Protection of recombinant glutathione reductase by Oryzacystatin-I in transgenic tobacco

By

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DECLARATON

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.

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ABSTRACT

Protein degradation poses a significant challenge for the efficient production of recombinant proteins in plants, affecting the stability and yield of the recombinant protein. In this study the *E. coli*-derived enzyme glutathione reductase (GR) was transiently expressed in transgenic tobacco plants constitutively expressing the cysteine protease inhibitor OC-I and non-transgenic plants. A protein resembling the GR was detected in infiltrated leaves. Transiently expressing GR in transgenic *N tabacum* plants resulted in almost two fold significant increases in GR activity. Transgenic tobacco plants expressing the rice cysteine protease inhibitor OC-I had significantly lower cysteine protease activity when compared to non-transgenic tobacco plants. Lower cysteine protease activity in transgenic plants was directly related to higher GR activity and also higher GR amounts in transgenic plants. The study has demonstrated that OC-I is an effective companion protease inhibitor candidate with the potential to protect other high value proteins such as GR, from cysteine protease degradation.

THESIS COMPOSITION

Chapter 1 of this thesis elaborately discusses the expression systems for the production of recombinant proteins as well as transformation methods more commonly used such as transient agro-infiltration. This chapter also outlines the current knowledge about the various plant host systems for recombinant protein production. The last part of this chapter provides information on the influence of cysteine proteases on recombinant protein stability and the use of protease inhibitors to prevent protein degradation, with the aim and objectives of the study outlined at the end of the introduction. **Chapter 2** comprises the materials and methods used in this study. The agro-infiltration process and the use of various molecular biology techniques such as SDS-PAGE, Fluoroimetric assays, western blotting are described. In **Chapter 3** the results obtained for transient expression of GR are presented. This chapter further reports on the protease profiling and leaf phenotypic changes after agro-infiltration. Finally the detection of recombinant bacterial GR and OC-I is reported in this chapter. In **Chapter 4** the results obtained are discussed and key results, which have contributed to a better understanding of how specific endogenous plant proteases contribute to recombinant protein degradation and whether this process can be prevented by co-expression of a protease inhibitor, are highlighted. Under **References** a list of the literature used and cited in this thesis is provided.

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ABBREVIATIONS AND SYMBOLS

AACT	α-antichimotrypsin
aadA	aminoglycoside resistance protein
A. tumefaciens	*Agrobacterium tumefaciens*
AMC	Aminomethyl coumarin
APS	Ammonium persulfate
BoPI	*Brassica oleracea* protease inhibitor
bp	Base pair
BSA	Bovine Serum Albumin
cm	Centimetre
CP	Cysteine protease
CT	Cholera toxin
Cv.	Cultivar
Cys	Cysteine
dH_2O	Sterile distilled water
DMSO	Dimethyl sulfoxide
DTNB	5, 5'-dithiobis (2-nitrobenzoic acid)

E. coli	*Escherichia coli*
e.g.	Example
E-64	Trans-(epoxysuccinyl)-L-leucylamino-4-guanidine butane
EDTA	Ethylenediamine tetra acetic acid
ER	Endoplasmic reticulum
FMDV	Foot and mouth disease virus
FU	Fluorescence unit
g	Gram
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidised glutatione
GUS	β-glucoronidase
H+	Hydrogen ion
H_2O	Water
HBsAg	Hepatitis B surface antigen
Hrs	Hour
IgA	Immunoglobin A
IgGs	Immunoglobins G

Kan	Kanamycin
kDa	Kilo Dalton
L	Litre
LB	Luria broth
LT-B	Labile toxin-B
M	Molar
mA	Milliamperes
mg	Milligrams
$MgCl_2:6H_2O$	Magnesium chloride hexahydrate
min	Minute(s)
mL	Millilitre
mM	Millimolar
MS	Murishage and Skoog
MU	4-methyl umbelliferone
MUG	4-methyl umbelliferyl glucoronide
Na_2CO_3	Sodium carbonate
Na_2EDTA	Diaminetetraacetic acid
	Phosphate (oxidised)
NaH_2PO_4	Sodium hypophosphate

$NaPO_4$	Sodium phosphate
$(NH_4)2SO_4$	Ammonium phosphate
nm	Nanometer
OC-I	Oryzacystatin-I
OD	Optical density
PCR	Polymerase chain reaction
PI	Protease inhibitor
PVDF	Polyvinylidene difluoride
Rif	Rifampicin
RNA	Ribonucleic acid
rpm	Rotations per minute
SAGs	Senescence associated genes
ScFvs	Single chain antibodies
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium adodecyl sulphate polyacrylamide gel electrophoresis
sec	Seconds
SEKDEL	seryl-glutamyl-lysyl-aspartyl-glutamyl leucine
TAE	Tris-acetate EDTA

TBS	Tris-buffered Saline
TEMMED	N, N, N-tetramethyl ethylenediamine
Ti-plasmid	Tumour inducing plasmid
TNB	5'-thio-2-benzene
Tris	2-amino-2-(hydromethyl) propane-1.3
TTBS	Tween 20-Tris buffered saline
U	Unit
UV	Ultra violet
V	Voltage
v/v	Volume per volume
Z-Phe-Arg-AMC	Benzyloxycarbonyl-phenylanine-arginine-aminomethyl coumarin
µg	Microgram
µL	Microlitre
µM	Micromolar

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plasmid DNA carrying the OC-I coding sequence. The control containing no added DNA did not amplify any OC-I fragment (Lane 13). M represents the 100bp marker.

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CHAPTER ONE

INTRODUCTION

1.1 Recombinant protein production

Historically, bacterial cells have been widely used for protein production and human insulin being the first recombinant human protein produced in the 1970's on a commercial scale was accomplished using a bacterial-based expression system. Although bacteria are a convenient production system, they are lacking in ability to perform important mammalian post-translational modifications (Fischer *et al*., 2003) and they also can produce endotoxins that are difficult to remove. Further, recombinant proteins produced using a bacterial system are often occur in inclusion bodies requiring post purification processes to produce a native and active product (Schillberg *et al*., 2003; Yin *et al*., 2007). Mammalian cell cultures are further used for the production of most commercially available monoclonal antibodies but this process is often costly and also requires safety measures to remove oncogenic sequences and viral contaminants when the protein is for therapeutic usage (Fischer *et al*., 2000).

Plants are increasingly becoming an attractive system for the production of recombinant proteins. Plants, as expression systems, have certain advantages over other systems which include:- low cost of production; easy adjustment to large scale production and absence of human pathogens (Goulet *et al*., 2006). One further advantage, in comparison to yeast and *E. coli*, is the ability of the plants cell to carry out translational modifications required for protein activity and kinetic properties which has been illustrated by their capacity to produce functional forms of complex mammalian proteins such as secretory IgA (Faye *et al*., 2005).

In comparison to bacterial and mammalian systems, in plant-based production systems the production rate can also be effectively increased by increasing the agricultural land used to cultivate for example transgenic plants, such as tobacco, for protein production. Also plants

have not infectious agents harmful to end-users such as humans or mammals. Avidin and β-glucuronidase, which are both commercially available, have been produced in plants at low and competitive cost (Kusnadi *et al*., 1998). The production potential of plants for foreign protein production was also illustrated by Daniell *et al*. (2001) where cost per gram IgA antibody produced using transgenic plants was significantly lower than for other expression systems.

Although plant-based production systems are becoming economically viable, several challenges still remain. This includes optimization of protein yield and also the quality of the recombinant protein produced. Several factors influence the potential yield of the recombinant protein and these include: - (i) choice of production plant; (ii) stability of the recombinant protein produced; (iii) expression vector used to drive transgene expression when a transgenic plant approach is applied and finally (iv) purification of the recombinant protein from plant tissues (Du Santos and Wigorovitz., 2005; Twyman *et al*., 2003; Yin *et al*., 2007).

1.2 Plant transformation

Plant transformation methods involve transferring foreign DNA, such as protein coding sequences, into the plant cell where the coding sequence is under the control of regulatory elements (promoter and terminator sequence) (Stitt and Sonnewald, 1995). There are two major transformation systems which are stable genetic transformation and transient gene expression.

1.2.1 Stable genetic transformation

Agrobacterium-mediated gene transfer is the most widely used technique for stably transforming plants. *Agrobacterium tumefaciens,* which is a soil borne bacterium, carries virulence genes located on the tumor-inducing (Ti) plasmid. This plasmid also contains transfer DNA (T-DNA), which can carry foreign DNA, allowing integration into the plant genome after Agrobacterium infection (Tzfira and Citozsky, 2006). In Agrobacterium-mediated transformation, foreign DNA is incorporated into the nuclear genome of a transgenic plant. Transgenic plants can also be generated by introducing the DNA into plastids, such as the chloroplast genome, usually by particle bombardment. Because plastids (chloroplast) are inherited maternally, transgenes expressed in chloroplast are not transmitted through pollen, therefore reducing the risk of gene transfer from transgenic plants to non-transgenic plants (Ruf *et al*., 2007). Chloroplast transformation is further advantageous due to the high copy number and absence of gene silencing (Daniell *et al.*, 2002).

One problem with Agrobacterium-mediated stable transformation is that the process to obtain stably transformed plants can be time consuming. It can take several months before any regenerated transgenic plants are available and selection of high expressing plants can be carried out for expression of the foreign protein of interest. While Agrobacterium-mediated transformation is a more efficient transformation method for dicotyledonous species, particle bombardment is more suited for monocot plants (Christou, 1993; Ko and Koprowski, 2005).

1.2.2 Transient gene expression

A rapid method for recombinant production is transient gene expression (Kapila *et al*., 1996). Three major transient expression techniques are applied for gene delivery into a plant cell including: (i) particle bombardment; (ii) agro-infiltration and (iii) infection of plant cells with a viral vector (Negrouk *et al.*, 2005). While particle bombardment can only produce rather small quantities of recombinant protein, due to affecting only a few cells, transient gene expression application of viral vectors produces high amounts of proteins due to high replication of viral particles in 2-4 weeks (Lico *et al*., 2008) However, viral vector infection results in a systemic spread of the virus throughout the entire plant. Agro-infiltration affects a much higher number of cells when compared to particle bombardment (Kapila *et al.*, 1996) and the transgene can be further transferred into the nucleus (Porta and Lomonossoff, 1996).

In agro-infiltration plant cells are suspended in modified *A. tumefaciens* which mechanically impregnate under vacuum plant cells with the gene construct for expression of the desired protein (Kapila *et al*., 1996). Although the T-DNA carrying a gene of interest enters the cell and is expressed for a short period, the gene will not be integrated into the plant genome and is ultimately degraded by nucleases. This transient gene expression occurs almost immediately after gene transfer into the plant cell and expression is at maximum 60-70 hrs after the infiltration process (D'aoust *et al*., 2009; Altpeter *et al.*, 2005). Agro-infiltration has the general advantage of being rapid and yielding high quantities of expressed protein and proteins have no size limitation (Fischer *et al*., 2000). A further major advantage of agro-infiltration is that more than one gene can be expressed. The fact that more than one vector can be used in the same plant allows complex multimeric proteins to be assembled simultaneously. This was previously demonstrated by Vaquero *et al.* (1999) who

simultaneously transiently expressed ScFvs, an individual heavy light chain as well as the full size mouse-human chimeric anti-carcinoembryonic antigen (CEA) antibodies in plant leaves (Vaquero *et al*., 1999).

A second transient gene expression method is syringe agro-infiltration. This is carried out by injecting an Agrobacterium cell suspension directly into the abaxial airspace of a leaf tissue (Maclean *et al*., 2007). This method has been first successfully used with *Nicotiana benthamiana* (Goodin *et al*., 2008), and has also been successfully used with other plant species such as lettuce (Wroblewski *et al*., 2005), tomato (Orzaez *et al*., 2006) grapevine (Santos- Rosa *et al*., 2008) and potato (Bhaskar *et al*., 2009). Agro-infiltration using a syringe has also been applied as a technique for rapidly evaluating gene function including, for example, evaluation of the *Brassica oleracea* protease inhibitor (BoPI) for insect resistance (Leckie and Steward Jr., 2011).

1.3 Plant host systems

Several seed crops, such as maize, rice, wheat and barley, have been used as host plants for recombinant protein expression (Table 1.1). Maize seeds have been used for commercial production of several proteins, such as avidin and aprotinin (Zhong *et al*., 1999), over other cereals due to the fact that it has the highest annual grain yield (83000 kg/ha). The seeds are relatively high in protein content (10%) and there are pre-existing techniques for transformation which facilitate the ease for transformation (Hood *et al*., 1997; Twyman *et al*., 2003). The seeds can serve as a storage reservoir for accumulation of the recombinant protein. This requires using a seed specific promoter to drive the transgene expression. Although the seeds are stable at room temperature, the overall yield is low. Furthermore, the

plant has to develop to a flowering stage before seeds can be produced increasing the risk of gene transfer through pollen dispersal (Twyman *et al*., 2003; Fischer *et al*., 2004).

Table 1.1: Recombinant proteins expressed in transgenic plants

Host plant	Plant tissue	Recombinant protein	Reference
Nicotiana tabacum	leaf	scFv anticutinase	Schouten *et al.*, 1997
Nicotiana benthamiana	leaf	Human growth hormone	Gils *et al.*, 2005
Medicago sativa	Leaf	FMDV VP1 structural protein	Wigdorovitz *et al.*, 1999
Hordeum vulgare	Endosperm	β-glucanase	Horvath *et al*., 2000
Zea mays	Seed	*E. coli* heat labile enterotoxin B	Streatfield *et al.*, 2003
Triticum aestivum	apoplast	ScFV CEA	Stoger, 2000
Musa sp.	Fruit/leaf	HBsAg	Kumar *et al.*, 2005
Oryza sativa	Cell suspension	Human growth hormone	Kim *et al.*, 2008
Lycopersicon esculentum	Leaf/fruit	Rabies virus glycoprotein	McGrarvey *et al.*, 1995
Brassica napus	Chloroplast	*aadA*	Cheng *et al.*, 2010
Solanum tuberosum	Potato tubers	Norwalk virus capsid protein	Mason *et al.*, 1996
Daucus carota	Tap roots	*E. coli* heat labile enterotoxin	Rosalez-Mendozas *et al.*, 2008

Both fruit, e.g. banana (Kumar *et al*., 2005), and vegetable crops, including potato (Haq *et al*., 1995), tomato (McGarvey *et al*., 1995) and carrot (Rosalez-Mendozas *et al*., 2008), have been previously investigated as production systems for edible vaccines. Particularly banana has been investigated as a possible oral vaccine (Kumar *et al.*, 2005). A major benefit of fruits is that fruits can be consumed raw, or partially cooked, to administer the vaccine avoiding heat-denaturation of the vaccine protein during cooking and reducing the need for cold storage (Hood *et al.*, 1997). This eliminates any further downstream processing of plant material. Although oral delivery reduces the costs associated with processing of the plant material to obtain pure proteins, delivery of vaccine in adequate doses to confer immunity is usually challenging (Daniell *et al*., 2009). Banana can be seen as an ideal source of an oral vaccine because it is consumed raw in many developing countries (Mason et *al*., 1992).

Mason *et al.* (1992) successfully expressed the hepatitis B subunit antigen in tobacco leaves. However HBsAg was found to be 0.01% of soluble leaf proteins which was considered inadequate to use plants as efficient production systems. They further expressed the HBsAg in potato tubers and optimized accumulation of the protein. The resulting material proved to boost an immune response of oral immunogenic in mice (Kong *et al.*, 2001). Thanavala *et al.* (2005) also showed that HBsAg transgenic potatoes administered orally to mice can elicit a humoral immune response. Further, plant-derived vaccines, including labile toxin B subunit (LT-B) of enterotoxigenic *E. coli* and capsid protein of Norwalk virus, were explored. The plant-derived proteins correctly assembled into functional oligomers that could elicit an immune response when administered orally to animals (Haq *et al.*, 1995; Mason *et al.*, 1996; Mason *et al*., 1998). McGarvey *et al*. (1995) reported about the use of transgenic tomatoes to produce the first plant-derived rabies-vaccine. The rabies virus glycoprotein (G-protein) was

successfully expressed in leaf and fruit tissues and the amount of G-protein precipitated was approximately 1-10 ng/mg soluble protein McGarvey *et al*. (1995). In a study by De Wilde *et al.* (2002) for bulk production of antibodies and *Fab* fragments production in transgenic potatoes, constructs were engineered for targeting full size IgGs and Fab fragments to the ER and plant cell apoplast. Antibodies targeted to the ER had accumulated at 0.5% of total soluble protein whereas those targeted for secretion had a 5-fold lower accumulation of IgG antibodies. Transgenic tubers could be stored for up to 6 months without the antibodies losing their activity (De Wilde *et al.*, 2002). The production of the human milk protein beta-casein was also successfully expressed in transgenic potatoes as reported by Chong *et al*. (1997).

Oil crops, such as rapeseed (*Brassica napus*), are further considered as useful hosts for protein production because the oil bodies can be manipulated to facilitate protein isolation and processing (van Rooijen and Moloney, 1995). A limiting factor of using oil bodies is that proteins directed to oil-bodies do not pass through the secretory pathway and are therefore not glycosylated (Twyman *et al*., 2003).

Leafy plants, such as tobacco, alfalfa and lettuce, are currently widely used as expression hosts for the production of recombinant proteins (Negrouk *et al*., 2005; Kapusta, 1999). The usage of tobacco is mainly due to the high leaf biomass yields and the well established technology for plant transformation via the Agrobacterium transformation system (Table 1.1). Tobacco has also the advantage being a non-food and non-feed plant, and transgenic material will neither contaminate the feed nor or human food chain. One of the drawbacks of using tobacco for recombinant protein production is, however, protein instability after harvesting due to unwanted proteolysis in plant samples (Michaud *et al*., 1998). Leaf tissues have to be rapidly frozen or dried immediately prior to processing to preserve the stability of the

recombinant protein and to minimize degradation by plant proteases. Further, many tobacco cultivars can produce high levels of toxic alkaloids (nicotine), but there are currently low alkaloid varieties (e.g. *Nicotiana benthamiana)* also available that might be more suitable for recombinant protein production (Du Santos *et al*., 2005; Twyman *et al*., 2003; Fischer *et al*., 2004).

Table 1.2: Advantages and disadvantages of various plant host systems for recombinant protein production (Fischer *et al*., 2004).

Species	Advantages	Disadvantages
Model plants		
Arabidopsis thaliana	Range of available mutants, accessible genetics, ease of transformation	Not useful for commercial production (low biomass)
Simple plants		
Physcomitrella patens, Chlamydomonas reinhardtii, Lemna	Containment, clonal propagation, secretion into medium, regulatory compliance, homologous recombination in *Physcomitrella*	Scalability
Leafy crops		
Tobacco	High yield, established transformation and expression technology, rapid scale-up, non-food/feed	Low protein stability in harvested material, presence of alkaloids
Alfalfa, clover	High yield, useful for animal vaccines, clonal propagation, homogenous N-glycans (alfalfa)	Low protein stability in harvested material, presence of oxalic acid
Lettuce	Edible, useful for human vaccines	Low protein stability in harvested material
Cereals		
Maize, rice	Protein stability during storage, high yield, easy to transform and manipulate	
Wheat, barley	Protein stability during storage	Low yields, difficult to transform and manipulate
Legumes		
Soybean	Economical, high biomass, expression in seed coat	Low expression levels, difficult to transform and manipulate
Pea, pigeon pea	High protein content	Low expression levels
Fruits and vegetables		
Potato, carrot	Edible, proteins stable in storage tissues	Potato needs to be cooked
Tomato	Edible, containment in greenhouses	More expensive to grow, must be chilled after harvest
Oilcrops		
Oilseed rape, *Camelina sativa*	Oleosin-fusion platform, sprouting system	Lower yields?

1.4 Transgene expression

Transgene expression is affected by mRNA stability, transcriptional and translational efficiency as well as protein stability. In addition, the selected tissue or organ for recombinant protein expression can greatly influence final protein yield (Benchabane *et al*., 2008). A common approach to increase protein yield includes directing transgene expression to specific tissues or organelles, attaching the expressed protein to a stabilizing fusion protein partner, and also the use of host plants with reduced proteolytic activity.

1.4.1 Tissue/organ specific expression

Leaf tissues have been used for the production of recombinant proteins. This is due to the leaf's fast growth rate, likelihood to harvest plant material more than once during the growth season and well-established technologies for transgene expression in leaves (Daniell *et al*., 2001). However, a major problem is the often high protease activity causing protein degradation and denaturation.

Increased recombinant protein production can be achieved by targeting a transgene to organelles, such as the ER (endoplasmic reticulum), by using a C-terminal H/KDEL signal peptide. The endoplasmic reticulum has been found having the potential for increased accumulation of proteins relatively to cytoplasmic protein retention (Spiegel *et al*., 1999; Napier *et al*., 1998). Previous studies have also shown that directing of a foreign protein to the ER minimizes proteolytic degradation resulting in higher recombinant protein yield (Conrad and Fieldler, 1998; Sainsbury and Lomonoshoff, 2008). Furthermore results by Kang *et al.* (2004) showed that protein expression levels were enhanced 200-fold in *N. tabacum* L.

transformed with the β-subunit of *E. coli* heat- labile toxin fused to a SEKDEL ER retention signal.

Recombinant antibodies produced have also been successfully produced in other sub-cellular organelles including the apoplast (Badri *et al*., 2008); vacuole (Yang *et al*., 2003) and chloroplast (Gils *et al*., 2005). Targeting recombinant protein production in particular to protein storage vacuoles with a slightly acidic to neutral pH presents a suitable environment for protein accumulation. The lower proteolytic activity of storage vacuoles especially in seeds where they are prominently found makes them well adapted for storing proteins (Stoger *et al*., 2005). Expression of recombinant proteins in the chloroplast can also greatly enhance transgene expression, with a yield of 46.1% of total soluble protein achieved using this method (Daniell *et al*., 2005, Fletcher *et al*., 2007). Chloroplast transformation allows a uniform rate of transformation with a high number of gene copies integrated into the genome. Also, multiple genes can be co-expressed using the same gene construct and further gene silencing is minimal in chloroplasts (Danielle *et al*., 2002). However, a disadvantage of using recombinant protein expression in the chloroplast is that plastids do not carry out any glycosylation and, therefore, cannot be used for production of glycoproteins where the glycan-structure is vital for protein activity (Ko and Koprowoski, 2005; Rice *et al*., 2005).

1.4.2 Recombinant protein stabilization

Approaches for stabilizing proteins include site-directed mutagenesis and protein domain grafting and stability of the γ-zein proline-rich domain at the C-terminus was for example achieved through protein grafting (Mainieri *et al*., 2004). Further, proteins can be designed to have improved stability by identifying those amino acid structures that are susceptible to degradation by proteases in the transgenic host. Such mutant proteins can be structurally stable and resist proteolysis (Benchabane *et al*., 2008). An example is the expression of the sea anemone protein equistatin in potato, where susceptible amino acid sites were mutated via site directed mutagenesis to produce truncated forms of potato leaf cells (Outchkourov *et al*., 2003). Creation of a fusion protein by attaching a recombinant protein to a second protein is often applied as a strategy to enhance protein stability and folding (Arakawa *et al*., 1998). The heat labile endotoxin (LT) from *E. coli* and the cholera toxin (CT) from *Vibrio cholera* have been used as fusions to other recombinant proteins for protein stability (Mason *et al*., 1998, Kang *et al*., 2004). Rigano *et al.* (2004) also used this strategy to enhance the stability of the tubercolosis antigen by fusing it to the receptor binding subunit of the heat labile toxin of *E. coli*. In addition, foreign proteins fused to oleosins have been expressed in *Brassica napus* seeds, where they can stably accumulate in the oil bodies (van Rooijen and Moloney, 1995).

1.5 Protein degradation

1.5.1 Proteolytic degradation of recombinant proteins

For recombinant protein production, a major challenge is optimizing yield and quality of the recombinant protein stabilizing polypeptide chains expressed in a heterologous expression system (Faye *et al*., 2005). Proteases severely affect stability both *in planta*, during protein expression, and also *ex planta* during the protein extraction process (Michaud *et al*., 1998; Rivard *et al*., 2006). Proteolysis can therefore directly impact heterologous protein yield and activity. Proteolysis (protein degradation) is naturally required for the processing and turnover of proteins (Abraham *et al*., 2006). Degradation is associated with plant development processes such as germination, senescence, programmed cell death (PCD), differentiation and morphogenesis. Proteolysis can also occur during oxidative stress (Palma *et al*., 2002). Further, protein degradation accelerates when nitrogen is limited in tissues, or when tissues senesce, and stored amino acids are needed. Therefore, signaling pathway(s) possibly link the supply of free amino acids (or nitrogen) with intracellular proteolysis (Ho *et al*., 2001).

1.5.2 Proteases and leaf senescence

Leaf senescence is a highly regulated developmental process ultimately leading to plant death (Swidzinski *et al*., 2002). The senescence process includes remobilization and degradation of proteins (Martinez *et al*., 2007). Two leaf senescence types have been identified. The first is developmental or natural senescence, where specific tissues, organs and the whole plant undergoes senescence, and induced, or premature, where senescence occurs under environmental stress (Nooden *et al*., 1997). Stresses include drought, temperature extremes, nutrient deficiency, and pathogen infection (Hensel *et al*., 1993; Lee *et al.*, 2001).

Leaf yellowing, due to chlorophyll degradation, is often considered to be the main marker for leaf senescence indicating the disintegration of the photosynthetic apparatus in the chloroplast (Gepstein *et al*., 2004). During senescence in plants the proteolytic activity also increases with degradation of endogenous proteins (Desimone *et al*., 1996). Proteolysis is caused by proteases and includes different types of proteases including cysteine proteases which have a major role in protein degradation within senescent leaves (Gou *et al*., 2005). In particular papain-like cysteine proteases are often found in senescing plant tissues (Ueda *et al*., 2000; Gepstein *et al*., 2003). A papain like sequence, NtCP1, has been previously isolated from senescent tobacco leaves (Beyene *et al*., 2006). Results from this study also show that NtCP1 is specifically up-regulated during developmental senescence.

1.5.3 Cysteine proteases

The most abundant group of plant endo-peptidases are the cysteine proteases, which are characterized by the conserved Cys and His residues which form the active site (Vincent *et al*., 2000). Cysteine proteases are involved in a variety of developmental processes in plants including programmed cell death. Programmed cell death (PCD) also referred to as apoptosis, is a physiological process by which cells or organs that have reached a certain age are spontaneously killed to preserve the integrity of the whole organism (Solomon *et al*., 1999). Cysteine proteases are involved in the degradation and remobilization of stored proteins. During germination this provides nitrogenous nutrients to sustain growth of the young seedlings (Grudkowska *et al*., 2004; Schaller *et al*., 2004). The relatively acidic pH optima of many of the endogenous cysteine proteases indicate that they are localized in the vacuole *in vivo* (Callis, 1995).

Proteolysis represents a major obstacle to the efficient production of recombinant proteins in plants. This occurs both *in vivo* during expression, as well as *in vitro* during recovery from the source tissue (Rivard *et al*., 2006). It has been suggested that ectopic expression of specific proteases inhibitors could improve the yield of recombinant proteins in vegetative organs of plants (Goulet *et al*., 2006).

1.5.4 Plant protease inhibitors

Protease inhibitors (PI) are proteins which bind tightly to proteases inhibiting proteolytic activity. They have been identified in animals, plants as well as microorganisms (Laskowski *et al*., 1980). Protease inhibitors are classified according to the classes of proteases they inhibit, serine, cysteine, aspartic and metallo-proteases (Koiwa *et al.*, 1997). Serine inhibitors are reversible inhibitors of serine proteases mainly trypsin and chymotrypsin. They tend to accumulate in storage organs such as tubers and seeds (Gatehouse *et al*., 2002). The Bowman-Birk inhibitors, which are common in legumes, were first isolated in soybean seeds. Currently eight families of serine protease inhibitors are known and one family of cysteine protease inhibitors.

In plants, cysteine protease inhibitors, also known as cystatins, are reversible inhibitors of the cysteine class of proteases that include papain and its related proteases (cathepsin B, H, L) (Massonneau *et al*., 2005). The cystatin family contains members that have molecular masses ranging from 13-24 kDa. The first well characterized plant cystatin was oryzacystatin (OC-I) (Abe *et al*., 1987). Cystatins have been reported to be expressed in plants in response to wounding, insect herbivory and chemical signals such as jasmonic acid (JA) derivatives (Ryan, 1990; Koiwa *et al*., 1997). Cysteine protease inhibitors are involved in the regulation of protein turnover and play an important role in resistance against insects and pathogens (Belengi *et al*., 2003).

1.5.5 Co-expression of protease inhibitors

Low yield of the recombinant protein due to degradation by proteases is a major problem in plants (Doran *et al*., 2006). Limiting protease activity is, therefore, a potential strategy to improve recombinant protein yield (Rivard *et al*., 2006). Ectopic co-expression of a specific protease inhibitor has been suggested as a strategy for improvement (Goulet *et al*., 2006). The *in vivo* expression of the tomato cathepsin D inhibitor (S/CDI) in potato has already demonstrated the potential of co-expression of protease inhibitors; transgenic potato lines expressing the S/CDI inhibitor showed a 35-40% increase in leaf protein content. Further, transient expression of the human α1-antichymotrypsin (AACT) increased by ~2.5-fold in lines expressing S/CDI inhibitor in the cytosol (Goulet *et al*., 2010).

Ectopic expression of a protease inhibitor in transgenic tobacco also increases total protein amounts. Van der Vyver *et al*. (2003) reported that expression of the rice cysteine protease inhibitor, oryzacystatin I (OC-I), in the cytosol results in an increase in the total soluble protein amount by ~20%. Further, expression of the tomato cathepsin D inhibitor in the cytosol of transgenic potato increased the total soluble protein content by 20% -30% in leaves (Rivard *et al*., 2006).

1.6 Glutathione reductase

GR (EC 1.6.4.2) is a commercially interesting protein for the measurement of glutathione in diagnostic kits. The enzyme is further used in medicinal test kits, measuring human tissue for reactive oxygen species (ROS), as an indicator of cardiovascular health. GR is also an essential antioxidant enzyme in plants that catalyzes the reduction of oxidised glutathione (GSSG) to reduced (GSH) using NADPH as a reducing cofactor ($GSSG + NADPH + H^+ \rightarrow NADP^+ + 2GSH$). It is a dimeric flavoprotein (Fig 1.1) and belongs to the important class of flavoprotein enzymes, the disulfide oxido-reductases (Greer and Perham, 1986). GR is mainly localized in the cytosol, mitochondria and chloroplast (Ulusu and Tandogan, 2007) There are several isozymes of GR in different compartments of plants (Edwards *et al*., 1990) with the chloroplastic GR being the most predominant in tobacco leaves (Aono *et al*., 1993).

Fig 1.1: Three-dimensional representation of *E.coli* GR obtained from Protein Data Bank.

1.7 Research aim and objectives

Protein degradation poses a significant challenge for the efficient production of recombinant proteins in plants, affecting the stability and yield of the recombinant protein. In this study the *E. coli*-derived enzyme GR was transiently expressed in transgenic tobacco plants expressing the cysteine protease inhibitor OC-I and non-transgenic plants. GR was further specifically used due to easy measurement of activity and natural expression of the enzyme in the cytosol comparable to OC-I in transgenic tobacco plants.

The research aim of the study was therefore to determine whether a cysteine protease inhibitor (OC-I) expressed in a transgenic plant protects transiently expressed GR against

cysteine protease mediated degradation, thus allowing greater accumulation of the protein. In order to achieve this aim, the study had the following objectives:

1) To determine whether GR is susceptible to cysteine protease mediated degradation.
2) To transiently express recombinant GR in either transgenic OC-I expressing or non-transgenic tobacco plants.
3) To measure cysteine protease activity and determine leaf phenotypic changes after agro-infiltration.
4) To measure GR activity and amounts produced after agro-infiltration in either transgenic OC-I expressing or non-transgenic tobacco plants.

CHAPTER TWO

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

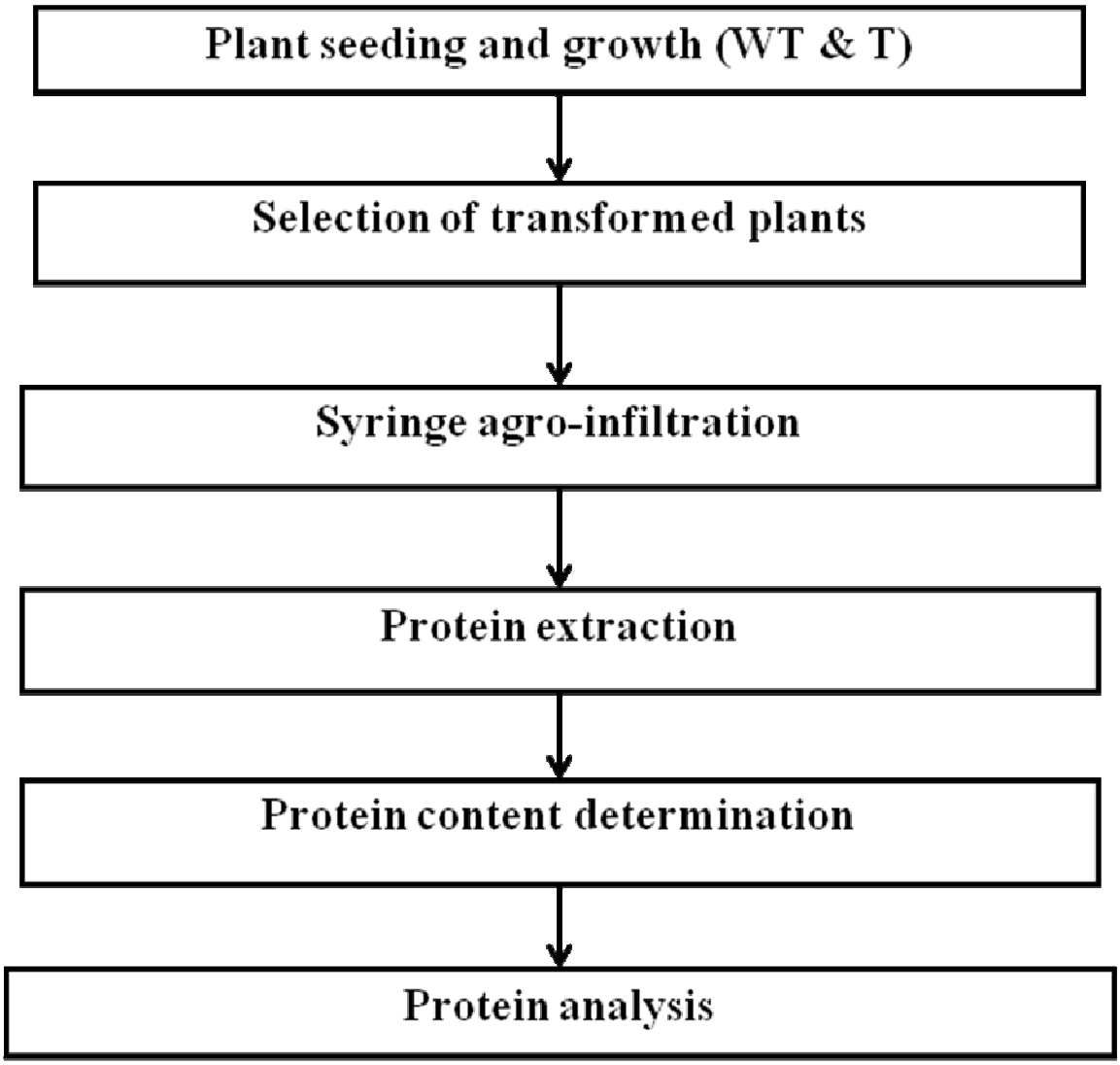


Figure 2.1: A flow diagram of the experimental design in order to achieve the research objectives. WT and T represent wild-type plants and transgenic plants, respectively.

2.1 Plant material and plant growth

Seeds of wild-type non-transformed tobacco (*Nicotiana tabacum* L. Cv. Samsun) and the transformed tobacco line (T4/5) expressing both the gene coding for oryzacystatin-I (OC-I) and the *gus* gene coding for β-glucuronidase (GUS) both under the control of a constitutive CaMV 35S promoter were germinated on a commercially available germination soil mixture in trays. Plants were grown at a 12/12 hrs light/dark cycle with a day/night temperature of 26ºC/20ºC and a 80% (v/v) relative humidity. At 4 weeks, the germinated seedlings were transferred to 25 cm diameter pots. The plants were further grown for 12 weeks until they had a size of approximately 25 cm.

2.2 Gene detection by PCR analysis

2.2.1 DNA isolation and quantification

Genomic DNA was isolated from young leaves of putative transformed plants using a commercial isolation kit (ZR plant/Seed DNA kit, Zymo Research, UK). The quality of the DNA was verified visually by agarose gel electrophoresis. For that, isolated genomic DNA (5 µL) was mixed with 1 µL loading dye and loaded onto a 1% agarose gel. The gel was prepared by mixing 90 mg agarose with 90 mL TAE buffer (0.04 M Tris-base, 0.1 M of glacial acetic acid, 7 mM EDTA) and the solution was heated for 3 min in a microwave oven to dissolve the agarose. The agarose solution was allowed to cool and then poured into a commercial gel casting system with a comb inserted to produce wells for loading the DNA. Marker dye (2 µL) was loaded to monitor the movement of the gel front. Agarose gel electrophoresis was carried out for 20 min at 100 V. After electrophoresis, the DNA was

analysed visually using an ultraviolet (UV) transilluminator. The quantity of the DNA in each sample was determined with 2 µL of DNA using the Nanodrop spectrophotometer (Fermentas, Canada) following the manufacturer's instructions.

2.2.2 Polymerase chain reaction (PCR) assay

Isolated genomic DNA from transformed tobacco plants were screened by PCR to verify the presence of the OC-I coding sequence using the OC-I forward primer (5'-TCACCGAGCACAACAAGA-3') and the OC-I reverse primer (5-CATCGACAGGCTTGAACT-3') (Inqaba Biotec, SA). A PCR reaction containing the following was employed: 0.5 µL of genomic DNA (100 ng/µL), 2.5 µL of PCR buffer containing 20 mM $MgCl_2$, (Fermentas, Canada) 2 µL of 1.25 µM dNTPs, 2.5 µL of 10 mM OC-I-R, 2.5 µL of 10 mM OC-I-L and 14.5 µL sterile distilled water to obtain a total reaction volume of 25 µL. Amplification of DNA was performed using a Palm Cycler (Corbett Life Science cycler, Australia), the PCR cycle conditions were as follows: DNA denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 45 sec and a final extension at 72°C for 10 min to obtain fully-double stranded DNA. Amplified PCR products were then visualized on a 1% agarose gel to verify DNA bands of the expected band size of 250 base pairs (bp).

2.3 Plant treatment and analysis

2.3.1 Agro-infiltration

2.3.1.1 *Preparation of bacterial culture and suspension medium*

Agrobacterium tumefaciens cells of strain LBA4404 harbouring the plasmid pKG2, which carried the coding sequence for *E. coli* derived glutathione reductase (GR) under the control of the cauliflower mosaic virus (CaMV) 35S promoter sequence and the CaMV terminator sequence cloned into the plasmid pBIN19 (Foyer *et al.*, 1994), were sub-cultured on a petri-dish containing Luria Broth (LB) solid medium containing 50 mg/l kanamycin and 25 mg/l rifampicin. Cells were grown at 28˚C for two days. Bacterial cultures were prepared by inoculating 5 mL of LB medium containing 50 mg/l kanamycin and 25 mg/l rifampicin.

Cells were grown overnight at 28˚C under constant agitation at 220 rpm on a shaker. Overnight culture (1 mL) was added to 100 mL fresh LB medium containing 50 mg/l kanamycin and 25 mg/l rifampicin and cells were again incubated overnight at 28˚C under constant agitation at 220 rpm to allow cell growth to an optical density (600 nm) of 0.8-1.2. Once an optical density of 1.2 was reached, 50 mL of the cell culture were centrifuged at 10000 g for 10 min at 4˚C and the supernatant was discarded. The cell pellet was re-suspended in 50 mL of a solution containing 10 mM $MgCl_2$:$6H_2O$ and 100 µM acetosyringone and cells were incubated in this solution for 1 hr at room temperature.

2.3.1.2 *Syringe agro-infiltration and plant incubation*

Wild-type non-transformed *Nicotiana tabacum* plants (12-weeks old) and transformed plants expressing both OC-I and GUS were used for agro-infiltration. For infiltration, a 10 mL syringe (without needle) was filled with an Agrobacterium suspension containing cells carrying plasmid pKG2 and the solution was gently infiltrated into the abaxial side of the leaf by maintaining an even pressure during the infiltration in the leaf tissue. Wetting of the leaf was observed as the suspension solution entered the tissue. Several points of infiltration were made to completely inoculate each leaf. The first three fully expanded leaves were inoculated on each plant. The plants with infiltrated leaves were kept for up to 6 days in the greenhouse and were watered every second day.

2.3.1.3 *Protein extraction from tobacco leaves*

Leaf material (100 mg) was ground into a powder in liquid nitrogen with a pestle and mortar with 20% PVPP (polyvinylpolypyrrolidone) added to the powder. Soluble proteins were extracted by addition of 1 mL of an ice-cold solution containing 50 mM Tris-HCl, pH 7.8 and 1 mM EDTA to the powder. The content of the mortar was transferred to an Eppendorf tube and centrifuged at 13000 rpm for 10 min at 4ºC. The resulting supernatant containing all soluble proteins was stored at -80ºC for further analysis.

2.3.1.4 *Determination of tobacco leaf protein content*

Total protein content of tobacco leaves extracts was determined using a commercial kit (BioRad, UK) following the Bradford method (Bradford, 1976) with bovine serum albumin

(BSA, Sigma SA) as a protein standard. This assay involves the addition of an acid dye to the protein solution and subsequent measurement of the reaction mixture at 595 nm in a spectrophotometer.

To obtain a protein standard curve, five concentrations of BSA (16 µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL and 1 µg/mL 0 µg/mL) were prepared through serial dilutions. From each standard solution, 10 µL was added to 200 µL of acidic dye and 790 µL of dH_20. For colour development, the assay was placed on the bench for 30 min. The same procedure was followed using tobacco extract instead of a standard protein solution. A blank sample was prepared by replacing the protein extract with water. Measurement of optical density was performed in a 1 mL cuvette (Whitehead Scientific, SA) at 595 nm with a spectrophotometer. The various clear supernatants containing soluble proteins were then directly used for detection of protease activity.

2.4 Enzymatic assays

2.4.1 GUS activity measurement

GUS activity in transgenic plants was detected by using the fluorogenic GUS substrate 4-methyl umbelliferyl glucoronide (MUG). This compound does not fluoresce until cleaved by GUS to release 4-methyl umbelliferone (MU) which is fluorogenic. For the assay, 50 µL of protein extract corresponding to 20 µg protein was mixed with 200 µL reaction buffer (50 mM $NaPO_4$ buffer, pH 7.0; 1% Triton X-100; 10 mM Na_2EDTA; 0.1% sodium lauryl sarkosine; 10 mM β-mercaptoethanol) containing 1mm MUG. Immediately after mixing, 50 µL of the reaction mixture was transferred to an Eppendorf tube and 950 µL of stopping buffer (0.2 M Na_2CO_3) was added to obtain the fluorescence value at time 0. The reaction mixture was incubated at 37°C for a further 30 min when 50 µL was again removed from the reaction mixture and added to 950 µL of stopping buffer added to obtain fluorescence value at time 30 min. The procedure was repeated at again at 60 min. All samples containing the stopping buffer were kept in the dark on ice.

A MU standard was prepared from a 1 mg/mL stock solution of MU. The following standard values were prepared: 0.25; 0.5; 1.0; 1.5; 2.0; 2.5 and 3.0 µg/mL MU. Fluorescence of these standards was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a Fluoroskan Ascent® fluorometer (Thermo Labsystems).

2.4.2 GR activity measurement

GR activity was measured colorimetrically using the NADPH-driven glutathione-dependent reduction of 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB, Ellman's reagent) (Sigma-Aldrich, UK). DTNB contains a disulphide, which can be reduced by thiols, and measured in its reduced form (5' thio-2-nitrobenzene; TNB) at 412 nm. In the presence of reduced glutathione (GSH), or GSSG in the presence of GR, a cyclic reaction is generated as follows:

$GSSG + NADPH + H^+ = 2\ GSH + NADP^+$ (catalysed by GR)

$2\ GSH + DTNB_{ox} = GSSG + DTNB_{red}$ (increase in absorbance at A_{412})

2.4.2.1 *Protein extraction*

Leaf samples were groud in liquid nitrogen using pestle and mortar and proteins extracted in 50 mM Tris-HCl pH 7.5, 1 mM EDTA. All extraction steps were performed at 4^{o}C. The homogenate was transferred to Eppendorf tubes and centrifuged at 13000 rpm for 10 min at 4^{o}C. The supernatant was removed and used for GR activity determination.

2.4.3.2 *GR assay*

GR was prepared fresh daily by centrifugation of a 22 µL $(NH4)_2SO_4$ suspension at 13000 g at 4^{o}C for 5 min. The supernatant was discarded and the pellet was re-suspended in 500 µL 0.12M NaH_2PO_4 (pH 7.5), 6 mM EDTA to obtain a concentration of 20 UmL^{-1}. DTNB solution was stored in the dark at 4^{o}C. Standards for GR were prepared from a 20 U/mL stock solution. The following standards were prepared 0.01, 0.02, 0.03, 0.04, 0.05 U/mL.

A leaf protein extract (20 µL) was added to a plate well containing 100 µL 0.12 M NaH_2PO_4 (pH 7.5), 6 mM EDTA, 10 µL of 12 mM DTNB, 10 µL of 10 mM NADPH, 40 µL dsH_2O, 10 µL of 4 mM GSSG. Reference samples contained the above mentioned solutions but lacked the standard and plant supernatant. The increase in absorbance at 412 nm was observed for 5 min using Soft Max Pro, Molecular Device.

2.4.4 Protease activity measurement

Frozen leaf samples were homogenized in liquid nitrogen and extracted in extraction buffer containing 50 mM Tris-HCl (pH 8.0). The homogenate was centrifuged at 13000 rpm at 4ºC for 10 min in an Eppendorf centrifuge and the resulting supernatant was used for the detection of cysteine protease activity after determination of the protein concentration.

A fluorometric assay was conducted to compare the amount of cysteine protease activity between transformed and non-transformed tobacco plants both infiltrated with the bacterial *gor* gene encoding GR. Cysteine protease activity was detected in plant extracts using the synthetic cysteine protease substrate Z-Phe-Arg-AMC. In a micro-titer plate-well total soluble protein (25 µg protein) was diluted to a final volume of 92 µL by addition of a 50 mM sodium phosphate buffer (pH 6.0) containing 1 mM L-cysteine. A 100 µM Z-Phe-Arg-AMC solution (8µL) substrate in DMSO was then added to the wells. The plate was then incubated at 37ºC for 30 sec, followed by shaking for 10 sec. Increase in fluorescence due to released AMC was measured using a fluorometer (Fluoroskan Ascent®) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a multi-well plate reader

and activity measurement over 10 min. As control a solution was used that only contained the substrate without protein extract.

2.5 Gel electrophoresis

2.5.1 SDS-PAGE analysis

Total soluble protein samples of infiltrated and non infiltrated leaves harvested on day 6 post infiltration were extracted in 50 mM Tris-HCl pH 7.8 and separated on sodium a dodecyl sulfate polyacrylamide gel (10% SDS-PAGE) under non-reducing conditions. Protein samples were mixed with 5X sample buffer (31.25 mL 1M Tris-HCl, pH 6.8, 1% SDS powder, 25 mL 50% glycerol, 75 µL of 1 mM bromo-phenol blue dissolved in 2% ethanol and 5 µL of 100 % 2-mercaptoethanol) and incubated at 95ºC for 15 min before loading onto the gel. Following electrophoresis at room temperature, the gel was subsequently transferred overnight to a staining solution (Coomassie Blue) at room temperature. The gel was then de-stained in de-staining solution.

2.5.2 Activity-PAGE

For detection of protease activity in infiltrated tobacco leaf samples, representing the progressive stage of senescence (0, 3, 6, 9 days), a 10% SDS-PAGE gel was prepared containing 1% gelatine, 0.01% sodium dodecyl sulfate (SDS), 3% polyacrylamide, 1% ammonium persulfate (APS), Temed (Bio-Rad, UK) with 1.5 M Tris-HCl, pH 8.8, and 1 M Tris-HCl, pH 6.8. For loading, 40 µg protein derived from homogenized tobacco leaves were mixed with 5X sample buffer. Protein separation was performed in a mini-PROTEAN II™

apparatus (Bio-Rad, UK) using a 1X SDS running buffer (0.038 M glycine, 0.1 M of Tris-HCl pH 8.0 and 1% of SDS) at 100 V on ice.

After electrophoresis, the gel was incubated in 2.5% Triton X-100 for 30 min at room temperature to re-nature the proteases. After 30 min, the gel was rinsed 3-times for 10 min with dH_2O to remove Triton-X 100. The gel was then incubated overnight in a proteolytic buffer (0.1 M citrate phosphate buffer pH 6.0 containing 10 mM L-cysteine) at 37°C for the developing the protease reaction. The gel was subsequently transferred overnight to a staining solution (25% isopropanol, 10% acetic acid and 0.5% Coomassie blue) at room temperature and then de-stained for 2 hr with a de-staining solution (10% acetic acid, 40% dH_20 and 50% methanol). The de-stained gel was captured using a digital camera.

2.6 Western blotting

2.6.1 Detection of OC-I

Tobacco leaves protein samples were mixed with 5X sample buffer. Extracts were boiled at 95°C for 15 min and shortly centrifuged for 30 sec at full speed (13000 rpm) in an Eppendorf centrifuge and then subjected to a 10 % SDS-PAGE for 3 hr at 100 V at room temperature. The separated proteins were then transferred onto a nitrocellulose Immuno Blot™ PVDF membrane (Bio-Rad, UK). The transfer buffer contained 25 mM of glycine, 192 mM Tris-base and 20 % methanol at pH 8.2. Transfer was done on ice for 2 hr at 45 V and 200 mA.

After 3 hr of protein transfer, the membrane was incubated overnight on ice in a 5% skimmed milk powder solution in TTBS (0.5 M of Tris-HCl, 1 M NaCl, 0.1% Tween 20, pH 7.6)

which blocks non-specific binding sites. The membrane was then incubated on ice for 5 hr with TTBS buffer containing 5% skim milk powder and an antibody raised against OC-I in a rabbit (1:10000 dilution). After incubation, the membrane was washed 3-times for 10 min with 5% skim milk powder, 0.1% Tween 20 and TBS (Tris-base saline). A goat anti-rabbit antibody conjugated to alkaline phosphatase (Santa Cruz Biotechnology, USA), which was used as a secondary antibody, was added (1:2000 dilution) to the membrane and the membrane was incubated with the antiserum for 1 hr at room temperature in 5% skimmed milk powder, 0.1% Tween 20 and TBS. Finally the membrane was washed 3-times for 30 min with 10 min intervals in 0.1% Tween 20 containing 30 mL of TBS. The presence of OC-I was detected using the alkaline phosphatase detection kit (Bio-Rad, UK).

2.6.2 Detection of GR

Tobacco leaves protein samples were extracted in 50 mM Tris-HCl (pH 7.8) containing 1 mM EDTA. Proteins were separated by SDS-PAGE electrophoresis gel and mixed with 5X sample buffer. Extracts were boiled at 95^{o}C for 5 min and shortly centrifuged for 30 seconds at full speed (13000 rpm) and then subjected to a 10% SDS-PAGE for 2 hr at 100 V at room temperature. The separated proteins were then transferred onto a nitrocellulose Immuno Blot^{TM} PVDF membrane (Bio-Rad, UK). The transfer buffer contained 25 mM of glycine, 192 mM Tris-base and 20% methanol at pH 8.2. Transfer was conducted on ice for 2 hr at 45 V and 200 mA.

After 2 hr of protein transfer, the membrane was incubated overnight on ice with 5% skimmed milk powder in TTBS (0.5 M of Tris-HCl, 1 M NaCl, 0.1% Tween 20, pH 7.6) to block all the proteins from extracts. Furthermore, the membrane was incubated on ice

overnight with TTBS buffer containing 5% skimmed milk powder and the GR antibody (diluted 1:1000). GR antibody was raised in rabbits by injection of a fusion protein consisting of staphylococcal protein A and bacterial GR and was purified by immunoglobulin G affinity chromatography as described by Foyer *et* al., 1991.After incubation, the membrane was washed 3-times for 10 min with 5% skimmed milk powder, 0.1% Tween 20 and TBS (Tris-base saline). A goat anti-rabbit antibody conjugated to alkaline phosphatase (Santa Cruz Biotechnology, USA), which was used as a secondary antibody, was added (1:2000 dilution) to the membrane incubated for 1 hr at room temperature in 5% skimmed milk powder, 0.1% Tween 20 and TBS. Finally the membrane was washed 3-times at 30 min intervals in 30 mL of TBS containing 0.1% Tween 20. The GR band (49 kDa) was detected by incubating the membrane for 10 min in an alkaline phosphatase detection solution (Bio-Rad, UK).

2.6.3 Detection of GR stability

2.6.3.1 *Western blotting*

To monitor the effect of proteases on GR stability, a Western blot analysis was conducted. A solution of pure bacterial GR (Sigma, UK) was prepared by centrifugation of a 22 µL $(NH_4)_2SO_4$ suspension at 13000 rpm at 4^{o}C for 5 min. The supernatant was discarded and the pellet was re-suspended in a protein extraction buffer (50 mM Tris-HCl, pH 7.8). The GR protein sample was incubated in the presence of different amounts of papain or trypsin (0.5, 1, 2 µg) at 37^{o}C for 5 min to activate protease activity. A control sample was incubated in the absence of proteases. Digested GR was then subjected to a 10% SDS-PAGE and a Western blotting was conducted as described in paragraph 2.5 & 2.6 using a bacterial GR antiserum.

2.6.3.2 GR enzymatic assay

To determine whether bacterial GR is sensitive to degradation by endogenous proteases, an *in vitro* assay was conducted comparing the degradation of GR in the presence of different amounts of a cysteine protease (papain) and serine protease (trypsin). GR was prepared fresh by centrifugation of a 22 µL $(NH_4)_2SO_4$ suspension at 13000 rpm at 4^oC for 5min. The supernatant was discarded and the pellet was re-suspended in 500 µL 0.12 M NaH_2PO_4 (pH 7.5) containing 6 mM EDTA to obtain a concentration of 20 UmL^{-1}. In order to activate papain and trypsin activity, GR was incubated at 37^oC for 5 min in the presence of different amounts of papain and trypsin (0, 1, 2 µg). A control sample was incubated in the absence of proteases. The incubated mixture (20µL) was then assayed for GR activity as described in paragraph 2.2.3.2.

CHAPTER THREE

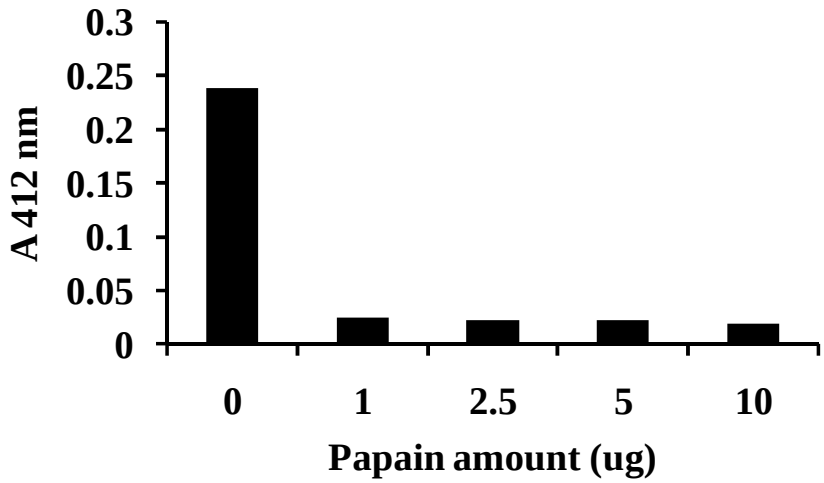
RESULTS

3.1 Protease effect on GR stability

To determine whether bacterial GR is susceptible to degradation by proteases, *in vitro* assays were conducted comparing degradation of GR in presence of different amounts of the cysteine protease papain and serine protease trypsin (Figures 3.1A and B). Treatment of GR with various amounts of papain showed that GR activity is sensitive to degradation by papain and GR activity dramatically decreased in the presence of papain when compared to the untreated control. A similar result was found when GR was treated with different amounts of trypsin.

Result of sensitivity of GR to papain and trypsin was further confirmed by Western blot analysis (Figure 3.2). When this technique was used to determine the stability of GR against protease treatments, the three different amounts of papain (0.5, 1, 2 µg) completely degraded GR. However, no lower molecular weight band for GR was found after protease treatment. A clear band representing GR with the predicted molecular weight was detected in the untreated control. When GR was treated with trypsin, a faint band was detected when GR was treated with the lowest concentration of trypsin used but no GR band was detected with the other trypsin amounts used.

A



B

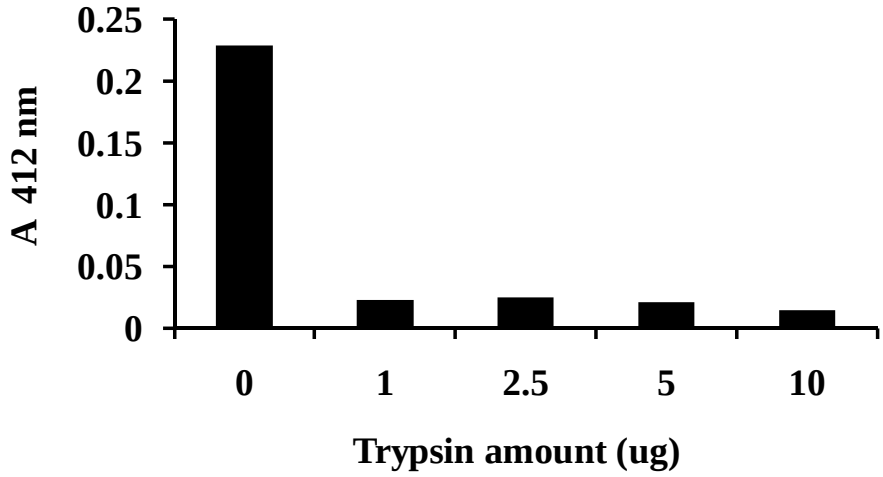


Figure 3.1: Effect of different amounts of proteases on the degradation of bacterial GR activity. GR treated with the cysteine protease papain (A) and the serine protease trypsin (B). GR activity was determined by increase in absorbance at 412 nm due to reduction of 5'5-dithiobis (2-nitrobenzoic acid) (DTNB) by GSH produced from GSSG.

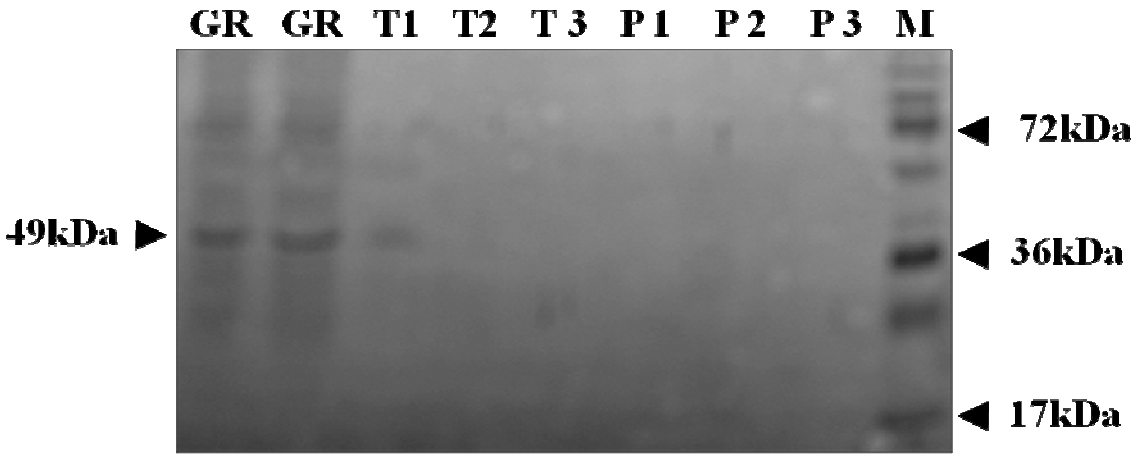


Figure 3.2: Western blot analysis to determine GR stability against protease treatment. (T) represents trypsin with T1, T2 T3 corresponding to (0.5, 1, 2µg) of trypsin respectively and (P) represents papain with P1, P2, P3 corresponding to (0.5, 1, 2µg) papain, respectively and GR with a predicted size band of 49 kDa as a control. M represents a protein marker.

3.2 Characterization of transformed tobacco plants

3.2.1 PCR analysis

To select for transformed plants, a total of 10 putative transformed plants were tested for the presence of the OC-I coding sequence by PCR analysis. PCR amplification of the OC-I coding sequence showed that 6 plants carried the OC-I fragment with the predicted size of 200 bp (Figure 3.3) and an identical fragment was amplified from genomic DNA obtained from a positive control transformed plant (lane 12). The negative control containing no added DNA did not amplify any OC-I fragment. The six plants that carried the OC-I fragment were designated as T1-T6 and were used for further analysis.

3.2.2 Detection of GUS expression

Putative transformed seedlings expressing the OC-I coding sequence were also tested for expression of the *gus* selectable marker gene. Gus enzyme activities were evaluated by fluorometric assay for putative transformed plants. All six putative transformed plants tested positive for GUS expression. The control wild-type plant (Figure 3.4 plant C) had no GUS expression. Since the *gus* gene is closely linked to the OC-I gene on the T-DNA, all *gus* positive plants should also carry the OCI-transgene encoded protein. Putative transformed plants that tested positive for OC-I PCR fragment and the *gus* selectable marker gene were further screened for the expression of OC-I protein.

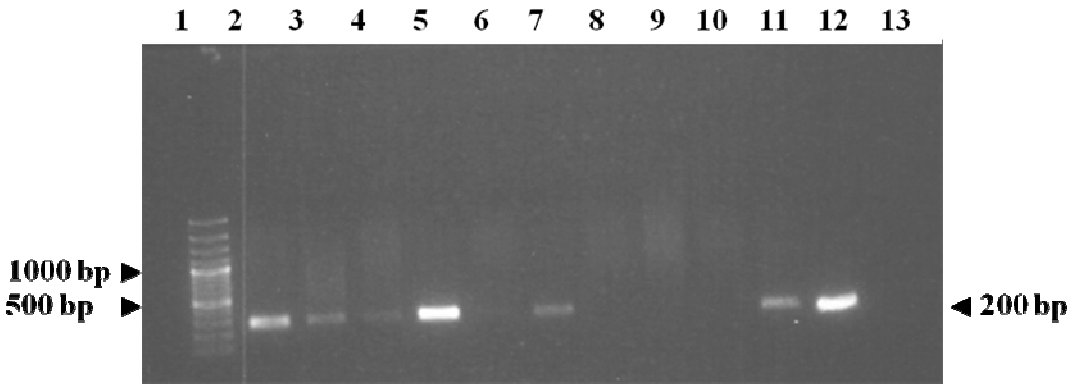


Figure 3.3: PCR analysis to identify transformed tobacco plants carrying OC-I. Lanes 2-11 represent PCR amplified products from putative OC-I transformed plants and lane 12 represents plasmid DNA carrying the OC-I coding sequence. The control containing no added DNA did not amplify any OCI fragment (Lane 13). Lane 1 represents the 100 bp marker.

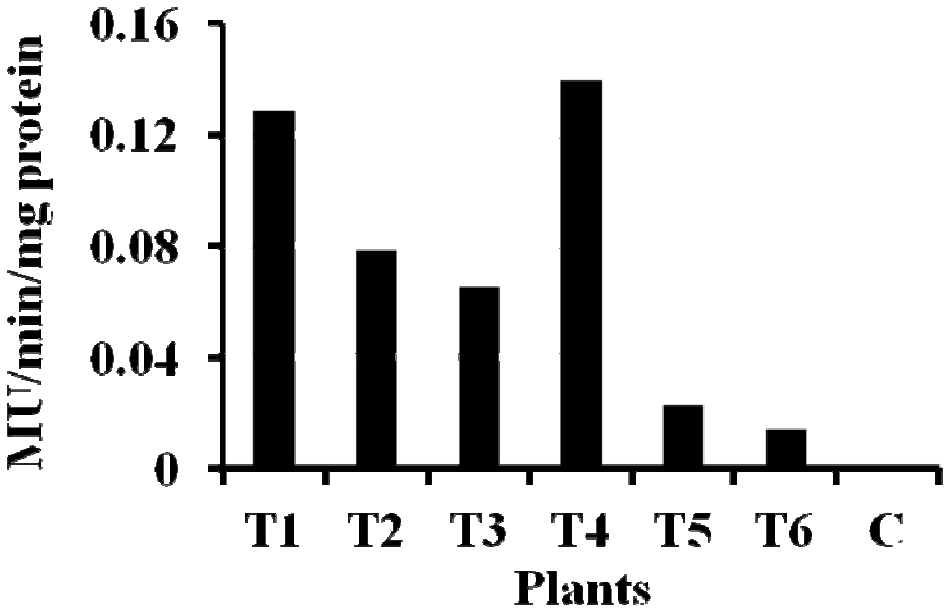


Figure 3.4: Fluorometric analysis of GUS activity in OC-I/GUS transformed tobacco plants. GUS activity was measured in transformed (T1-T6) and wild-type control (C) non-transformed tobacco and expressed as MU produced/min/mg protein of total protein.

3.2.3 OC-I expression detection by Western blot analysis

To detect for the expression of the rice cysteine protease inhibitor coding sequence (OC-I) in putative transformed tobacco plants, leaf protein extracts were separated by SDS-PAGE. An immuno-blot analysis was carried out using an antiserum raised against the rice cysteine protease inhibitor OC-I. Figure 3.5 (lanes T1-T6) shows the predicted OC-I protein band of about12 kDa detected from six putative transformed plants.

3.3 Transient GR expression in tobacco leaves

3.3.1 Agro-infiltration

Agrobacterium cells (LBA4404) containing plasmid pKG2 were grown on LB solid medium containing appropriate antibiotics for selection of plasmid containing cells. The culture was grown overnight to allow growth of cells to an optical density of 0.9 measured at wavelength 600 nm in a spectrophotometer. This suspension was infiltrated into the leaf as illustrated in Figure 3.6. Wetting of the leaf (Figure 3.7) was observed after infiltration with the bacterial suspension culture and infiltrated plants were then placed for 6 days into a green house.

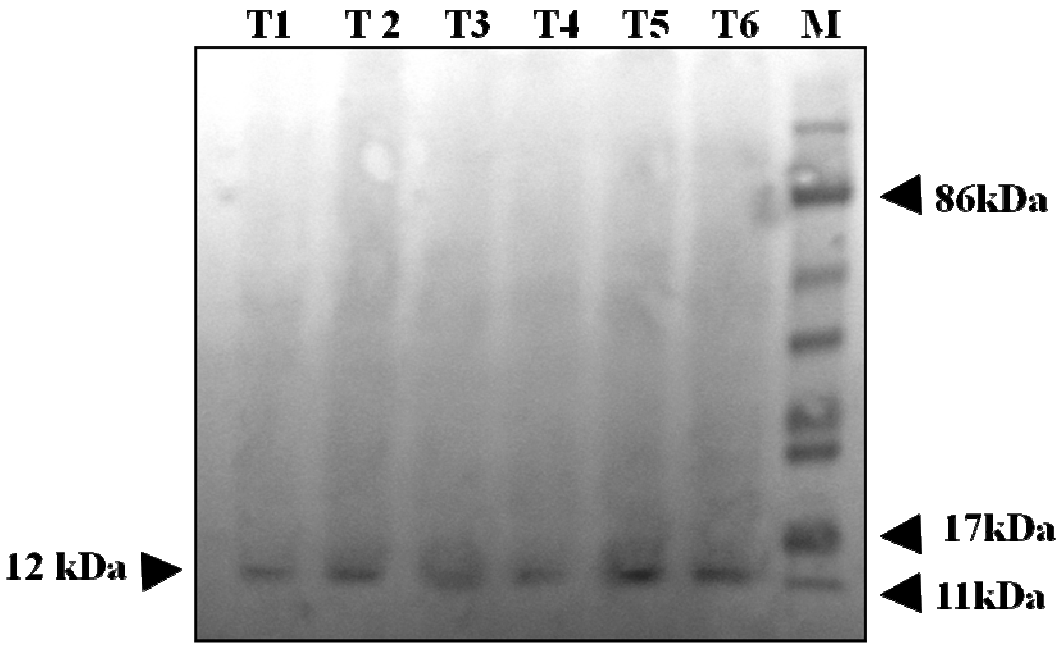


Figure 3.5: Western blot analysis to detect OC-I expression using an antiserum raised against OC-I in rabbit and detection of OC-I bands by alkaline phosphatase activity staining. Tobacco leaf extracts (20 µg protein) were separated on 10% SDS-PAGE. T1-T6 represents six putative OC-I transformed plants. The molecular mass of OC-I of about 12 kDa is indicated. M represents pre-stained protein marker

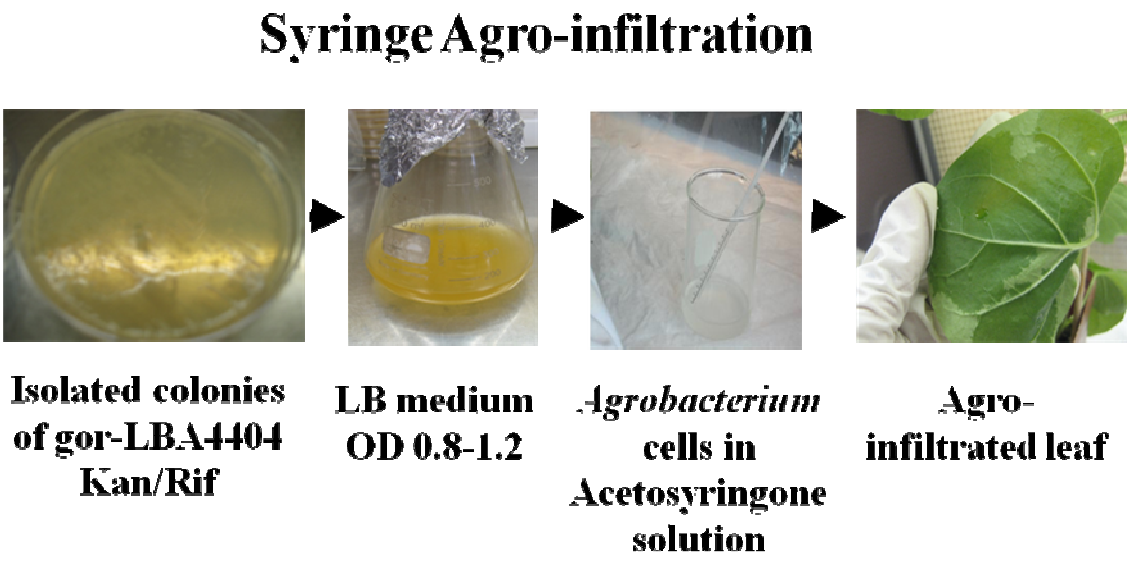


Figure 3.6: Syringe agro-infiltration using *Agrobacterium tumefaciens* cells carrying the plasmid pKG2 with the *E. coli* GR coding sequence. After centrifugation of LB medium containing *Agrobacterium* cells, the resulting pellet was dissolved in a acetosyringone solution. The cell suspension containing the *Agrobacterium* cells was infiltrated on the abaxial side of the leaf and the agro-infiltrated leaf was used for further analysis.

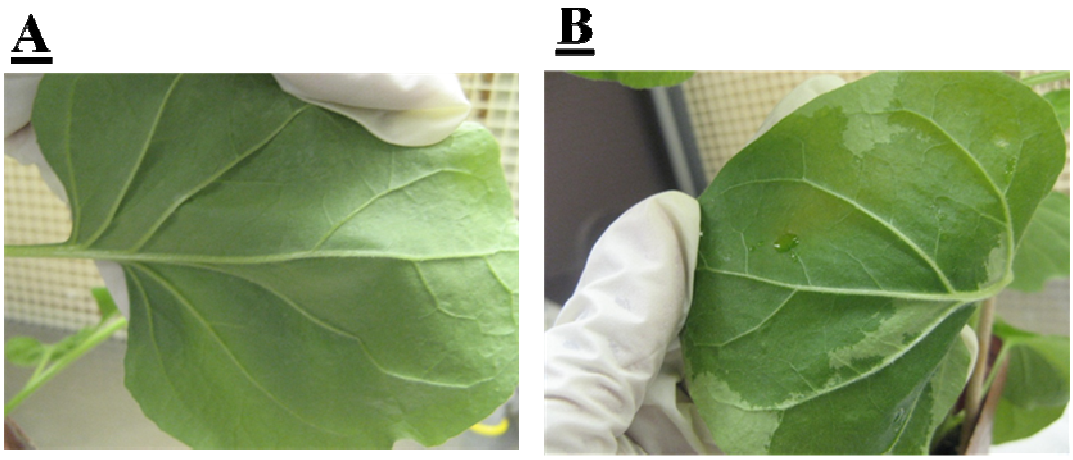


Figure 3.7: Non-infiltrated (A) and infiltrated (B) leaves showing wetting of the leaf tissue as a result of infiltration with bacterial cell solution.

3.3.2 Phenotypic leaf analysis

Infiltrated leaves could be distinguished from non-infiltrated leaves by the wet appearance of the leaf tissue as well as infiltration lesions formed when small incisions were made with a blade to allow easier infiltration of the bacterial solution into the leaf tissue. Leaf tissues harvested 6 days post-inoculation showed the development of localized chlorosis around the point of infiltration (Figure 3.8). A yellow to brown color developed on some of the infiltrated leaves. Leaves that were similar in size were used for further analysis.

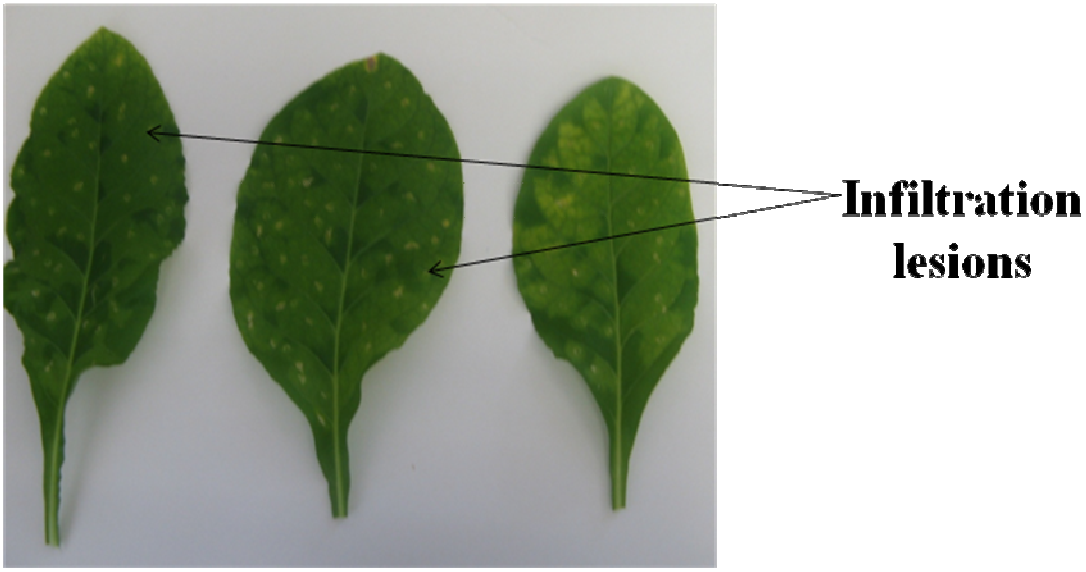


Figure 3.8: Tobacco leaves 6 days post-infiltration showing chlorotic tissue around infiltration points.

3.3.3 GR detection

Western blot analysis was performed to confirm the expression of GR in all infiltrated leaves. A band corresponding to a size between 45 and 50 kDa for GR was detected in infiltrated leaves (Figure 3.9). Also, a band with stronger intensity was detected in agro-infiltrated transgenic OC-I leaves compared to non-transgenic leaves indicating some protective effect of OC-I expression on GR accumulation. No GR expression was detected in non-infiltrated leaves.

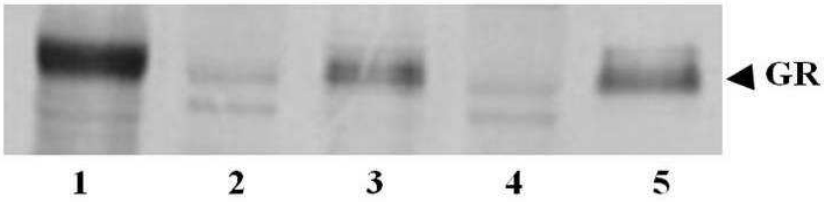


Figure 3.9: Western blot analysis of leaf protein extracts to detect GR expression with a GR antiserum (1:1000 dilution) by alkaline phosphatase activity staining in infiltrated leaves. Lane 1 represents transient GR expression in OC-I expressing transgenic tobacco leaves, lane 2 represents non-infiltrated transgenic leaves, lane 3 represents infiltrated non-transgenic leaves, lane 4 represents non-infiltrated non-transgenic leaves and lane 5 represents purified GR. For detection, a GR antiserum in a 1:1000 dilution was used and the position of detected GR is indicated.

3.3.4 GR activity measurements

When GR activity was measured in infiltrated and non-infiltrated tobacco leaves, a higher GR activity was found in all infiltrated leaves than in non-infiltrated leaves (Figure 3.10). Wild-type non-infiltrated leaves had a GR activity of 80±1.6 U/mg protein/min. This was significantly lower ($p<0.05$) than the GR activity in infiltrated leaves with an activity of 112±10.8 U/mg protein/min. Infiltrated OC-I leaves had further a significantly, almost 2-times, higher ($p<0.05$) GR activity (215±12 U/mg protein/min) than non-infiltrated OC-I leaves (114±6.2 U/mg protein/min).

3.3.5 Total protease activity

When infiltrated leaves were analyzed for differences in the total leaf protein profile, proteins were more degraded in agro-infiltrated leaves (Figure 3.11). In order to identify any proteolytic activity during leaf senescence after infiltration, protease activity was determined by SDS-PAGE containing gelatine as a protease substrate. Following protease re-activation and protein staining, the proteolytic activity is visualized as clear bands where gelatine had been hydrolysed by a protease on the gel. Three bands (P1, P2 and P3) with proteolytic activity were detected (Figure 3.12). Proteolytic activity further increased until 9 days after inoculation where a further clearance of bands was found. At this time point leaves were also almost completely yellow in color. Protease activity of band P3 showed consistent activity over the 9 day period whereas activities of P1 and P3 steadily increased over the 9 days period.

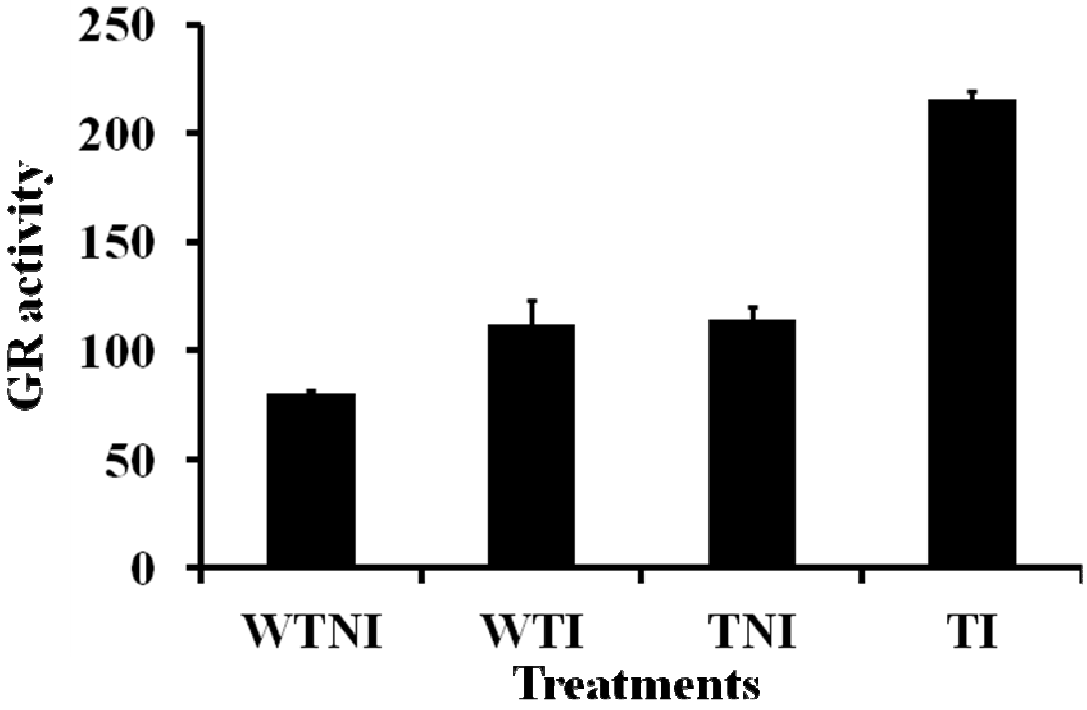


Figure 3.10: GR activity in wild type non-infiltrated (WTNI), wild-type infiltrated (WTI), transformed non-infiltrated (TNI) and transformed infiltrated (TI) leaf extracts. GR activity expressed as units/mg protein/min.

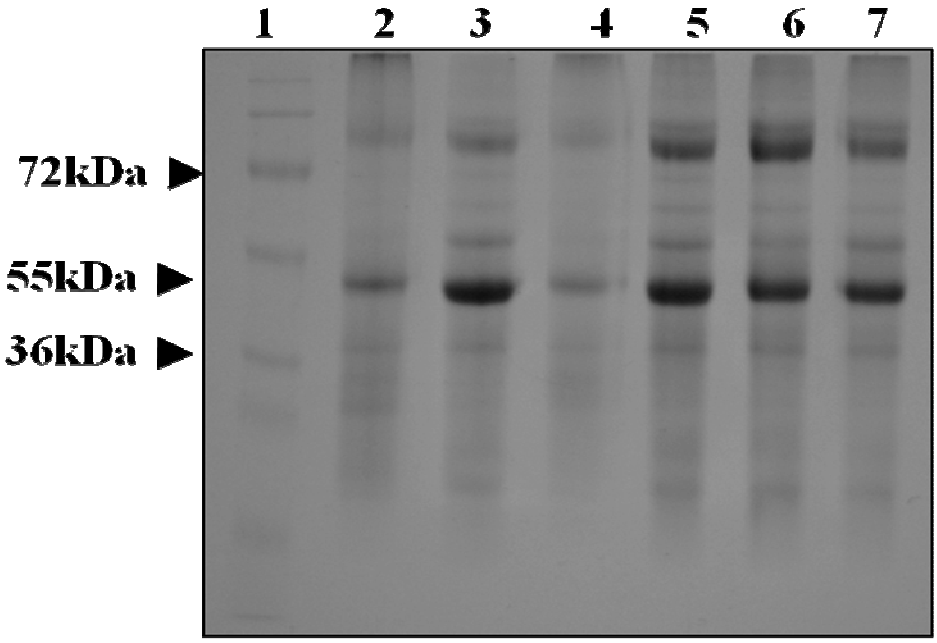


Figure 3.11: SDS-PAGE analysis of agro-infiltrated tobacco leaves. Total soluble proteins extracted 6 days after infiltration were separated on a 10% SDS-PAGE and stained with Coomassie blue. Lane 1 represents the pre-stained molecular weight marker, lanes 2: represents transformed infiltrated leaves, lane 3 represents transformed non infiltrated, lane 4 represents wild-type infiltrated and lane 5, 6, and 7 represent wild-type non-infiltrated leaves.

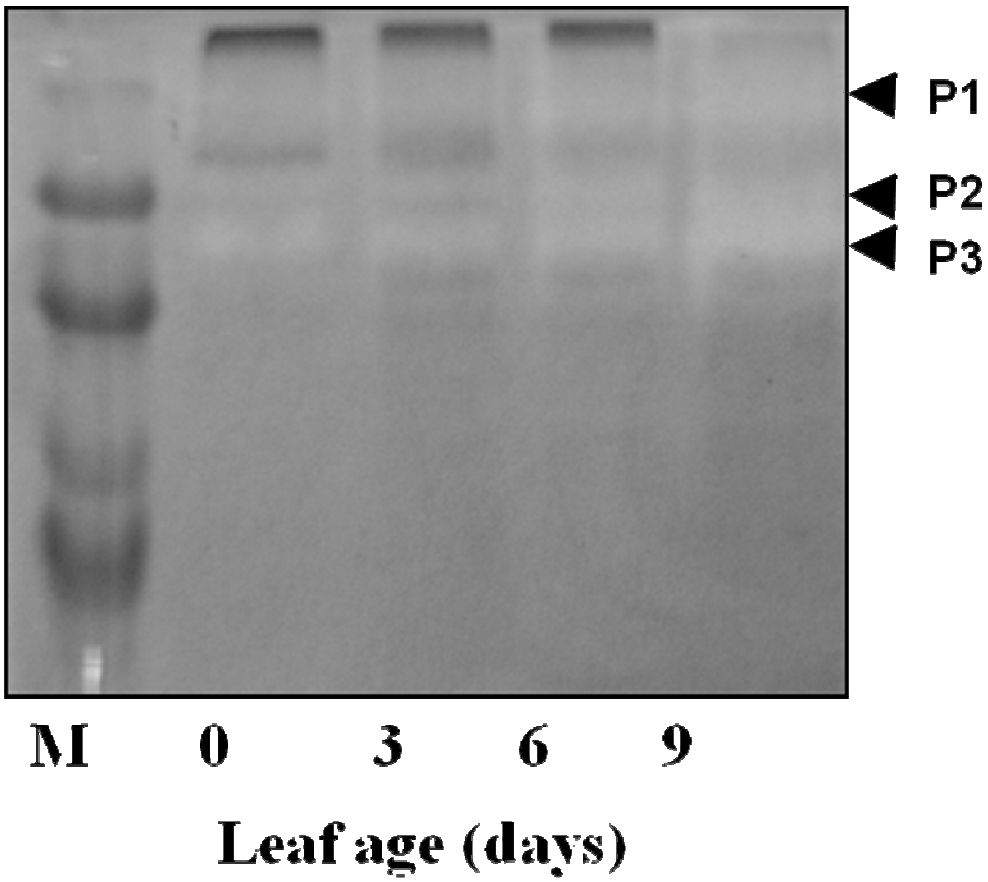


Figure 3.12: Activity gel analysis of changes in protease activity during senescence of agro-infiltrated wild-type tobacco leaves. Protein samples (25 µg) extracted at different days after inoculation and induced by agro-infiltration were separated on a 10% SDS-PAGE gel containing 1% gelatine as a protease substrate. Following protease re-activation and protein staining, protease activities (P1, P2, and P3) were detected by the appearance of a clear band.

3.3.6 Measurement of cysteine protease activity

Cysteine protease activity was determined using Z-phe-arg-AMC as a substrate for detection of cathepsin-L like activity. When protease activities of control, wild-type and OC-I transformed tobacco plants (Figure 3.13) were measured, cysteine protease activity was found to be significantly higher ($P<0.001$) in wild-type leaves (665±14.7 FU/mg protein/min) than in OC-I expressing leaves (301±12 FU/mg protein/min). Further, for wild-type leaves, cysteine protease activity was significantly higher ($p<0.05$) in infiltrated leaves (WTI) than in non-infiltrated wild-type leaves (WTNI). Also, infiltrated OC-I leaves (301±12 FU/mg protein/min) had higher cysteine protease activity that non-infiltrated leaves (201±8.2 FU mg/protein/min). Cysteine protease activity in extracts of all four types of treatments was significantly inhibited by E-64 (Table 3.1) with an efficiency of 82% and 77% for wild-type infiltrated and wild-type non-infiltrated respectively and 90% for both infiltrated and non-infiltrated OC-I expressing plants.

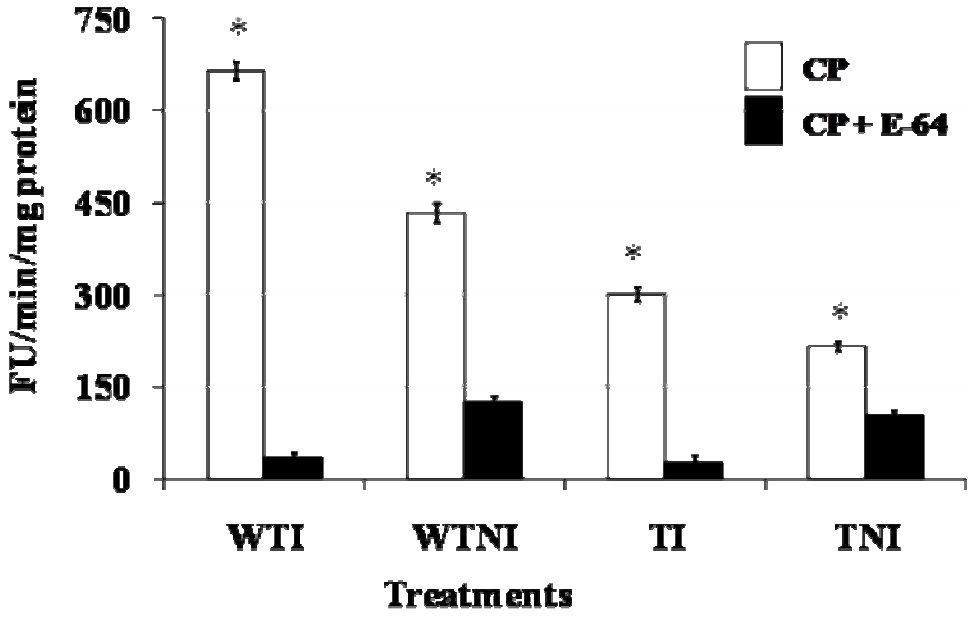


Figure 3.13: Cysteine protease activity expressed as fluorescence units in wild-type infiltrated (WTI) and non infiltrated (WTNI) as well as OC-I expressing transformed infiltrated (TI) and OC-I transformed non-infiltrated (TNI) tobacco plants and inhibition by E-64. Protein samples of 25 µg were used in each assay. Data represents the means ± SE of 4 replicates and significance level was determined by the students t-test ($p<0.001$).

Table 3.1: Inhibition of leaf cysteine proteinase activity by E-64. Proteolytic activity was measured as fluorescence units (FU)/mg protein/min. Data represents the means ±SE of 4 replicates.

Leaf type	FU/mg protein/min	E-64 inhibition (%)
WTI	665±14*	82
WTNI	434±15*	77
TI	301±11*	90
TNI	216±6*	89

*Signifcant differences among the leaf types was determined by the student's t test and are indicated by the asterisks ($p<0.001$)

CHAPTER FOUR

DISCUSSION

Transient gene expression in plants is an alternative to stable plant transformation allowing rapid production of a recombinant protein. In this study, bacterial GR was transiently expressed in *Nicotiana tabacum* leaves. A protein corresponding to a size between 45 and 50 kDa to *E. coli*-derived GR was detected after infiltrating tobacco leaves with *Agrobacterium* cells carrying the coding sequence for GR. In contrast, expression of bacterial GR was not found in non-infiltrated leaves. Furthermore, both GR activity and amounts were higher in transgenic OC-I expressing tobacco leaves than in non-transgenic leaves. Since OC-I prevents papain-like cysteine protease activity, this result suggests that OC-I expression provides GR protection against cysteine protease degradation. Sensitivity of GR to papain, a plant cysteine protease, was also found in this study.

Proteolytic processing of proteins by proteases can dramatically alter the structural integrity and overall accumulation of a recombinant protein using a plant expression system. This can occur both *in planta*, during expression, and also *ex planta* after extraction. It has been suggested that ectopic expression of specific protease inhibitors could improve the yield of proteins in vegetative organs of plants (Goulet *et al.*, 2006). Therefore, co-expression of a "companion" protease inhibitor has been recently proposed as a suitable strategy to protect plant-expressed recombinant proteins (Benchabane *et al.*, 2008). A protective OC-I effect on endogenous Rubisco and superoxide dismutase has been previously already reported (Prins *et al.*, 2008; Demirevska *et al.*, 2010). However, using transgenic tobacco plants engineered with OC-I, in particular for use in transient recombinant protein production, is still novel.Expression of an exogenous protease inhibitor in transgenic plant material can reduce endogenous protease activity. Kim *et al.* (2007) reported that serine protease inhibitor II, active against chymotrypsin and trypsin, reduced relative serine protease activity by 23% in a transformed rice cell suspension when compared to a non-transformed suspension. In this

study, transformed tobacco plants had lower cysteine protease (papain-like) activity, when OC-I was expressed in the cystosol of transgenic tobacco plants compared to non-transgenic tobacco plants. This confirms previous results reported by Van der Vyver *et al.* (2003) and Prins *et al*. (2008) of decreased cysteine protease activity in transgenic OC-I expressing tobacco plants that were also used in experiments. Papain-like cysteine proteases are generally involved in many developmental processes. This includes protein degradation and nitrogen-mobilization during the processes of seed germination (Callis, 1995) as well as during leaf senescence (Ueda *et al.*, 2000). There are, however, differences in the amounts and types of proteases expressed during plant development and that are induced by various stress factors. Proteases are progressively expressed with the onset of leaf senescence. This causes a progressive degradation of proteinaceous cellular components with recycling to other organs within the plant (De Michele *et al.*, 2009). In particular, increased activities of cysteine proteases (Wagstaff *et al*., 2002) as well as high cysteine protease transcript levels (Lohman *et al*., 1994; Buchanan-Wallaston, 2003) have been found during leaf senescence. In this study, agro-infiltration of leaves caused an increase in total proteolytic activity and activity progressively increased after infiltration. Increase in proteolytic activity was clearly visible on a gelatine-containing polyacrylamide gel where more intense de-stained bands appeared due to greater degradation of gelatine by proteases. Inclusion of gelatine into gels detects multiple proteolytic activities in crude plant extracts (Michaud *et al.*, 1993) and three major proteolytic activity bands were detected in extracts from infiltrated leaves. However, when leaf protein extracts were incubated with the cysteine protease inhibitor E-64 the band intensity indicating that cysteine (or trypsin-like) protease activity was significantly decreased in leaf material. E-64 is an irreversible cysteine protease inhibitor and will not inhibit serine proteases except trypsin and causes inhibition by forming a tight reversible

complex with cysteine proteases, acting as a pseudo-substrate entering the active site of the target enzyme (Sreedharan *et al.*, 1996).

Transient expression of GR in *N. tabacum* plants induced localized chlorosis in leaf tissues after agro-infiltration. Further, when the highest proteolytic activity was measured on a gelatine-containing polyacrylamide gel, leaves had a brown to yellow colour indicating the onset of leaf necrosis. GR harvesting was therefore carried out 6 days after agro-infiltration before severe leaf necrosis occurred. A similar result of leaf colour change was previously reported by Sawers *et al*. (2006), where transient expression of a mutant form of the maize CHLI protein induced localised chlorosis in mature leaves of *N. benthamiana*.

When immuno-detection of GR, using an antiserum raised against GR, was carried out to determine GR accumulation in agro-infiltrated leaves, a band corresponding to a size between 45 and 50 kDa for GR (Scrutton *et al.*, 1987) was detected in infiltrated leaves. Also, a band with a stronger intensity was detected in agro-infiltrated transgenic OC-I leaves compared to non-transgenic leaves indicating some protective effect of OC-I expression on GR accumulation. However, leaves possibly accumulated only a small amount of GR. Low accumulation might be partly due to transient GR expression in the cytosol. Since no signal peptide was attached to the GR sequence, GR has very likely not migrated out from the cytosol after its mRNA was translated (Benchabane *et al*., 2008). Recombinant proteins often accumulate only at a very low amount in the cytosol despite that the transgene is efficiently transcribed and also stable in this cellular compartment (Conrad and Fiedler, 1998). Possible reasons are incorrect folding of the protein and the presence of the ubiquitin–proteasome proteolytic pathway for recognition and degradation of incorrectly folded proteins (Goulet and Michaud, 2006; Vierstra, 1996). Sub-cellular compartments, such as the chloroplast or

different sub-compartments of the cell secretory pathway, are therefore regarded as more suitable locations for protein accumulation (Daniell, 2006; Ma *et al.*, 2005). For example, production of the human growth hormone in the cytosol of *Nicotiana benthamiana* leaf cells resulted in about 0.01% total soluble protein whereas 10% of total soluble protein for the hormone was obtained when targeted to the apoplast (Gils *et al.*, 2005). Foyer *et al.* (1995) previously found also much higher bacterial GR accumulation when the GR transgene was expressed in the chloroplast instead of the cytosol.

The ectopic expression of OC-I in transgenic tobacco plants has also been found to increase the protein content of tobacco leaves by 40% (Van der Vyver *et al.*, 2003). Higher protein content has also been found for transgenic potato leaves expressing a tomato cathepsin D inhibitor in the cytosol (Rivard *et al.*, 2006). However, the exact reason for higher protein production is still unclear and what role cysteine proteases, mostly located in the vacuole (Callis, 1995), are playing in protein biosynthesis. Therefore, it cannot be excluded that transgenic OC-I plants used might not have prevented GR degradation but synthesized more protein including GR than non-transgenic plants. Further, it could not be exactly determined from this study if OC-I acts either *in planta* or *ex planta*, or both (Rivard *et al.*, 2006). OC-I might have acted *ex planta* during GR recovery after tissue harvesting and leaf homogenization preventing extracted cysteine proteases directly reacting with GR. Therefore, a more detailed investigation is required to determine the exact mode of OC-I action on expressed proteins in transgenic OC-I plants.

Recently, major progress has been made to improve the yield of recombinant proteins in plant-based expression systems. However, given the individual characteristics of each recombinant protein and expression host, a case-by-case evaluation is always necessary for

the efficient production of such protein. This study has particularly provided evidence that the use of OC-I to control recombinant protein degradation, using GR as an example, is feasible. The study further contributed to provide an advanced understanding on how specific endogenous plant proteases contribute to recombinant protein degradation and whether this process can be prevented by the expression of an exogenous protease inhibitor. However, more research is still required to confirm whether these observations hold true for other cysteine protease inhibitors, or combinations of inhibitors, and also other transiently expressed transgenes. Furthermore, obtaining a better understanding of the plant protease/protease inhibitor system might contribute elucidating the role of this system in plant-based recombinant protein production. Future research might also focus on establishing which particular cysteine proteases are involved in the degradation of a specific recombinant protein. Subsequently, each inhibitor necessary for protection of a specific recombinant protein might have to be determined and routine characterization of different hosts might be required in order to recommend the most effective protease inhibitor gaining maximal recombinant protein yield.

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