Expression And Characterization Of Antimicrobial Peptides Retrocyclin-101 And Protegrin-1 In Chloroplasts To Control Viral And Bacterial Infections

2010

Baichuan Li
University of Central Florida

EXPRESSION AND CHARACTERIZATION OF ANTIMICROBIAL PEPTIDES RETROCYCLIN-101 AND PROTEGRIN-1 IN CHLOROPLASTS TO CONTROL VIRAL AND BACTERIAL INFECTIONS

by

BAICHUAN LI
B.S. China Agricultural University, 2007

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Department of Molecular Biology and Microbiology
in the College of Medicine
at the University of Central Florida
Orlando, Florida

Summer Term
2010

Major Professor: Henry Daniell

ABSTRACT

Retrocyclin-101 (RC101) and Protegrin-1 (PG1) are two important antimicrobial peptides that can be used as therapeutic agents against bacterial and/or viral infections, especially those caused by the HIV-1 or sexually-transmitted bacteria. Because of their antimicrobial activity and complex secondary structures, they have not yet been produced in microbial systems and their chemical synthesis is prohibitively expensive. Therefore, we created chloroplast transformation vectors with the RC101 or PG1 coding sequence, fused with GFP to confer stability, furin or Factor Xa cleavage site to liberate the mature peptide from their fusion proteins and a His-tag to aid in their purification. Stable integration of RC-101 into the tobacco chloroplast genome and homoplasmy were confirmed by Southern blots. RC-101 and PG1 accumulated up to 32-38% and 17~26% of the total soluble protein. Both RC-101 and PG1 were cleaved from GFP by corresponding proteases *in vitro* and Factor Xa like protease activity was observed within chloroplasts. Confocal microscopy studies showed location of GFP fluorescence within chloroplasts. Organic extraction resulted in 10.6 fold higher yield of RC 101 than purification by affinity chromatography using His-tag. In planta bioassays with *Erwinia carotovora* confirmed the antibacterial activity of RC101 and PG1 expressed in chloroplasts. RC101 transplastomic plants were resistant to TMV infections, confirming antiviral activity. Because RC101 and PG1 have not yet been produced in other cell culture or microbial systems, chloroplasts can be used as bioreactors for producing these proteins. Adequate yield of purified antimicrobial peptides from transplastomic plants should facilitate further pre-clinical studies.

ACKNOWLEDGMENTS

This investigation was supported by NIH R01 GM 63879 and USDA 3611-21000-021-02S grants to Henry Daniell. Authors are thankful to Dr. Alexander Cole and his laboratory colleagues for providing data on Figure 4a and helpful discussions, Dr. Jerry Bartz (University of Florida, Gainesville) for providing Erwinia carotovora culture and Dr. William Dawson (Citrus Research and Education Center, UF, Lake Alfred) for providing the cDNA clone of TMV, Dr. Seung-Bum Lee for providing the plant materials and data on purification, Dr. Jin for confirming the functionality of antimicrobial peptides expressed in chloroplasts.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ACRONYMS/ABBREVIATIONS

RC101	Retrocyclin-101
PG1	Protegrin-1
ELISA	Enzyme-Linked Immunosorbent Assay
CTB	Cholera Toxin B Subunit
TMV	Tobacco Mosaic Virus
STDs	Sexually Transmitted Diseases
PTD	Protein Transduction Domain

INTRODUCTION

Antimicrobial peptides are evolutionarily conserved components of the innate immune response and are found in different organisms, including bacteria, vertebrates, invertebrates and plants (Boman, 1995; Broekaert *et al*., 1997; Hancock and Chapple, 1999; Nicolas and Mor, 1995). Antimicrobial peptides are also called peptide antibiotics. When compared with conventional antibiotics, development of resistance is less likely with antimicrobial peptides. Many bacteria species remain sensitive to antimicrobial peptides after a long time of evolution (Nizet, 2006; Yeaman and Yount, 2003). Adaptive immune systems can remember the pathogen and elicit a much faster and stronger immune response against that pathogen at subsequent encounters (Boman, 1995). Without such specificity and memory, antimicrobial peptides evolved a different mechanism against pathogen infections. Most antimicrobial peptides are efficient against a broad-spectrum of pathogens rather than specific against one pathogen, which makes them especially suitable for use against local and systematic infections (Bals, 2000; Schaller-Bals *et al*., 2002). Other than the antimicrobial activities, some antimicrobial peptides are shown to have immunomodulatory activities. Some studies show that antimicrobial peptides like defensins are likely to play a role in recruiting effector T cells to inflammatory sites, thereby contributing to the effector phase of adaptive immunity (Yang *et al*., 2001). These intriguing characteristics of antimicrobial peptides facilitate development of novel antibiotics. However, the high cost of production of antimicrobial peptides and lack of suitable expression systems could be potential barriers for their development and clinical studies.

The chloroplast, as a bioreactor, is able to express foreign proteins at high levels because of their high copy numbers. When a transgene is integrated into the inverted repeat region of the chloroplast genome, up to 20,000 copies of the transgene per cell could be expressed. Several therapeutic proteins have been expressed in chloroplasts, including human blood proteins somatotropin (Staub *et al*., 2000), insulin like growth factor (Daniell et al., 2009), proinsulin (Ruhlman *et al*., 2007), IFN-α2b (Arlen *et al*., 2007), serum albumin (Fernandez-San *et al*., 2003), IFN-γ (Leelavathi and Reddy, 2003), cardiotrophin-1 (Farran *et al*., 2008), alpha1-antitrypsin (Nadai *et al*., 2009) and glutamic acid decarboxylase (Wang *et al*., 2008). In addition, several vaccine antigens have been expressed in chloroplasts against several bacterial pathogens including cholera toxin B subunit (Daniell *et al*., 2001), tetanus toxin (Tregoning *et al*., 2003), anthrax protective antigen (Koya *et al*., 2005; Watson *et al*., 2004), plague F1-V fusion antigen (Arlen *et al*., 2008), outer surface lipoprotein A (OspA) for Lyme disease (Glenz *et al*., 2006) and their functionality have been evaluated in cell culture systems or animal models after pathogen or toxin challenges. Antigens produced against protozoan pathogens were immunogenic against amoeba (Chebolu and Daniell, 2007) or effective against the malarial parasite (Davoodi-Semiromi *et al*., 2009). Although several viral antigens have been expressed in chloroplasts, neutralizing antibodies were shown only against human papillomavirus (Fernandez-San *et al*., 2008) and canine parvovirus 2L21 peptide (Molina *et al*., 2004). Other proteins expressed in chloroplasts include bovine mammary-associated serum amyloid (Manuell *et al*., 2007), aprotinin (Tissot *et al*., 2008) and monoclonal large single-chain (lsc) antibody against glycoprotein D of the herpes simplex virus (Mayfield *et al*., 2003). The expression levels of these proteins are mostly 2~20% of TSP, but could be even higher than RuBisCo (Oey *et al*.,

2009; Ruhlman *et al*., 2010). Other advantages of chloroplast transformation include multigene engineering, transgene containment, lack of position effect, gene silencing and maternal inheritance (Daniell *et al*., 2005; 2009).

Retrocyclin is a cyclic octadecapeptide, which is artificially synthesized based on a human pseudogene that is homologous to rhesus monkey circular minidefensins. Retrocyclin contains six cysteines, and has largely β-sheet structure that is stabilized by three intramolecular disulfide bonds. Structure-function studies indicate that the cyclic backbone, intramolecular tri-disulfide ladder, and arginine residues of retrocyclin contributed substantially to its protective effects (Jenssen *et al*., 2006; Trabi *et al*., 2001). Retrocyclin peptides are small antimicrobial agents with potent activity against bacteria and viruses, especially against HIV retrovirus or sexually-transmitted bacteria. Previous studies have shown that RC-101 and other retrocyclins can protect human $CD4^+$ cells from infection by T- and M-tropic strains of HIV-1 in vitro (Cole *et al*., 2002) and prevent HIV-1 infection in an organ-like construct of human cervicovaginal tissue (Cole *et al*., 2007). The ability of RC-101 to prevent HIV-1 infection and retain full activity in the presence of vaginal fluid makes it a good candidate for topical microbicide to prevent sexual transmission of HIV-1.

Protegrin-1 (PG1) belongs to the protegrin family, which is discovered in porcine leukocytes (Kokryakov *et al*., 1993). PG1 is a cysteine-rich, 18-residue β-sheet peptide. It has a high content of arginine, an amidated C-terminus, and four conserved cysteines at positions 6, 8, 13, and 15 which would form two disulfide bonds. The antimicrobial activity of PG1 is strongly related to the stability of β-hairpin conformation and the β-hairpin conformation of PG1 is

stabilized by the two disulfide bonds. Removal of both disulfide bonds would result in substantial reduction of PG1's activity (Chen *et al*., 2000; Harwig *et al*., 1996). Therefore, the disulfide bridges are very important to the activity of PG1. It was shown that PG1 had potent antimicrobial activity against a broad spectrum of microorganisms, including bacteria, fungi and yeasts (Kokryakov *et al*., 1993; Steinberg *et al*., 1997). *Chlamydia trachomatis* and *Neisseria gonorrhoeae* are two kinds of pathogenic bacteria which can cause sexually transmitted diseases (STDs) in humans. Two previous studies that compare the efficiency of PG1 with human neutrophil defensins demonstrated that PG1 is more potent than human neutrophil defensins in inactivating *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (Qu *et al*., 1996; Yasin *et al*., 1996). Therefore, a combination of RC-101 and PG1 should be able to inactivate most bacterial and viral pathogens and they will be especially effective against bacteria and viruses that cause STDs**.**

In a previous study, our lab expressed the antimicrobial peptide MSI-99, an analog of magainin 2, via the chloroplast genome to obtain high levels of protection against bacterial and fungal pathogens (DeGray *et al*., 2001). Recently, a proteinaceous antibiotic, PlyGBS lysin was also expressed in the chloroplast and it was shown that the protein synthesis capacity of the chloroplast was exhausted by the massive production of the foreign protein (Oey *et al*., 2009). However, antimicrobial peptides containing multiple intramolecular disulfide bonds have not yet been expressed in chloroplasts. In this study we investigated expression of functional disulfide-bonded antimicrobial peptides in chloroplasts. The rationale is that chloroplasts have already been shown in previous studies to be fully functional in expressing biologically active, disulfide-

bonded therapeutic proteins, such as human somatotropin (Staub *et al*., 2000), cholera toxin B (Daniell *et al*., 2001), human interferon-α2b (Arlen *et al*., 2007) and alkaline phosphatases (Bally *et al*., 2008). Because of the high cost associated with chemical synthesis and inability of cell culture or microbial systems to produce these proteins, expression of RC101 or PG1 antimicrobial peptides in chloroplasts would be an ideal solution for their large scale economic production.

EXPERIMENTAL PROCEDURES

PCR Analysis to Confirm Transplastomic Plants

Total plant DNA was isolated from transplastomic tobacco leaves using the DNeasy Plant Mini Kit from Qiagen Company. PCR was set up with two pairs of primers, 3P-3M and 5P-2M (Verma *et al*., 2008) to confirm the successful transformation of tobacco chloroplasts. The 3P primer (AAAACCCGTCCTCAGTTCGGATTGC) anneals with the native chloroplast genome while 3M primer (CCGCGTTGTTTCATCAAGCCTTACG) anneals with the *aadA* gene. Therefore this pair of primers was used to check site-specific integration of selectable marker genes into the chloroplast genome. The 5P primer (CTGTAGAAGTCACCATTGTTGTGC) anneals with the *aadA* gene while 2M primer (TGACTGCCCACCTGAGAGCGGACA) anneals with the *trn*A gene, which was used to check integration of the transgene expression cassette.

Southern Blot to Confirm Homoplasmy

Total plant DNA was digested with *Apa*I enzyme and then separated on a 0.8% agarose gel. After electrophoresis, the gel was soaked in 0.25N HCl depurination solution for 15 minutes, and then rinsed 2 times in water, 5 minutes each. After that, the gel was soaked in transfer buffer (0.4N NaOH, 1M NaCl) for 20 minutes, and then the dry transfer was set up. After transfer, the membrane was rinsed with 2×SSC 2 times for 5 minutes each. After the membrane was dry, it was cross-linked using GS GeneLinker UV Chamber at C3 setting. The 0.81 kbp flanking sequence probe was prepared by digesting pUC-CT vector with *Bam*HI and *Bgl*II (Figure 1c). After the probe was labeled with ^{32}P, hybridization of the membrane was done by using Stratagene QUICK-HYB hybridization solution and protocol (Stratagene, La Jolla, CA).

Factor Xa and Furin Cleavage Assays

RC-101 tobacco transplastomic leaves (100 mg) were ground in liquid nitrogen and homogenized in 200 µl of plant extraction buffer (0.1 N NaOH, 1 M Tris-HCl, pH4.5) using a mechanical mixer. The homogenized plant extract was then centrifuged for 5 minutes at 14,000rpm at 4 °C. The extract (10 µg) was then incubated with 1 µg of Factor Xa protease in 20mM Tris-HCl (pH 8.0 @ 25 °C) with 100 mM NaCl and 2 mM $CaCl_2$ overnight at 23 °C. The cleaved products were loaded with uncleaved RC-101 protein extracts on the same gel to

investigate cleavage of RC101-GFP fusion protein. Western blot analysis was performed as described below.

Total protein from the PG1-GFP transplastomic tobacco leaves were extracted the same way as RC101-GFP described above. The extract (10 µg) from PG1-GFP transplastomic tobacco leaves was incubated with 1 unit of furin in a total reaction volume of 25 µl containing 100 mM Hepes (pH7.5, 25 °C), 0.5% Triton X-100, 1 mM CaCl2, 1 mM 2-mercaptoethanol at 25 °C.

Native Polyacrylamide Gel Electrophoresis and Densitometric Analysis

Total protein from the RC101-GFP and PG1-GFP transplastomic plants were extracted as described above. The TSP concentration was determined by the Bradford assay and then different amount of TSP was loaded with native gel loading buffer (60 mM pH 6.8 Tris-HCl, 25% glycerol and 0.01% Bromophenol blue) into the 12% native polyacrylamide gel. After electrophoresis, the gel was scanned and analyzed for the presence of GFP fusion proteins using AlphaImager® and AlphaEase® FC software (Alpha Innotech, San Leandro, CA, USA). The integrated density values (IDVs) of the GFP standards and samples were recorded and analyzed further.

Western Blot Analysis

Frozen leaf materials (100 mg) were ground in liquid nitrogen and then resuspended in 200 µl of plant extraction buffer. The supernatant was collected after centrifuging the sample for 5 minutes at 14,000rpm. The plant extract was mixed with 2x sample loading buffer and then boiled for 5 minutes before loading. The transformed, untransformed plant extracts and recombinant GFP standard (Vector Labs) were loaded onto the 12% SDS-PAGE gel. The proteins in the gel were then transferred to the nitrocellulose membrane at 100V for 1 hour. After transfer, the membrane was first blocked in PTM (1X PBS, 0.1% Tween-20, 3% milk) for 2 hours at room temperature and then incubated with chick anti-GFP primary antibody (Chemicon) at 1:3000 dilution in PTM for 2 hours at room temperature. After the membrane was washed 3 times with PBS-T (1X PBS, 0.1% Tween-20), 5 minutes each time, rabbit anti-chick secondary antibody conjugated with HRP was added at 1:3000 dilution in PTM and then incubated for 1 hour at room temperature.

PG-1 Furin Cleavage Assay and Silver Staining

After furin digestion, PG1 was cleaved off from GFP. Because of non-availability of PG1 antibody, we used silver staining to investigate the presence of the 2.1 kDa PG1 protein after furin cleavage. The cleaved products of PG1-GFP fusion protein were separated in a 16.8% tris-tricine gel to get the maximum resolution in the ≤10 kDa range. Untransformed plant extracts, Marker 12 unstained standard (Invitrogen), PG1-GFP plant protein extracts before and after furin digestion were mixed with sample loading buffer and loaded on the 16.8% gel. After electrophoresis, the gel was stained by silver staining.

Confocal Microscopy

Untransformed, RC101-GFP and PG1-GFP transplastomic tobacco leaves were harvested fresh before microscopic analysis. They were cut into 5 mm× 5 mm small pieces and fixed on slides. Confocal microscope (Olympus FluoView) with adjustable bandwidths of the detected fluorescence wavelength was used. The filter used was 505-525nm. GFP fluorescence from the samples was detected and saved as digital format files.

ELISA Quantification of RC101-GFP and PG1-GFP Fusion Proteins

All the untransformed, transplastomic plant protein extracts (all the extracts used here were the same as used in Bradford assay) and recombinant GFP standard (Vector Laboratories, MB-0752) were diluted using the ELISA coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6). The recombinant GFP standard was serially diluted from 100 ng/ml to 3.125 ng/ml. Different dilutions of test samples were prepared ranging from 1:1000 to 1:9000. The wells of a 96-well microtiter EIA plate were coated with 100 µl of diluted test samples and standards. The plate was covered with an adhesive plastic and incubated for 2 hours at room temperature. After incubation, the coating solution was removed and the plate was washed twice by filling the wells with 200 µl PBS and once by water. The coated wells were blocked by adding 200 µl of blocking buffer (3% dry milk in PBS). Then the plate was covered and incubated for 2 hours at room temperature. After removing the blocking buffer, the plate was washed again as described before.

Mouse anti-GFP IgG monoclonal antibody (Chemicon, MAB3836) at 1:2000 dilution was added and incubated for 2 hours at room temperature. After washing twice with PBS and once with water, HRP conjugated goat anti-mouse IgG antibody (American Qualex) at 1:2000 dilution was added and incubated for 2 hours at room temperature. After washing, the plate was developed with TMB (3, 3', 5, 5'-Tetramethylbenzidine). The absorbance of each well was read with a microplate reader (Biorad, model 680).

RESULTS

Construction of Chloroplast Transformation Vectors

Two chloroplast transformation vectors were designed for expressing RC-101 and PG1 in chloroplasts. They were constructed by Dr. Seung Bum Lee using the basic pLD vector, which was developed in our laboratory for chloroplast transformation (Daniell *et al*., 1998; Verma *et al*., 2008). Both PG1 and RC-101 genes were fused with GFP because of their small size (18 amino acids). Besides, GFP was used as a reporter and in quantification of the fusion proteins. A 6-histidine tag was also engineered upstream of RC-101/PG1 to facilitate purification of these fusion proteins. A furin protease cleavage site was engineered between PG1 and GFP while a Factor Xa protease cleavage site was engineered between RC-101 and the 6-histidine tag to facilitate release of PG1/RC-101 from these fusion proteins. The promoter and 5'-untranslated region (UTR) of the tobacco *psb*A gene was placed upstream of the His_6-GFP-Furin-PG1/GFP-

His_6-Xa-RC101 transgene cassette to enhance expression of these fusion proteins. The *aad*A gene, which conferred resistance to spectinomycin, was driven by the constitutive P*rrn* promoter. The flanking sequences of *trn*I and *trn*A facilitated recombination with the native chloroplast genome (Figure 1b-c). The transgene fragment sequences and the disulfide bonds of RC101 and PG1 are shown in Figure 1d-e.

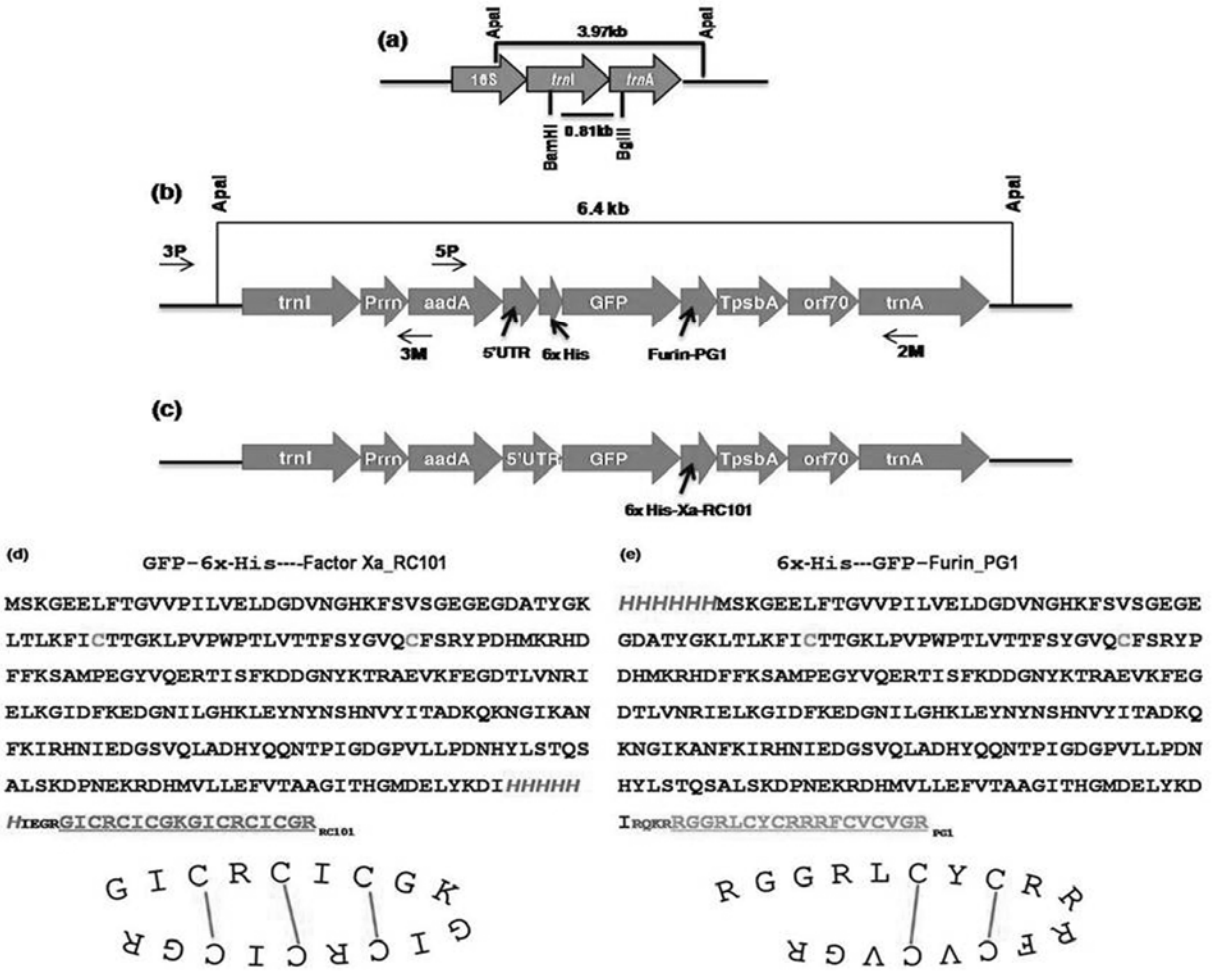

Figure 1: Schematic representation of the chloroplast vectors.

(a) The native chloroplast genome showing both homologous recombination sites (*trn*I and *trn*A) and the restriction enzyme sites used for Southern blot analysis. (b) The pLD-His_6-GFP-Furin-PG1 vector map

with the primer annealing sites. (c) The pLD-GFP-His_6-Factor Xa-RC101 vector map; primer annealing sites are the same as shown on the PG1 vector map. (d) The nucleotide sequence of GFP-6xHis-Factor Xa-RC101 and the schematic representation of disulfide bonds in RC101. (e) The nucleotide sequence of 6xHis-GFP-Furin-PG1 and the schematic representation of disulfide bonds in PG1.

Confirmation of Transgene Cassette Integration and Homoplasmy

The bombardments of RC101 and PG1 tobacco plants were performed by Dr. Seung-Bum Lee. Several primary shoots appeared from the RC101 and PG1 bombarded tobacco leaves and they were developed through three rounds of selection. To confirm integration of transgene cassettes into the chloroplast genome, the putative transformed shoots were screened by PCR. Two pairs of primers were used for screening. The 3P and 3M primers were used to check site-specific integration of the selectable marker gene (*aad*A) into the chloroplast genome. The 5P and 2M primers were used to check integration of the transgene expression cassette (Figure 1b-c). DNA template from the RC101-GFP and PG1-GFP transplastomic shoots yielded PCR products with both primers (Figure 2a-b). The 3P-3M PCR products for both the RC101 and PG1 transformants were 1.65 kbp and 5P-2M PCR products were 2.6 kbp. Because the sizes of the RC101 and PG1 transgene expression cassette (including GFP) were of similar size, PCR product sizes were also similar. These PCR products could be generated only from transformed chloroplasts and not nuclear transformants or spontaneous mutants.

Because there are thousands of copies of chloroplast genomes in each plant cell, some of them may not be transformed. Therefore, Southern blot was performed to investigate whether

RC101 and PG1 transplastomic plants achieved homoplasmy. The probe used was made by digesting the flanking sequences *trn*I and *trn*A with *Bam*HI and *Bgl*II (Figure 1a). Flanking sequence probe identified a single 4.0 kbp fragment in the untransformed tobacco, as expected. In the RC-101 and PG1 transplastomic lines, only one 6.4 kbp fragment was observed (Figure 2c). Absence of the 4.0 kbp fragment confirmed that all the chloroplast genomes were transformed (to the detection limit of Southern blots) and therefore they are considered to be homoplasmic.

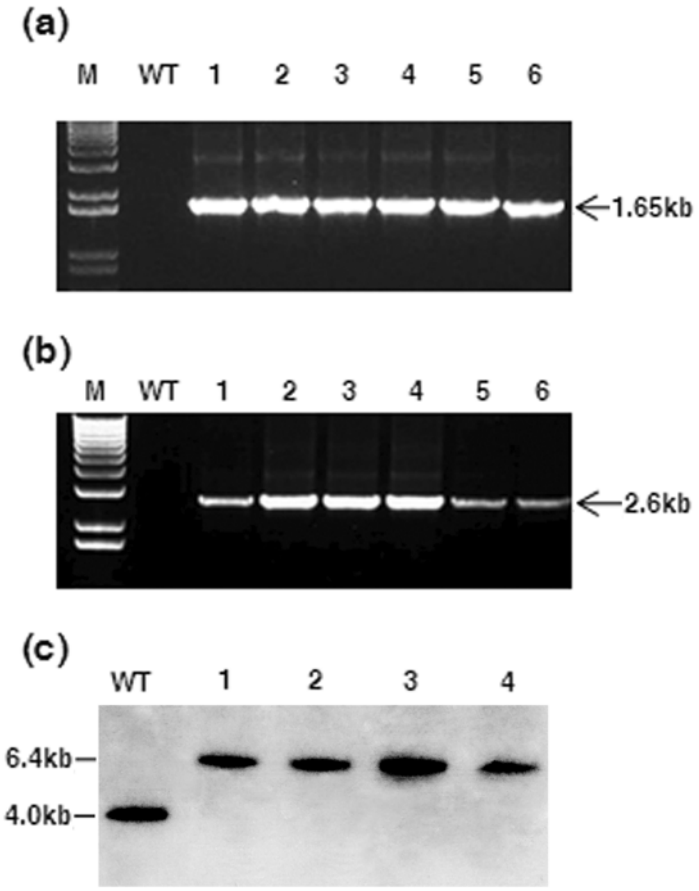

Figure 2. PCR and Southern blot analysis to confirm trangene integration and homoplasmy.

(a) PCR analysis of the untransformed and transplastomic lines using the primer pair 3P/3M. Lanes 1-3: RC-101 transplastomic lines; 4-6: PG1 transplastomic lines. (b) PCR analysis of the untransformed and transplastomic lines using the primer pair 5P/2M. Lanes 1-3: RC-101 transplastomic lines; 4-6: PG1 transplastomic lines. (c) Southern blot hybridized with the flanking sequence *trn*I-*trn*A probe to investigate the homoplasmy of RC101 and PG1 transplastomic lines. Lanes 1-2, DNA samples from RC101 transplastomic plants; lanes 3-4, PG1 transplastomic plants. M, 1 kbp DNA plus ladder; WT, untransformed tobacco.

Evaluation of RC101 or PG1 Expression in Transgenic Chloroplasts

To evaluate expression of foreign genes in chloroplasts of RC101-GFP and PG1-GFP transplastomic lines, immunoblots using GFP antibodies were performed. Based on the TSP concentration, same amount of protein extracts from RC-101 and PG1 transplastomic lines (before and after protease digestion) were resolved on 12% SDS-PAGE gels. The size of RC-101 is 1.9 kDa while the size of PG1 is 2.1 kDa. Therefore, the sizes of RC101-GFP and PG1-GFP are both ~29 kDa. After cleavage of RC101 and PG1 from GFP, we should observe only the 27 kDa GFP polypeptide. The immunoblot result is shown in Figure 3a. Clearly, the fusion proteins were cleaved after protease digestion.

An alternative approach to confirm the expression of RC101-GFP and PG1-GFP proteins is to observe the green fluorescence emitted by GFP. After crude protein extracts were resolved on the native polyacrylamide gel, the green fluorescence emitted by GFP fusion proteins was observed under the UV light. The green peptides shown correspond to the GFP fusion proteins. The strong green fluorescence observed indicated that GFP fusion proteins were expressed at high levels (Figure 3b). The expression of RC101 and PG1 transplastomic plants were quantified using the GFP fluorescence by densitometric analysis. The integrated density values (IDVs) of

GFP fluorescence were measured by spot densitometry. The linear GFP standard curve was established using 150 - 600 ng of GFP standard protein (Figure 3c). Based on this GFP standard curve, the expression levels of RC101 and PG1 transplastomic plants were estimated to be approximately 35% and 25% of TSP (Figure 3d). To confirm the expression levels of the transplastomic plants, ELISA was also performed to determine the quantities of RC101-GFP and PG1-GFP fusion proteins in transplastomic tobacco plants. Because the antimicrobial peptides RC-101 and PG1 were fused with GFP proteins, ELISA was performed using the GFP antibodies to quantify the RC101-GFP and PG1-GFP fusion proteins. RC101-GFP accumulated to 32~38% of TSP and PG1-GFP accumulated to 17~26% of TSP. This variation of expression levels could be due to leaf samples harvested from plants under different periods of illumination.

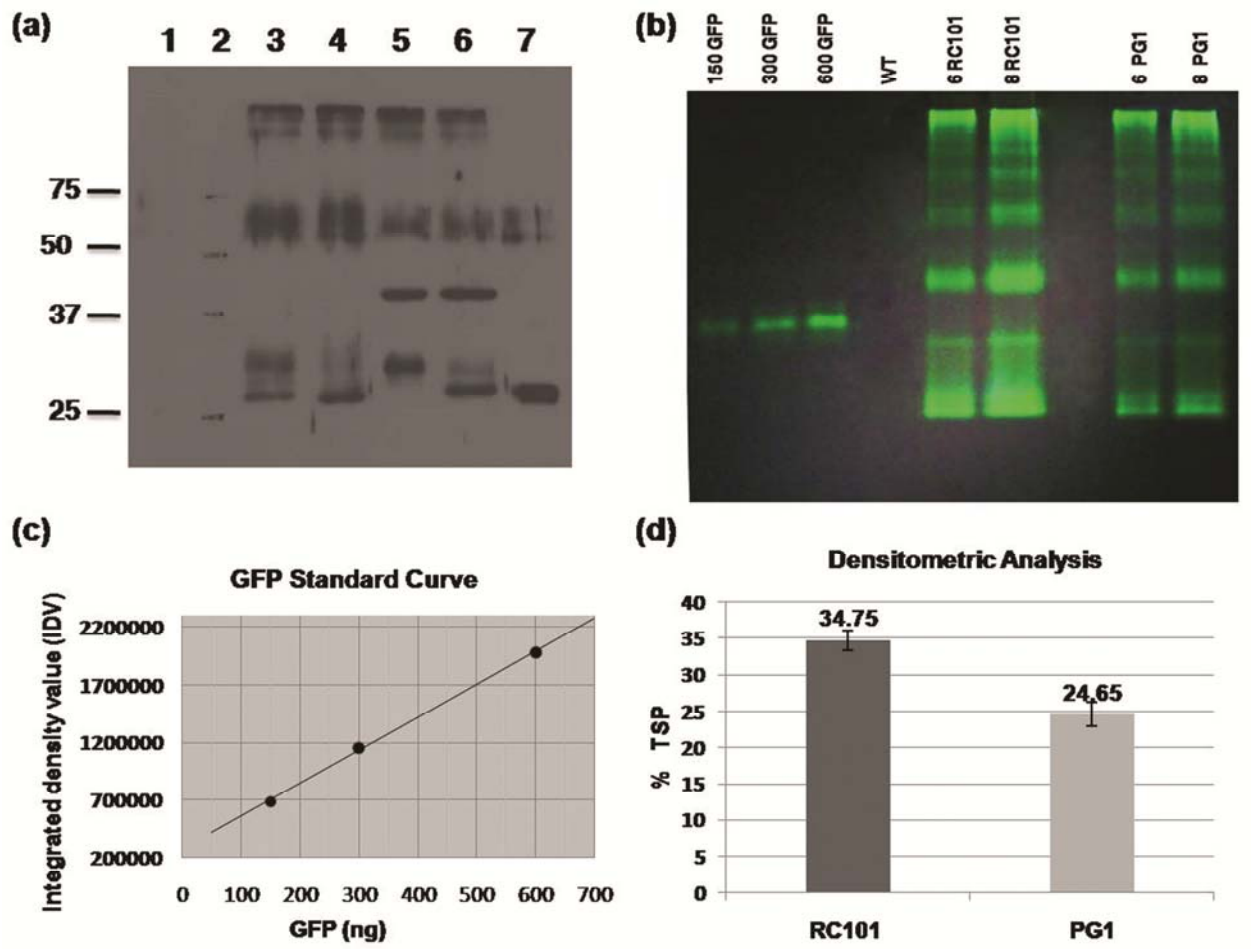

Figure 3. Protease cleavage of the fusion proteins by immunoblot and quantification of expression by densitometric analysis.

(a) Immunoblot analysis of RC101-GFP and PG1-GFP expression and cleavage. 1: Untransformed protein extract, 10 µg; 2: Precision Plus protein marker, 5 µg; 3: RC101-GFP transplastomic line protein extract, 3 µg; 4: RC101-GFP protein extract digested by Factor Xa protease, 3 µg; 5: PG1-GFP protein extract, 6 µg; 6: PG1-GFP protein extract digested by furin protease, 6 µg; 7: GFP standard, 100 ng. (b) Native polyacrylamide gel electrophoresis of RC101-GFP and PG1-GFP protein extracts. Lanes 1-3, GFP standard (150, 300, 600 ng); lane 4, untransformed plant extract, 10 µg; lanes 5-6, RC101 transplastomic extracts (6, 8 µg); lanes 7-8, PG1 transplastomic extracts (6, 8 µg). (c) GFP standard curve based on the IDVs of 150, 300 and 600 ng of GFP standard. (d) Estimation of RC101-GFP and PG1-GFP expression levels in transplastomic plants.

Dot blot analysis was performed by Dr. Cole's lab to evaluate the expression of RC-101 in transgenic chloroplasts. Factor Xa cleaved samples and uncleaved samples from RC-101 transplastomic plants were tested by dot blots. It is shown that both uncut and cut samples of RC101-GFP appeared positive (Figure 4a). As shown in previous experiments (Figure 3a), RC101-GFP fusion proteins were already partially cleaved by Factor Xa within chloroplasts. Because PG1 was not immunogenic, dot blot analysis could not be done with PG1 transplastomic plants. Instead, PG1 protein expression was examined by silver staining. By comparison of cut and uncut samples from PG1 transplastomic plants, it is clear that there is a 2 kDa polypeptide present in the furin digested sample but absent in the uncut sample and untransformed tobacco protein extract (Figure 4b). The size of PG1 is 2.16 kDa and therefore this polypeptide should correspond to the PG1 protein.

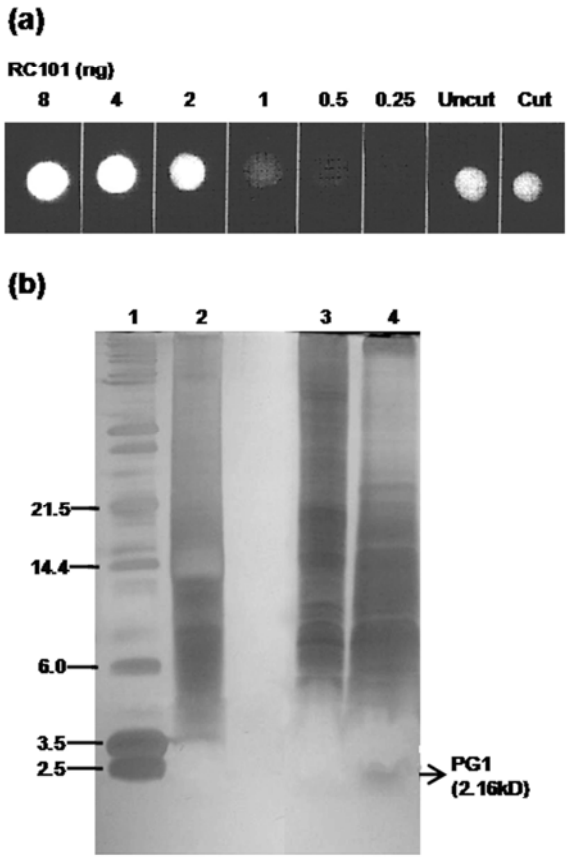

Figure 4. Dot blot analysis and silver staining to investigate expression of RC101 and PG1.

(a) Dot blot analysis of RC101 before and after cleavage. Indicated amount of RC101 was used as standards. Uncut, RC101-GFP without Factor Xa cleavage; Cut, RC101-GFP after Factor Xa cleavage. (b) Silver stained gel of plant extracts before or after furin cleavage of PG1-GFP protein. 1: Marker 12 (Invitrogen); 2: Untransformed plant protein extract, 40 μg; 3: PG1-GFP protein extract without furin digestion, 40 μg; 4: PG1-GFP protein extract digested by furin protease, 40 μg.

RC101 and PG1 were Expressed and Contained within Chloroplasts

In order to investigate whether the chloroplasts remained intact when RC101 or PG1 antimicrobial peptides were highly expressed in chloroplasts, fresh leaves were examined under the confocal microscope. Strong green fluorescence was emitted from the RC-101 and PG1

transplastomic lines (Figure 5a-b). We observed that chloroplasts emitting green fluorescence formed circles around each cell. There was no GFP fluorescence outside chloroplasts. This observation confirmed that chloroplasts remained intact because GFP fused antimicrobial proteins were not released into the cytoplasm in any detectable quantity.

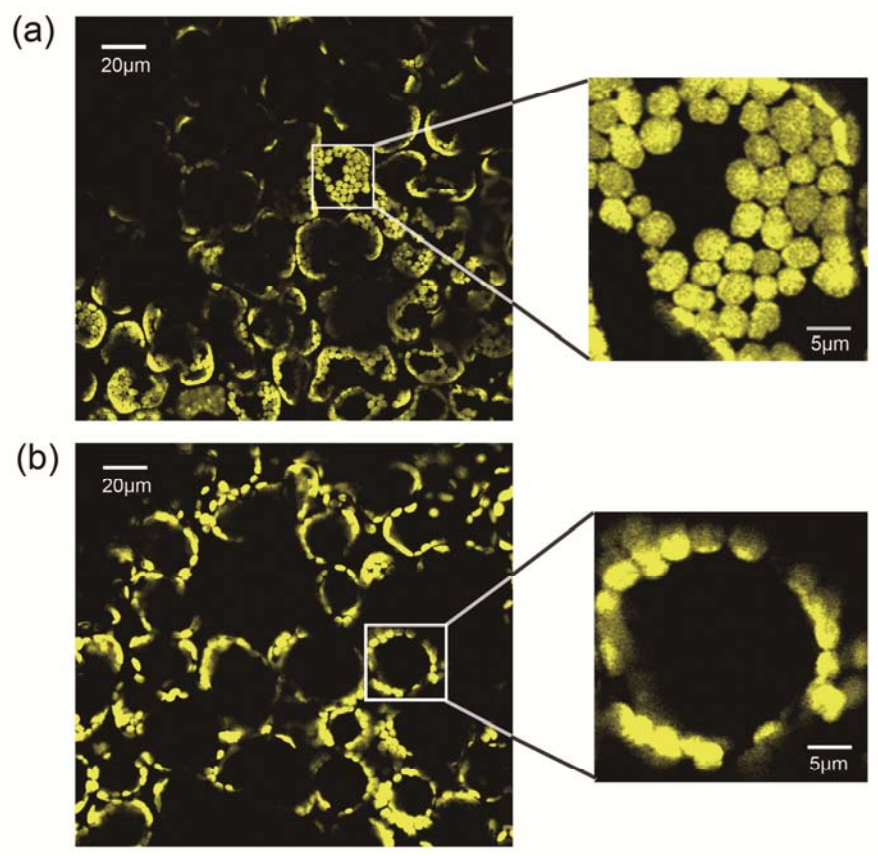

Figure 5. Confocal microscopy of RC101-GFP and PG1-GFP transplastomic plants.

The left panels shows chloroplasts from RC101-GFP (a) or PG1-GFP (b) transplastomic lines (bars = 20 μm). The right panels showed four times higher magnification of the boxed regions (bars = 5 μm).

Comparison of Proteins Containing Different Number of Disulfide Bonds

RC-101 is a cyclic antimicrobial peptide with three disulfide bonds and PG1 is a β-sheet peptide with two disulfide bonds. In both RC101 and PG1, the intramolecular disulfide bonds are important for their antimicrobial activity. To investigate formation of disulfide bonds in proteins expressed within chloroplasts, we compared proteins expressed in chloroplasts with different number of disulfide bonds by immunoblot of native PAGE, including PTD-GFP (0), PG1-GFP (2), RC101-GFP (3), and CTB-GFP (1). CTB is known to form pentamers and that would be 5 disulfide bonds in total. The numbers denote the number of disulfide bonds in each protein. Because the expression levels of these transplastomic plants were different, different amount of plant extracts were loaded (Figure 6). There are no multimers present in the PTD-GFP lane while there are multimers in the CTB-GFP, RC101-GFP and PG1-GFP lanes.

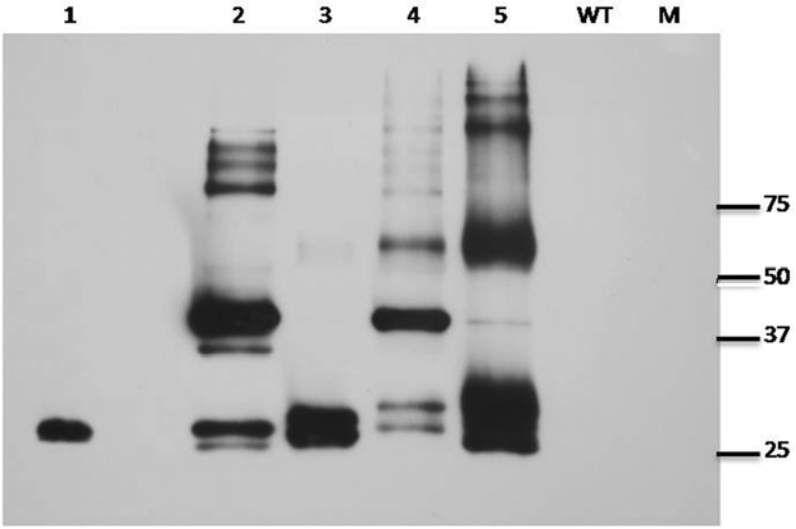

Figure 6. Native gel western blot to compare proteins with different numbers of disulfide bonds expressed in chloroplasts.

Lane 1: Recombinant GFP standard protein, 40 ng; 2: protein extract from CTB-GFP transplastomic plant, 3 μg; 3: PTD-GFP extract, 1 μg; 4: PG1-GFP extract, 3.5 μg; 5: RC101-GFP extract, 2 μg; WT, untransformed tobacco; M, Marker. Mouse anti-GFP primary antibody and goat anti-mouse secondary antibody was used.

Purification of RC101-GFP and PG1-GFP Fusion Proteins

Purification of the RC101-GFP and PG1-GFP fusion proteins was performed by Dr. Lee. The engineered His-tag and GFP protein facilitated purification of RC101-GFP and PG1-GFP fusion proteins. We tried to purify the fusion proteins by affinity chromatography using His-tag or organic extraction through GFP. Results of purification using both methods are shown in Figure 7. Approximately 8 μg of purified PG1-GFP and 5 μg of purified RC101-GFP were obtained from one gram of fresh tobacco leaf by using the affinity chromatography method. In contrast, purification of RC101-GFP using the organic extraction method resulted in a yield of 53 μg purified RC101-GFP per gram of fresh tobacco leaf. The organic extraction method resulted in much higher yield than the affinity chromatography method. It is evident that monomers, dimers and multimers of the RC101-GFP were recovered by organic extraction method, resulting in 10.6 fold higher yield whereas only the monomer was recovered using the affinity chromatography. The highly enriched fraction was the RC101-GFP monomer, ~29 kDa in size. The upper bands should be dimers and multimers formed by RC101-GFP proteins. This same pattern was observed in the native gel electrophoresis of RC101-GFP transplastomic plant protein extracts (Figure 3b). PG1-GFP protein was purified only by affinity chromatography, and we could observe a single band, which should be the monomer form of PG1-GFP.

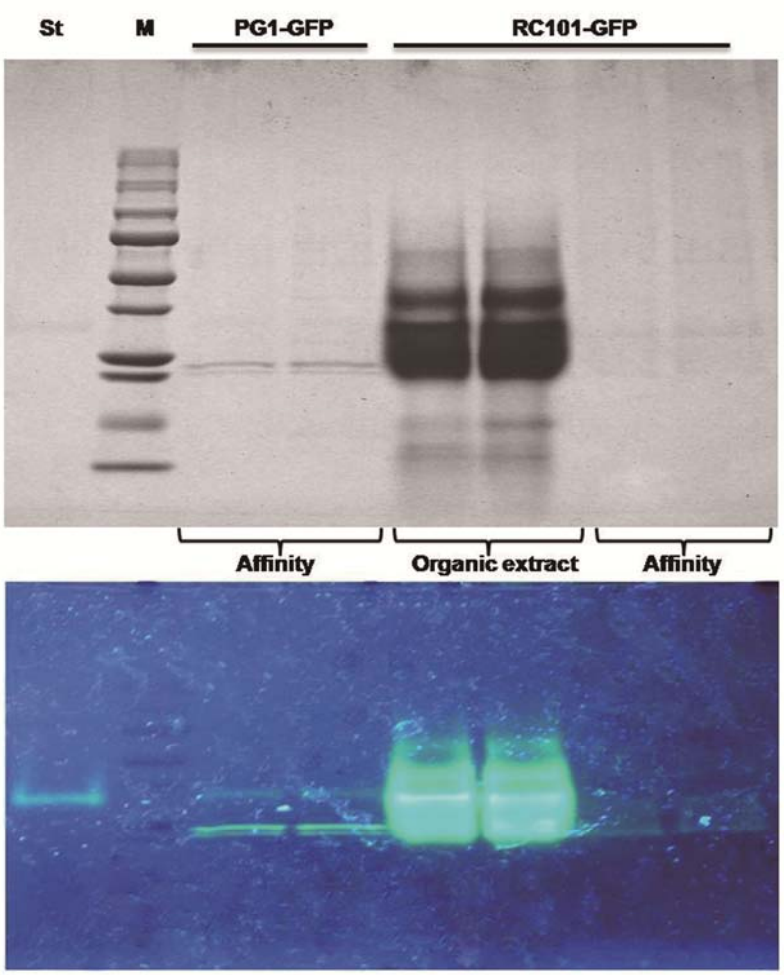

Figure 7. Purified RC101-GFP and PG1-GFP fusion proteins were separated on native PAGE and observed by Coomassie staining or fluorescence under UV light.

PG1-GFP was purified by affinity chromatography and RC101-GFP was purified by both affinity chromatography and organic extraction method. Samples were loaded in duplicate. M, Precision Plus protein marker, 5 μg; St, GFP standard, 500 ng. The same gel was observed under UV light (bottom) or stained by Coomassie staining (top). The yield of RC101-GFP was 5 μg/g leaf by affinity chromatography and 53 μg/g leaf by organic extraction; PG1-GFP yield was 8 μg/g leaf by affinity chromatography purification.

RC101 and PG1 Retained Their Antimicrobial Activity when Expressed in Chloroplasts

Retrocyclin-101, as a member of the θ-defensin family, possesses antibacterial activity as well as antiviral activity (Tang *et al*., 1999). To investigate the functionality of RC101 and PG1 expressed in the tobacco chloroplasts, both antibacteria and antivirus assays using plant pathogens were performed (these studies were performed by Dr. Jin in our lab) because use of HIV and other human bacterial pathogens require higher levels of containment than our current facilities. The antibacterial activity of RC101 and PG-1 was studied by investigating enhanced resistance to *Erwinia* soft rot either by using the syringe or sand paper method. One day after inoculation with *Erwinia*, the first signs of damage were observed on leaves of untransformed plants in the regions of inoculation. On the 3rd day, virtually all inoculated untransformed leaf surfaces underwent necrosis whereas in leaves of RC101 or PG1 transplastomic plants, no or minimally damaged zones were observed depending on the number of bacteria inoculated. Inoculation of potted plants with *E. carotovora* using a syringe method resulted in areas of necrosis surrounding the point of inoculation in untransformed control for all cell densities (Figure 8b, f), whereas transplastomic RC101 and PG-1 mature leaves showed no areas of necrosis (Figure 8a, e). Even inoculation of 10^8 cells resulted in no or minimal necrosis in mature transplastomic leaves. In contrast, untransformed plants inoculated with 10^2 cells displayed obvious necrosis. Similar results were obtained with *E. carotovora* inoculated by the sand paper method. Transplastomic mature leaves inoculated with *E. carotovora* showed no necrosis (Figure 8c) or a mild discoloration at the site of inoculation of 10^8 cells (Figure 8g) and untransformed plants inoculated with 10^2 cells or higher density displayed obvious necrosis (Figure 8d, h).

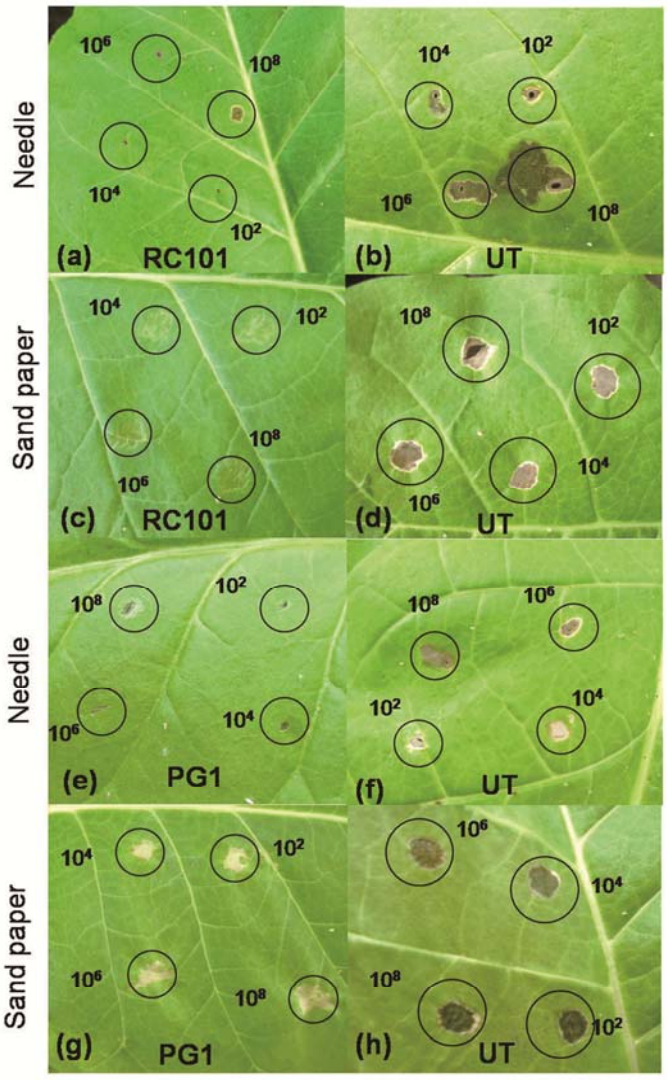

Figure 8. In planta antimicrobial bioassays to investigate functionality of RC101 and PG1 expressed in chloroplasts.

Twenty µl of the 10^8, 10^6, 10^4 and 10^2 cells from an overnight culture of *E. carotovora* were injected into leaves of (a) RC101, (e) PG-1 transplastomic, and (b, f) untransformed (UT) plants using a syringe with a precision glide needle. Five- to 7-mm areas of (c) RC101, (g) PG-1 and (d, h) untransformed leaves were scraped with fine-grain sandpaper. Twenty µl 10^8, 10^6, 10^4 and 10^2 cells of *Erwinia* were inoculated to each prepared area. Photos were taken 5 days after inoculation.

The bacteria count in inoculated plants was also estimated. Bacterial suspensions (1.0×10^5 cfu/ml) of *E. carotovora* were inoculated into transplastomic and untransformed leaves by a syringe. Following inoculation, the density of *E. carotovora* in untransformed, RC101 and PG-1 transplastomic leaves was less than 1×10^5 cfu/cm^2 at 0 day post-inoculation. Three days after inoculation, the population of *E. carotovora* in untransformed tobacco leaves reached 2.0×10^8 cfu/cm^2 (Figure 9a, b). In comparison, the density of *E. carotovora* was less than 1×10^4 cfu/cm^2 in both RC101 (Figure 9a) and PG1 (Figure 9b) transplastomic leaves three days after inoculation, a 10,000 fold reduction in bacterial burden. In addition, no apparent symptoms of necrosis were observed in any of the RC101 or PG1 plants. These results demonstrated that the RC101 and PG1 transplastomic plants are resistant to *E. carotovora.* Therefore, RC101 and PG1 maintained their antibacterial activity when expressed in chloroplasts.

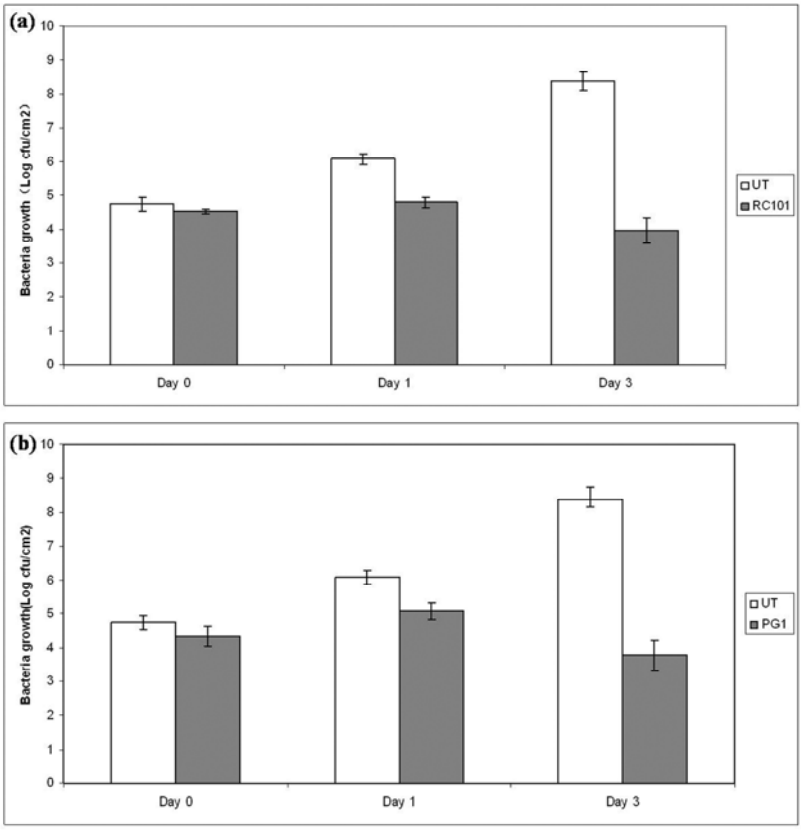

Figure 9. Bacteria density in the PG1, RC101 and untransformed (UT) plants inoculated with *E. carotovora*.

(a) Bacteria density in RC101 and untransformed leaves. (b) Bacteria density in PG1 and untransformed leaves. The bacteria density in plants on 0, 1 and 3 days after inoculation. All values represent means of 6 replications with standard deviations shown as error bars.

To determine the antiviral activity of PG1 and RC101 when expressed in tobacco chloroplasts, transplastomic and untransformed control plants were tested for tobacco mosaic virus (TMV) infection for 20 days. In susceptible untransformed control and PG1 plants, TMV multiplied and spread throughout the plants, causing typical mosaic, necrosis and wrinkle symptoms within 20 days after inoculation (Figure 10a, b). However, the RC101 transplastomic

plants didn't show obvious symptoms of TMV infection, and the plants grew well (Figure 10c). These results confirmed the antiviral activity of RC101 by conferring resistance to TMV when expressed in chloroplasts.

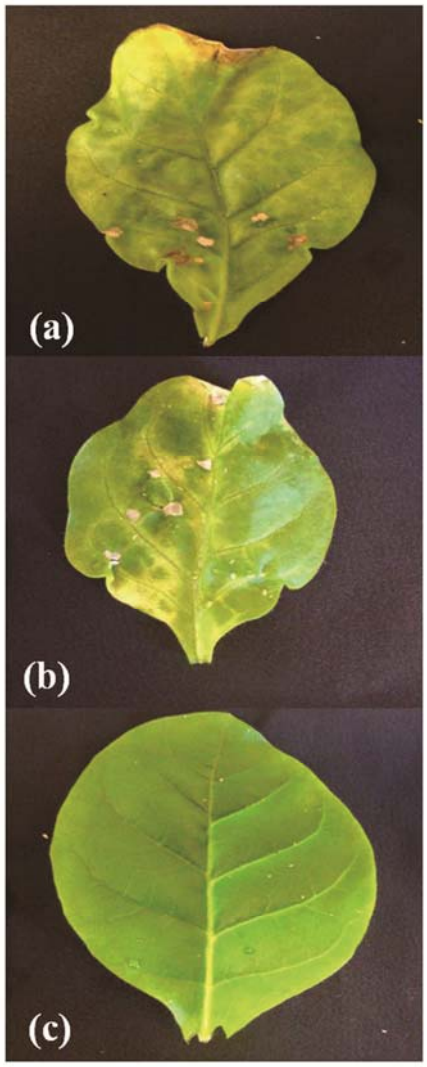

Figure 10. Response of untransformed and RC101/ PG1 transplastomic plants to TMV.

(a) TMV inoculated leaf from untransformed plant; (b) TMV inoculated leaf from transplastomic PG1 plant. (c) TMV inoculated leaf from transplastomic RC101 plant. Pictures were taken on 20 days after inoculation.

DISCUSSION

RC101 and PG1 are antimicrobial peptides that have potent antimicrobial activities against a broad spectrum of microorganisms. Both RC101 and PG1 are disulfide-bonded proteins. RC101 contains three and PG1 contains two intramolecular disulfides bonds that are important for their antimicrobial activities (Chen *et al*., 2000; Harwig *et al*., 1996; Jenssen *et al*., 2006; Trabi *et al*., 2001). Because RC101 and PG1 are microbicidal and contain multiple disulfide bonds, they have not yet been produced in microbial or cell culture systems. The goal of our study is to produce low cost and functional RC101 and PG1 antimicrobial peptides in transgenic tobacco chloroplasts.

Our lab has previously expressed antimicrobial peptide MSI-99 in transgenic tobacco chloroplasts without harmful effects to transplastomic plants. MSI-99 is an analog of a naturally occurring peptide (magainin 2) found in the skin of the African frog (Jacob and Zasloff, 1994). In another study, a proteinaceous antibiotic called PlyGBS lysine was expressed in tobacco chloroplasts to high levels (>70% TSP, (Oey *et al*., 2009). The PlyGBS transplastomic plants showed delayed growth and a slightly pale-green phenotype when compared to the untransformed plants. The authors suggested that it was due to the exhaustion of protein synthesis capacity of transgenic chloroplasts by the massive over-expression of PlyGBS although expression of >70% TSP of CTB-proinsulin yielded healthy transplastomic plants (Ruhlman et al., 2010). Previously expressed antimicrobial peptides did not contain disulfide bonds whereas the RC101 and PG1 antimicrobial peptides have β-sheet structures and contain multiple

intramolecular disulfide bonds. Therefore, efforts to express RC101 and PG1 in transgenic chloroplasts should further expand the applications of the chloroplast transformation system.

To facilitate expression of small antimicrobial peptides RC101 and PG1 in tobacco chloroplasts, each peptide was translationally fused with the GFP. This also facilitated detection and quantification of RC101-GFP and PG1-GFP in chloroplasts. The expression of GFP fusion proteins was visualized by examination under UV light or in immunoblots using the anti-GFP antibody. ELISA was also performed using anti-GFP antibody to quantify the expression of fusion proteins. Factor Xa protease cleavage site was inserted between RC101 and GFP and the furin cleavage site was inserted between PG1 and GFP so that they could be cleaved from their fusion proteins by appropriate proteases. It is interesting to note that RC101-GFP protein was already partially cleaved within chloroplasts, suggesting the presence of Factor Xa like protease activity within chloroplasts.

The smaller green fluorescent peptides observed in RC101 and PG1 lanes in figure 3b should be the monomer forms of RC101-GFP or PG1-GFP. The monomers ran faster than the GFP standard, probably because GFP when fused with RC-101 or PG1, has higher electrophoretic mobility in native gels. Different sizes correspond to the multimers formed by the GFP fusion proteins. GFP protein did not form multimers. Therefore, the formation of multimers by RC101-GFP or PG1-GFP fusion proteins is probably because of folded antimicrobial peptides RC-101 or PG1, which are both disulfide-bonded proteins. Similar folding pattern has also been observed before, when proteins containing multiple disulfide bonds were expressed in chloroplasts, including CTB-proinsulin (Ruhlman *et al*., 2007) and interferon-α2b (Arlen *et al*., 2007).

The toxicity of antimicrobial peptides is specific against microbial membranes and therefore can be safely applied to mammals, including human beings. The composition of the membranes is likely to be the determining factor for their selectivity. Biomembranes of prokaryotic or eukaryotic cells differ significantly. Mammalian cytoplasmic membranes are mainly composed of phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (Sph) and cholesterol, which are all generally neutrally charged. In contrast, in many bacterial pathogens, the membranes are composed predominantly of phosphatidylglycerol (PG), cardiolipin (CL) and phosphatidylserine (PS), which are highly electronegative (Yeaman and Yount, 2003). Most antimicrobial peptides, including RC101 and PG1, are positively charged under physiological pH because they are rich in Arginine. Therefore, the net negative charge of the biomembranes makes them the preferred target sites of antimicrobial peptides. The chloroplast envelope and thylakoid membranes predominantly possess three glycolipids: monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG) and sulfoquinovosyl diacylglycerol (SQDG), and a sole phospholipid: phosphatidylglycerol (PG). SQDG and PG, distinct from the non-charged MGDG and DGDG, are negatively charged. However, MGDG makes up 50% of chloroplast membrane lipid and DGDG makes up 30%, suggesting that the major components of chloroplast membranes are neutral. In this study, we examined fresh leaves of RC101 and PG1 transplastomic plants under confocal microscope. Confocal images showed that GFP fusion proteins were contained within chloroplasts and were not released into the cytoplasm. Cationic antimicrobial peptides including RC101 and PG1 kill bacteria by disrupting their membranes. Although the chloroplast membrane structure cannot be

resolved from the confocal images shown in Figure 5, no GFP fluorescence was detected outside the chloroplasts, suggesting that chloroplasts are not disrupted.

We compared RC101 and PG1 with other proteins we have expressed before in chloroplasts. We compared proteins expressed in chloroplasts with different number of disulfide bonds by immunoblot of native PAGE, including PTD-GFP (0), PG1-GFP (2), RC101-GFP (3), and CTB-GFP (1). CTB is known to form pentamers and that would be 5 disulfide bonds in total. As can be seen from Figure 6, proteins with more disulfide bonds tend to form more multimers when expressed in chloroplasts. PTD-GFP, which doesn't have any disulfide bonds, didn't form multimers. In contrast, the other disulfide-bonded proteins form multimers. Although this is not direct evidence that disulfide bonds were properly formed in chloroplasts, it indicated us that disulfide-bonded proteins can form more complex quaternary structures in chloroplasts. These complex structures may be important for the functionality of these proteins. Combining with the fact that RC101 and PG1 were proved to be fully functional when expressed in chloroplasts, we believe that disulfide bonds can be properly formed when disulfide-bonded proteins are expressed in chloroplasts.

RC101-GFP and PG1-GFP accumulated up to 32~38% and 17~26% of TSP and they were purified by affinity chromatography or organic extraction method. The results showed that organic extraction resulted in nearly ten-fold higher yield than the affinity chromatography method (53 μg/g vs 5μg/g fresh leaf). PG1 was only purified by affinity chromatography and the yield was 8 μg/g fresh leaf. We did not observe dimers or multimers in RC101-GFP or PG1-GFP samples purified by affinity chromatography, which indicated that they were lost during the purification process. The His-tag was not accessible in the dimer or multimer forms of RC101-

GFP and PG1-GFP. Therefore, most of the fusion proteins were not bound to the affinity column and lost during purification.

Previous study reported that the minimum inhibitory concentrations of PG-1 against gram-positive or gram-negative bacteria ranged from 0.12 to 2 μg/ml (Steinberg *et al.*, 1997). Retrocyclin (10-20 μg/ml) can inhibit proviral DNA formation and protect human CD4+ lymphocytes from in vitro infection by both T-tropic and M-tropic strains of HIV-1 (Cole *et al.*, 2002). RC-101, as low as 2 μg, can prevent HIV-1 infection in an organ-like construct of human cervicovaginal tissue (Cole *et al.*, 2007). In another study, it was reported that Retrocyclin-1, an analogue of RC101, can kill vegetative *B. anthracis* cells with an minimum effective concentration < 1 μg/ml (Wang *et al.*, 2006). As can be seen from these published data, antimicrobial peptides are highly potent and their effective dosage is only few μg/ml. Although our purification yield is relatively low, tobacco can be scaled up to yield up to 40 metric tons of biomass/acre/year. One acre of RC101 transplastomic tobacco plants could potentially yield up to 2 kg purified RC101 by organic extraction. Therefore, adequate quantities of RC101 or PG1 could be purified from transplastomic plants for preclinical or clinical studies.

RC101 and PG1 are shown to be functional when expressed in chloroplasts. Both RC101 and PG1 protected the transgenic tobacco plants from bacterial infection caused by Erwinia carotovora. In the antiviral assays, RC101 transgenic plants were resistant to TMV infection, but PG1 transgenic plants showed the symptoms of mosaic, necrosis and wrinkle as untransformed plants. Although PG1 has a broad-spectrum antimicrobial activity against bacteria, virus and fungus, it is most effective against bacterial infections, especially antibiotic-resistant bacteria (Kokryakov *et al.*, 1993; Qu *et al.*, 1996; Steinberg *et al.*, 1997; Yasin *et al.*, 1996). In our study,

PG1 is not effective in protecting plants from TMV infection. RC101 is an analog of retrocyclin and it is especially effective in protecting against viral infections. Several previous studies have shown that RC101 can be used to prevent HIV-1 infection (Cole *et al*., 2002; Cole *et al*., 2007). Our study shows that RC101 is active against the retrovirus TMV when expressed in chloroplasts. The antimicrobial activities of RC101 and PG1 can protect plants from phytopathogen infections, which make them good candidates to engineer disease resistant plants. Because the use of HIV and other human bacterial or viral pathogens require higher levels of containment than our current facilities, these studies were not performed. Future studies will include testing RC101 and PG1 in suitable animal models against bacterial or viral pathogens.

REFERENCES

Arlen, P. A., Falconer, R., Cherukumilli, S., Cole, A., Cole, A. M., Oishi, K. K. and Daniell, H. (2007) Field production and functional evaluation of chloroplast-derived interferon-alpha2b. Plant Biotechnol. J. **5**, 511-525.

Arlen, P. A., Singleton, M., Adamovicz, J. J., Ding, Y., voodi-Semiromi, A. and Daniell, H. (2008) Effective plague vaccination via oral delivery of plant cells expressing F1-V antigens in chloroplasts. Infect. Immun. **76**, 3640-3650.

Bally, J., Paget, E., Droux, M., Job, C., Job, D. and Dubald, M. (2008) Both the stroma and thylakoid lumen of tobacco chloroplasts are competent for the formation of disulphide bonds in recombinant proteins. Plant Biotechnol. J. **6**, 46-61.

Bals, R. (2000) Epithelial antimicrobial peptides in host defense against infection. Respir. Res. **1**, 141-150.

Boman, H. G. (1995) Peptide antibiotics and their role in innate immunity. Annu. Rev. Immunol. **13**, 61-92.

Broekaert, W. F., Cammue, B. P. A., De Bolle, M. F. C., Thevissen, K., De Samblanx, G. W. and Osborn, R. W. (1997) Antimicrobial Peptides from Plants. Crit. Rev. Plant. Sci. **16**, 297-323.

Chebolu, S. and Daniell, H. (2007) Stable expression of Gal/GalNAc lectin of Entamoeba histolytica in transgenic chloroplasts and immunogenicity in mice towards vaccine development for amoebiasis. Plant Biotechnol. J. **5**, 230-239.

Chen, J., Falla, T. J., Liu, H., Hurst, M. A., Fujii, C. A., Mosca, D. A., Embree, J. R., Loury, D. J., Radel, P. A., Cheng, C. C., Gu, L. and Fiddes, J. C. (2000) Development of protegrins for the treatment and prevention of oral mucositis: structure-activity relationships of synthetic protegrin analogues. Biopolymers. **55**, 88-98.

Cole, A. M., Hong, T., Boo, L. M., Nguyen, T., Zhao, C., Bristol, G., Zack, J. A., Waring, A. J., Yang, O. O. and Lehrer, R. I. (2002) Retrocyclin: A primate peptide that protects cells from infection by T- and M-tropic strains of HIV-1. Proceedings of the National Academy of Sciences **99**, 1813-1818.

Cole, A. L., Herasimtschuk, A., Gupta, P., Waring, A. J., Lehrer, R. I. and Cole, A. M. (2007) The retrocyclin analogue RC-101 prevents human immunodeficiency virus type 1 infection of a model human cervicovaginal tissue construct. Immunology **121**, 140-145.

Daniell, H., Datta, R., Varma, S., Gray, S. and Lee, S. B. (1998) Containment of herbicide resistance through genetic engineering of the chloroplast genome. Nat. Biotechnol. **16**, 345-348.

Daniell, H., Lee, S. B., Panchal, T. and Wiebe, P. O. (2001) Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. J. Mol. Biol. **311**, 1001-1009.

Daniell, H., Singh, N. D., Mason, H. and Streatfield, S. J. (2009) Plant-made vaccine antigens and biopharmaceuticals. Trends Plant Sci. **14**, 669-679.

Daniell H, Ruiz G, Denes B, Sandberg L and Langridge W (2009) Optimization of codon composition and regulatory elements for expression of human insulin like growth factor-1 in transgenic chloroplasts and evaluation of structural identity and function. *BMC Biotechnology* **9**:33.

Daniell, H., Chebolu, S., Kumar, S., Singleton, M. and Falconer, R. (2005) Chloroplast-derived vaccine antigens and other therapeutic proteins. Vaccine **23**, 1779-1783.

Davoodi-Semiromi, A., Samson, N. and Daniell, H. (2009) The green vaccine: A global strategy to combat infectious and autoimmune diseases. Hum. Vaccin. **5**, 488-493.

DeGray, G., Rajasekaran, K., Smith, F., Sanford, J. and Daniell, H. (2001) Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. Plant Physiol **127**, 852-862.

Farran, I., Rio-Manterola, F., Iniguez, M., Garate, S., Prieto, J. and Mingo-Castel, A. M. (2008) High-density seedling expression system for the production of bioactive human cardiotrophin-1, a potential therapeutic cytokine, in transgenic tobacco chloroplasts. Plant Biotechnol. J. **6**, 516-527.

Fernandez-San, M. A., Mingo-Castel, A., Miller, M. and Daniell, H. (2003) A chloroplast transgenic approach to hyper-express and purify Human Serum Albumin, a protein highly susceptible to proteolytic degradation. Plant Biotechnol. J. **1**, 71-79.

Fernandez-San, M. A., Ortigosa, S. M., Hervas-Stubbs, S., Corral-Martinez, P., Segui-Simarro, J. M., Gaetan, J., Coursaget, P. and Veramendi, J. (2008) Human papillomavirus L1 protein expressed in tobacco chloroplasts self-assembles into virus-like particles that are highly immunogenic. Plant Biotechnol. J. **6**, 427-441.

Glenz, K., Bouchon, B., Stehle, T., Wallich, R., Simon, M. M. and Warzecha, H. (2006) Production of a recombinant bacterial lipoprotein in higher plant chloroplasts. Nat. Biotechnol. **24**, 76-77.

Hancock, R. E. W. and Chapple, D. S. (1999) Peptide Antibiotics. Antimicrob. Agents Chemother. **43**, 1317-1323.

Harwig, S. S., Waring, A., Yang, H. J., Cho, Y., Tan, L. and Lehrer, R. I. (1996) Intramolecular disulfide bonds enhance the antimicrobial and lytic activities of protegrins at physiological sodium chloride concentrations. Eur. J Biochem. **240**, 352-357.

Jacob, L. and Zasloff, M. (1994) Potential therapeutic applications of magainins and other antimicrobial agents of animal origin. Ciba Found. Symp. **186**, 197-216.

Jenssen, H., Hamill, P. and Hancock, R. E. W. (2006) Peptide Antimicrobial Agents. Clin. Microbiol. Rev. **19**, 491-511.

Kokryakov, V. N., Harwig, S. S. L., Panyutich, E. A., Shevchenko, A. A., Aleshina, G. M., Shamova, O. V., Korneva, H. A. and Lehrer, R. I. (1993) Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. FEBS Letters **327**, 231-236.

Koya, V., Moayeri, M., Leppla, S. H. and Daniell, H. (2005) Plant-based vaccine: mice immunized with chloroplast-derived anthrax protective antigen survive anthrax lethal toxin challenge. Infect. Immun. **73**, 8266-8274.

Leelavathi, S. and Reddy, V. S. (2003) Chloroplast expression of His-tagged GUS-fusions: a general strategy to overproduce and purify foreign proteins using transplastomic plants as bioreactors. Molecular Breeding **11**, 49-58.

Manuell, A. L., Beligni, M. V., Elder, J. H., Siefker, D. T., Tran, M., Weber, A., McDonald, T. L. and Mayfield, S. P. (2007) Robust expression of a bioactive mammalian protein in Chlamydomonas chloroplast. Plant Biotechnol. J. **5**, 402-412.

Mayfield, S. P., Franklin, S. E. and Lerner, R. A. (2003) Expression and assembly of a fully active antibody in algae. Proc. Natl. Acad. Sci. U. S. A. **100**, 438-442.

Molina, A., Hervas-Stubbs, S., Daniell, H., Mingo-Castel, A. M. and Veramendi, J. (2004) High-yield expression of a viral peptide animal vaccine in transgenic tobacco chloroplasts. Plant Biotechnol. J. **2**, 141-153.

Nadai, M., Bally, J., Vitel, M., Job, C., Tissot, G., Botterman, J. and Dubald, M. (2009) High-level expression of active human alpha1-antitrypsin in transgenic tobacco chloroplasts. Transgenic Res. **18**, 173-183.

Nicolas, P. and Mor, A. (1995) Peptides as weapons against microorganisms in the chemical defense system of vertebrates. Annu. Rev. Microbiol. **49**, 277-304.

Nizet, V. (2006) Antimicrobial peptide resistance mechanisms of human bacterial pathogens. Curr. Issues Mol. Biol. **8**, 11-26.

Oey, M., Lohse, M., Kreikemeyer, B. and Bock, R. (2009) Exhaustion of the chloroplast protein synthesis capacity by massive expression of a highly stable protein antibiotic. Plant J. **57**, 436-445.

Qu, X. D., Harwig, S. S., Oren, A. M., Shafer, W. M. and Lehrer, R. I. (1996) Susceptibility of Neisseria gonorrhoeae to protegrins. Infect. Immun. **64**, 1240-1245.

Ruhlman, T., Ahangari, R., Devine, A., Samsam, M. and Daniell, H. (2007) Expression of cholera toxin B-proinsulin fusion protein in lettuce and tobacco chloroplasts--oral administration protects against development of insulitis in non-obese diabetic mice. Plant Biotechnol. J. **5**, 495-510.

Ruhlman, T., Verma, D., Samson, N. and Daniell, H. (2010) The role of heterologous chloroplast sequence elements in transgene integration and expression. Plant Physiol. [Epub ahead of print].

Schaller-Bals, S., Schulze, A. and Bals, R. (2002) Increased levels of antimicrobial peptides in tracheal aspirates of newborn infants during infection. Am. J Respir. Crit Care Med. **165**, 992-995.

Staub, J. M., Garcia, B., Graves, J., Hajdukiewicz, P. T., Hunter, P., Nehra, N., Paradkar, V., Schlittler, M., Carroll, J. A., Spatola, L., Ward, D., Ye, G. and Russell, D. A. (2000) High-yield production of a human therapeutic protein in tobacco chloroplasts. Nat. Biotechnol. **18**, 333-338.

Steinberg, D. A., Hurst, M. A., Fujii, C. A., Kung, A. H., Ho, J. F., Cheng, F. C., Loury, D. J. and Fiddes, J. C. (1997) Protegrin-1: a broad-spectrum, rapidly microbicidal peptide with in vivo activity. Antimicrob. Agents Chemother. **41**, 1738-1742.

Tang, Y. Q., Yuan, J., sapay, G., sapay, K., Tran, D., Miller, C. J., Ouellette, A. J. and Selsted, M. E. (1999) A Cyclic Antimicrobial Peptide Produced in Primate Leukocytes by the Ligation of Two Truncated -Defensins. Science **286**, 498-502.

Tissot, G., Canard, H., Nadai, M., Martone, A., Botterman, J. and Dubald, M. (2008) Translocation of aprotinin, a therapeutic protease inhibitor, into the thylakoid lumen of genetically engineered tobacco chloroplasts. Plant Biotechnol. J. **6**, 309-320.

Trabi, M., Schirra, H. J. and Craik, D. J. (2001) Three-Dimensional Structure of RTD-1, a Cyclic Antimicrobial Defensin from Rhesus Macaque Leukocytes. Biochemistry **40**, 4211-4221.

Tregoning, J. S., Nixon, P., Kuroda, H., Svab, Z., Clare, S., Bowe, F., Fairweather, N., Ytterberg, J., van Wijk, K. J., Dougan, G. and Maliga, P. (2003) Expression of tetanus toxin Fragment C in tobacco chloroplasts. Nucleic Acids Res. **31**, 1174-1179.

Verma, D., Samson, N. P., Koya, V. and Daniell, H. (2008) A protocol for expression of foreign genes in chloroplasts. Nat. Protoc. **3**, 739-758.

Wang, W., Mulakala, C., Ward, S. C., Jung, G., Luong, H., Pham, D., Waring, A. J., Kaznessis, Y., Lu, W. Y., Bradley, K. A. and Lehrer, R. I. (2006) Retrocyclins kill bacilli and germinating spores of Bacillus anthracis and inactivate anthrax lethal toxin. J. Biol. Chem. **281**, 32755-32764.

Wang, X., Brandsma, M., Tremblay, R., Maxwell, D., Jevnikar, A. M., Huner, N. and Ma, S. (2008) A novel expression platform for the production of diabetes-associated autoantigen human glutamic acid decarboxylase (hGAD65). BMC. Biotechnol. **8**, 87-90.

Watson, J., Koya, V., Leppla, S. H. and Daniell, H. (2004) Expression of Bacillus anthracis protective antigen in transgenic chloroplasts of tobacco, a non-food/feed crop. Vaccine **22**, 4374-4384.

Yang, D., Chertov, O. and Oppenheim, J. J. (2001) The role of mammalian antimicrobial peptides and proteins in awakening of innate host defenses and adaptive immunity. Cell Mol Life Sci. **58**, 978-989.

Yasin, B., Harwig, S. S., Lehrer, R. I. and Wagar, E. A. (1996) Susceptibility of Chlamydia trachomatis to protegrins and defensins. Infect. Immun. **64**, 709-713.

Yeaman, M. R. and Yount, N. Y. (2003) Mechanisms of Antimicrobial Peptide Action and Resistance. Pharmacol Rev **55**, 27-55.