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PRODUCTION OF BIOLOGICALLY ACTIVE HUMAN SECRETORY LEUKOCYTE PROTEASE INHIBITOR IN PLANTS: A PROTEIN WITH ANTI-VIRAL INCLUDING ANTI-HIV, ANTI-BACTERIAL, ANTI-INFLAMMATORY AND WOUND HEALING PROPERTIES

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ABSTRACT

The human Secretory Leukocyte Protease Inhibitor (SLPI) is widely viewed as an anti-HIV vaccine or treatment potential due to its broad spectrum of antimicrobial activities. However, human SLPI has not been pre-clinically tested yet for its' therapeutic treatments because of its high production costs ($245/100ug; R & D System Corp), when produced in *Escherichia coli*. To overcome this limitation, we employed a plant production system for SLPI. We developed a series of plant expression vectors containing the human SLPI gene sequences driven by a green tissue specific (rubisco) promoter to produce this protein only in plant green tissues. We also used signal peptides to secure its' accumulation in different sub-cellular compartments away from the cytoplasm, where normally most biological activities take place. Our results demonstrated that the recombinant human SLPI (rhSLPI) gene was successfully integrated into the plant genome, and transcribed and translated, and accumulated in plant apoplast

(plant cell wall areas). Biological activity tests indicated that the plant produced-rhSLPI had inhibitory activities against serine protease α-chymotrypsin, suggesting that the end terminal partial cleavage may not have affected the rhSLPI catalytic activities. Our research demonstrates the feasibility of producing rhSLPI in plant system instead of its production in *E. coli*. The successful production of biologically active rhSLPI in plants might be a step forward to facilitating the potential use of rhSLPI in preclinical testing.

Keywords: *Nicotiana tabacum*, Tobacco, Secretory Leukocyte Protease Inhibitor (SLPI), Plant Expression Vector, AIDS/HIV, Biological Activity

INTRODUCTION

Tremendous demands for the development of safe and effective treatments and vaccines against HIV/AIDS have been raised since the breakthrough of the disease in early 1980's. It is estimated that approximately 35 million people were living with human immunodeficiency virus (HIV) in 2013 worldwide and 1.5 million people died of acquired immunodeficiency syndrome (AIDS)-related illnesses. [1] Surprisingly, 3.2 million are children under 15 years of age. [1] In addition, 2.1 million more people were infected with HIV in 2013. [1] The number is steadily increasing in developing countries while the number of AIDS patients in Europe and the United States has slowed down due to greater public awareness and education. Nevertheless, poor economic conditions in Africa and other developing countries limit the use of precautionary needs, resulting in the spread of the HIV/AIDS disease.

The human secretory protein, SLPI, is considered to be one of the most potent future potential drugs to control human HIV. [2, 3] HIV transmission through oral-genital sexual practice is far less than through genital-genital contact because of the antiviral activity of SLPI and its combination with other antimicrobial/viral factors such as defensins, salivary agglutinin, and thrombospondin. [2] The anti-viral activity of SLPI is demonstrated when it is filtered out of saliva, i.e. the remaining saliva sample loses most of its inhibitory activity against HIV-1. However, modest inhibition of HIV-1 remains, suggesting the presence of other anti-HIV-1 agents in oral secretions. These studies clearly conclude that SLPI is a major anti-HIV-1 component as human secretory SLPI blocked more than 90% of HIV-1 infectivity at endogenous concentrations (1-10 ug/ml). [4]

The anti-viral activities of SLPI against HIV are attributed to several mechanisms. First, SLPI interferes with HIV-1 transmission by disrupting the formation of a bridge between HIV-1

and human innate immune cells or human target cell surfaces. SLPI may form a complex with human scramblase, which is a membrane protein required for the movement of membrane phospholipids, thereby interfering with the fusion of the HIV-1 virus and the host cell membrane. [5] Second, SLPI may also interfere by blocking the entry of HIV-1 via binding to the phospholipid-binding protein annexin II required to promote HIV-1 infection of human macrophage cells. [6] Third, SLPI possibly interferes with the interaction between the HIV protein gp120 and the target cell surface. [2, 7] In addition to the protective role of SLPI against HIV-1, SLPI also inhibits herpes simplex virus (HSV) infection in vitro by binding to epithelial cell surface and preventing viral infection. [8]

The human secretory SLPI protein possesses distinctive physiochemical properties. The protein is a low-molecular weight (11.7 kDa) serine protease inhibitor that is non-glycosylated, basic, and cysteine rich. [9, 10] The protein consists of 107 amino acids with a high affinity for the neutrophil serine proteases including elastase, proteases 3, and cathepsin G. [9] Human secretory SLPI is comprised of two structurally homologous domains; one having a transglutaminase substrate (Domain 1, N-terminal), and the other (Domain 2; C-terminal) having an elastase inhibitory domain. Each domain contains eight cysteines, resulting in four disulfide bonds. The disulfide bonding and helical structure of each domain make SLPI a very stable protein. Also, the acid stability of SLPI allows the protein to maintain its function in an acidic environment such as the human mouth.

SLPI is naturally expressed in a variety of cell types including phagocytic cells, lung epithelial cells, pulmonary vascular endothelial cells, parotid tissue, and hepatocytes. [11] Its' wildly distributed anti-protease appears to have different biological functions in the human body. Also, as a major protease inhibitor of the mucous secretions of the respiratory and genital tracts, the SLPI protein plays an important role as a primary defense mechanism and is the first barrier against microorganisms. [12] For instance, SLPI is known to be the third most abundant anti-microbial protein in upper airways, showing anti-microbial activity *in vitro* against *E. coli* and lung pathogens. [13] SLPI also shows fungicidal activities on human fungal pathogens, such as *Aspergillus fumigatus* and *Candia albicans.* [14, 15] This protein not only plays these roles in the immune system, but also acts as a major endogenous mediator for skin injury and oral mucosal wound healing by promoting cellular proliferation in the wound area. [16-18]

Due to the broad spectrum of anti-microbial activities, SLPI has potential for a wide array of therapeutic applications. Its' multifaceted functions have been successfully tested and proven to be effective on human diseases including HIV [19], herpes simplex virus [8], *Neisseria gonorrhoea* [20], tumor growth [21-23], allergic asthma [24], chronic obstructive pulmonary disease [25], and ovarian cancer cells. [26] SLPI is shown to be a potent cure for patients with multiple infections or in patients with unidentified infections. Most importantly, the human derived SLPI is not expected to trigger an allergenic response; therefore, it can be applied to most patients experiencing different medical conditions.

Despite its potential for a wide array of therapeutic applications, no preclinical testing of human SLPI has yet been achieved due to the high cost ($245/100ug; see: http://www.rndsystems.com/product_results.aspx?m=2100) of its' production in the *E. coli* expression system. In fact, production of SLPI as a non-glycosylated cationic protein in *E. coli* requires extensive denaturation and renaturation processes to refold this disulfide-rich protein into its normal biologically active form. Also, it is reported that intracellular expression of SLPI in *E. coli* causes severe reductions in overall bacterial nucleotides and protein synthesis, resulting in declined bacterial viability (~25%), presumably due to interference of ribosome-mRNA interactions. [27] To overcome limitations with production of human SLPI in *E. coli*, SLPI was produced in its active form in the yeast *Pichia pastoris*, displaying fivefold higher production level with the overproduction of the molecular chaperon, protein disulfide isomerase (PDI). [28] The human SLPI was also successfully produced in its biologically active form in insect cells using the baculovirus promoter. In this experiment, a recombinant human SLPI produced in insect cells presented much higher cellular viability (~90%) than when produced in E. coli expression system. [29]

At least 20 grams of SLPI is needed in order to conduct a comprehensive preclinical test against HIV (Sticklen's communication with Dr. Andrew Badley, Director, HIV Research Center, Mayo Clinic, Rochester, MN). Transgenic plants has been used for large-scale production of recombinant biopharmaceuticals as insulin, erythropoietin, α-interferon, human serum albumin, as well as glucocerebrosidase, granulocyte–macrophage colony-stimulating factor, and production of industrial enzymes, such as multi-cellulases required for biofuel productions. [30, 31] Plants are considered an ideal biofactory for the production of recombinant drugs because of the economic and qualitative benefits. Plant systems are relatively cheaper and recombinant protein yields are relatively high. In addition, the

recombinant protein can be targeted into different plant sub-cellular compartments away from cytoplasmic activities. [32] Kusnadi et al. [33] estimated that the cost of producing recombinant proteins in plants could be 10- to 50-fold lower than their production in *E. coli*.

This is the first report on the successful production of biologically active rhSLPI with potential for preclinical testing.

MATERIALS AND METHODS

Plant Expression vector constructs

A set of sub-cellular targeting plant expression vectors (ImpactVector™) containing human SLPI regulated by the *Asteraceous chrysanthemum* rubisco small subunit (RbcS1) promoter and the RbcS1 terminator sequences were produced to be genetically engineered into tobacco (*Nicotiana tabacum* L. cv Samsun) genome using the *Agrobacterium tumefactions* system.

The transit peptide sub-cellular targeting sequences used in the plant expression vectors were to target the rhSLPI into the secretory pathway endoplasmic reticulum (ER), chloroplast or mitochondria. A plant expression vector was also produced without the presence of any signal peptide to allow the rhSLPI to accumulate in cytoplasm. In addition, all expression vectors contained a cmyc-tag allowing the identification of expressed rhSLPI protein using commercially available monoclonal antibody, and a six-histidine tag for protein purification using a nickel column (Figure 1 and Table 1).

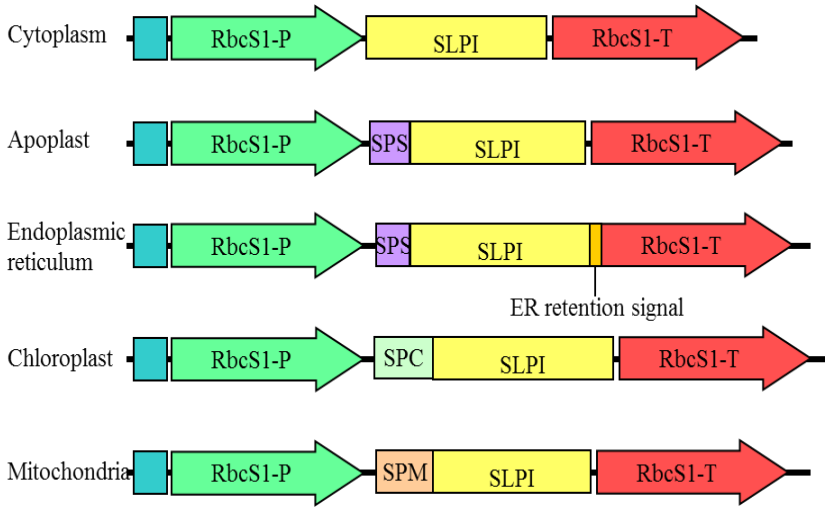


Figure 1. Plant expression vectors containing the rhSLPI gene regulated by the green-tissue specific promoter and containing no targeting signal peptide sequences for recombinant protein accumulation in cytoplasm; with an apoplast-specific, an ER-specific signal peptide, a chloroplast-specific, and a mitochondria-specific signal peptides.

Abbreviations: P-RbcS1: Ribulose bisphosphate carboxylase promoter from the Asteraceous chrysanthemum. SPS: signal peptide sequences, T-RbcS1: Ribulose bisphosphate carboxylase small unit terminator from the *Asteraceous chrysanthemum*.

Table 1. ImpactVector™ series for plant sub-cellular targeting.

	IV1.1-tag	IV1.2-tag	IV1.3-tag	IV1.4-tag	IV1.5-tag
Target organelle	Cytoplasm	Apoplast (Secreted expression)	ER	Chloroplast stroma	Mitochondrial matrix
Origin of the signal peptide	No signal peptide	Sea anemone equistatin	Sea anemone equistatin with KDEL retention	Chrysanthemum morifolium	Yeast CoxIV secretion signal

Agrobacterium-mediated plant transformation

Leaves of *in-vitro* cultured tobacco plants (4-8 week old) were used for *Agrobacterium*-mediated transformation. Each plant expression vector (Figure 1) was inserted into a pBINPLUS binary vector and transformed into the *Agrobacterium tumefaciens* strain LBA4404 (Invitrogen, Carlsbad, CA). For tobacco transformation, healthy expanded leaves from 4-5 wk old plants were cut into 0.6~0.8 cm squares sections and co-cultivated in a regeneration medium (RM; 4.43 g/L MS with vitamin, 2.0 mg/L BAP, 0.5 mg/L IAA, 8 g/L bacto-agar, pH 5.6) containing suspended *Agrobacterium*. The cultures were maintained under gentle shaking at 100 rpm at 28 ºC for 30 min. Then the co-cultivated tobacco leaf sections explants were kept in dark for four days, and the *Agrobacterium* infected explants were washed with liquid RM including 1000 mg/L timentin for 3 min, and washed twice with liquid RM for 3 min. The washed explants were then blotted onto sterile filter-paper to remove excess bacteria and transferred into selection RM medium (SRM; 4.43 g/L MS salts and vitamins, 2.0 mg/L BAP, 0.5 mg/L IAA, 300 mg/L timentin, 100 mg/L Kanamycin, 8 g/L Bacto-agar, pH 5.6).The explants were subcultured onto fresh SRM medium every two wks for callus formation. Once the callus regenerated into shoots containing leaves (~8 wks), the whole shootlets were transferred to rooting medium containing the selectable marker (4.43 g/L MS with vitamin, 300 mg/L timentin, 100 mg/L kanamycin, 2.5 g/L GELRITE® Gellan Gum, pH 5.6). The shootlets were then sub-cultured under a 16 hr photoperiod for 3 wks for further rooting. The rooted shoots were transferred to potting soil, acclimated to the greenhouse conditions, and grown to maturity in a 16 hr photoperiod greenhouse under 24°C.

Confirmation of Integration and transcription of rhSLPI in plants

Polymerase chain reaction (PCR) analyses were performed on transgenic tobacco plants to confirm the integration of rhSLPI using the primer: SLPI_NcoI-F: 5'-CCATGGGATCTGGAAAGTCCTTCAAA-3' (Tm: 58.8^{o}C)

SLPI_BglII-R: 5'-AGATCTCCAGCTTTCACAGGGGAAA-3' (59.9 ºC). Northern blotting was performed to confirm the transcription level of the rhSLPI. Total RNA was isolated from putatively transgenic and untransformed wild-type control plants using Trizol reagent following the manufacturer instructions (Invitrogen, Carlsbad, CA). RNA gel blot analysis was carried out following the modifications of our previous procedure. [33]

Preparation of crude plant protein extracts and Western blotting

Proteins were extracted from untransformed wild-type and transgenic plant leaf tissues using 2 x SDS protein extraction buffer (100 mM Tris-Cl pH 6.8, 200 mM DTT, 4 % SDS, 20 % glycerol). For crude protein extraction, two leaf discs (0.5 cm in diameter) were collected and ground in 100 ul of 2 x SDS protein extraction buffer. The Invitrogen NuPAGE® Bis-Tris Discontinuous Buffer System with a 10 % NuPAGE® Novex Bis-Tris Pre-Cast Gel was used for Western blotting according to the manufacturer instructions (Invitrogen, Carlsbad, CA) using human SLPI polyclonal antibody, goat IgG (R&D systems, Minneapolis), human SLPI monoclonal antibody, Mouse IgG (R&D systems, Minneapolis) and histidine monoclonal antibody (R&D systems, Minneapolis, MN). The signals were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo scientific, Rockford, IL).

SLPI purification via dialysis

Prior to the purification, crude extracts from tobacco leaves were centrifuged at 14,000 rpm for 10 min and the supernatant was filtered using 2.0 um filter. Then the plant produced rhSLPI was dialyzed using Slide-A-Lyzer® G2 Dialysis Cassette to remove low molecular weight contaminants according to manufacturer's protocol (Thermo scientific, Rockford, IL). Briefly, the dialysis cassette was hydrated in a dialysis buffer for 2 min, 2.5 ml of tobacco leaf crude extracts was injected into the cassette, and most of the air was removed. Then, the cassette was dialyzed for 2 hours at room temperature or 4 ºC; the dialysis buffer was changed and dialyzed for another 2 hours, then the dialysis buffer was changed again and dialyzed overnight. The dialyzed tobacco leaf crude extracts were collected from the cassette using a syringe.

Biological activity tests

Previous studies demonstrated that *E. coli*-produced rhSLPI effectively inhibited the activity of serine protease, α-chymotrypsin, which breaks down a peptide substrate, N-succinyl–Ala–Ala–Pro–Phe q-nitroanilide. Once the substrate is cleaved, yellow fluorescence (q-nitroanilide) is released under an alkaline condition. [34, 45] Fluorescence at 405 nm was used as an indicator of the activity of serine protease.

Therefore, the plant-produced purified rhSLPI was tested for biological activity against the proteases α-chymotrypsin. Four ug of purified rhSLPI was added to wells of a 96-well plate containing 90 ul of reaction buffer (0.1M Tris-HCl, 0.01 M $CaCl_2$, pH 7.8). Ten ul of 1uM α-chymotrypsin was added to each well. The final volume was adjusted into 110 ul with reaction buffer, and pre-incubated at 37 ^oC for 20 min. Then ten ul of substrate (0.4mM N-succinyl-Ala-Ala-Pro-Phe-nitroanilide was added and the absorbance monitored at 405 nm to determine the substrate degradation activity of the proteases using Gen5 (Biotek, Winooski).

RESULTS

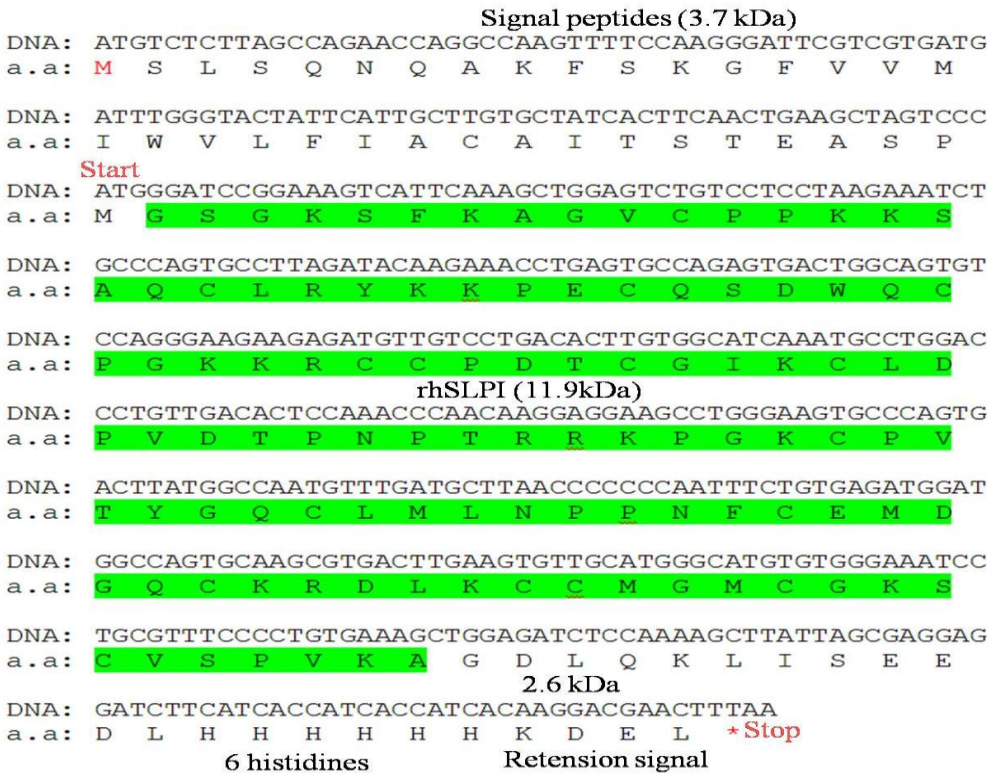


Figure 2. Recombinant human SLPI nucleotide and amino acid sequences. ImpactVectorTM 1.3-tag (IV1.3-tag) contains signal peptides (3.7 kDa), rhSLPI (11.9 kDa), c-myc, polyhistidines and retention signal (2.6 kDa). DNA: diooxynucleic acid; a.a: amino acid.

Recombinant human SLPI gene constructs

Figure 2 shows the DNA sequences of ImpactVector™ cloned-rhSLPI nucleotide, and its encoded amino acids. The theoretical pI/MW of the rhSLPI protein is 7.83 / 19.8 kDa. The amino acid sequences also include a signal peptide (3.7 kDa), rhSLPI (11.9 kDa) and the rest of the C-terminal (2.6 kDa) containing c-myc, six histidines and an ER retention signal (KDEL).

Tobacco transformation and molecular analyses

More than 30 different tobacco lines resulted from the transfer of each construct into the plant genome using the *Agrobacterium*-mediated transformation system.

The regenerated tobacco leaves were subjected to a series of molecular analyses to confirm the integration and transcription of the rhSLPI gene in plant genome. Figure 3a shows the PCR analyses of 45-day old regenerated transgenic tobacco leaves, showing the expected size (337 bp) of the rhSLPI integration (Fig 3).

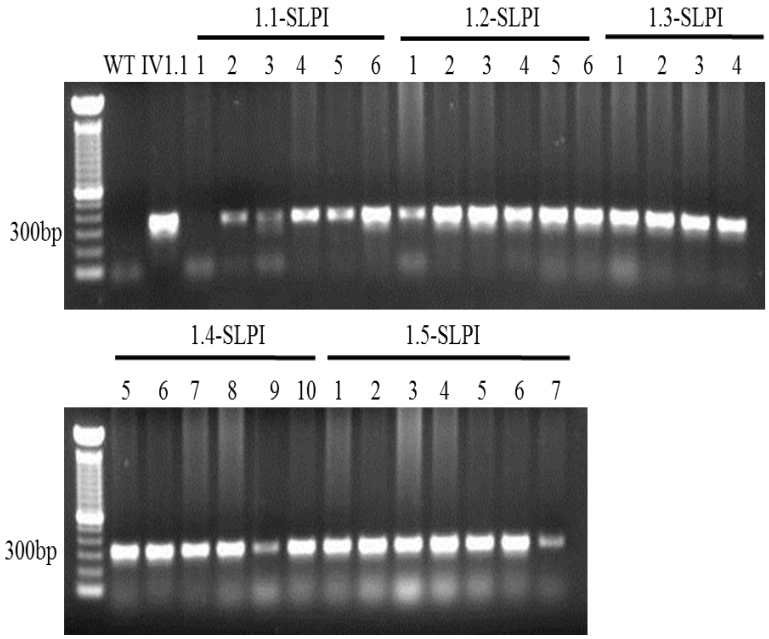


Figure 3. PCR amplification of the 337 bp fragment for rhSLPI confirms the integration of human SLPI in tobacco plants. Note: 1.1: rhSLPI without any targeting signal peptide; 1.2: rhSLPI targeted into apoplast; 1.3: rhSLPI targeted into ER; 1.4: rhSLPI targeted into chloroplast; 1.5: rhSLPI targeted into mitochondria.

Transcription analysis

Figure 4 shows different transcription levels of rhSLPI produced in the cytoplasm, apoplast and three different sub-cellular compartments in transgenic tobacco plants.

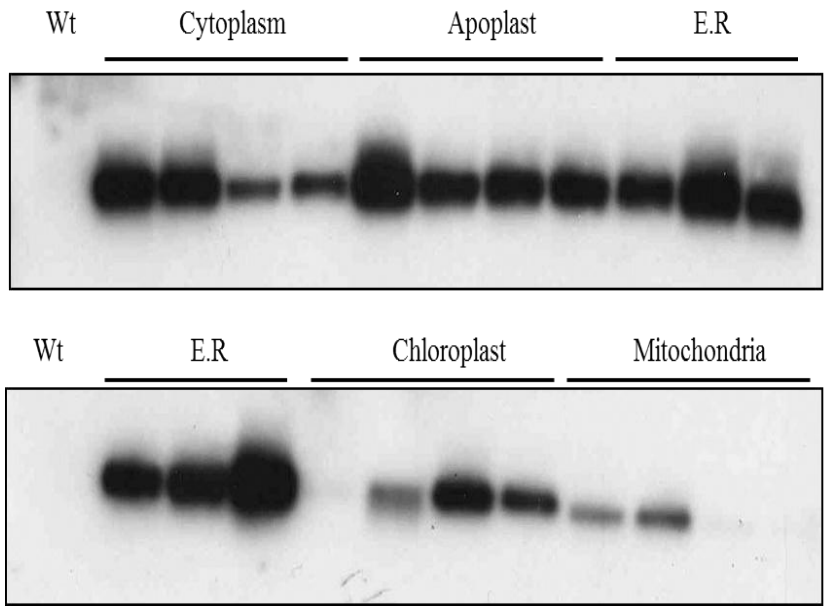


Figure 4. Northern blot analysis of rhSLPI in transgenic tobacco leaves. Different levels of rhSLPI transcription are shown in transgenic tobacco plants.

Abbreviations: Wt: wild-type tobacco; Cytoplasm: rhSLPI kept in cytoplasm; Apoplast: rhSLPI targeted into apoplast; ER: rhSLPI targeted into ER; Chloroplast: rhSLPI targeted into chloroplast; Mitochondria: rhSLPI targeted into mitochondria.

Recombinant human SLPI protein analyses

More than ten independent transgenic lines from each set of ImpactVector^{TM} series were analyzed to confirm the production of rhSLPI protein using a human SLPI polyclonal antibody (R&D systems, Minneapolis). The rhSLPI putatively targeted into tobacco apoplast consistently showed the accumulation of rhSLPI protein (Fig 5 and Fig 6). Among 14 independent transgenic lines putatively targeting the heterologous protein into apoplast, 13 transgenic lines showed a high expression of rhSLPI protein (>90%).

Abbreviations: SM: protein standard (MagicMarkXP, Invitrogen); Wt: wild-type tobacco; 1.1: rhSLPI without any targeting signal peptide; 1.2: rhSLPI targeted into apoplast; 1.3: rhSLPI targeted into ER; 1.4: rhSLPI targeted into chloroplast; 1.5: rhSLPI targeted into mitochondria. Ten ng of rhSLPI produced in *E.coli* was used as a positive control.

Abbreviations: SM: standard protein (MagicMarkXP, Invitrogen); Wt: wild-type control plant; SLPI (apoplast): SLPI targeted into plant cell apoplast.

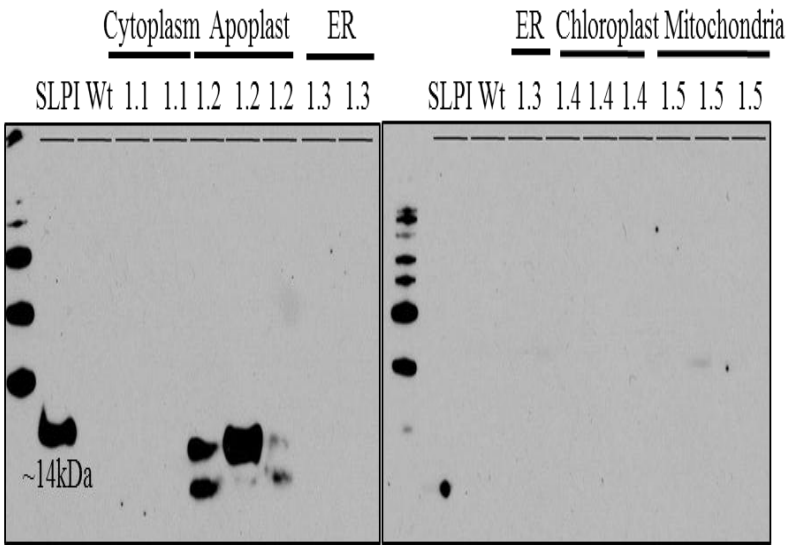


Figure 5. Western blot analysis of rhSLPI produced in the cytoplasm and different sub-cellular compartments in transgenic tobacco lines.

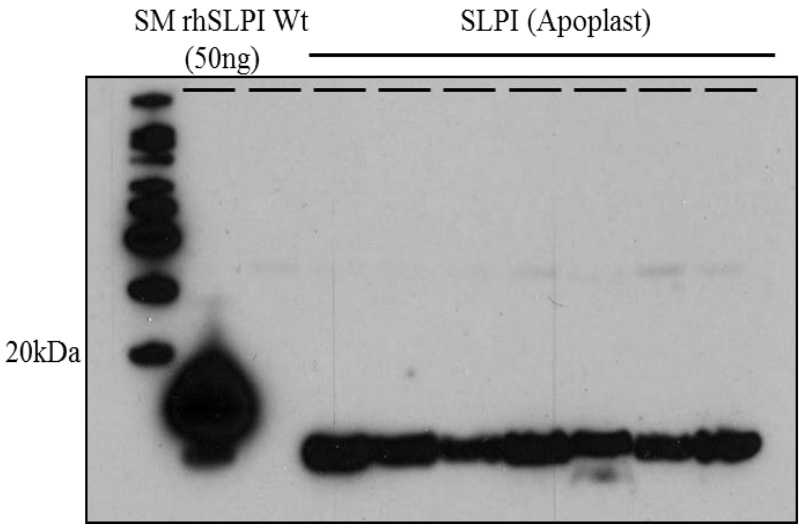


Figure 6. Western blot analysis of rhSLPI. The confirmation of rhSLPI protein production in more transgenic lines targeting rhSLPI into apoplast.

Note: There was no IgG conjugated protein standard for the range below 20 kDa for Western blot analysis, therefore MagicMark^{XP} (the smallest size is 20 kDa) was used as a protein size marker in Fig 7B. The Novex Sharp protein standard, which ranges from 3.5 kDa to 230 kDa, was used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) commassie blue staining to identify protein size more accurately on the protein gel. Even though the rhSLPI protein expression was not distinctively visualized in the SDS-PAGE gel, the gel clearly showed the rhSLPI protein (marked with a red asterisk). The amount could have been estimated by comparing with the positive control (100 ng of *E. coli* produced

rhSLPI). The SDS-PAGE positive tobacco line was also used for a Western blot analysis using rhSLPI specific polyclonal antibody, and the protein expression was confirmed (Fig 7B). Therefore, both protein analyses confirmed the rhSLPI protein expression of tobacco transgenic lines and their approximate sizes in SDS-PAGE gel (rhSLPI produced in *E. coli* : ~15 kDa and rhSLPI produced in tobacco: ~14 kDa).

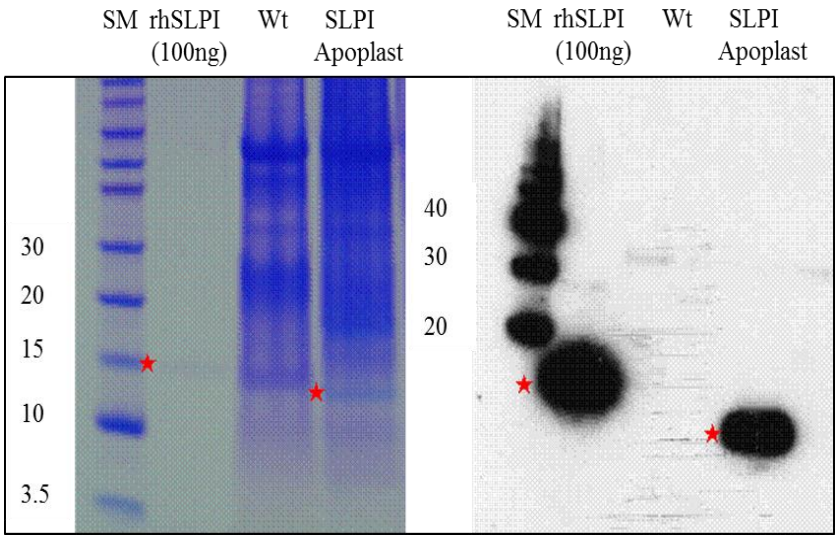


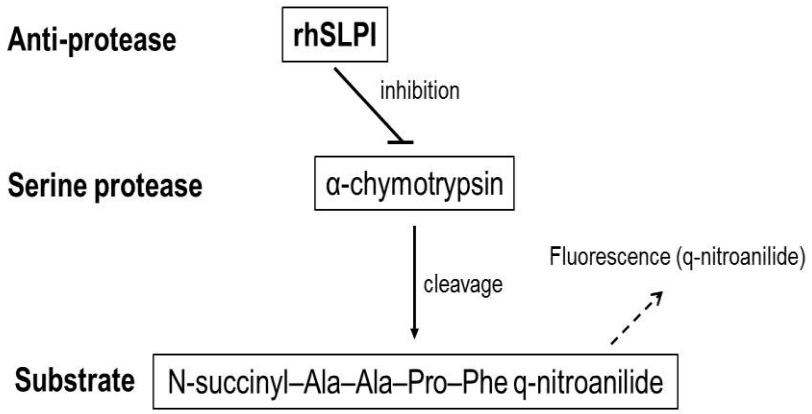
Figure 7. SLPI protein analyses in transgenic tobacco (45 days old).

(A) SDS-PAGE. SM: protein standard (Novex Sharp protein standard, Invitrogen, Carlsbad, CA); 100 ng rhSLPI: positive control; Wt: wild-type tobacco; SLPI apoplast: SLPI targeted into plant cell apoplast. (B) Western blot analysis. SM: protein standard (MagicMarkXP, Invitrogen); Wt: wild-type tobacco; SLPI apoplast: SLPI targeted into plant cell apoplast.

Recombinant human SLPI anti-protease activity assay

Fig 8A shows a schematic drawing of an SLPI anti-protease activity assay. Fig 8B shows the fluorescent absorbance of five samples: one positive control (*E.coli* purified-rhSLPI), two negative controls (wild-type tobacco leaf extract and reaction buffer), and two tobacco produced-rhSLPI samples (rhSLP-1 and rhSLPI-2). Two negative controls show high fluorescent absorbance at 405nm. This result indicates that the protease activity of α-chymotrypsin was not disrupted. In contrast, relatively low fluorescent absorbance was detected when *E. coli* purified-rhSLPI (positive control) and two tobacco extracted-rhSLPI samples were added. This result indicates that, because of the inhibitory effects of rhSLPI on

α-chymotrypsin activity, the q-nitroanilide was not released in the way it had been in the negative control. Therefore, the authors concluded that the rhSLPI produced in two tobacco transgenic lines (rhSLPI-1 and rhSLPI-2) exhibits significant anti-protease activities against the serine protease.



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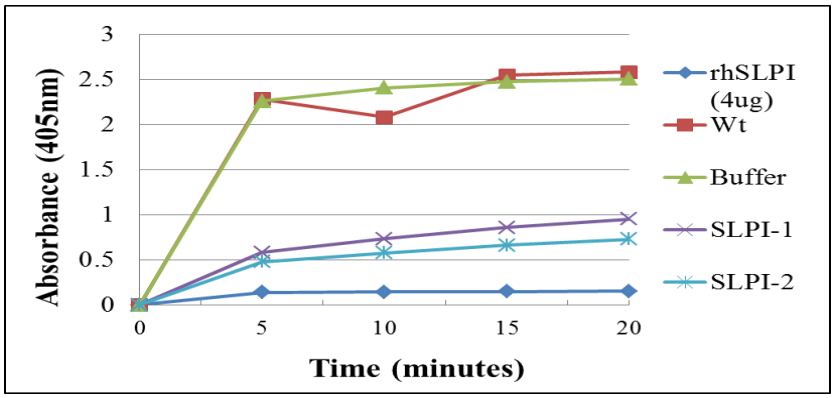

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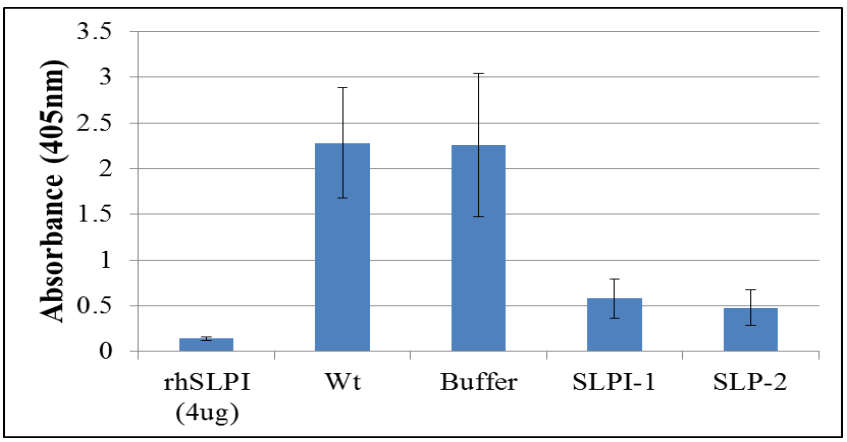

C

Figure 8. The rhSLPI serine protease inhibition assay. (A) Schematic drawing of anti-protease inhibition assay (B) Fluorescent absorbance of tobacco produced-rhSLPI at the 5 min interval. (B) Fluorescent absorbance of plant produced-rhSLPI at 5 min. Mean ± standard deviation (P<0.05, n=6).

DISCUSSIONS

Recombinant human SLPI has been expressed in *E.coli.* However, the rHSLPI produced in a bacterial system requires expensive denaturation and renaturation processes because this protein does not remain properly folded in *E. coli*. The recombinant human SLPI has also been produced in insect cells using a *Baculovirus* promoter [34] and in the yeast *Pichia pastoris* via the protein folding aid chaperone, protein disulfide isomerase (PDI). [35] However, both systems are expensive and are not capable of producing high volume rhSLPI sufficient for preclinical testing.

To overcome these limitations, we employed plant expression system of producing the rhSLPI. To avoid public concerns, we also produced the rhSLPI only in green tissues (e.g., leaves and stems), not in pollen, grains, or roots.

Successful applications of plants as a high-value protein production system is described in previous studies. [31, 36] In this research, the rhSLPI was targeted into different apoplast, ER, chloroplast, aor mitochondria to determine the best location for the stability of the heterologously produced rhSLPI, potentially away from anti-protein degradation. Western blot analyses (Figures 5 and 6) demonstrated that the rhSLPI transgene was successfully translated into protein and apparently targeted into apoplast for accumulation.

Although all different sub-cellular targeted rhSLPI lines showed transcription of the rhSLPI gene (Fig 4), the Western-blot analysis showed rhSLPI being accumulated only in apoplast (Figures 5 and 6), probably because the apoplast contains fewer contaminants and protein degrading enzymes, and the protein could be readily purified by vacuum infiltration of the plant leaf. [37] On the contrary, a report indicates that the plant apoplast is considered to be one of the potential sites of proteolytic degradation of recombinant proteins. [38]

Although apoplast was the ideal compartment to accumulate the rhSLPI, the plant- produced-rhSLPI appeared to be slightly smaller than the E.coli-produced refolded rhSLPI. Showing a small ((~ten kDa) cleaved fragment (Figure 5). The truncation of the rhSLPI might be ascribed to the cleavage activity of plant proteases on recombinant the rhSLPI. Thi is not surprising, as It is estimated that other plants such as rice and *Arabidopsis* contain 678 and 826 proteases respectively.

The plant proteases function in diverse biological mechanisms. [39] The proteases major role is to scavenge/recycle improperly folded and nonfunctional proteins into amino acids by breaking the peptide bonds. At the same time, the plant proteases also act as the counterparts against proteins released from microorganisms and pests. Therefore, the cleaved forms of rhSLPI in plant extracts might be the result of the cleavage of improperly folded rhSLPI and partial cleavage of rhSLPI C- or N terminals, not the internal (endopeptidases). Likewise, the cysteine rich rhSLPI production in plants system is not an easy process. Any improperly folded rhSLPI molecules are subjected to the protein degradation processes occurring in proteasome after ubiquitylation.

The peptidyl-prolyl *cis/trans* isomerase (PPI), the protein disulfide isomerase (PDI) and the ER luminal binding protein (BiP) molecular chaperons are known to be associated with protein folding, assembly, and with the prevention of the transport of immature protein molecules. [28, 40-43] Therefore, assistance from molecular chaperones, might be needed to promote protein folding and assembly of recombinant peptides into functioning proteins [35] might be needed enhance the folding process of the rhSLPI in plants.

Tobacco leaf crude extracts contain not only macro-molecules, but also micro-molecules such as water soluble sugars, amino acids, vitamins, salts, and certain toxic compounds, all of which can interfere with enzymatic activity. [44] Therefore, the plant-produce rhSLPI was purified by dialysis and filtration prior to its biological activity test in order to remove any contaminant below 2 kDa.

Fig 8B indicates that two plant produced samples, rhSLPI-1 ($A_{405\ nm}$: 0.5803) and rhSLPI-2 ($A_{405\ nm}$: 0.4798), show a statistically significant increase in anti-protease activity compared to the negative controls (Wt; $A_{405\ nm}$: 2.2802 and buffer only; $A_{405\ nm}$: 2.2585). Since the accurate rhSLPI concentration of dialyzed crude extracts was not measured in this experiment, it was difficult to conclude that rhSLPI-2 has a higher inhibition activity over rhSLPI-1 line. However, when the equivalent amount of total crude extracts (40 ug) of wild-type, rhSLPI-1 and rhSLPI-2 was used for the protease inhibition assay, it was concluded that the protease inhibition assay of dialyzed plant produced rhSLPI also retained serine protease activity.

Beyond the scope of this report, mass spectrometry (MS) is necessary to validate the chemical structure and mass of the plant produced rhSLPI molecule. Most importantly,

intensive purification methods might be required for reaching the level of protein purity required for pre-clinical testing.

We report the production of biologically active rhSLPI in a crop plant using the *Agrobacterium*-mediated transformation method. This report might represent a major step forward in the development of a system for the potent treatment drug as rhSLPI in plants.

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