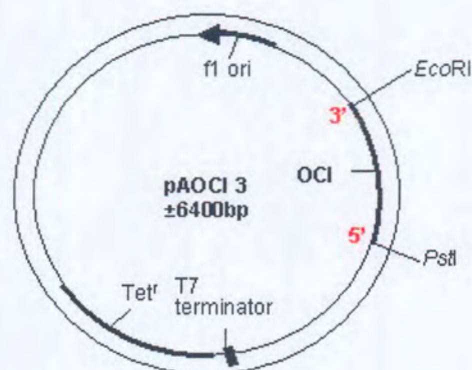

Fig. 3.5.1 Map of plasmid pAOCI 3 containing the OCI coding region. F1 ori – origin of replication. Tet^r tetracycline resistance gene allowing selection of bacterial cells carrying the plasmid.

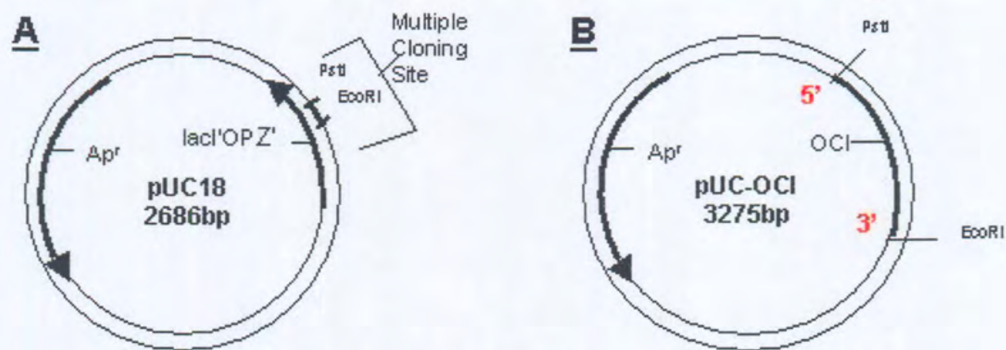

Fig. 3.5.2 Map of the pUC18 plasmid (A) and pUC-OCI (B), created by inserting the OCI fragment from pAOCI 3 into pUC18. Ap^r – ampicillin resistance gene (β-lactamase). lacI'OPZ' - α-peptide of the *lacZ* (β-galactosidase) gene allowing blue/white selection.

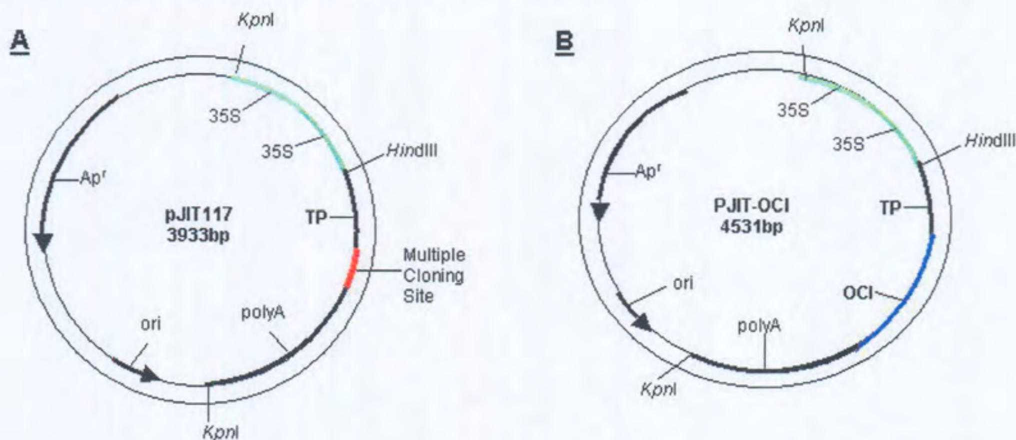

Fig. 3.5.3 Map of the pJIT117 plasmid (A) and pJIT-OCI (B) (pJIT117 with the OCI fragment inserted into the multiple cloning site *Pst*I/*Eco*RI). Ap^r – ampicillin resistance gene (β-lactamase). TP represents transit peptide sequence, 35S – 35S represents double CaMV 35S promoter sequence and poly A the CaMV transcription termination sequence.

Bacterial cells containing pJIT-OCI were selected on a medium containing ampicillin. All plasmids were screened for insertion of the fragment after digestion with appropriate restriction enzymes to release the cloned OCI coding sequence. Finally, a *Kpn*I fragment excised from plasmid pJIT-OCI

containing all regulatory elements for OCI expression in plants, the transit sequence and the OCI coding sequence was cloned into the unique *Kpn*I site in both orientations of the binary vector pBIN19 (Bevan, 1984; Figure 3.5.4 and 3.5.5) allowing *Agrobacterium* transformation. The resulting plasmid (pBIN-TP-OCI) was used for *Agrobacterium*-mediated plant transformation.

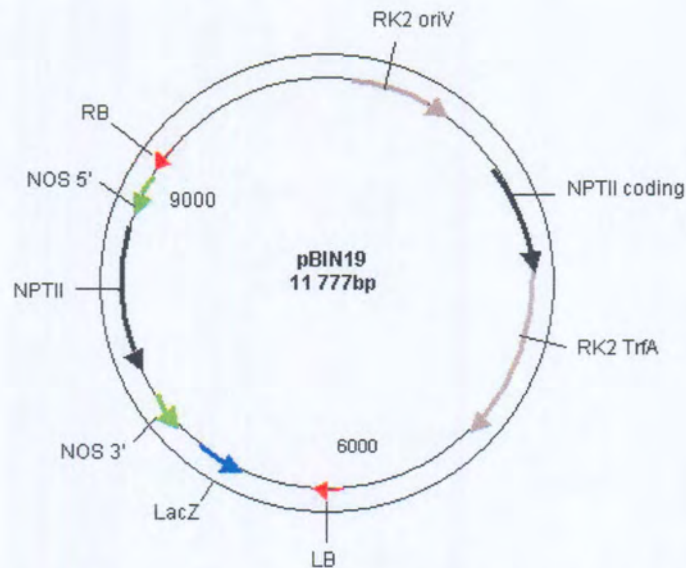

Fig. 3.5.4 Diagram of plasmid pBIN19. RK2 oriV – origin of replication from parent plasmid pRK2. NPTII – kanamycin resistance gene. RK2 TrfA – TrfA locus from pRK2, which promotes plasmid replication. LB – Left Border of T-DNA. LacZ - α-peptide of the *lacZ* (β-galactosidase) gene allowing blue/white selection for inserts cloned into the multiple cloning site. NOS 3' – nopaline synthase terminator region. NPTII – neomycin (kanamycin) resistance gene allowing growth of transformed plant material on an antibiotic-containing medium. NOS 5' – nopaline synthase promoter region. RB – Right border of T-DNA.

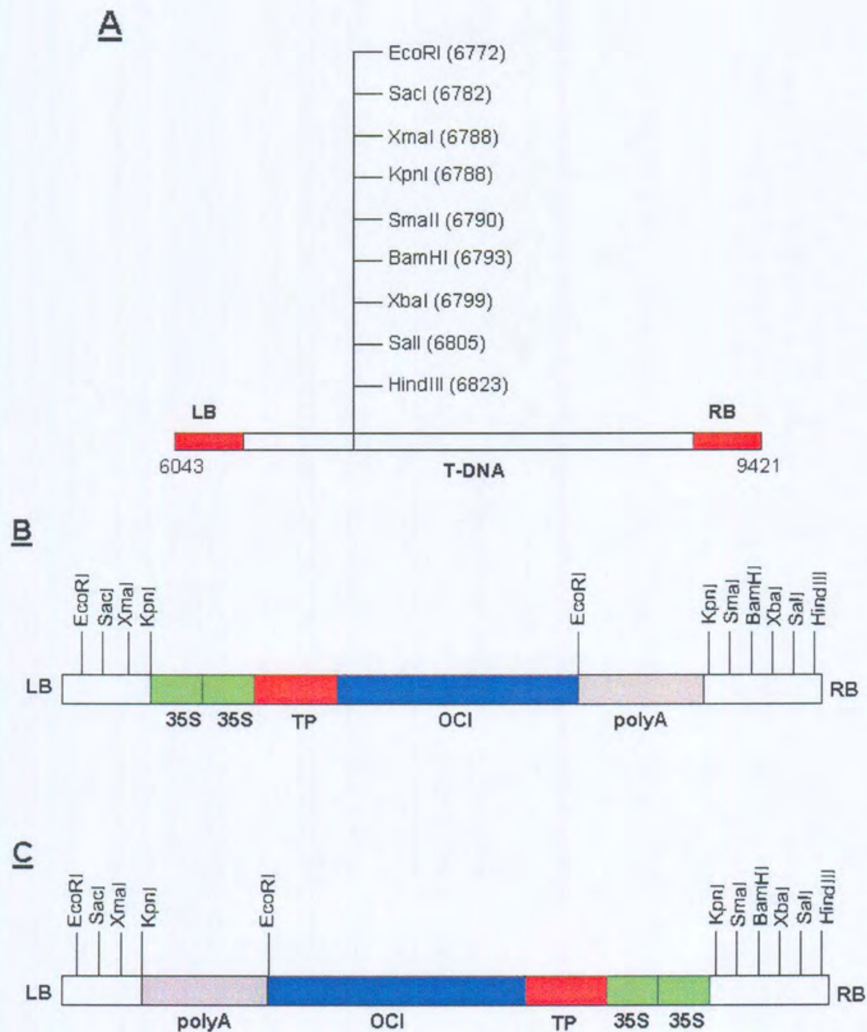

Fig. 3.5.5 Plasmid pBIN19 T-DNA (A) and multiple cloning site carrying the TP-OCI fragment in both orientations (B) and (C). LB – Left border of T-DNA. RB – Right border of T-DNA. TP – trans-peptide sequence. OCI – oryzacystatin I coding sequence. PolyA - CaMV transcription termination sequence.

3.5.2.2 *Transformation and plant selection*

Transformation and regeneration of tobacco was carried out following the protocol outlined under Materials and Methods (Chapter 2), using the plant transformation vector pBIN-TP-OCI and the *Agrobacterium* transformation method. Selection on a kanamycin-containing medium produced 15 shoots,

which were screened by PCR of the OCI sequence, as well as immunoblotting using an antiserum raised against OCI to detect presence and possible expression of the OCI coding sequence in the tobacco genome. For OCI detection by PCR, primers were designed from the OCI sequence reported by Abe *et al.* (1987) using the Web Primer program (http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer) for primer design. The forward primer used for OCI detection was OCI-LI-KK: 5' –TCA CCG AGC ACA ACA AGA AG- 3' and the reverse primer OCI-RI-KK: 5' –CAT CGA CAG GCT TGA ACT CC- 3' (Figure 3.5.6). The melting temperature for primer OCI-LI-KK was 57.3ºC, and for primer OCI-RI-KK it was 59.4ºC.

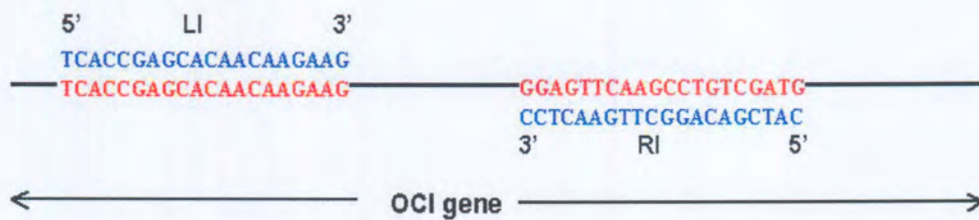

Fig. 3.5.6 Representation of primer binding sites on OCI-encoding sequence. LI – OCI-LI-KK primer; RI – OCI-RI-KK primer.

3.5.3 Results

3.5.3.1 *Vector construction*

A 590bp DNA fragment containing the OCI coding sequence was successfully isolated from the pAOCI-3 plasmid (Figure 3.5.7) by digestion with the restriction enzymes *Pst*I and *Eco*RI and separation on a 1% agarose gel which was used in further cloning procedures.

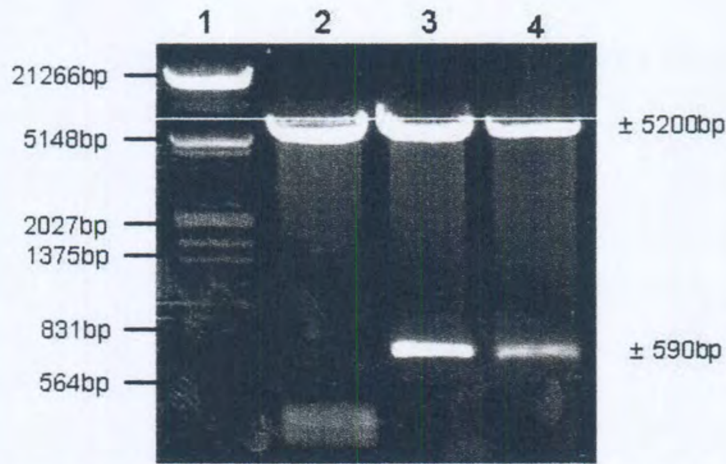

Fig. 3.5.7 Restriction enzyme digestion of plasmid pAOCI-3 separated on a 1% agarose gel and stained with ethidium bromide. Lane 1 represents molecular weight marker III (Roche, Switzerland). Lane 2 represents pAOCI-3 cut with *Kpn*I. Lanes 3 and 4 represent pAOCI-3 cut with *Pst*I and *Eco*RI.

The fragment isolated from plasmid pAOCI-3 was cloned into plasmid pUC18 at the *Pst*I/*Eco*RI sites to create plasmid pUC-OCI. Plasmid pUC-OCI was digested with the restriction enzymes *Pst*I and *Eco*RI (Figure 3.5.8) to identify the cloned OCI fragment. Only plasmids with an insert were used for the further cloning procedures.

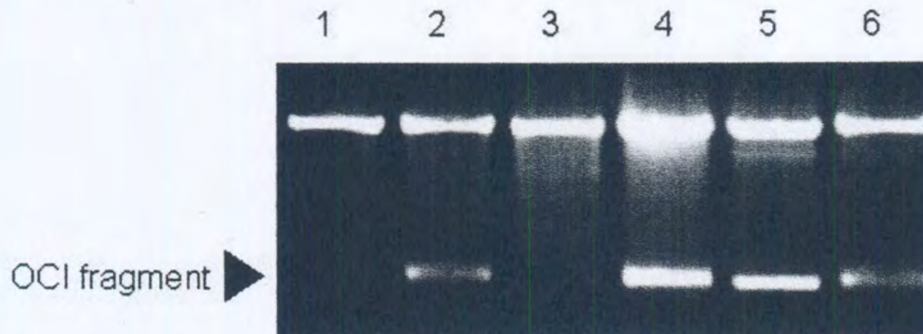

Fig. 3.5.8 Screening of pUC18 plasmids containing the OCI fragment. Plasmids digested with *Pst*I and *Eco*RI, separated on a 1% agarose gel, and stained with ethidium bromide.

Sequencing of one of the pUC-OCI plasmids was successfully carried out (Figure 3.5.9). The OCI fragment (556 bp) was identified from sequence data, and confirmed to be in frame (Figure 3.5.10). This plasmid was used in further cloning procedures.

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  1 GCAAAGCTTGCATGCCTGCAGGTCCCTCATGGCGAGCGACGGAGGGCCGG
 51 TGCTTGGCGGCGTCGAGCCGGTGGGGAACGAGAACGACCTCCACCTCGTC
100 GACCTCGCCCGCTTCGCCGTCACCGAGCACAACAAGAAGGCCAATTCTCT
150 GCTGGAGTTCGAGAAGCTTGTGAGTGTGAAGCAGCAAGTTGTCGCTGGCA
200 CTTTGTACTATTTCACAATTGAGGTGAAGGAAGGGGATGCCAAGAAGCTC
250 TATGAAGCTAAGGTCTGGGAGAAACCATGGATGGACTTCAAGGAGCTCCA
300 GGAGTTCAAGCCTGTCGATGCCAGTGCAAATGCCTAAGGCCCATCTCGTA
350 TCTTATGTGTATCAAGTTATCAAGAAGATGGGGAATAATATGGTGTGGAT
400 ATAGCTATTGGACATGTTAATTATCCACATGATAATATGGCTTGGATATA
450 AGGATCTCACACGATAATATGGCTTGGATATATAGCTATTAAAGATTTTA
500 CCTATGGCATATTTCAATGTGTATTAGTACTAAGTAAGAATGATTGCAAG
550 GTGTATTAACTACAAATATTGCAATAAAAGTCCCATGGGTATATCTCCTT
600 CTTAAAGTTAACGAATTCGTAATCATGGTCATAGCTGTTTCCTGTGTGAA
650 ATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAG
700 TGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTT
750 GCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATT
800 AATGAATCGGCCAA
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Fig. 3.5.9 DNA sequence of pUC-OCI insert using the M13 forward primer for sequencing. OCI coding sequence in blue with start and stop codon (red).

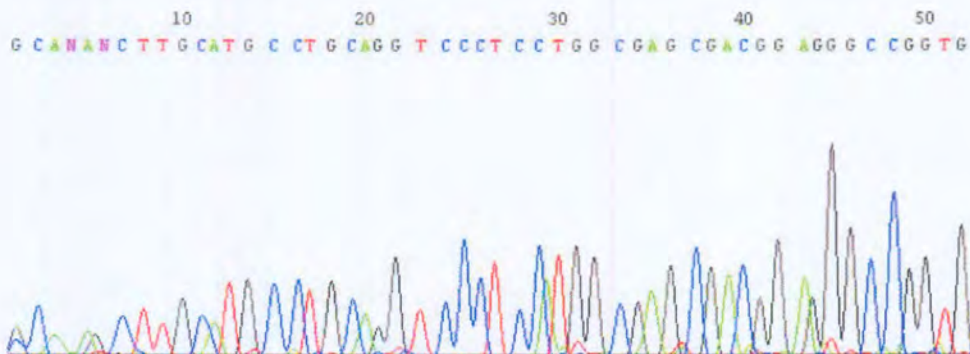

Fig. 3.5.10 Electropherogram showing the first 52 bases of plasmid pUC-OCI sequenced with the M13 forward primer sequenced to confirm correctness of ATG start codon and ATG flanking sequences.

Presence of restriction sites suitable for OCI coding sequence subcloning and transfer of the coding sequence with regulatory elements into plasmid pBIN19 was confirmed in plasmid pJIT117 using restriction enzyme digestions (Figure

3.5.11). Digestion with restriction enzyme *Kpn*I produced two fragments of approximately 1700 bp and 2200 bp (lane 2) demonstrating the two expected *KpnI* sites in pJIT117 (Figure 3.5.11). Both *Eco*RI and *Hin*dIII cut the plasmid only once and produced a fragment of approximately 3900bp (lanes 3 and 4 respectively) which corresponds to the size of plasmid pJIT117 (Figure 3.5.3A).

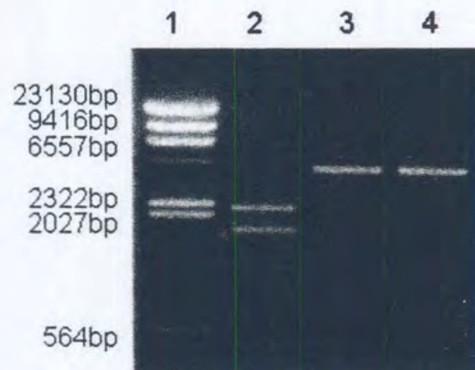

Fig. 3.5.11 Plasmid pJIT117 digested with restriction enzymes. DNA molecules were separated on a 1% agarose gel and stained with ethidium bromide. Lane 1 represents molecular weight marker II (Roche, Switzerland). Lane 2 represents pJIT117 cut with *Kpn*I. Lane 3 represents pJIT117 cut with *Eco*RI. Lane 4 represents pJIT117 cut with *Hind*III.

After digestion of plasmid pUC-OCI with *Pst*I and *Eco*RI to release the OCI fragment, and isolation of the fragment from an agarose gel, the OCI fragment was inserted into plasmid pJIT117 into the *Pst*I/*Eco*RI restriction sites of the multiple cloning site to create plasmid pJIT-OCI. Insertion of the OCI coding sequence into pJIT117 was confirmed by restriction enzyme digestion using *Pst*I/*Eco*RI. Restriction enzyme digestion of pJIT-OCI with *Kpn*I produced 2 fragments (±2360 bp and ±2200 bp) which indicated the presence of an extra ±600 bp in the plasmid, representing the OCI fragment. Restriction enzyme digestion with *Kpn*I and *Hind*III produced expected DNA fragments of approximately 2200 bp, 1400 bp, 700 bp and 300 bp. Restriction enzyme digestion with *Kpn*I and *Eco*RI produced fragments of approximately 2200 bp, 1700 bp, and 700 bp (Figure 3.5.12).

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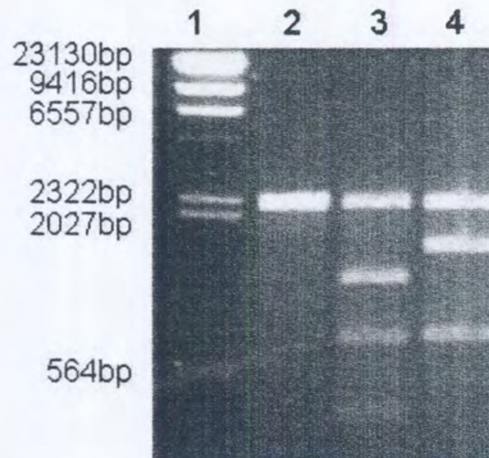

Fig. 3.5.12 Plasmid pJIT-OCI digestion with restriction enzymes. DNA molecules were separated on a 1% agarose gel and visualised under UV light after staining with ethidium bromide. Lane 1 represents molecular weight marker II (Roche, Switzerland). Lane 2 represents pJIT-OCI cut with *Kpn*I. Lane 3 represents pJIT-OCI cut with *Kpn*I and *Hind*III. Lane 4 represents pJIT-OCI cut with *Kpn*I and *Eco*RI.

After plasmid pJIT-OCI was digested with restriction enzyme *Kpn*I, the released fragment (TP-OCI) containing the OCI coding sequence, the targeting sequence, the regulatory elements for OCI expression in plants, and the polyadenylation signal was cloned as a *Kpn*I fragment into the *Kpn*I site of plasmid pBIN19 (Figure 3.5.4) to create plasmid pBIN-TP-OCI. The presence of the TP-OCI fragment was confirmed by restriction enzyme digestion using *KpnI* (Figure 3.5.13 and Figure 3.5.14). Digestion of plasmid pBIN-TP-OCI with *Kpn*I released an expected fragment of approximately 2300bp, corresponding to the TP-OCI fragment. To determine the cloning orientation in pBIN19, plasmid pBIN-TP-OCI was digested with restriction enzyme *Eco*RI (Figure 3.5.13 and Figure 3.5.14), which cuts in the cloned fragment. Determination of fragment size indicates the cloning orientation of the TP-OCI fragment in pBIN19 (Figure 3.5.5B and C).

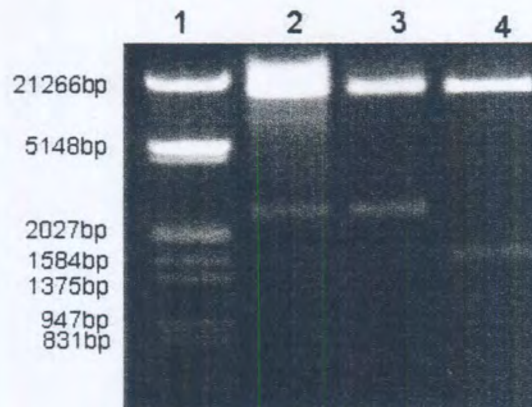

Fig. 3.5.13 Identification of plasmid pBIN-TP-OCI by restriction enzyme digestion and separation on a 1% agarose gel after staining with ethidium bromide. Lane 1 represents molecular weight marker III (Roche, Switzerland). Lanes 2 and 3 represent pBIN19-TP-OCI cut with *Kpn*I. Lane 4 represents pBIN19-TP-OCI cut with *Eco*RI.

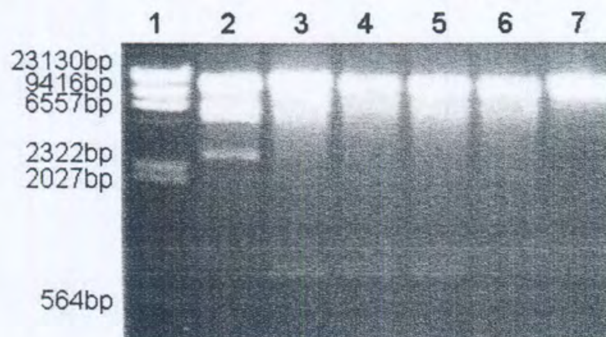

Fig. 3.5.14 Plasmid pBIN-TP-OCI digested with restriction enzyme *Eco*RI, separated on a 1% agarose gel, and stained with ethidium bromide. Lane 1 represents molecular weight marker II (Roche, Switzerland). Lanes 2-7 represent pBIN-TP-OCI digested with *Eco*RI.

3.5.3.2 *Plasmid transfer into Agrobacterium and plant transformation*

After triparental mating in which plasmid pBIN-TP-OCI was transferred into *Agrobacterium*, non-transformed tobacco leaf material was placed in an *Agrobacterium* solution for infection, and subsequent transformation. After disks were left on selective medium for 4 weeks, callus tissue formed on transformed leaf disks. Shoots developed from the callus tissue after 9 weeks,

and putative transformed shoots were placed on rooting medium for root formation. A total of 15 putative transformed shoots were obtained after rooting, and were placed in individual containers on half-strength MS medium for plant growth. After shoots had grown to a height of approximately 4 cm, leaves were detached from the shoots and tested for the presence of the OCI coding sequence in the tobacco genome by PCR using the primers OCI-LK and OCI-RK (Figure 3.5.15). PCR amplification of the OCI coding sequence showed that 8 plants carried the OCI-coding sequence. (Figure 3.5.15 lanes 8 and 9).

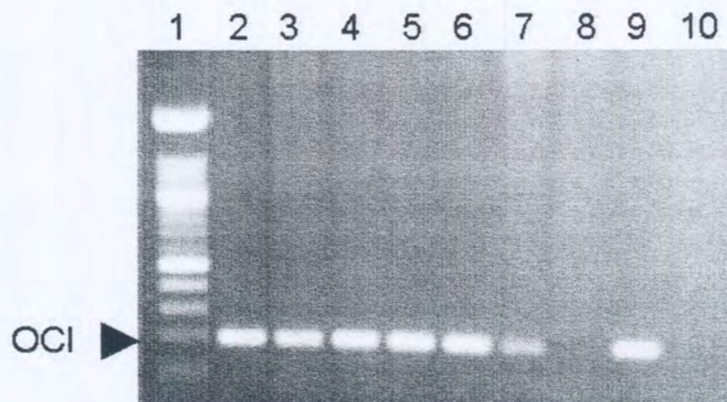

Fig. 3.5.15 PCR screening of putative transformants transformed with pBIN-TP-OCI. Lane 1 represents a hundred basepair marker (Roche, Switzerland). Lanes 2-10 represent PCR products from putative transformed shoot DNA amplified with primers specific for the OCI coding sequence.

Leaves of all putative transformed shoots carrying the OCI-coding sequence were also tested for OCI expression by immuno-blotting with antiserum raised against OCI (Figure 3.5.16). None of the putative transformed plants showed visible expression of OCI at the expected size of about 12kDa using immuno-blotting for OCI expression and an antiserum dilution of 1:10,000.

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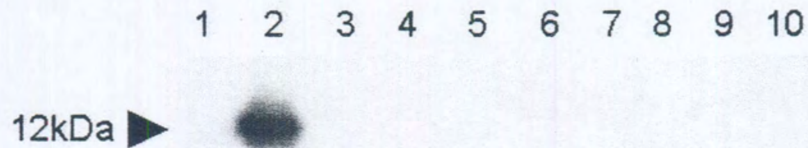

Fig. 3.5.16 Detection of OCI by immuno-blotting (Western blot analysis) with an antiserum after SDS PAGE of plant extracts derived from putative transformed shoots. Lane 1 represents a non-transformed control plant. Lane 2 represents a transformed plant. Lanes 3-10 represent putative transformed shoots.

CHAPTER 4

4. DISCUSSION

Several research groups have studied the role of cysteine proteinases during senescence of plants (Lohman *et al.*, 1994; Buchanan-Wollaston, 1997; Navarre and Wolpert, 1999; Wagstaff *et al.*, 2002) and it is known that especially the large subunit (LSU) of Rubisco, the major photosynthetic and most abundant enzyme in plants, is sensitive to cysteine proteinase degradation (Yoshida and Minamikawa, 1996; Navarre and Wolpert, 1999). Previous studies have further shown that transformed tobacco expressing an exogenous cysteine proteinase inhibitor (OCI) recovers its photosynthetic activity much better after chilling than non-transformed plants (Van der Vyver *et al.*, 2003). It was therefore hypothesized that increased ability to recover from chilling might be due to protection of Rubisco by OCI. This study was therefore aimed to provide evidence whether exogenous OCI expressed in transformed tobacco could possibly interact with endogenous cysteine proteinase activity and prevent degradation of Rubisco. This would ultimately lead to a better understanding of the physiological role of a cysteine proteinase inhibitor in plants under stress.

4.1 Phenotypic Plant Characterization

In this study, transformed tobacco seedlings expressing OCI displayed a ratio of inheritance of 0.76 based on both antibiotic selection and histochemical screening procedures. This ratio is near the expected Mendelian ratio of inheritance of 0.75 for offspring of a selfed, heterozygous parent plant carrying a transgene controlling a single-gene trait (Conner and Christey, 1994). However, the detection of OCI expression in these plants by immuno-blotting showed that a certain number of transformed plants clearly expressing both antibiotic resistance and GUS, did not express OCI at all or at various levels. It was therefore vital to test all plants used in the experiments for OCI expression to avoid use of any non-OCI expressing plants in the experiments.

It is well documented that such variable transgene expression levels occur in plants, including variable expression levels of different transgenes in a single plant, which also frequently does not correlate with the transgene copy number (Dean *et al.*, 1988; Peach and Velten, 1991; Foyer *et al.*, 1995; Matzke and Matzke, 1995). Research has shown that the level of transgene expression can be influenced by the position of integrated T-DNA within the genome (position effects) of a transformed plant (Beilman *et al.*, 1992; Campisi, *et al.*, 1999). This includes the integration of T-DNA near to or far from transcriptional activating elements or enhancers, resulting in the activation (or lack thereof) of a T-DNA-carried transgene. T-DNA can also integrate into a transcriptionally competent or transcriptionally silent region of the plant genome and transgene silencing can also be due to transgene DNA methylation (Day *et al.*, 2000) where the transgene has integrated into a region of the plant genome susceptible to DNA methylation. On the post-transcriptional level the transgene could be transcribed, but the resulting RNA is unstable (Meins, 2000). Such post-transcriptional gene silencing is frequently associated with multiple transgene copies within a cell. Although *Agrobacterium*-mediated transformation usually results in a low copy number of integrated transgenes in many plant species, it is common to find tandem copies of a few T-DNAs integrated at a single locus (Jorgensen *et al.*, 1987). Integration of T-DNA repeats, especially "head-to-head" inverted repeats around the T-DNA right border, frequently results in transgene silencing (Cluster *et al.*, 1996; Stam *et al.*, 1997). However, Elmayan and Vaucheret (1996) found that transgene silencing could also occur in plants harbouring a single integrated T-DNA.

4.2 Protein Degradation

In this study, cysteine proteinases were more active in an acidic buffer system (buffer B) than in a basic buffer system (buffer A). Furthermore, native gel electrophoresis showed that protein degradation in buffer B was less obvious in transformed plants expressing OCI when compared to non-transformed plants. This indicates that expression of exogenous OCI in transformed

tobacco prevents degradation by endogenous cysteine proteinases in transformed tobacco.

Previous results in which the sensitivity of the LSU to degradation by cysteine proteinases was shown were also confirmed in this study (Peñarrubia and Moreno, 1990; Yoshida and Minamikawa, 1996). When purified Rubisco was incubated with the cysteine proteinase papain and Rubisco LSU degradation was monitored using immuno-blotting, LSU was rapidly degraded by the papain. The incubation of crude plant extracts from transformed and non-transformed plants in an acidic extraction buffer, and immuno-blotting to detect LSU degradation products also showed that the LSU is highly degraded in non-transformed plants, but less severely degraded in transformed plants. Also, in this study it was confirmed by both papain and plant crude extract incubation and detection with immuno-blotting that, in comparison to the Rubisco LSU, the SSU is resistant to degradation by cysteine proteinases as already found by Yoshida and Minamikawa (1996).

4.3 Cold Stress and Cystatins

In this study it was found that non-transformed plants have a higher cysteine proteinase activity than transformed tobacco expressing exogenous OCI confirming previous findings by Van der Vyver *et al.* (2003). Furthermore, cysteine proteinase activity, as measured by the fluorometric enzyme activity assay, did not increase in either non-transformed or transformed plants after chilling although transformed plants had consistently lower cysteine proteinase activity than non-transformed plants regardless of being chilled or not. Research has shown that abiotic stress, such as low temperature, increases the rate of protein degradation and that acidic proteinase activity, which includes cysteine proteinase activity, increases after exposure to stress (Cooke *et al.,* 1980). However, this rise is seemingly transient, and generally the activities of the proteinases decline following the transfer to adverse growth conditions as already shown several years ago (Cooke *et al.*, 1979 and 1980). It was hypothesized that the increased protein degradation found is due to stress-induced changes in the properties of the vacuolar tonoplast,

and that various stress conditions alter the permeability of the tonoplast allowing an increased efflux of vacuolar amino acids into the cytoplasm. The permeability of the tonoplast, however, changes only under severe stress conditions, such as in the late stages of senescence.

Although a higher cysteine proteinase activity in non-transformed plants compared to transformed plants was found in this study, native PA gel quantification of the Rubisco holoprotein indicates that both types of plants have almost unchanged amounts of Rubisco holoprotein regardless of the temperature treatment. This was also confirmed by spot densitometric quantification. This technique and also radioactive quantification of Rubisco further confirmed results obtained with native gel quantification that expression of exogenous OCI in transformed tobacco does not significantly prevent Rubisco degradation during chilling. Therefore, even though there was always a higher cysteine proteinase activity in non-transformed tobacco than in transformed tobacco, this, very likely, does not directly affect Rubisco levels during chilling. Therefore, the plant cell might not utilise cysteine proteinases for Rubisco degradation during chilling. Also, the Rubisco LSU degradation profile of plants did not significantly differ under chilling in transformed and non-transformed plants. This shows that there is no direct detectable benefit regarding protection of LSU by OCI against proteinase action in transformed plants during chilling. Furthermore, a recent study done at IACR-Rothamsted investigating Rubisco activity and activation state before exposure to a chill, after a chill, and after a 2-day recovery period also indicated no difference between transformed and non-transformed plants in both activity and activation state (Foyer, personal communication). This means that improved recovery rates in photosynthesis in transformed tobacco plants cannot be ascribed to detectable differences in Rubisco activity or activation state between transformed and non-transformed tobacco. This also shows that OCI does not affect the transformed plants on Rubisco activity level. The lack of any clear evidence for OCI protection of Rubisco found in this study stands despite the fact that several studies have previously shown a linkage between oxidative modification of Rubisco occurring during chilling and enhanced susceptibility of the enzyme to proteolysis by cysteine

proteinases (Peñarrubia and Moreno, 1990; Mehta *et al.*, 1992; Desimone *et al.*, 1996). Also, in a previous study on the regulation of cell death by cysteine proteases, ectopic expression of a soybean cystatin led to an effective block in H_2O_2-induced cysteine proteinase activity involved in programmed cell death (PCD) (Solomon *et al.*, 1999). It might therefore be speculated that OCI has a low binding affinity to the type of tobacco cysteine proteinase involved in Rubisco degradation or that the amount of cysteine proteinases employed by tobacco is too low to be significant. Cellular compartmental barriers, such as the chloroplast membranes and the tonoplast, also prevent interaction of OCI expressed in the cytosol, Rubisco LSU encoded in the chloroplast, and cysteine proteinases which are most likely located in the vacuole. Membranes might remain intact despite exposure to a stressful condition, thereby preventing interaction of components of the cytosol, chloroplast, and vacuole.

4.4 Plant Senescence and Cystatins

In this study, senescent tobacco leaf material of both transformed and non-transformed tobacco wilted and turned yellow. This is a distinct characteristic of the senescence programme, indicating the loss of chlorophyll and the disassembly of the chloroplast (Lohman *et al.*, 1994). Under senescent conditions the total cysteine proteinase activity increased to the same level in both transformed and non-transformed leaves. This result is in contrast to the result obtained during chilling, where cysteine proteinase activity did not increase in either transformed or non-transformed plants. This study induced senescence by detaching leaves. It is known that there are differences in the way attached and detached leaves senesce, including differences in the amounts and types of proteinases that develop (Miller and Huffaker, 1985) and differences in ultrastructural changes in chloroplasts (Hurkman, 1979). Detachment of leaves to induce senescence was used due to the experimental convenience of accelerated uniform senescence and independence from hormonal and nutritional influences of other organs (Morris *et al.*, 1996).

Although loss of total amount of Rubisco protein was partially prevented under senescent conditions by OCI expression in transformed plants, the degradation pattern of Rubisco LSU between transformed and non-transformed plants was not significantly different under senescent conditions, as shown by immuno-blotting. In general, proteolysis of chloroplast proteins begins in an early stage of senescence and the liberated amino acids can be exported to growing parts of the plant (e.g. maturing fruits) (Hörtensteiner and Feller, 2002). Endopeptidases, such as metallo-endopeptidases and amino-peptidases, have been detected in the chloroplast, and they contribute to the complete degradation of stromal proteins (Miller and Huffaker, 1985; Bushnell *et al.*, 1993). However, carboxypeptidases, such as cysteine proteinases, have so far not been detected in intact plastids and these proteinases are believed to be located in the vacuole (Feller and Fischer, 1994). The vacuole is also the destination of the final products of chlorophyll catabolism (Matile *et* al., 1999; Thomas *et al.*, 2001). High cysteine proteinase and carboxypeptidase levels are present in the vacuole, and increased transcript levels (Lohman *et al.*, 1994; Buchanan-Wollaston, 1997) as well as increased activities (Feller *et al.*, 1977; Wagstaff *et al.*, 2002) of cysteine proteinases have been observed during senescence. The function of these cysteine proteinases may be restricted to degradation processes occurring after the rupture of the tonoplast at the end of senescence.

For Rubisco, there are two main views held about the degradation of the enzyme in senescing leaves. Firstly, it is believed that the vacuole is the major intracellular compartment responsible for the degradation of Rubisco (Wittenbach *et al.*, 1982). Secondly, it is believed that Rubisco is hydrolysed by proteolytic enzymes inside the chloroplast, with the digestion products being exported (Huffaker, 1990). Wittenbach and co-workers (1982) reported that virtually all of the endoproteinase activity remains in the vacuole throughout the senescence of wheat leaves. Furthermore, Matile and co-workers (1988) reported that degradation products of chlorophyll are found in vacuoles as barley leaves senesce. They proposed that vacuoles serve as lysosomes in plant cells being involved in the degradation of proteins by invagination of the cytoplasm. Membrane blebbing is also a result of

senescence (Hadfield and Bennet, 1997), and might provide another means by which chloroplastic proteins, such as Rubisco, are degraded by vacuolar proteinases. Results in this study also confirm previous studies showing sensitivity of Rubisco LSU to degradation by cysteine proteinases. However, it remains to be elucidated in future research how different cellular compartments might interact in case a cytosol-expressed cystatin protects a chloroplast-located protein like Rubisco from degradation by a vacuole-located proteinase.

4.5 OCI Expression in the Chloroplast

Since this study gave no clear evidence that an exogenous cytosol-expressed cysteine proteinase inhibitor can protect a chloroplast-located protein under stress, a study was initiated as a second part of this thesis with the aim to determine whether targeting OCI expression into the chloroplast might provide any protection of Rubisco against degradation in the chloroplast itself. Although a construct was used allowing protein targeting to the chloroplast and several putative transformed tobacco shoots were regenerated on a selective medium from transformed tobacco leaf disks and screened by PCR for the presence of the OCI coding sequence, no OCI-expressing transformed plant could so far be detected. Failure can be explained in a number of ways: First, it is difficult to predict expression levels and stability of a transgene (Conner and Christey, 1994). The position of integration in the plant genome affects transgene expression greatly, and this can vary in a population of plants independently transformed with the same transgene (Gelvin, 1998). This position effect is believed to be a consequence of each transformation event involving random integration of the transgene into different sites of the plant chromatin (Dean *et al.*, 1988). Also, the presence or absence of transcriptional activating elements or enhancers which results in the transcription of T-DNA, or the lack thereof might be important (Beilman *et al.*, 1992; Campisi, *et al.*, 1999). Post-transcriptionally, the transgene might be silenced by the presence of multiple RNA transcripts of the gene that bind to each other and prevent translation (Cluster *et al.*, 1996; Stam *et al.*, 1997). Also, T-DNA integration is not accurate, and vector DNA has been known to

integrate into plant genome sequences along with the transgene (Martineau *et al.*, 1994; Matzke and Matzke, 1998), which is associated with aberrant transgene expression. Further investigation should be launched into the transcriptional state of the transit peptide-OCI sequence, since it might also be possible that the sequence is transcribed, but not translated. The level of transcription might also be so low that the amount of protein possibly expressed by the plant is not detectable by immuno-blotting under the conditions applied in this study. Furthermore, it is difficult to predict whether the OCI protein can be successfully transported across the chloroplast membrane, whether the processing enzymes within the chloroplast will correctly modify the protein by cleaving the transit peptide from the pre-protein, and whether the OCI protein will have the correct structure in the environment of the chloroplast.

4.6 Achievements and Future Perspective

4.6.1 Achievements

In this study previous research results could be confirmed and several new technical and scientific achievements have been made. This study confirmed the results by Yoshida and Minamikawa (1996) that Rubisco LSU is sensitive and SSU resistant to cysteine proteinase degradation. Furthermore, it confirmed that there is an increase in cysteine proteinase activity in plants under cold stress, as found by Schaffer and Fischer (1988) and Van der Vyver and co-workers (2003). The study also confirmed that cysteine proteinases play a role in senescence (Granell *et al.,* 1992; Morris *et al.*, 1996; Wagstaff *et al.*, 2002).

Several technical achievements were also made in this study. In particular this includes the design, construction and cloning of a vector (pBIN-TP-OCI) allowing (1) transformation of tobacco with a transgene directing expressed OCI to the chloroplast and (2) the selection of transformed tobacco plants carrying the OCI transgene. Also, a number of new techniques for measuring Rubisco content and stability were acquired and successfully implemented

into the research group. These include gel-based protein quantification, whereby dye was extracted from stained protein bands excised from a native PA gel after electrophoresis, and subsequent quantification of the dye. Radioactive quantification of Rubisco was applied, in which the radioactively labelled inhibitor of Rubisco, ^{14}CABP, was used to quantify the amount of enzyme in tobacco plants before and after exposure to cold stress. This procedure was acquired during a two-month visit to Rothamsted Research in Harpenden, Hertfordshire, England. Spot densitometric protein quantification was also acquired during my visit to Rothamsted Research, in which the intensity of protein bands were compared using computer software, after proteins had been extracted from plants and separated by SDS-PAGE.

Finally, two scientific achievements have been made in this study. First, it could be demonstrated that there is no significant protective effect of an exogenous cystatin expressed in the cytosol on the degradation of Rubisco in plants under cold stress as recently hypothesized by Van der Vyver and co-workers (2003). This clearly points the way to further investigations regarding the effect an exogenous cystatin might have on recovery of photosynthesis in transformed tobacco, as well as the other physiological changes described in the publication of Van der Vyver and co-workers (2003). Secondly, this study gave first evidence of a possible protective effect of exogenous OCI on Rubisco holoprotein under a severe stress condition, such as prolonged senescence. This protective action of exogenous OCI requires, however, a much more detailed investigation in the future.

4.6.2 Future perspective

This study formed part of a continuous body of research in molecular plant physiology and abiotic stress. In future, the results obtained from this study might contribute to develop plants that are more resistant/tolerant to unfavourable environmental conditions, such as chilling, as well as providing plants that are more nutritionally dense. In particular, a more advanced understanding of any benefit/disadvantage of expression of an exogenous

cystatin on endogenous cysteine proteinase activity during senescence and under cold stress was gained.

Future research might focus in greater detail on (1) the metabolic involvement of OCI in providing better photosynthesis recovery in transformed tobacco after being exposed to chilling. A better understanding of the interaction of OCI with other cytosolic proteins that are important in photosynthesis and stress response is necessary. For example, OCI might protect the chaperone proteins that transport nuclear-encoded proteins to other cellular organelles where they are active. Furthermore, OCI could protect important anti-oxidants, such as ascorbate peroxidase. Another important cytosolic enzyme that might be protected by OCI is sucrose-phosphate synthase (SPS), which is the key regulatory enzyme in sucrose synthesis in the cytosol. SPS is known to be very sensitive to chilling stress (Jones *et al.*, 1998). Inhibition of SPS activity and sucrose synthesis by chilling stress results in phosphate limitation within the chloroplast, which limits photosynthesis.

Future research might also focus on (2) the possible implications of delayed senescence in OCI-expressing plants, as well as (3) any further beneficial role an exogenous cystatin might have in a plant. Since senescence has an important function in plant homeostasis (Vierstra, 1996), future research has also to answer the question if prevention by a cystatin of protein breakdown by cysteine proteinases in a plant is, at all, a desirable aim. Prevention of protein breakdown might be disadvantageous when the plant is using this process as a means to protect itself from attack by fungi or other pathogens and further as a source of amino acids for further protein synthesis and re-growth after a stress period. Entering senescence prematurely and sacrificing a part of a plant's biomass might actually benefit the plant by providing nutrients, which can be recycled from senescing tissue, while simultaneously depriving the pathogen of living cells and sustenance (Navarre and Wolpert, 1999). In contrast, Solomon *et al.* (1999) suggest that proteinase inhibitors might have a crucial role in preventing unwanted cell death in plants, such as that which may occur after wounding caused by insect chewing, or during chilling-induced oxidative stress. In case the plant in question is an

economically important crop then resistance to unnecessary cell death caused by pathogens or abiotic stress would be beneficial. In addition, an increase in the protein content of a plant, due to the prevention of protein degradation by proteinases, would further mean a more nutrient dense crop, which might benefit humans and animals.

ANNEXURE

BUFFERS FOR PROTEIN WORK

Extraction buffer for protein degradation studies:

50mM Tris-HCl (pH 8.9) (Buffer A)

606mg Tris was added to 180mL distilled water. The pH was adjusted to pH 8.9 with 0.1M HCl, and the final volume made up to 200mL.

50mM sodium acetate (NaAc)/ 10mM β-mercaptoethanol (pH 5.4) (Buffer B)

820mg NaAc was added to 180mL distilled water. The pH was adjusted to pH 5.4, and the final volume made up to 200mL. β-mercaptoethanol was diluted to a 50mM stock solution on the day of use (35uL β-mercaptoethanol added to 10mL dH_2O), and 100uL of this stock solution was added to 400uL of the 50mM NaAc extraction buffer to get a final concentration of 10mM β-mercaptoethanol.

Table A.1 Extraction buffer for radioactive labelling of Rubisco (pH 8.2)

Chemical	Concentration	Mass/ Volume
Bicine	50 mM	4.08g/ 500mL buffer
$MgCl_2$	20 mM	0.95g/ 500mL buffer
EDTA	1 mM	0.146g/ 500mL buffer
β-mercaptoethanol	50 mM	350 uL/ 100mL buffer
Protease inhibitor cocktail	1%	10 uL/ 1mL buffer

A stock solution of bicine (Sigma, UK), $MgCl_2$, and EDTA was prepared a day before use. β-mercaptoethanol was added on the day of use. The protease inhibitor cocktail was added to individual samples.

Table A.2 Activation buffer (pH 8.0)

Chemical	Concentration	Mass/ Volume
Bicine(Sigma, UK)	100mM	8.16g/ 500mL buffer
$MgCl_2$	20mM	0.95g/ 500mL buffer
$NaHCO_3$	10mM	0.42g/ 500mL buffer
Na_2SO_4	0.6M	42.6g/ 500mL buffer
^{14}CABP		
β-mercaptoethanol	50 μM	1 μL of a 50mM stock solution/1mL buffer

A stock solution of bicine (Sigma, UK), $MgCl_2$, $NaHCO_3$ and Na_2SO_4 was prepared a day before use. β-mercaptoethanol was added on the day of use.

Transfer buffer for Western Blot (pH 8.2)

25mM Tris, 192mM glycine, 15% methanol. 3.03g Tris and 14.4g glycine were dissolved in 150mL methanol. The solution was brought to 1 litre with dH_2O. The solution was stored at 4°C.

10x Tris buffered saline (TBS) (pH 7.6)

24.23g Tris and 292g NaCl were dissolved in 700mL dH_2O, pH was adjusted to pH 7.6 with HCl, and the final volume was brought to 1L with dH_2O. For further use the buffer was diluted 10-times and stored at 4°C.

GEL ELECTROPHORESIS SYSTEMS

30% Acrylamide mix (stock solution):

29g acrylamide and 1g bisacrylamide were dissolved in warm, distilled water and brought to 100mL with dH_2O, to give a final ratio of 29:1 (w/w) acrylamide:bisacrylamide. The solution was stored at 4°C.

1M Tris-HCl, pH 6.8 (stock solution):

12g Tris was dissolved in 80mL dH_2O, titrated to pH 6.8 with 1M HCl and brought to a final volume of 100mL with dH_2O.

1.5M Tris-HCl, pH 8.8 (stock solution):

18.17g Tris was dissolved in 80mL dH_2O, titrated to pH 8.8 with 1M HCl and brought to a final volume of 100mL with dH_2O.

Table A.3 Polyacrylamide gel (non-denaturing) 6%

Stock solution	**6% Resolving gel**
30% acrylamide mix	2mL
1.5M Tris (pH8.8)	2.5mL
10% Ammonium persulphate	0.1mL
TEMED	8μL
Water	5.4mL

Tris glycine electrophoresis buffer for non-denaturing PAGE gel:

Electrophoresis buffer stock (5x) (0.125M Tris, 1.25M glycine, pH 8.3): 15.1g Tris and 94g glycine were dissolved in 900mL dH_2O. The pH was adjusted to pH 8.3, and the final volume made up to 1L. The solution was stored at 4°C and diluted 5 times before use.

Loading buffer for non-denaturing PAGE gel:

1 x buffer [50mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 100mM dithiothreitol (DTT), 0.01% (w/v) bromophenol blue]: 1.7mL 1.5M Tris (pH 6.8) and 5mL glycerol were mixed. Bromophenol blue (2.5mg) was added and the solution was brought to a final volume of 50mL with dH_2O. DTT was added to the solution on the day of use from a 1M stock solution, to bring the final concentration of DTT to 100mM.

Table A.4 SDS-PAGE gel (5-10%) (Sambrook *et al.*, 1989)

Stock solution	Stacking gel	**10% Resolving gel**
30% acrylamide mix	0.33mL	1.7 mL
1M Tris (pH 6.8)	0.25mL	-
1.5M Tris (pH 8.8)	-	1.3 mL
10% SDS	0.02 mL	0.05 mL
10% Ammonium persulphate	0.02 mL	0.05
TEMED	2 μL	0.002 mL
Water	1.4 mL	1.9 mL

Table A.5 SDS-PAGE gel (5-12%)

Stock solution	**Stacking gel**	**12% Resolving gel**
30% acrylamide mix	0.33mL	2.0 mL
1M Tris (pH 6.8)	0.25mL	-
1.5M Tris (pH 8.8)	-	1.3 mL
10% SDS	0.02 mL	0.05 mL
10% Ammonium persulphate	0.02 mL	0.05 mL
TEMED	2 μL	2 μL
Water	1.4 mL	1.6 mL

Electrophoresis buffer for SDS PAGE gel:

Electrophoresis buffer stock (5x) [0.125M Tris, 1.25M glycine, pH 8.3, 0.1% (w/v) SDS]: 15.1g Tris and 94g glycine were dissolved in 900mL dH_2O. The pH was adjusted to pH 8.3 and 50mL of a 10% (w/v) stock solution of SDS was added. The final volume was made up to 1L with dH_2O. The solution was stored at 4°C and diluted 5 times before use.

Loading buffer for SDS PAGE gel:

1 x buffer [50mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 100mM DTT, 0.01% (w/v) bromophenol blue]: 1.7mL 1.5M Tris (pH 6.8) and 5mL glycerol were mixed. 10mL from a 10% (w/v) SDS stock solution was added.

Bromophenol blue (2.5mg) was added and the solution was brought to a final volume of 50mL with dH_2O. DTT was added to the solution on the day of use from a 1M stock solution, to bring the final concentration of DTT to 100mM

Table A.6 Staining solution for PAGE gels:

Chemical	Concentration	Mass/ volume
Coomassie Brilliant Blue R-250	0.1%	0.1g
Water	65%	65mL
Propan-2-ol	25%	25mL
Glacial Acetic Acid	10%	10mL
Total Volume		100mL

1% Agarose gel for separation of DNA

1g Agarose was added to 100mL 1xTAE buffer and heated in a microwave until boiling. After solution had cooled down to 60°C, 2μL of a 10 mg/mL stock solution ethidium bromide was added. The ethidium bromide intercalates between the DNA basepairs, and fluoresces under UV light, so that the DNA can be visualised.

BUFFERS FOR DNA WORK

Table A.7 TAE buffer (50x stock)

Chemical	Concentration	Mass/ Volume
Tris	2M	242g
EDTA disodium salt	0.1M	37.2g
Glacial acetic acid	5.71% (w/v)	57.1mL
Total volume		1L

A 50x stock solution was prepared by mixing the ingredients and adjusting the pH to pH 8.5. This stock solution was stored away from light, and diluted 50 times for use.

Table A.8 DNA loading buffer

Chemical	Concentration	Mass/ Volume
Glycerol	50%	5mL
1x TAE buffer	1x	200 μL of 50x stock
Bromophenol blue	1%	0.1g
Total volume		10mL

Loading buffer was prepared as indicated in table A.8, and stored at 4°C.

Polymerase chain reaction

PCR was done using a reaction mixture containing a 1x PCR reaction buffer (TaKaRa, Japan), 0.5mM of each deoxynucleotide triphosphate (dCTP, dGTP, dATP, and dTTP) (TaKaRa), 0.4μM of each primer (OCI-LI-KK and OCI-RI-KK) (Roche), and 1 unit Taq polymerase (TaKaRa, Japan) per 50μL reaction mixture.

GROWTH MEDIA

Bacterial growth medium (Luria-Bertani broth):

To 1L distilled water, 10g Tryptone, 5g Yeast extract, and 10g NaCl were added and the pH was adjusted to 7.4. For solid LB medium on which bacterial colonies were grown, agar was added at a concentration of 10g/L.

Plant growth medium (Murashige and Skoog medium):

Full-strength Murashige and Skoog basal salts including vitamins (Highveld Biological LTD, South Africa) were used (Murashige and Skoog, 1962). Sucrose (2%) was added. In order to get solid growth medium, agar was added at a concentration of 8g/L.

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