EXPRESSION OF HUMAN PROTEIN C IN TRANSGENIC TOBACCO

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(ABSTRACT)

Human Protein C (hPC) is a vitamin K-dependent serine protease that has a critical role in the naturally-occurring anticoagulant pathway. Upon activation of the zymogen by thrombin at the endothelial cell surface, the active form of hPC has anticoagulant activity in hemostasis due to its ability to inactivate factors Va and VIIIa. For biological activity, hPC requires several post-translational modifications including proteolytic cleavage, disulfide bond formation, β-hydroxylation, γ-carboxylation, and N-linked glycosylation. Plants have the eukaryotic protein modifying mechanisms required for many human proteins and may provide a safe, cost-effective system for producing hPC on a large-scale basis. Tobacco (*Nicotiana tabacum L.*) is particularly well suited for use as a bioreactor for high-value recombinant proteins. Tobacco is one of the easiest plants to transform, it is an excellent biomass producer and can produce up to a million seeds from a single genetically engineered plant. Previous attempts to produce hPC in tobacco were limited by expression levels.

The overall goal of the research was to develop transgenic plants that express hPC at higher levels. A cDNA encoding hPC was fused to an enhanced constitutive 35S promoter ($35S^{DE}$) and introduced into a plant transformation vector. The hPC construct was introduced into tobacco leaf disks using *Agrobacterium tumefaciens*-mediated transformation, and 30 transgenic plants were generated.

Stable integration of the hPC gene construct into the tobacco genome and transgene copy number were determined by genomic Southern hybridization and segregation analyses. The majority of transgenic plants expressed the hPC transgene based on RNA analyses by northern hybridization. Plants utilizing the enhanced 35S promoter had equivalent levels of expression to previously generated hPC-containing plants. A variety of polyclonal and monoclonal antibodies raised against hPC were tested for detection of hPC standards and tobacco-synthesized hPC by western immunoblotting. Novel proteins in the size range of hPC heavy chain cross-reacted with anti-heavy chain hPC antibodies in $35S^{DE}$:hPC plants. Thus, plants may be capable of synthesizing hPC and proteolytically processing it to light and heavy chains. Although further experiments will be required to confirm the identity of these putative hPC proteins in tobacco, these result suggest that analyses of hPC expressed in plants have been limited by effective tools for detecting the hPC gene product rather than expression levels determined by the transgene promoter.

Dedication

I am dedicating this thesis to my parents,

Jun and Shijun Ni,

for everything they give to me.

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Abbreviations

ATA	aurintricarboxylic acid
CaMV	cauliflower mosaic virus
ConA	concanavalin A
CRIM	cross-reactive immunodetected material
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FPLC	fast protein liquid chromatography
Gla	γ-carboxyglutamic acid
HIV	human immunodeficiency virus
hPC	human protein C
HSA	human serum albumin
K	lysine
L	leucine
LB	left border of transfer DNA
MAb	monoclonal antibodies
PCR	Polymerase chain reaction
R	arginine
RB	right border of transfer DNA
rHb	recombinant hemoglobin
rhPC	recombinant human protein C
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
S IgA	secretory IgA
TCA	trichloroacetic acid
T-DNA	transfer DNA
TEV	tobacco etch virus
Ti genes	tumor-inducing genes of *Agrobacterium tumefaciens*

Chapter I

Literature Review

I.1 INTRODUCTION

Protein C is a vitamin K-dependent plasma protease which plays an essential role in the regulation of blood coagulation. Coagulation/anticoagulation is a carefully controlled process in the blood (Figure I.1). Injury to blood vessels must be rapidly sealed off to stop blood loss. Yet, the clotting must be local and not effect blood flow throughout the body. A complex regulatory cascade of proteins exist to control this process involving the activation and inactivation of a series of proteases (Factor I to Factor XIII). Those proteases exist in inactive forms (zymogens) and are activated by a proteolytic clip.

The final protease in the pathway is thrombin, which has a dual role in hemostasis (Grinnell, 1997). On one hand, thrombin is a procoagulant enzyme involved in the formation of blood clots by inducing platelet aggregation and converting soluble fibrinogen to insoluble fibrin. On the other hand, it is also a potent anticoagulant that activates protein C. Activated protein C cleaves essential peptide bonds in the heavy chains of factors Va and VIIIa which result in their inactivation and consequently in inhibition of the coagulation cascade. The relative rates of cleavage of thrombin's various substrates determine the extent of blood clotting. In addition to the cleavage of procoagulant substrates, thrombin can proteolytically activate factor VIIIa and Va to amplify thrombin synthesis to enhance the coagulant function. However, thrombin-activated protein C is the balance that inhibits the coagulation by cleaving and inactivating the factors VIIIa and Va.

The balance between thrombin cleavage of fibrinogen or activation of protein C is controlled by two major interactions (reviewed in Grinnell, 1997). The first interaction is with the endothelial membrane protein thrombomodulin, which can block the efficient binding of thrombin to fibrinogen and result in enhanced activation of protein C. When thrombin forms a 1:1 high affinity complex with thrombomodulin, activation of protein C is accelerated approximately 20,000 fold (Kisiel, 1979).

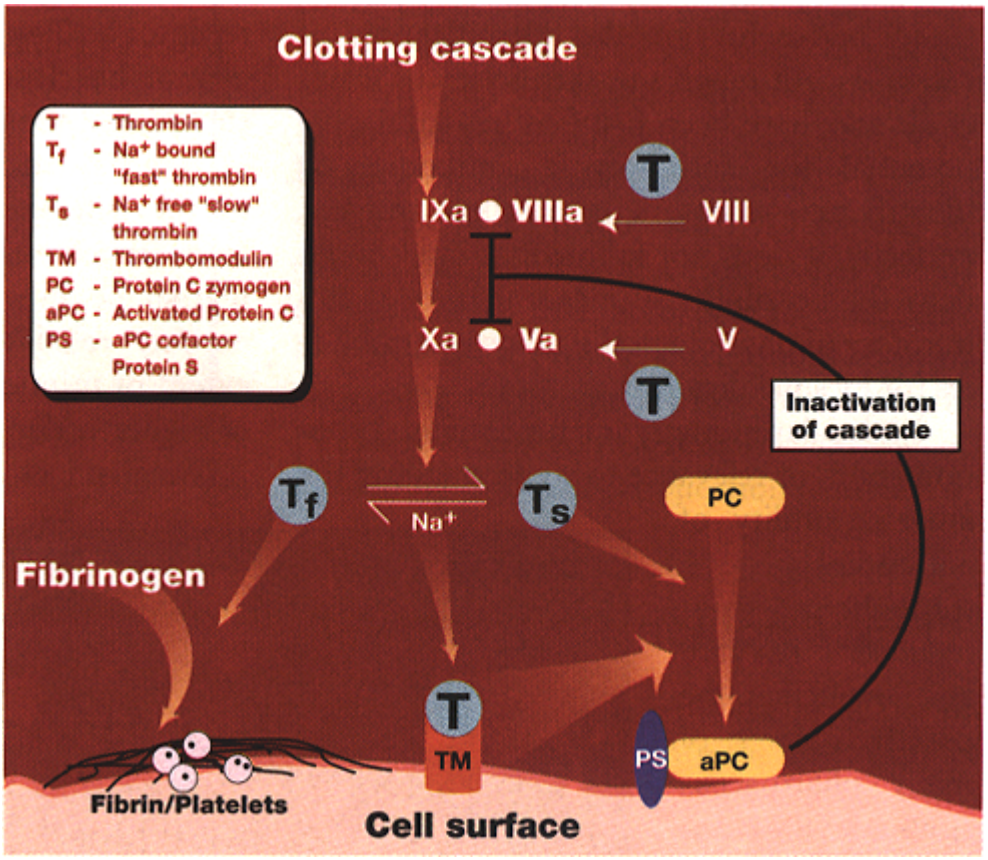

Figure I.1 The anticoagulant activity of human protein C in the clotting cascade (cited with permission from Grinnell, 1997). Thrombin is the final protease in the cascade, which plays a dual-role. It can enhance clot formation by converting circulating fibrinogen to insoluble fibrin. In addition, it will generate activated protein C to cleave and inactivate the cofactors VIIIa and Va, resulting in the inhibition of the clotting pathway.

The second interaction, Na^+ binding to thrombin residue Tyr_{225}, enhances on efficient cleavage of fibrinogen. If the binding site is not occupied by Na^+, thrombin cleaves fibrinogen very poorly. Mutational elimination of the Na^+ coordination site inhibits thrombin's ability to cleave fibrinogen (Dang et al., 1997). Thus, protein C is one factor of the complex process of hemostasis, that is involved in very rapid localized activation and inactivation of a whole cascade of proteins.

Protein C has potential use as a therapy for many medical conditions, such as stroke, myocardial infarction, and venous thrombosis. The plasma concentration of protein C is approximately 4 μg/ml with a half-life of about 15 hours. The nucleotide sequence of the gene that codes for protein C has been determined (Foster et al., 1985). Protein C is synthesized by liver parenchymal cells as a single chain polypeptide, but in plasma it consists mainly of a heavy chain (41 kD) linked by a disulfide bond to a light chain (21 kD).

Protein C is a highly processed protein (Figure I.2). As shown in Figure I.2, there are a prepeptide and a propeptide at the N-terminus of protein C encoded by its mRNA. The prepeptide is a signal peptide that directs the protein into the endoplasmic reticulum (ER) for post-translational modifications. The propeptide is required for γ-glutamyl carboxylase to direct the γ-carboxylation of glutamic acid residues in the amino-terminal region. The nine γ-carboxyglutamic acid (Gla) residues at the N-terminal domain of the light chain are highly conserved among vitamin K-dependent proteins. A vitamin K-dependent γ-glutamyl carboxylase catalyzes the carboxylation reactions and this post-translational modification is required for calcium-dependent membrane binding and functional activity of protein C. The light chain also contains a β-hydroxylation site and a N-glycosylation site. It is also believed that the β-hydroxylation of Asp_{71} on light chain is a calcium-binding site and required for protein C activity. The glycosylation site at the light chain (Asn_{97}) is critical for efficient secretion of hPC and affects the degree of glycosylation at Asn_{329}, one of the three glycosylation sites at the heavy chain (Grinnell et al., 1991). Cleavage of the single chain involves removal of an internal lysine-arginine

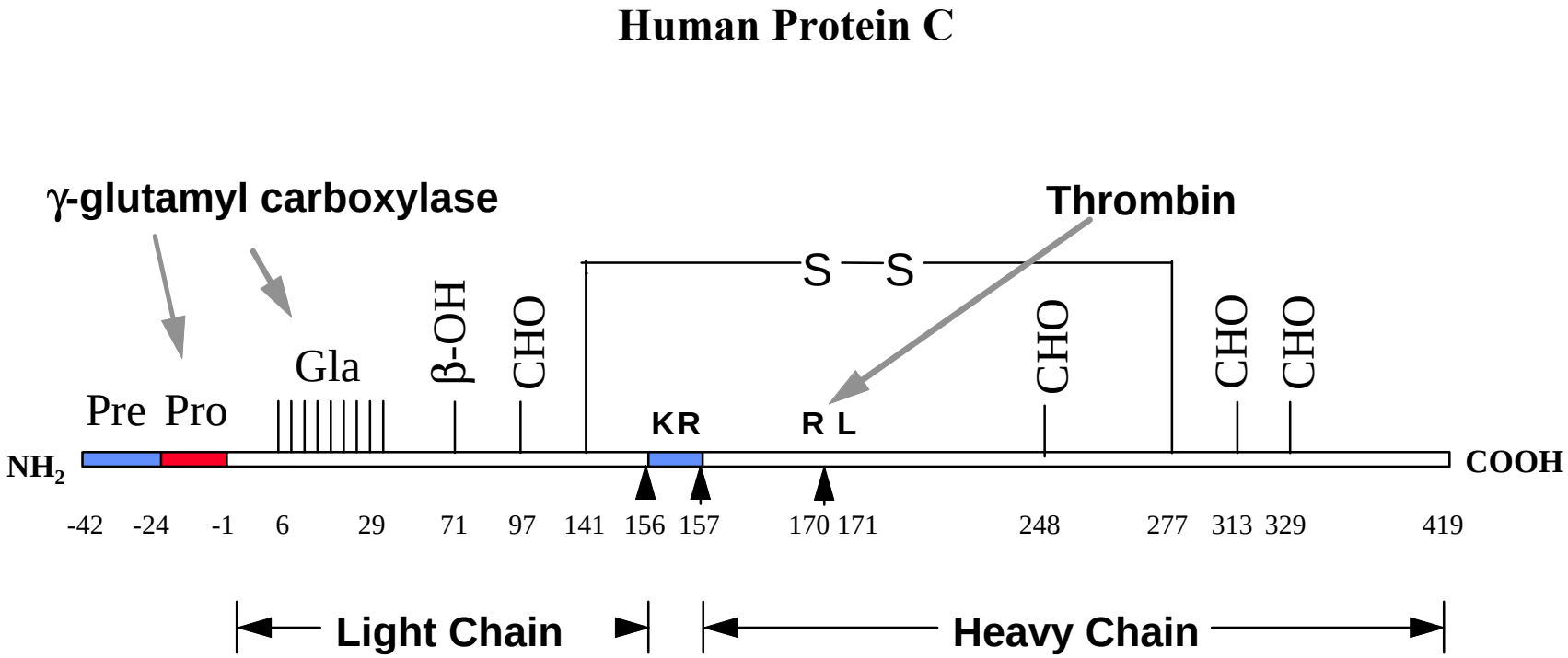

Figure I.2 Post-translational processing of human Protein C.
Pre: prepeptide; Pro: propeptide; Gla: γ-carboxyglutamic acid residues; β-OH: β-hydroxylation; CHO: N-glycosylation; S-S: the single inter-molecular disulfide bridge; K: Lysine; R: Arginine; L: Leucine.

dipeptide (K_{156} R_{157}) to yield the disulfide-linked heterodimer. In addition to the single inter-molecular disulfide bridge connecting the light chain and heavy chain, there are also ten intra-molecular disulfide bonds within the light chain or heavy chain. The heavy chain contains the serine protease domain and three N-glycosylation sites (Asn_{248}, Asn_{313}, and Asn_{329}). They affect internal KR dipeptide cleavage and activation efficiency of hPC (Grinnell et al., 1991). Activation of human protein C involves the release of a 12-residue peptide from the N-terminal domain of the heavy chain which accomplished by thrombin cleavage at an Arg_{170}-Leu_{171} bond. In summary, protein C needs extensive modifications for activity, including four proteolytic cleavages, eleven disulfide bond formation, nine γ-carboxylations, one β-hydroxylation and four N-glycosylations.

I.2 TRANSGENIC PLANTS FOR RECOMBINANT PROTEIN PRODUCTION

The availability of expression systems for the production of valuable recombinant proteins is an important issue in biotechnology. Some well-established biotechnology companies utilize *Escherichia coli* (or yeast) fermentation and mammalian cell systems (e.g., Chinese hamster ovary cells) to produce recombinant proteins. Both of these systems have inevitable limitations (Cramer et al., 1998). Bacteria lack machinery to perform the complex post-translational modifications required for bioactivity of many human proteins and high-level expression can yield insoluble protein aggregates. Mammalian cell cultures have difficulties reaching high levels of transgene expression, in maintaining stable selected cell lines, and in scale-up. Using transgenic plants for human protein production has significant advantages compared to other expression systems (Cramer et al., 1996). 1) Transgenic plants are easy to manipulate, i.e., gene transfer and recovery of transformants is simple. Tobacco is considered among the easiest plant to genetically engineer. 2) Product safety is the most important advantage of transgenic plants. Plants are unable to serve as hosts or carriers of human pathogens. Hence, transgenic plants avoid the chance of product contamination with human pathogens such as hepatitis and human immunodeficiency virus (HIV). 3) Transgenic plant bioproduction

Table I.1. Plant-based production of human (or other animal) proteins (Cramer et al., 1998)

Transgene product	Potential Use Disease Target	Plant Host	Structural Integrity	Functional Activity	Reference
Serum proteins					
Haemoglobin (α and β)	Blood substitute	Tobacco	YES (dimer)	YES (O_2 binding)	Dieryck et al. 1997
Human serum albumin	Blood extender	Potato	YES	Not tested	Sijmons et al. 1990
Protein C	Anticoagulant	Tobacco	most processing steps performed	Not tested	Cramer et al. 1996
Cytokines/Lymphokines					
α-Interferon	Viral protection Anti-cancer	Rice	YES	YES (viral resistance assay)	Zhu et al. 1994
γ-Interferon	Phagocyte activator	Tobacco	YES (glycans[a])	YES (in vitro assay)	Grill 1997
CM-CSF	Leukopoiesis in bone marrow transplants	Tobacco	YES (glycans[a])	YES (growth stimul. of TF-1 cells)	Ganz et al. 1996
Epidermal growth factor	Mitogen	Tobacco	CRIM[b]	Not tested	Higo et al. 1993
Lysosomal enzymes					
α-Galactosidase	Fabry Disease	Tobacco	YES (glycans[a])	YES (enzyme act.)	Grill 1997
Glucocerebrosidase	Gaucher Disease	Tobacco	YES (glycans[a])	YES (enzyme act.)	Cramer et al. 1996
Other proteins					
Hirudin	Anticoagulant	Canola	YES	YES (thrombin inh.)	Parmenter et al. 1995
NP1 defensin	Antibiotic	Tobacco	CRIM[b]	YES (antibiotic act.)	Grill 1997
Glutamic acid decarboxylase	Diabetes	Tobacco	CRIM[b]	YES (mouse model)	Ma et al. 1997

a. Proteins were glycosylated but the glycan composition may differ from those produced in humans
b. Detected as cross-reactive immunodetected material by Western blots or ELISA

is cost-effective. It has lower cost of cultivation and high capacity for large volume production. 4) Plant systems have eukaryotic cell machinery for complex protein processing. In fact, transgenic plants have been used to produce a number of complex serum proteins, cytokines, lysosomal enzymes and other pharmaceutical value proteins (Table I.1). Promisingly, most of these proteins appear fully functional and structurally comparable to the analogous proteins synthesized from animal cell cultures or humans.

However, transgene expression levels can be highly variable among different independently generated transgenic plants. The variability in transgene expression could be explained by differences in transgene copy number and/or insertion site (reviewed by Finnegan and McElroy, 1996). Multiple copies of the transgene contained with a plant can result in post-transcriptional silencing of the transgene, which is termed co-suppression. Following co-suppression, expression of the transgene mRNA can be detected, but the transgene product levels are severely reduced. Co-suppression is independent of the promoter used to drive the transgene. The mechanism of co-suppression appears to involve gene methylation, which may be a natural protection mechanism in plant system to defend against viruses or transposons.

The site of insertion of the foreign gene also plays an essential role in the transgene expression, which is termed position effect. The position of the transgene with respect to neighboring genes may affect functional transcription of transgene from chromatids, in which the transgene expression is reduced. In contrast, at particular loci, the expression of the transgene may be enhanced due to the extra promoter function from neighboring genes.

The possibilities of variability among transgenic plants and potential instability of the transgene creates additional challenges in using plant systems for the production of valuable proteins. Large numbers of independently transformed plants need to be generated in order to identify those showing high-level, stable transgene expression. Both the RNA and protein expression levels of the transgene need to be tested to determine the

stable expression of the transgene.

I.3 SERUM PROTEINS PRODUCED IN PLANTS

Plant genetic engineering has potential for the production of valuable compounds, such as serum proteins, several of which have been expressed in transgenic plants. Four examples of proteins with important serum activities are described below.

Human serum albumin (HSA) is a 66.5 kD heat-coaguable, water-soluble protein in serum that is initially synthesized as preproalbumin by the liver. HSA has potential medical applications for certain hemophiliac diseases and as a carrier protein for many drugs, but is most widely used as a blood extender (e.g. for blood transfusions). After an 18-amino-acid prepeptide is removed in the endoplasmic reticulum, the pro-albumin is further processed in the Golgi complex where the 6-amino-acid propeptide is cleaved by a serine proteinase. The mature polypeptide of 585 amino acids is secreted into serum. Two different kinds of signal sequences were used to generate recombinant HSA gene for transformation into potato plants (Sijmons et al., 1990). In one construct, the native human prepro-sequence was used; in the second, the human prepro peptide was replaced with the signal sequence from the extracellular tobacco protein PR-S. In transgenic potato plants expressing the HSA sequence fused to the constitutive CaMV 35S promoter, the 66.5 kD HSA protein constituted up to 0.02% of total soluble leaf protein. HSA protein was detected in all parts of the transgenic plants, although the highest expression was found in stems and upper leaves. Almost all transgenic plants were phenotypically normal. Interestingly, both the human and plant signal sequences facilitated targeting of the protein outside the plant cell. But for the human prepro-sequence, only the 18 amino acid presequence was cleaved during the secretion process; the 6 amino acid prosequence still remained at the N-terminus. The construct using the plant-derived signal generated authentic mature protein in transgenic potato plants. Based on comparing the chromatographic behavior of commercial HSA and HSA from

transgenic plants, the recombinant protein was indistinguishable from the commercial HSA. These experiments demonstrated that human prepro-sequences can function in targeting the protein out of the plant cell, although the recombinant protein was incompletely processed and yielded an intermediate precursor protein. It was also shown that prosequence was not essential for protein secretion. This report established the potential use of transgenic crop plants as a source of secreted recombinant proteins.

Hemoglobin in erythrocytes binds oxygen, which makes erythrocytes transport oxygen throughout the circulatory system. A hemoglobin molecule is composed of four polypeptide chains (2 α-globin, 141 amino acid each; 2 β-globin, 146 amino acid each), each containing a prosthetic heme group. Each heme group is capable of binding one molecule of oxygen. Thus, each hemoglobin molecule is capable of binding four molecules of O_2. The binding of O_2 at the first heme group induces a conformational change that facilitates the binding of O_2 at the other three heme groups. Similarly, the unloading of O_2 at one heme group facilitates the unloading of O_2 at the other three heme groups. This cooperation between the heme groups is an allosteric effect. Hemoglobin produced from human or bovine blood, yeast and transgenic animal has been used to make hemoglobin-based blood substitutes for medical use. Though the oxygen affinity and stabilization of the purified hemoglobin can be controlled, other problems, such as heme oxidation and the presence of infectious agents, still remain. It has been demonstrated that α- and β-globins of human hemoglobin (HbA) can be co-expressed in transgenic tobacco plants, resulting in functional tetrameric hemoglobin (Dieryck et al., 1997). The coding regions of α- and β-globins were fused to the sequence of the chloroplastic transit peptide of the small subunit of Rubisco from *Pisum sativum* L. Tobacco-synthesized recombinant globin had a similar molecular mass to the native globins, indicating cleavage of the transit peptide. Recombinant hemoglobin (rHb) was extracted from transgenic tobacco seeds and purified to test the functional properties. Based on flash photolysis, the kinetics of ligand recombination with hemoglobin tetramers, both human blood HbA and tobacco-synthesized Hb displayed a characteristic biphasic shape. All the results showed that functional recombinant human hemoglobin, a

complex tetramer protein, can be synthesized and assembled in active form in transgenic plants.

Antibodies, especially monoclonal antibodies (MAb), have been widely used as diagnostic or therapeutic agents in medical applications. They are also used in basic research as specific probes or immunoaffinity tools. A wide range of heterologous systems have been shown to produce functional recombinant MAbs and antibody fragments, such as *E. coli*, mammalian cells, insect cells, yeast, fungi and plants. The plant system offers a number of unique advantages, like ease of handling and low cost of biomass production and scale-up (Ma and Hiatt, 1996).

The expression of a complete antibody molecule in plants was first described by Hiatt et al. (1989). Different transgenic tobacco lines were constructed to express either γ-chain cDNA or κ-chain cDNA of IgG1 by using their native signal sequences. Subsequently, different lines of transgenic tobacco were crossed to select F_1 plants which expressed cDNAs for both the heavy and light chains of IgG1. Significant amount of assembled IgG1 was synthesized in the F_1 plants, approximately 1% of the total soluble protein. It was also observed that the plant-assembled IgG1 bound to its antigen with the same specificity as the native IgG1, and was located in the intercellular spaces (Hein et al., 1991). So it was demonstrated that the native signal sequences directed the antibody chains to the ER leading to secretion out of the plant cell. Production of secretory IgA (SIgA) in plants is another exciting example. The assembly of SIgA is complex, involving ten subunits (4 κ light chains, 4 α heavy chains, 1 joining chain, and 1 secretory component). It was reported that transgenic plants could produce assembled SIgA (Ma et al., 1995). Four transgenic tobacco lines were generated to express κ light chain, α heavy chain, joining chain, and secretory component, respectively. After a series of sexual crosses, the final quadruple transgenic plant produced efficiently assembled SIgA (up to 500 μg per gram of fresh leaf tissue). The plant-produced SIgA still bound its native antigen. Tobacco-synthesized SIgA directed against the streptococcal antigen I/II cell surface adhension molecule of *Streptococcus mutans* and *S. sobrinus* (Ma, et al., 1995) is

currently in clinical trials for treatment of gum disease (Larrick, 1997). These results demonstrated that plants can correctly assemble and process very complex mammalian proteins.

Hirudin is a 7kD, highly acidic small protein isolated from the salivary glands of the leech *Hirudo medicinalis* (reviewed in Parmenter et al., 1996). It can form a very stable non-covalent complex with thrombin and its high specificity makes it the most potent thrombin inhibitor known (Stone et al., 1986). To date, three naturally-occurring hirudin variants have been sequenced. They shared about 85% sequence identity and all contained six cysteines at conserved positions in the N-terminal portion (Dodt et al., 1984; Harvey et al., 1986; Dodt et al., 1986). The N-terminus of hirudin, which binds to the catalytic site of thrombin (Rydel et al., 1990), is compact and apolar because of the formation of disulfide bridges between those cysteine residues. The C-terminus of hirudin also binds to the fibrinogen-recognition exosite of thrombin. Altogether, these two domains of hirudin can inhibit almost all major thrombin activities (Rydel et al., 1990). Besides its high specificity for thrombin, hirudin is a very weak immunogen (Klocking, 1991) and can be eliminated by the kidneys and shows no deposition in any organs (Markwardt et al., 1982). These features makes hirudin a very promising medicine used for blood anticoagulation.

Plant seeds could be a particularly attractive alternative host for the production of recombinant proteins (Parmenter et al., 1995): 1) storage proteins accumulate to high levels and comprise up to 40% of total seed weight, 2) product contamination is minimal due to the sterile environment of seeds in the ovary, and 3) the stability of proteins in seeds facilitates the product management in the market. Parmenter et al. (1995) have produced recombinant hirudin in transgenic canola (*Brassica napus*) seeds as a oleosin:hirudin fusion. Oleosins are small lipophilic proteins associated with oil bodies in all oil seeds, and they are potential carriers for recombinant protein in oil seeds. First, oleosins accumulate in oil seeds at quite high levels, up to 20% of total cellular protein in canola (Murphy et al., 1989). Secondly, the oil bodies with their complement of oleosins

float to the surface when oil seeds are extracted in aqueous buffer, which provides an easy method to separate and enrich the recombinant proteins from the complex mixture of storage protein and other protein components of seed cells (Murphy et al., 1989). Furthermore, recombinant proteins may be cleaved from oil bodies by using endoproteases. This has been tested by extracting bacterial β-glucuronidase (GUS) protein from seeds of transgenic canola harboring the oleosin-thrombin site-GUS construct with thrombin treatment (van Rooijen and Moloney, 1995).

The construction of an oleosin:hirudin fusion was engineered as a translational fusion of the *Arabidopsis* oleosin gene followed by the hirudin coding region with sequences encoding the recognition site for the protease Factor Xa inserted between the two genes. The fusion gene was introduced into canola plants via *Agrobacterium*-mediated transformation. The oleosin-hirudin fusion protein accumulated stably and was located almost entirely within the oil body fraction in transformed seeds. After Factor Xa enzymatic digestion of the fusion protein, soluble hirudin was released from the fusion protein. The inhibition of thrombin activity from hirudin was observed, and this inhibition was dose-dependent (Parmenter et al., 1995). Following anion and reverse-phase chromatography, approximately 30% of the soluble fraction from the fusion protein enzymatic digestion was recovered as the active form of hirudin (Parmenter et al., 1995). These experiments demonstrated that the oleosin partitioning system has great potential for production of high-value biopeptides.

I.4 HUMAN PROTEIN C PRODUCED IN PLANTS

Human protein C (hPC) has potent anticoagulant activity and significant potential as a therapy for many medical conditions, including stroke, myocardial infarction and venous thrombosis. Because of limited plasma supply and risk of blood-borne pathogen contamination (e.g., hepatitis B and HIV), recombinant approaches have been developed to produce hPC.

Recombinant human protein C has been expressed in transgenic mammalian cells (Grinnell et al., 1990), mice (Velander et al., 1992a) and swine (Velander et al., 1992b). Velander and colleagues (1992a) used a fusion gene containing hPC cDNA to generate transgenic pigs, and found that recombinant human protein C can be produced at high levels in the milk of transgenic pigs. Up to 380 μg/ml per hour of recombinant hPC were produced in transgenic pig milk, and it had the equivalent anticoagulant activity to the hPC from human plasma (Velander et al., 1992b). Proper post-translational modifications of hPC were also observed in recombinant hPC from transgenic pigs. However, the glycan composition of pig produced hPC was different from native hPC, which resulted in lower molecular mass. In addition, a large portion of single-chain hPC was present in the pig milk, indicted the limitation in posttranslational removal of the internal KR dipeptide (Velander et al., 1992b).

As mentioned above, plants may be suitable alternatives to animal or microbial expression systems, so it presents an interesting challenge to test whether transgenic plants can express and process human protein C. Transgenic tobacco plants were used to express a hPC cDNA (Cramer et al., 1996). In a binary vector, the full length hPC cDNA was inserted downstream of a constitutive plant promoter, the cauliflower mosaic virus 35S promoter from the vector pBI221 (Clontech). The binary vector, pBIB-Kan (Becker, 1990. Appendix A), provided the T-DNA border sequences which can facilitate efficient delivery of the recombinant DNA construct into plant cells via *Agrobacterium*-mediated transformation. The vector also contained an NPTII gene conferring kanamycin resistance to select the transformed plants. Tobacco leaf disks were co-cultivated with *Agrobacterium tumefaciens* containing the CaMV 35S:hPC vector.

Twenty four independently derived transgenic tobacco plants were regenerated. Expression of tobacco-synthesized hPC was confirmed at both RNA and protein levels. However, less than 0.002% of total soluble protein in tobacco seedlings was detected as protein C, based on Enzyme-Linked Immunosorbent Assay (ELISA) using commercially available (Sigma) polyclonal antibody raised against serum hPC glycoprotein. Cross-

reactive peptides on western immunoblots could only be detected following several purification steps (ammonium sulfate precipitation and ConA affinity chromatography). Cross-reacting peptides in the size range of the single chain and heavy chain were detected. Presence of heavy chain suggested that tobacco cells performed the internal cleavage to release heavy and light chains. Other experiments demonstrated that glycosylation and sulfhydryl bridge formation occurred in tobacco-synthesized hPC. Since plant systems presumably lack γ-glutamyl carboxylase, the carboxylation of nine γ-carboxyglutamic acid (Gla) residues at N-terminal domain of light chain had not been examined nor anticoagulant activity of the hPC. These results suggest that tobacco is capable of performing many of the post-translational modifications required for synthesis of hPC. However, a thorough examination of hPC processing was severely limited by the low expression levels in these transgenic plants. In this study, 24 independent transgenic plants were analyzed suggesting that the low expression of hPC was not due solely to position effects. There are several possibilities of resulting in low expression: 1) low gene expression due to limitations in the promoter; 2) instability of hPC in tobacco cells; and 3) low hPC protein detection. In this thesis, a more active plant promoter was facilitated to increase the levels of hPC produced in tobacco to test the hypothesis that stronger promoter can result in higher hPC expression in tobacco.

I.5 SUMMARY OF PRODUCTION OF RECOMBINANT PROTEINS IN PLANTS

All the examples listed above suggested that plant systems can provide the proper machinery to facilitate serum protein processing. Recombinant proteins produced from transgenic plants are properly assembled and processed, i.e., secretion, signal peptide cleavage, disulfide bond formation are all performed to some degree. However, there still remains some limitations. In the case of hPC in transgenic tobacco, hPC was synthesized and partially processed. Because plant systems lack γ-glutamyl carboxylase, the carboxylation of nine γ-carboxyglutamic acid (Gla) residues does not look promising. Also, the protein expression was very low. Concerning the low expression level, the

promoter driving the engineered gene plays a very important role in determining the expression level of the corresponding protein. The previous results on tobacco-synthesized hPC relied on the constitutive CaMV 35S promoter. However, other researchers have demonstated that a modified CaMV 35S promoter, a double enhanced CaMV 35S promoter fused to a TEV translational enhancing leader sequence, could increase the transgene expression levels 30- to 100-fold (Carrington and Freed, 1990). Thus, using a modified CaMV 35S:hPC fusion to produce human protein C in tobacco may result in higher expression.

I.6 RESEARCH OBJECTIVES

In order to more efficiently assess the potential of transgenic tobacco to produce and process hPC, new gene constructs will be generated to enhance hPC expression levels. The modified CaMV 35S promoter will be engineered to drive human protein C cDNA expression, and the post-translational modification of tobacco-derived hPC will be examined. My specific objectives are:

1. to construct plant transformation/expression vectors which fuse the hPC coding region to the CaMV double enhanced/TEV leader 35S promoter.
2. to generate transgenic tobacco plants which have stably integrated the new hPC transgene.
3. to analyze the hPC transcript and protein produced in transgenic tobacco leaf material.

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Chapter II

Production of transgenic plants expressing a dual enhanced CaMV 35S promoter : human protein C construct

II.1 INTRODUCTION

Human protein C (hPC) is a 65 kD vitamin K-dependent plasma glycoprotein that circulates as an inactive zymogen. Upon activation of the zymogen by thrombin at the endothelial cell surface, the active form of hPC functions as an anticoagulant in hemostasis due to its ability to proteolytically inactivate factors Va and VIIIa (Grinnell, 1997). Protein C also plays an important role in the regulation of thrombin generation, which was demonstrated by the recurrent thrombosis in patients with low levels of hPC, and the fatal outcome of homozygous hPC deficiency (Esmon, 1989).

Human protein C is a critical factor in the naturally-occurring anticoagulant pathway, suggesting its potential use as a therapy for many disease states (Esmon, 1987). In fact, hPC was therapeutically used as an effective antithrombotic agent for purpura fulminans (Dreyfus et al., 1991), deep vein thrombosis (De Stefano et al., 1993), septic shock (Taylor et al., 1987), coumarin-induced skin necrosis (Schramm et al., 1993), stroke, disseminated intravascular coagulation, and prevention of reocclusion in patients treated with fibrinolytic agents (Esmon, 1987).

Due to limited supply of human plasma and potential risk of contamination by blood-borne pathogens, recombinant protein expression systems have been developed to produce hPC, including transgenic mammalian cell lines (Grinnell et al., 1990), mice (Velander *et al*., 1992a), swine (Velander *et al*., 1992b) and tobacco (Cramer et al., 1996). Transgenic tobacco has remarkable advantages compared to other expression systems, including ease of manipulation, safety and cost-effectiveness (see Chapter I).

Cramer and colleagues successfully generated transgenic tobacco plants that express an hPC cDNA (Cramer et al., 1996). The *Agrobacterium tumefaciens*-mediated leaf disk transformation-regeneration method (Horsch et al., 1985) was used to generate transgenic tobacco lines. *Agrobacterium tumefaciens*, a soil bacterium, has natural gene transfer abilities that facilitate transfer of its tumor-inducing (Ti) genes from the T-DNA

(transfer DNA) region of the Ti plasmid into plant cells to cause crown gall disease in plants (Horsch et al., 1985). Horsch et al. developed an approach that integrates the gene-transfer capability of disarmed *A. tumefaciens* and general regeneration capability of leaf explants. Modified *A. tumefaciens* strains were used in which the Ti genes had been deleted from T-DNA and replaced with a chimeric gene for kanamycin resistance (Horsch et al., 1985). The ability to select for transgenic plants using kanamycin resistance makes the approach generally used for transformation of dicotyledonous plants. As commonly used, the Ti genes on the T-DNA are replaced by foreign genes that contain the necessary regulatory elements for expression in plant cells. Infection of a plant with *Agrobacterium tumefaciens* containing the engineered plasmid will result in stable integration of the foreign genes into the plant genome.

In previous work (Cramer et al., 1996), the CaMV 35S:hPC cDNA fusion was inserted into a binary vector pBIB-Kan (Becker, 1990), which provided the T-DNA border sequences that facilitate efficient delivery of the recombinant DNA construct into the plant genome. The vector also contained a kanamycin resistance gene to select the transformed plants. Twenty four independently-derived transgenic tobacco plants were regenerated, in which the expression level of hPC was very low, less than 0.002% of total soluble protein in tobacco seedlings based on Enzyme-Linked Immunosorbent Assays. The low expression of hPC in the transgenic tobacco plants made it very difficult to perform effective product analysis to assess internal dipeptide cleavage, glycosylation and sulfhydryl bridge formation.

The promoter is an important determinant in the transgene expression levels attainable for recombinant proteins. A constitutive plant promoter, the cauliflower mosaic virus 35S promoter (from pBI221, Clontech), was used to drive hPC expression in previous transgenic tobacco lines. Although the CaMV 35S promoter is considered a strong constitutive promoter in plants (Benfey et al., 1989; Fang, et al., 1989), the hPC expression level detected by polyclonal antiserum cross-reaction was very low. A modified promoter containing a double enhanced CaMV 35S promoter fused to a tobacco

etch virus translational enhancing leader sequence has been shown to increase the transgene expression levels 30- to 100-fold higher (Mason et al., 1992). Thus, use of a modified CaMV 35S:hPC fusion to produce human protein C in tobacco may result in higher expression.

As the first step in testing the potential of an enhanced CaMV 35S promoter to result in high level expression of hPC, an engineered binary vector pBIB-KAN containing a modified CaMV 35S promoter : hPC cDNA fusion was generated and transferred into *A. tumefaciens* for tobacco transformation.

II.2 MATERIALS AND METHODS

II.2.1 Vector construction

A construct consisting of the entire coding region of human protein C (hPC) fused to an enhanced CaMV 35S promoter was produced. The enhanced CaMV 35S promoter ($35S^{DE}$) was a CaMV 35 promoter with a dual enhancer and the 5'-untranslated leader sequence from tobacco etch virus (Carrington and Freed, 1990), which was obtained from vector pRTL2 (see Appendix B) provided by Dr. John Mullet (Texas A&M). A portion of the sequence from *Nco*I to *Sac*I in pRTL2 was deleted by Dr. Jia Li (Virginia Tech) to eliminate the ATG codon at the *Nco*I site yielding the modified vector pRTL2-NS (Li, 1995). The promoter was excised from pRTL2-NS as a *Hin*dIII – *Kpn*I fragment, gel-purified, and inserted into the *Hin*dIII – *Kpn*I site of *A. tumefaciens* binary vector pBIB-KAN (Becker, 1990). cDNA of hPC was isolated as a 1.4 kb *Kpn*I fragment from p35S:C2 (Dr. D.L. Weissenborn, Virginia Tech). The plasmid p35S:C2 resulted from inserting a *Hin*dIII – *Xba*I CaMV 35S promoter fragment from pBI221 (Clontech Laboratories, Palo Alto, CA) and a 1.4 kb *Kpn*I fragment of hPC cDNA derived from vector WAPPC-1 provided by Dr. W. Velander (Virginia Tech, see Appendix C) into vector pTZ18R (U.S. Biochemicals, Cleveland, OH). The 1.4kb hPC fragment was

inserted into the *Kpn*I site downstream of the CaMV 35S^{DE} in pBIB-KAN. Figure II.1 shows the steps of vector construction. The final construct, pHN-1, was transformed into library efficiency DH5α *E. coli* competent cells (Life Technologies, Gaithersburg, MD) by the heat-shock method (Cohen et al., 1972).

II.2.2 Colony hybridization

The 1.4 kb *Kpn*I fragment hPC cDNA isolated from p35S:C2 was used as hybridization probe, and labeled with ^{32}P-dCTP (NEN, Du Pont) by random primer labeling following the manufacturer's instructions (Life Technologies, Gaithersburg, MD). A method modified from Grunstein and Hogness (1975) was used to screen *E. coli* colonies carrying hPC cDNA (Sambrook et al., 1989). About 250 single colonies were transferred onto four nitrocellulose filters, which were placed on the top of LB media containing kanamycin (100 mg/L). Each colony was also copied onto master plates for recovery after the hybridization. After the colonies on the filters were incubated at 37^{o}C overnight, the DNA from individual colonies was liberated by denaturing solution (0.5 N NaOH, 1.5 M NaCl) and bound to the filter by UV-crosslinking, then hybridized to the ^{32}P-labeled probe. The filters were exposed to Kodak X-OMAT^{TM} AR film for 24 hours at –80^{o}C with Cronex intensifying screens.

II.2.3 Sequencing

Plasmid DNA was isolated for sequencing using the Plasmid Select^{TM}-250 purification kit following the manufacturer's instructions (5 Primer → 3 Primer Inc., Boulder, CO). Two oligonucleotide primers synthesized by National Biosciences Inc.(Plymouth, MN) were designed to sequence the junction sites of hPC cDNA in vector pHN-1. One primer was from the TEV translational enhancing leader sequence upstream of hPC cDNA: 5' – TAC TTC TAT TGC AGC AA – 3'. The other primer was the DNA sequence of hPC near the stop codon: 5' – CGC TAC CTC GAC TGG A – 3'. DNA was sequenced by the dideoxy - termination method using the Sequenase II DNA

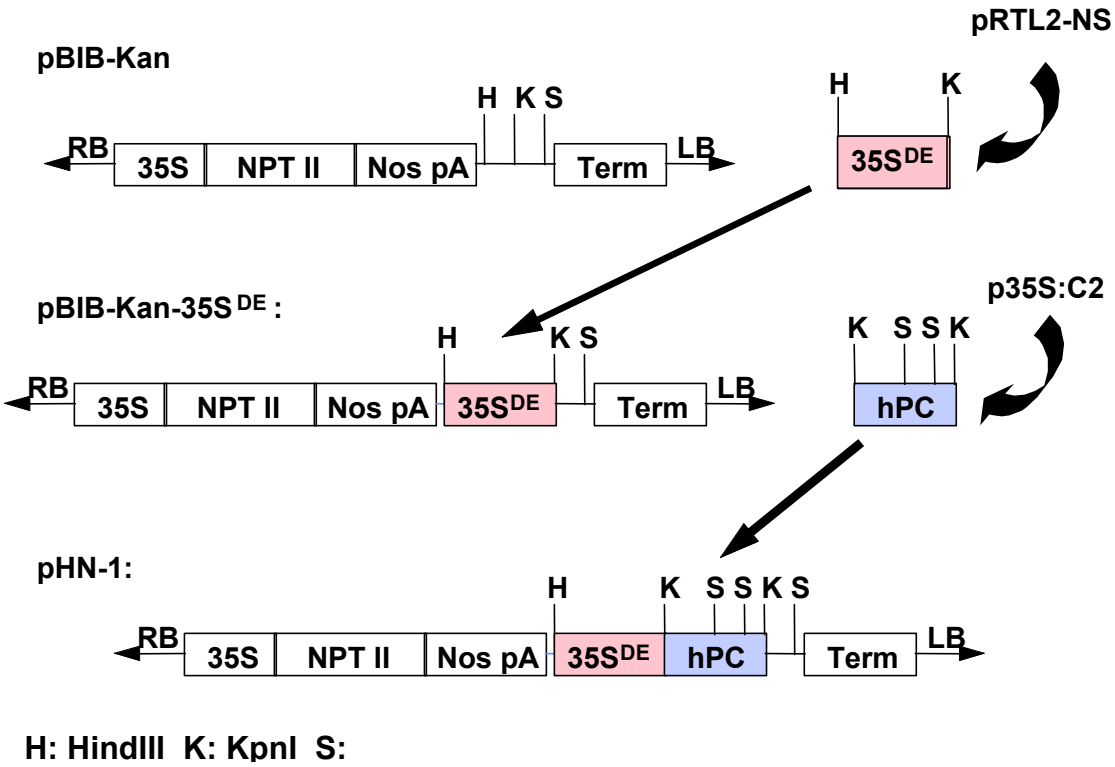

Figure II.1 Cloning strategy for pHN-1 vector. Human protein C cDNA (hPC) and the enhanced CaMV 35S promoter (CaMV $35S^{DE}$) were inserted into the binary vector pBIB-Kan. The vector also contained a kanamycin resistance gene (NPT II), plant terminators (nos pA, Term) and the T-DNA border sequences (LB, RB) that define the region to be transferred to the plant genome. H: *Hin*dIII, K: *Kpn*I, S: *Sst*I.

sequencing kit following the manufacturer's instructions (U.S. Biochemicals, Cleveland, OH).

II.2.4 *Agrobacterium tumefaciens*-mediated transformation of tobacco

The wild-type tobacco line used for transformation with the hPC cDNA was *Nicotiana tabacum* var. Xanthi nc. Plasmids pHN-1 were transformed into *A. tumefaciens* strain LBA4404 by a direct freeze-thaw method (An et al., 1988). The leaf disk transformation-regeneration method (Horsch et al., 1985) was used to generate transgenic tobacco lines. The transgenic plants were selected on selection/regeneration plates containing kanamycin (100 mg/L).

II.2.5 Southern hybridization

Genomic DNA was isolated from transgenic tobacco using a modified CTAB procedure (Rogers and Bendich, 1988). Three grams of leaf tissue were ground to fine powder with a mortar and pestle in liquid nitrogen. The powder was transferred to a 50 ml centrifuge tube which contained 15 ml of 65^{o}C prewarmed extraction buffer (2% cetyltrimethylammonium bromide [CTAB]), 100 mM Tris-HCl [pH 8.0], 20 mM EDTA [pH 8.0], 1.4 M NaCl, 0.2% β-mercaptoethanol). The mixture was incubated at 65^{o}C for 2 hours and was extracted with equal volumes of chloroform/isoamyl alcohol (24:1). Then the tubes were centrifuged at 3000 x g for 10 minutes (SA600 Rotor, Sorvall RC-5B Centrifuge, Du Pont). The top layer (aqueous phase) was transferred to new tubes, and 0.1 volume of 10% CTAB solution was added (10% CTAB, 0.7 M NaCl). After another chloroform/isoamyl alcohol extraction, the aqueous phase was transferred to new tubes. RNase Plus^{TM} (5 Primer → 3 Primer Inc., Boulder, CO) was added as 1:250 dilution to digest RNA, and the reaction mix was incubated at room temperature for 30 minutes prior to extraction with an equal volume of isopropyl alcohol. The precipitated DNA was recovered by centrifugation at 7500 x g for 15 minutes. The pellet was washed with 70% ethanol and resuspended in 400 μl TE (10 mM Tris-HCl, 1 mM EDTA, pH

8.0). DNA concentration was measured at OD_{260} with a DU640 spectrophotometer (Beckman, Fullerton, CA).

Ten micrograms of each genomic DNA sample were digested with *Hin*dIII or *EcoR*I overnight at 37^{o}C. The digested samples were separated by electrophoresis in a 0.8% agarose in 1X TAE buffer (Southern, 1975). The DNA was transferred by capillary blotting to a nylon membrane (Micro Separations Inc., Westboro, MA) and was crosslinked by UV light (Bioslink, Bios Corporation, CT). The 1.4 kb hPC cDNA isolated from p35S:C2 was used as probe, and labeled with ^{32}P-dCTP (NEN, Du Pont) by random primer labeling following the manufacturer's instructions (Life Techonologies, Gaithersburg, MD). The hybridizations were performed according to the membrane manufacturer's specifications (Micro Separations Inc., Westboro, MA). The blot was washed in 5X SSC, 0.5% SDS for 30 minutes at room temperature; in 1X SSC, 0.5% SDS for 30 minutes at 65^{o}C; followed by a final wash in 0.1X SSC, 1% SDS at 65^{o}C. The blot was exposed to Kodak X-OMAT^{TM} AR film for 7 days at -80^{o}C with Cronex intensifying screens.

II.3 RESULTS

II.3.1 Generation of CaMV 35S^{DE} : hPC fusion construct (pHN-1)

In order to enhance the expression of hPC in tobacco, a vector was constructed that fused the coding region of hPC to a modified CaMV 35S promoter consisting of a CaMV 35S promoter with a dual enhancer and the 5'-untranslated leader sequence from the tobacco etch virus. Vector construction was performed in two steps (Figure II.1). First the CaMV 35S^{DE} promoter was introduced into the binary transformation vector pBIB-KAN as a *Hin*dIII – *Kpn*I fragment. The hPC coding region was then introduced as a 1.4 kb *Kpn*I fragment.

Because of the lower efficiency of ligation and transformation of these large Ti-plasmid-derived vectors, clones containing the hPC insert were initially identified by colony hybridization. hPC sequences were detected in twenty three out of over 250 colonies screened (Figure II.2.a). Restriction endonuclease analyses were used to establish the orientation in which the hPC-containing *Kpn*I fragment inserted. Among these 23 colonies, five colonies contained the hPC cDNA in the desired orientation with respect to the CaMV $35S^{DE}$ promoter (Figure II.2.b). Colony #4-7 was chosen as the source of vector pHN-1 DNA for *Agrobacterium* transformation.

Selected regions of the DNA of pHN-1 from #4-7 were sequenced to check the *Kpn*I junction sites at both end of hPC cDNA (Figure II.3).

II.3.2 Introduction of CaMV $35S^{DE}$: hPC fusion into tobacco

pHN-1 plasmid DNA from colony #4-7 was purified from *E. coli* and transferred into *A. tumefaciens* strain LBA4404. The plasmid DNA was subsequently extracted from LBA4404 and digested with *Kpn*I. The appearance of 1.4 kb hPC cDNA in the digestion products (Figure II.4) indicated that the vector was successfully transferred into *Agrobacterium.* Following the leaf disk transformation, 30 kanamycin-resistant transgenic tobacco lines (T_0) were regenerated and designated phn0-1 to phn0-30.

Hundreds of T_1 seeds were harvested from plant phn 0-13 following self-fertilization and 72 seeds were used for a segregation assay based on kanamycin resistance (Table II.1). The observed segregation of 52 resistant to 20 sensitive was consistent with a 3:1 ratio. The chi-square value (χ^2), 0.30, was substantially less than the critical value needed for statistical significance. (With one degree of freedom, 0.05 probability, it is 3.84). In other words, the observed numbers were consistent with the 3:1 ratio expected under the principle of segregation of a single dominant locus. The 3:1 segregation ratio of kanamycin resistance to sensitivity, indicated that phn0-13 contained a single copy of the transgene.

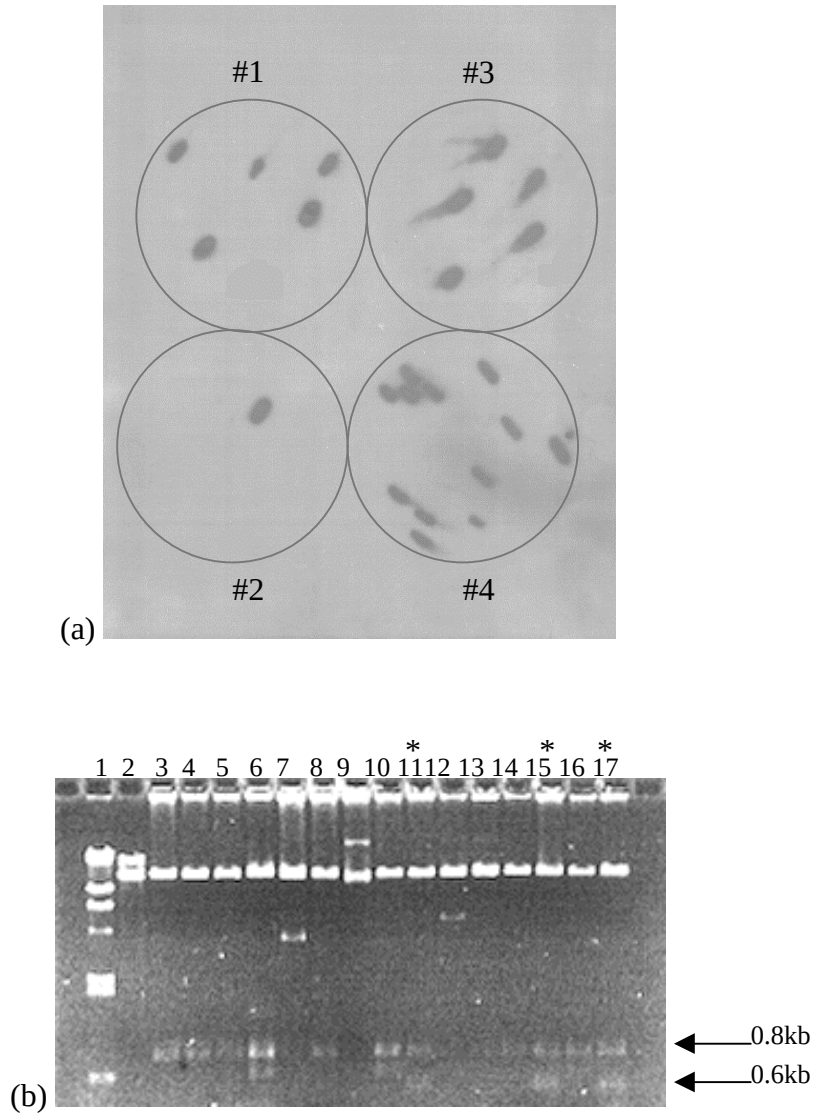

Figure II.2 Selection of colonies containing the CaMV 35S^{DE}:hPC fusion in the desired orientation. (a) Colony hybridization: Colonies generated from transformation with the pBIB-KAN-35S^{DE} and hPC ligation mix were streaked onto membranes and incubated on LB kanamycin plates. DNA from these colonies was denatured and probed with ^{32}P-labeled hPC sequences as described in the methods section. 23 colonies from four plates were positive for hPC hybridization. (b) Restriction analysis: Vector DNA from selected colonies was digested with *Sst*I, separated by 0.8% agarose gel electrophoresis, and stained with ethidium bromide. The correctly oriented hPC cDNA had 0.6 kb and 0.8 kb fragments, as shown in Lane 11 (Colony #3-20), Lane 15 (Colony #4-7) and Lane 17 (Colony #4-12). Lane 1: Molecular weight standards (*Hin*dIII digested lambda DNA) Lane 2: plasmid DNA of pBIB-Kan-35S^{DE}. Lane 3-17: DNA from colonies containing pHN-1 vector.

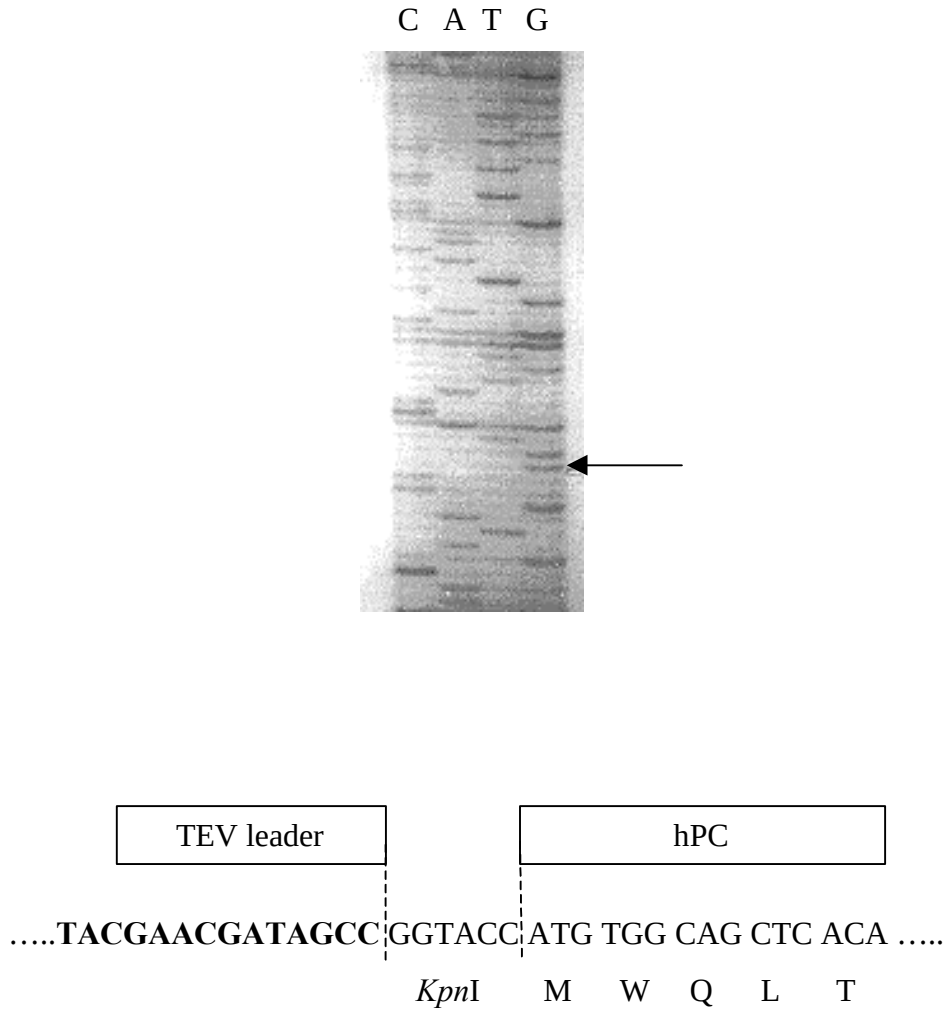

Figure II.3 Sequence analysis of CaMV 35S^{DE}:hPC construct fusion junction. The photo shows a selected region of the DNA sequence of the N-terminal junction site of hPC cDNA in vector pHN-1. The arrow identifies where *Kpn*I site starts. The illustration below identifies relevant regions within the DNA sequence and the encoded N-terminal amino acids of the hPC protein. Bolded sequences were derived from vector pRTL2-NS.

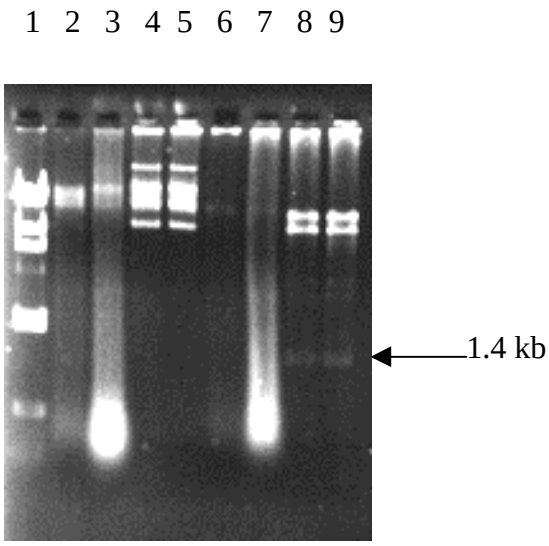

Figure II.4 Restriction analysis of plasmid DNA from *A. tumefaciens* strain LBA4404. Lane 1: Molecular weight standards (*Hin*dIII digested Lambda DNA). Lane 2&3: DNA from two non-transfected *A. tumefaciens* LBA4404 colonies. Lane 4&5: DNA from two kanamycin-resistant *A. tumefaciens* LBA4404 colonies following transformation with pHN-1. Lane 6&7: *Kpn*I digestion of same DNA samples as lane 2&3. Lane 8&9: *Kpn*I digestion of same DNA samples as lane 4&5. A 1.4 kb band was detected lanes 8&9 (arrow), indicating the presence of the pHN-1 in *A. tumefaciens* strain LBA4404.

Table II.1 Chi-square analysis of phn0-13 T_1 seedlings

Phenotype on kanamycin[a]	**Observed Numbers**	**Exppected Numbers[b]**	$\frac{(O-E)^2}{E}$ [c]
Resistant	52	72(0.75) = 54	$\frac{(-2)^2}{54} = 0.08$
Sensitive	20	72(0.25) = 18	$\frac{2^2}{18} = 0.22$
	72	72	$\chi^2 = 0.30$

a. T_1 seeds from plant phn0-13 surface-sterilized and germinated on germination media containing kanamycin (100 mg/L). Sensitive seedling produced no true leave and turned yellow within 4-5 weeks.

b. Expected ration of 3:1 based on segregating progeny of initial T_0 transgenic plant containing a single transgene insertion following self-fertilization.

c. χ^2 value consistent with a 3:1 segregation. (χ^2 of one degree of freedom give confidence to 0.5 level).

Southern hybridization analyses were also used to establish integration into the genome and copy number. Genomic DNA was digested with *Hin*dIII which cuts only once within the introduced T-DNA such that distinct integration events should yield hybridizing fragments of distinct sizes. As shown in Figure II.5.a, the kanamycin resistant lines all contained one or more copies of hPC. Results shown in Figure II.5.b confirmed that phn 0-13 contains a single copy of the hPC transgene.

II.4 CONCLUSION

Vector pHN-1 was successfully constructed and transferred into the tobacco genome by *Agrobacterium tumefaciens*-mediated transformation. Transgenic tobacco plants were selected based on kanamycin resistance and integration of one or more copies of the hPC transgene into the tobacco genome was established. Since multiple copies of the transgene contained with a plant can result in post-transcriptional silencing of the transgene (co-suppression), transgenic plants containing single copy transgene (i.e., phn0-13) were expected to have stable transgene expression. These plants provide the transgenic material needed to address the ability of tobacco to synthesize and process human protein C, which will be described in chapter III.

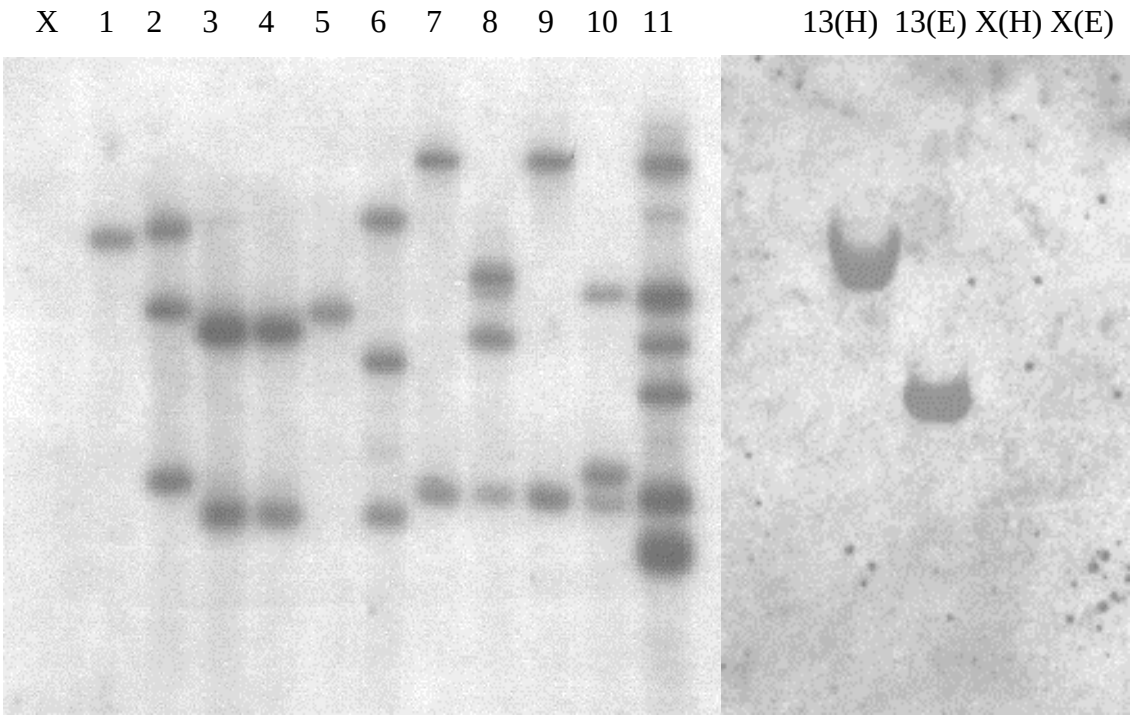

Figure II.5 Southern blots of DNA from transgenic tobacco lines. Ten micrograms of DNA were loaded for all samples. (a): Southern blots using genomic DNA from non-transformed *Nicotiana tabacum* var. Xanthi (X) and transgenic tobacco plants phn 0-1 to phn 0-11. DNA samples were digested with *Hin*dIII. phn 0-1 and phn 0-5 were shown to have a single copy of hPC gene in the genome, while other lines contained multiple copies. (b) Southern blot using plant phn 0-13 genomic DNA and non-transformed *Nicotiana tabacum* var. Xanthi DNA. Samples were digested with *Hin*dIII (H) and *Eco*RI (E). X: non-transformed *Nicotiana tabacum* var. Xanthi.

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Chapter III

Expression of human protein C in transgenic tobacco

III.1 INTRODUCTION

Since the discovery of *Agrobacterium tumefaciens*-mediated plant transformation sixteen years ago, plant biotechnology has had a tremendous impact on industry worldwide and scientists continue to develop innovative applications. Using transgenic plants for human protein production has significant advantages compared to other recombinant protein expression systems (Cramer et al., 1996), as described in Chapter I. In fact, transgenic plants have been used to express a number of proteins of pharmaceutical value (see Chapter I). Most of these proteins appear fully functional and structurally comparable to the analogous proteins synthesized from animal cell cultures or humans.

Human protein C (hPC) is a 65 kD serum protein that has anticoagulant activity in hemostasis, suggesting its potential use as a therapy for many disease states (Esmon, 1987). As described in Chapter II, transgenic tobacco plants have been generated to produce hPC. Human protein C cDNA was fused to an enhanced cauliflower mosaic virus (CaMV) 35S promoter comprising a dual enhancer and the 3'-untranslated leader sequence from tobacco etch virus (Carrington et al., 1990).

The purpose for generating the enhanced CaMV $35S^{DE}$: hPC fusion, replacing the CaMV 35S:hPC fusion in previous work (Cramer et al., 1996), was to elevate the expression levels of hPC in transgenic tobacco (see Chapter II). The low expression of hPC in CaMV 35S:hPC transgenic tobacco plants made it very difficult to perform effective product analysis to assess whether tobacco performs the post-translation modifications required to produce this complex serum protein.

The CaMV $35S^{DE}$: hPC fusion was inserted into the binary vector pBIB-KAN to generate the final construct, pHN-1. pHN-1 was transferred into *Agrobacterium tumefaciens* strain LBA4404. Following the leaf disk transformation, 30 kanamycin-resistant transgenic tobacco lines (T_0) were regenerated and designated phn0-1 to phn0-

30. Based on segregation assays and Southern hybridizations, all lines were found to contain CaMV $35S^{DE}$: hPC sequences. Integration of one or more copies of the hPC transgene into the tobacco genome was established. Results demonstrated that plant phn0-13 contains a single copy of the hPC transgene.

To address the ability of tobacco to synthesize and process human protein C, the goals of the current study are to identify the tobacco lines with the highest expression among the CaMV $35S^{DE}$: hPC transgenic tobacco lines and to initiate analysis of transgene product. The most convenient method to detect the expression of the transgene product is to perform an enzyme activity assay. However, this strategy is not suitable for hPC produced from transgenic tobacco. As mentioned in Chapter I, plant systems presumably lack γ-glutamyl carboxylase required for the carboxylation of nine γ-carboxyglutamic acid (Gla) residues at N-terminal domain of light chain, a necessary modification for hPC activity. Hence, the only method available to detect tobacco-synthesized hPC is to perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of plant proteins, followed by immunoblot detection with specific hPC antibodies. Thus, proteins extracted from transgenic tobacco leaves were subjected to western immunoblot analyses. Protein extracts of plants derived from XC2-17 expressing a CaMV 35S:hPC construct generated in previous work from Cramer et al. (1996) were also utilized in most analyses for comparative purposes.

III.2 MATERIALS AND METHODS

III.2.1 Identification of single insert line homozygous for CaMV 35S:hPC

In order to identify homozygous progeny of the previously generated CaMV 35S:hPC line XC2-17 (Cramer et al., 1996), six CaMV 35S:hPC transgenic tobacco lines, XC2-17T1-001 to XC2-17T1-006, were used to perform segregation assays. T2 seeds of each line were surface sterilized with 30% bleach solution (30% [v/v] bleach in distilled

water) for 30 minutes followed by sterile water rinse four times. The sterile seeds were germinated on germination plates (for 1 liter: 2.15 g of MS salts, 10 g of sucrose, 50 mg of myo-inositol, 8 g of agar) containing kanamycin (100 mg/L). Seedlings were grown under constant white light (150 mmol photons m^{-2} sec^{-1}). Two or three weeks after the germination, the numbers of kanamycin-resistant plants and kanamycin-sensitive plants were recorded. Seedlings of line XC2-17T1-005 were all kanamycin-resistant indicating homozygosity for the transgene.

III.2.2 RNA isolation from CaMV 35S^{DE}:hPC transgenic tobacco lines

Total RNA from young leaves of selected CaMV 35S^{DE}:hPC transgenic plants and wild type non-transformed *Nicotiana tabacum* var. Xanthi plants were extracted using a modified ATA (aurintricarboxylic acid) method (Thompson et al., 1983). One gram of leaf tissue was ground to fine powder with a mortar and pestle in liquid nitrogen. The powder was transferred to a 15 ml centrifuge tube which contained 2 ml of extraction buffer (50 mM Tris-HCl [pH 8.0], 2% β-mercaptoethanol [v/v], 4% ρ-aminosalicylate, 1% triisopropylnapthalene sulfonate, 1 mM ATA) and 2 ml of phenol/chloroform (1:1). The mixture was homogenized at setting 6 using PowerGen 700 (Fisher Scientific) homogenizer for 1 minute. The tubes were centrifuged at 7500 x g for 10 minutes (SA600 Rotor, Sorvall RC-5B Centrifuge, DuPont). The top layer (aqueous phase) was transferred to new tubes, and 20 μl of 0.1M ATA was added. After 12 M LiCl was added to final concentration of 2 M, the tubes were sealed and stored at 4^{o}C overnight. The tubes were centrifuged at 7500 x g for 20 minutes and the supernatant was discarded. The pellets were resuspended in 1 ml of resuspension buffer (10 mM Tris-HCl [pH 7.5], 2.5 mM EDTA, 50 μM ATA) and the solutions were transferred to microfuge tubes. After 12M LiCl was added to final concentration of 2M, the tubes were placed at 4^{o}C overnight. The tubes were microcentrifuged at 14,000 rpm at 4^{o}C. Each pellet was washed in 0.2 M sodium acetate/76% ethanol once and resuspended in 20 μl of 50 μM ATA solution. RNA concentration was measured at OD_{260} with a Beckman DU640 spectrophotometer.

The RNA sample of transgenic tobacco phn0-2 was further purified to mRNA by using the Micro Poly(A) Pure Kit following the manufacturer's instructions (Ambion Inc., Austin, TX) and measured at OD_{260}.

III.2.3 Northern hybridization

Thirty micrograms of total leaf RNA (or three micrograms of poly(A+) RNA) from different transgenic tobacco lines were denatured and separated on 1.2 % agarose-formaldehyde gels by the method of Sambrook et al. (1989). RNAs were visualized and photographed by staining with ethidium bromide to ensure that all lanes were loaded equally before being transferred to Maximum-Strength Nytran filter (Schleicher & Schuell, Keene, NH). The 1.4 kb hPC cDNA isolated from p35S:C2 was used as probe, and labeled with ^{32}P-dCTP (NEN, DuPont) by random primer labeling following the manufacturer's instructions (Life Technologies, Gaithersburg, MD). The hybridizations were conducted in 50% formamide, 6 X SSPE, 5 X Denhardt's solution, 0.5% SDS, and 100 μg/mL of sonicated denatured salmon sperm DNA at 42^0C. RNA blots were washed at 65^0C in 0.1 X SSC and 0.5% SDS for 30 min and the damp filters were exposed to Kodak X-OMAT^{TM} AR film at -80° C for 7 days with Cronex intensifying screens.

III.2.4 Isolation of protein from transgenic tobacco

For crude protein extraction, two to five grams of young tobacco leaf tissue from transgenic plants or non-transformed *Nicotiana tabacum* var. Xanthi control plants were ground to fine powder with a mortar and pestle in liquid nitrogen. The powder was mixed with Laemmli buffer (62.5mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% β-mercaptoethanol [v/v]) or hPC extraction buffer (50 mM Na_2HPO_4 [pH 7.0], 2 mM EDTA [pH 8.0], 0.1% Triton X-100, 400 μM phenylmethylsulfonyl fluoride [PMSF]) at a ratio of 0.5 ml buffer per gram leaf tissue. The mixtures were heated at 100^oC for 10 minutes and centrifuged at 13,000 x g for 20 minutes. The supernatants were saved for

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or further purification. Protein concentrations were determined with a Bio-Rad protein assay kit following the manufacturer's instructions (Bio-Rad Laboratories Inc., Hercules, CA) and measured at OD_{595} with a Beckman DU640 spectrophotometer.

III.2.5 Partial purification of hPC in crude protein samples

For ammonium sulfate precipitations, a crude protein sample was transferred to a small beaker with a stir bar. For the 0-45% salt cut, 27.7g ammonium sulfate/100ml solution was added slowly into the protein sample while stirring gently at 4^{o}C (Cooper, 1977). The beaker was placed at 4^{o}C for two hours and the solution was transferred into a 15 ml centrifuge tube. All tubes were centrifuged at 18,000 x g for 30 minutes at 4^{o}C (SA600 Rotor, Sorvall RC-5B Centrifuge, DuPont). The supernatant was transferred into dialysis tubing with 6,000-8,000 MW cutoff (Spectrum Medical Industries Inc., Laguna Hills, CA) and dialyzed overnight at 4^{o}C against 1X TBS containing 1mM EDTA. The dialyzed products were used for Concanavalin A (ConA) affinity chromatography purification or TCA precipitation prior to SDS-PAGE.

For trichloroacetic acid (TCA) precipitation, TCA was added into the dialyzed samples to a final concentration of 10% (v/v). After incubation at room temperature for 10 minutes and centrifugation at 12,500 rpm for 30 minutes, the pellet was resuspended in Laemmli buffer. The protein assay was the same as above.

A fast protein liquid chromatography (FPLC) system was used to further purify hPC through a ConA affinity column. The FPLC system (Pharmacia Biotech Inc., Piscataway, NJ) consists of two pumps (P-500), a control unit (UV-1), a programmer (GP-250 Plus), a optical unit (UV-1) and a detector (REC 102).

The ammonium sulfate cut protein samples were dialyzed against ConA buffer (100 mM NaOAc, 200 mM NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$) overnight at

4 ^{o}C. The ConA suspension mixture (18 ml) containing about 12 ml packed volume of ConA resin (Sigma) was poured into a straight column (diameter=10mm) and the column was washed with 120 ml of Tris buffer (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5), 120 ml of ConA buffer, 120 ml of NaOAc buffer (0.1M ammonium acetate, 0.5M NaCl, pH 4.5), and 120 ml of ConA buffer in order. The column was connected into the FPLC system. The dialyzed protein sample was loaded onto the ConA column and equilibrated with ConA buffer at a flow rate of 1 ml/min. The detector was set at 0.1 cm/ml. The column was extensively washed with ConA buffer until unbound protein no longer eluted from the column. Elution of bound protein was achieved by washing the column with ConA buffer containing 30% to 80% 200 mM α-L-methylglucopyranoside. The eluted protein was collected and run on SDS-PAGE for western blot analysis.

III.2.6 Antibody Preparation

Six different primary antibodies were used to perform the western immunoblots. Three are commercially available from Sigma: polyclonal anti-hPC rabbit IgG, monoclonal anti-hPC (heavy chain) mouse IgG clone HC-2, and monoclonal anti-hPC (light chain) mouse IgG clone HC-4.

Production of hPC peptides in *E.coli* and antibody production and purification for anti-hPCH (heavy chain) chicken serum, anti-hPCL (light chain) chicken serum and anti-hPCH chicken IgY were performed by Dr. Matthew Pelletier (Virginia Tech, Figure III.1).

DNA fragments encoding Gln_{-40} to His_{154} (hPCL) and Thr_{159} to Ala_{418} (hPCH) of hPC were synthesized by polymerase chain reaction (PCR) using oligonucleotides that generated flanking restriction sites. The PCR products were digested with *EcoRI* and *Hin*dIII and ligated into the corresponding sites of expression vector pET32a (Novagen, Inc., Madison, WI). The resulting vectors were transferred to *E. coli* strain BL21DE3 and recombinant proteins were purified by nickle affinity chromatography. Approximately 1

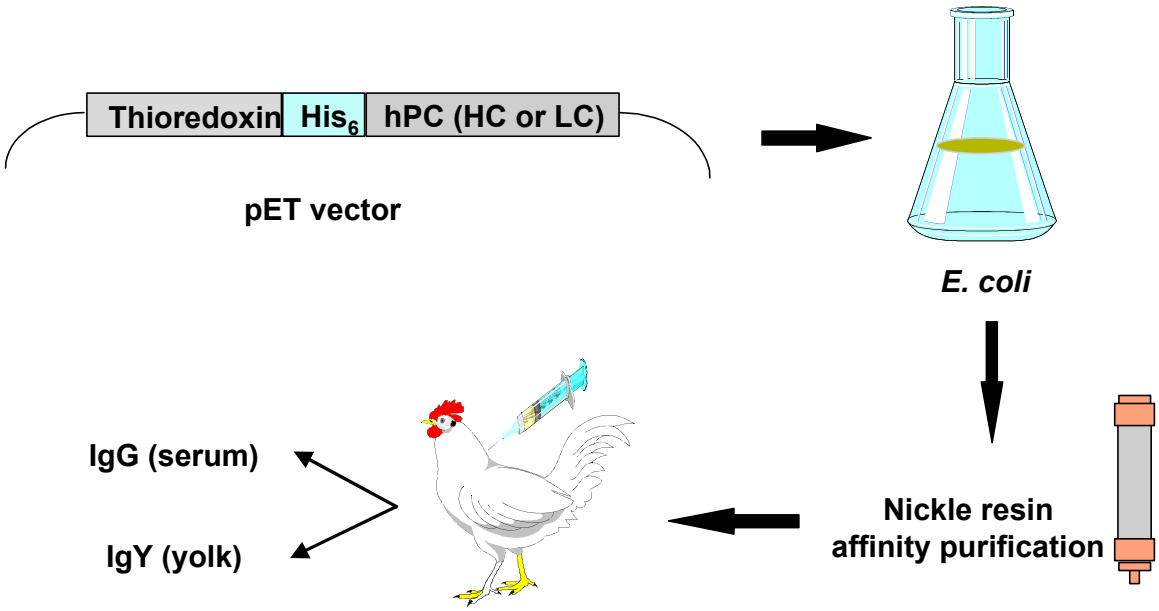

Figure III.1 Production of hPC peptides in *E. coli* for antibody production. hPC peptides for the heavy (HC) and light (LC) chain were synthesized in *E. coli* as thioredoxin, his-tagged fusions. Affinity purified HC and LC was used to inject chickens.

mg of hPCH and 2 mg of hPCL, each as a thioredoxin fusion, were sent to Cocalico Biologicals (Reamstown, PA) for the purpose of immunizing chickens.

Both sera and egg yolks were obtained from immunized chickens. IgY was purified from the yolks using the Promega Eggstract kit according to the manufacturer's recommendations (Promega Corporation, Madison, WI).

III.2.7 Western immunoblot analysis

SDS-PAGE was used to separate protein samples. Gel electrophoresis and transfer of proteins was carried out using the Mini-protein II system (Bio-Rad Laboratories Inc., Hercules, CA).

Protein samples (50 ng standards to 50 μg crude extracts) were heated at 100 C for 10 minutes in SDS-PAGE sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% β-mercaptoethanol [v/v], 0.025% bromophenol blue), and loaded onto 10% SDS polyacrylamide gels with a 4% stacking gel (Laemmli, 1970). After electrophoresis for approximately 1 hour at 200V (~40 mAmps), the electrophoresis was stopped as soon as bromophenol blue dye had exited. The resulting gel was stained with Coomassie blue to visualize the proteins or transferred to solid support.

Proteins were transferred to Trans-Blot pure nitrocellulose membrane (0.2μm) (Bio-Rad Laboratories Inc., Hercules, CA) in transfer buffer (192 mM glycine, 25 mM Tris-base, 20% (v/v) methanol, pH 8.3) using a Bio-Rad transfer apparatus at 100V for 1 hour or 25V overnight at 4^{o}C. The transfer sandwich consisted of a sponge, then a piece of 3MM Whatman paper, the gel, the nitrocellulose, another piece of 3MM Whatman paper, and the other sponge.

The resulting filter was used to perform immunological detection of immobilized proteins (western blot). The nitrocellulose filter was sealed in a seal-a-meal bag and

incubated in 50 ml of blocking solution (5% non-fat dry milk [Bio-Rad] in PBS-T [80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1% Tween-20, pH 7.5]) for 1 hour at room temperature with shaking. The filter was then incubated with primary antibody in 20 ml fresh blocking solution for 1 hour at room temperature on an orbital shaker. Six different primary antibodies were used. Unless noted, the working dilution for each antibody was as follows: 1) polyclonal anti-hPC rabbit IgG (Sigma), 1:500; 2) monoclonal anti-hPC (heavy chain) mouse IgG clone HC-2 (Sigma), 1:400; 3) monoclonal anti-hPC (light chain) mouse IgG clone HC-4 (Sigma), 1:1000; 4) anti-hPC (heavy chain) chicken serum (Cocalico Biologicals, Inc., Reamstown, PA), 1:250; 5) anti-hPC (light chain) chicken serum (Cocalico Biologicals, Inc.), 1:1000; 6) anti-hPC (heavy chain) chicken IgY (Cocalico Biologicals, Inc.), 1:500. The basic steps of immunoblotting were shown in Figure III.2.

For some experiments, primary antibodies were pre-absorbed before use in hPC detection. Primary antibody was incubated with a large amount of non-transformed *Nicotiana tabacum* var. Xanthi protein (fixed on pure nitrocellulose membrane) in blocking buffer at room temperature for one hour to reduce the non-specific cross-reaction to non-transformed plant proteins. After incubation with the primary antibody, the filters were washed in large volumes of PBS-T for 15 minutes, twice in PBS-T for 5 minutes each, and incubated with secondary antibody in blocking solution for 1 hour with shaking. The working dilutions for different secondary antibodies were as follows: 1) for polyclonal rabbit anti-hPC IgG, using horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma), 1:12,000; 2) for monoclonal mouse anti-hPC HC-2 IgG and HC-4 IgG, using horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham, Arlington Heights, IL), 1:4,000; 3) for all the primary antibodies from chicken, using horseradish peroxidase-conjugated rabbit anti-chicken IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), 1:5,000. Filters were washed with PBS-T as described above and detection of proteins was carried out using chemiluminescent reagents (ECL system) according to the manufacturer's protocol (Amersham, Arlington Heights, IL).

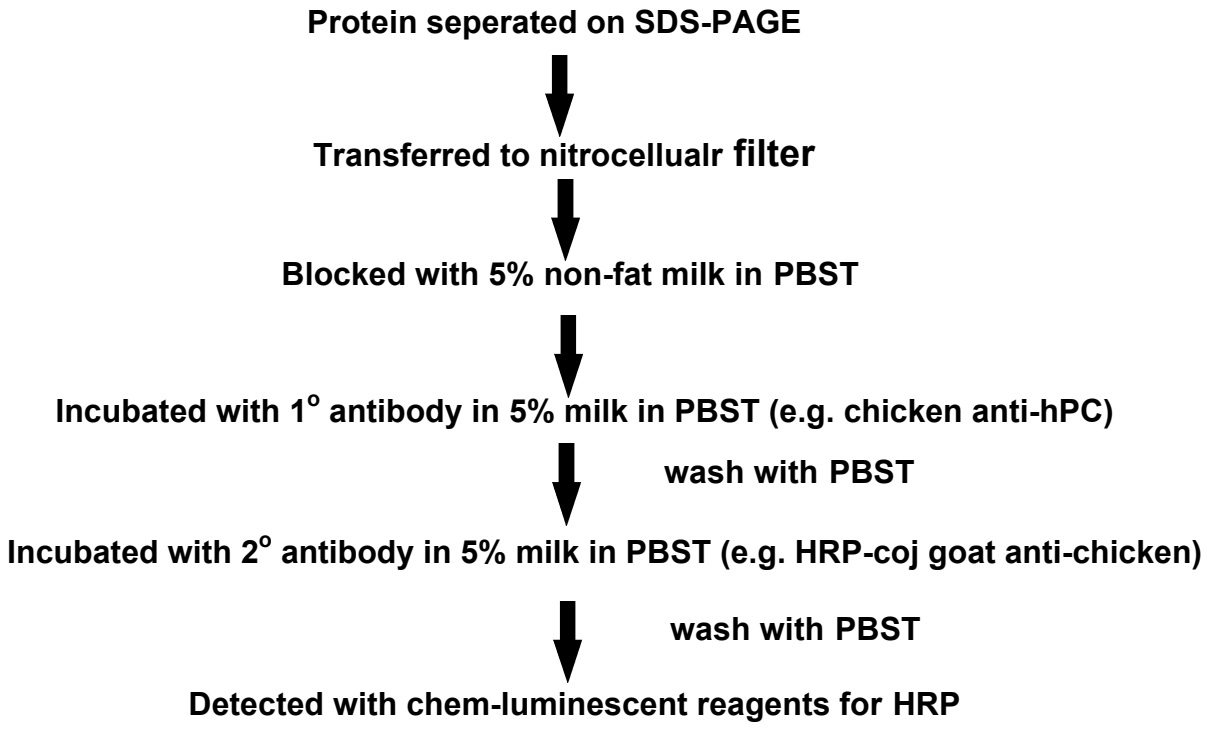

Figure III.2 Primary steps for western immunoblot analyses. PBST: [80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1% Tween-20, pH 7.5], HRP-conj: horse radish peroxidase conjugated.

All the antibodies and detection reagents on the resulting membrane of the western immunoblot could be stripped off and the membrane reused to perform another western immunoblot if needed. The membrane was incubated with stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl [pH 6.7]) at 50^{o}C for 30 minutes, followed by 50 ml of PBS-T wash for 2X 10 minutes at room temperature. The resulting membrane was used for immuno-detection as described above.

III.3 RESULTS

III.3.1 hPC expression at the RNA level

Eleven transgenic tobacco lines containing human protein C sequences (Chapter II) were tested for expression of the hPC transgene at the RNA level. Total RNA was extracted from young tobacco leaves (less than 2.5 inches in length) and used for northern blots. RNA from non-transformed wild-type plants was used as a negative control.

As shown in Fig. III.3a, the hPC probe cross-hybridized to an RNA species of approximately 1.7 kb from many of the transgenic lines. This transcript size is consistent with that expected from the hPC transgene. Plant phn0-10, a plant that had at least 3 inserts of the hPC transgene (Chapter II, Fig.II.4), showed the highest levels of expression. No cross-hybridization was detected with RNA from the non-transformed control (Fig.III.3a, lane 1; Fig.III.3a, lane 1).

Because the major hybridizing band was somewhat broad and of a similar mobility as the lower molecular weight rRNA bands, poly(A+) RNA was also isolated from total RNA of one of the plants (phn0-2) and analyzed by northern hybridization.

As shown in Fig.III.3c, the hPC-hybridizing band from poly(A+) RNA co-migrated with that from total RNA confirming that this material represents hPC mRNA

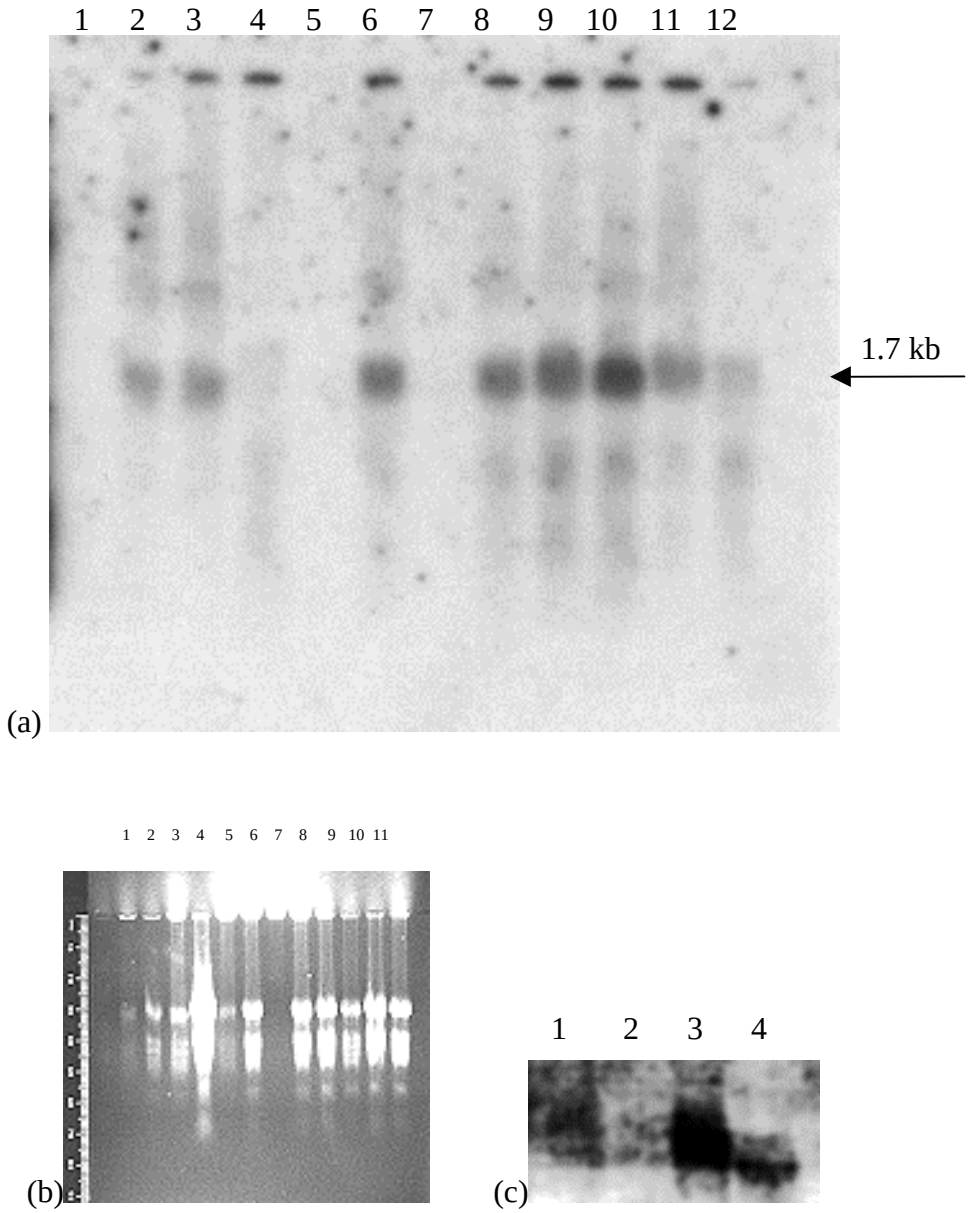

Figure III.3 Northern hybridization analyses of RNA from transgenic tobacco leaves. (a) Northern blot of total RNA (approximately 30 μg based on OD_{260}) was hybridized with ^{32}P-labeled hPC DNA (1.4 kb *Kpn*I fragment). Lane1: non-tranformed *Nicotiana tabacum* var. Xanthi; Lane2: phn0-1; Lane3: phn0-2; Lane4: phn0-3; Lane5: phn0-4; Lane6: phn0-5; Lane7: phn0-6; Lane8: phn0-7; Lane9: phn0-9; Lane10: phn0-10; Lane11: phn0-13; Lane12: phn0-16. (b): Ethidium bromide staining of the gel used for the blot shown in (a). (c): Northern blot of total and poly(A+) RNA probed with ^{32}P-labeled hPC DNA (1.4 kb *Kpn*I fragment). Lane1: 20 μg of total RNA from phn0-13; Lane 2: 20 μg of total RNA from phn0-10; Lane3: 3 μg of poly A RNA from phn0-2; Lane 4: 20 μg of total RNA from XC2-17T1-005.

rather than non-specific hybridization with the more abundant rRNA. It was also observed that the molecular weight of mRNA from XC2-17T1-005 (Fig.III 3c, lane 4) was smaller than the poly A RNA from phn0-2 (Fig.III 3c, lane 3). Both have poly A tails consistent with the presence of the TEV leader sequence in phn0 plants.

Northern analyses were also used to compare the levels of hPC RNA in the plants expressing the CaMV 35S ^{DE}: hPC constructs to previously generated CaMV 35S:hPC-expressing plants. Among the CaMV 35S:hPC lines, plant XC2-17 was identified as the highest expressor based on ELISA analyses (Cramer et al., 1996). Genetic segregation analyses (ratio of resistant to sensitive seedlings on kanamycin-containing medium) confirmed that XC2-17 contained a single copy of the transgene insert and were used to identify T_2 lines homozygous for the insert. Seedlings and young plants from this homozygous line (XC2-17T1-005) were used for RNA isolation and subsequent protein analyses.

Among the CaMV 35S ^{DE}: hPC-expressing plants, T_0 plant phn0-13 was the first to mature and produce seed and contained a single transgene insert (Chapter II). Transgenic T_1 seedlings of this plant were selected by growth on kanamycin-containing medium and transferred to potting mix. Because the CaMV 35S promoter is more active in young leaf tissue, matched leaves of less than 2.5 inches in length were harvested from young plants and used for total RNA isolation and northern hybridization analysis.

As shown in Fig.III.4a, plants expressing hPC driven by the enhanced CaMV 35S promoter (lane 3-5) did not show higher levels of hPC transcript, than line XC2-17T1-005.

III.3.2 Western immunoblot analyses

In order to detect hPC protein expression in transgenic plants containing CaMV 35S^{DE}:hPC fusion, SDS-PAGE was performed to separate proteins and followed by

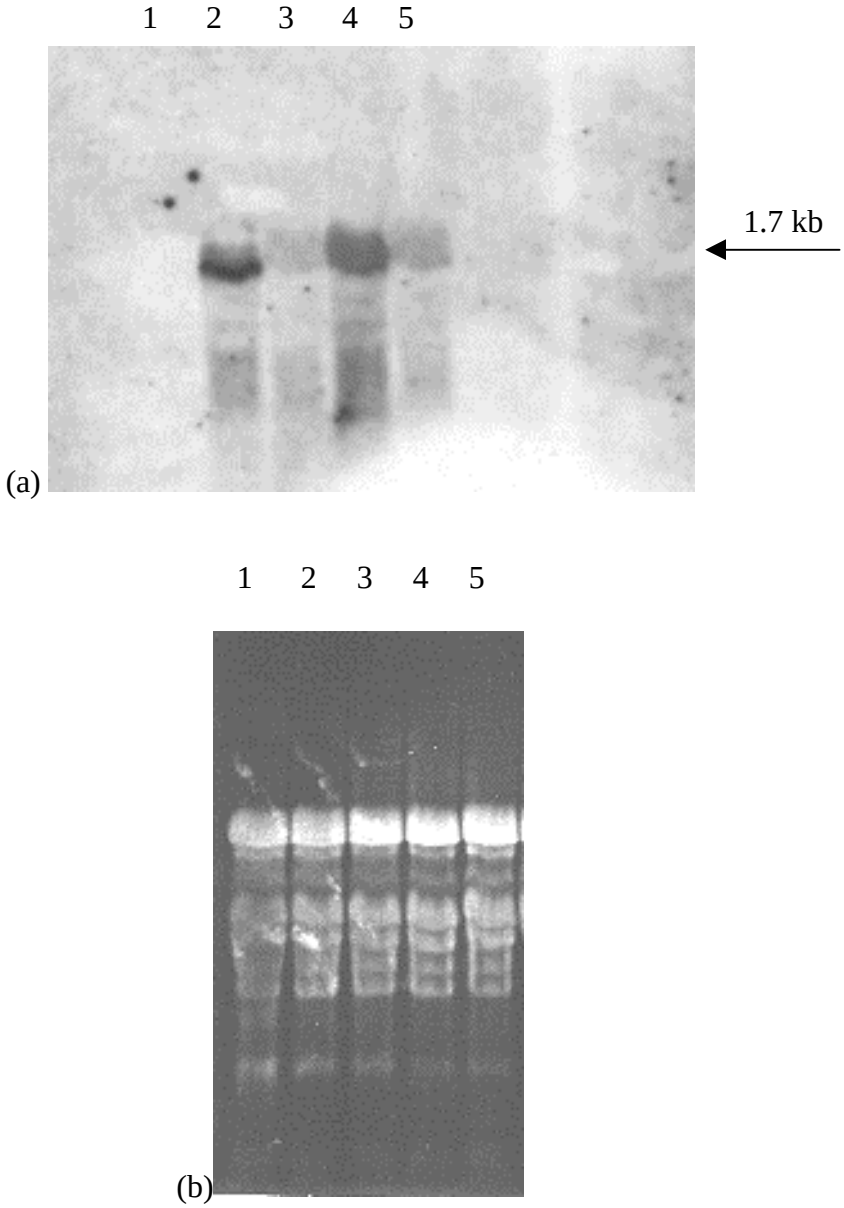

Figure III.4 Northern hybridization analysis comparing hPC transcript levels in plants utilizing the CaMV 35S versus the CaMV $35S^{DE}$ promoters. (a): 30 μg total RNA isolated from young leaves (less than 2.5 inches in length) of control or hPC-transgenic plants was used for northern blotting and probed with ^{32}P-labeled hPC DNA (1.4 kb *Kpn*I fragment). (b): Ethidium bromide-stained gel prior to transfer. Lane 1: non-transformed *Nicotiana tabacum* var. Xanthi; Lane2: XC2-17T1-005; Lane3: phn0-9; Lane4: phn0-10; Lane5: phn0-13.

western immunoblot and chem-luminescent detection. Recombinant hPC from the milk of trangenic pigs, generously provided by Dr. Velander (Velander et al., 1992), was used as a standard in all blots.

Western immunoblot analyses focused on three plant lines: the non-transformed *Nicotiana tabacum* var. Xanthi control, T_2 seedlings of XC2-17T1-005 (CaMV 35S:hPC), and kanamycin-resistant T1 seedlings of phn0-13 (CaMV $35S^{DE}$:hPC). The previous results showed XC2-17T1 seedlings produced hPC at very low level, only 0.002% of total soluble protein (Cramer et al., 1996). The low hPC protein level may have resulted from low gene expression, protein instability, or inadequate detection. The northern hybridization analyses described above suggested that hPC transcript was produced in tobacco leaves.

Detection of the hPC gene product is based on cross-reaction of antibodies to the hPC protein. The commercially available antibodies, monoclonal or polyclonal antibodies raised against either hPC single chain or heavy/light chain, were raised against the human hPC glycoprotein from serum and may be ineffective in recognizing hPC epitopes as synthesized in plants. The differences between common mammalian and plant glycan groups are shown in Figure III.5. Initial immunoblot analyses used the same polyclonal antisera (Sigma rabbit IgG raised against hPC glycoprotein isolated from serum) used for detection by Cramer et al. (1996). Subsequent analyses used newly produced chicken antibodies raised against *E. coli*-synthesized hPC polypeptides. Proteins synthesized in *E. coli* will not be glycosylated.

III.3.2.1 Western immunoblot analysis using polyclonal antibodies

Proteins from crude leaf extracts (soluble proteins after 45% ammonium sulfate precipitation) and hPC standards were size-separated on SDS-PAGE under reducing conditions and used for western immunoblots. The polyclonal antibodies against serum hPC detected pig recombinant hPC effectively (Figure III.6), showing single chains,

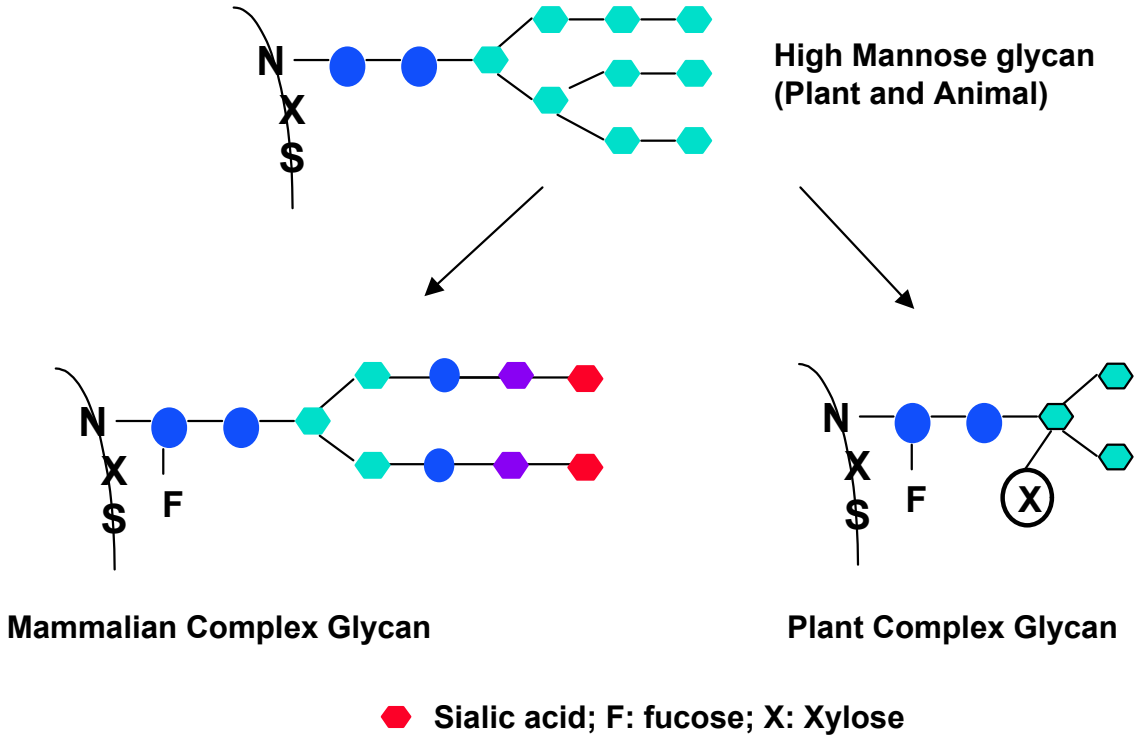

Figure III.5 Differences between mammalian and plant glycan groups.

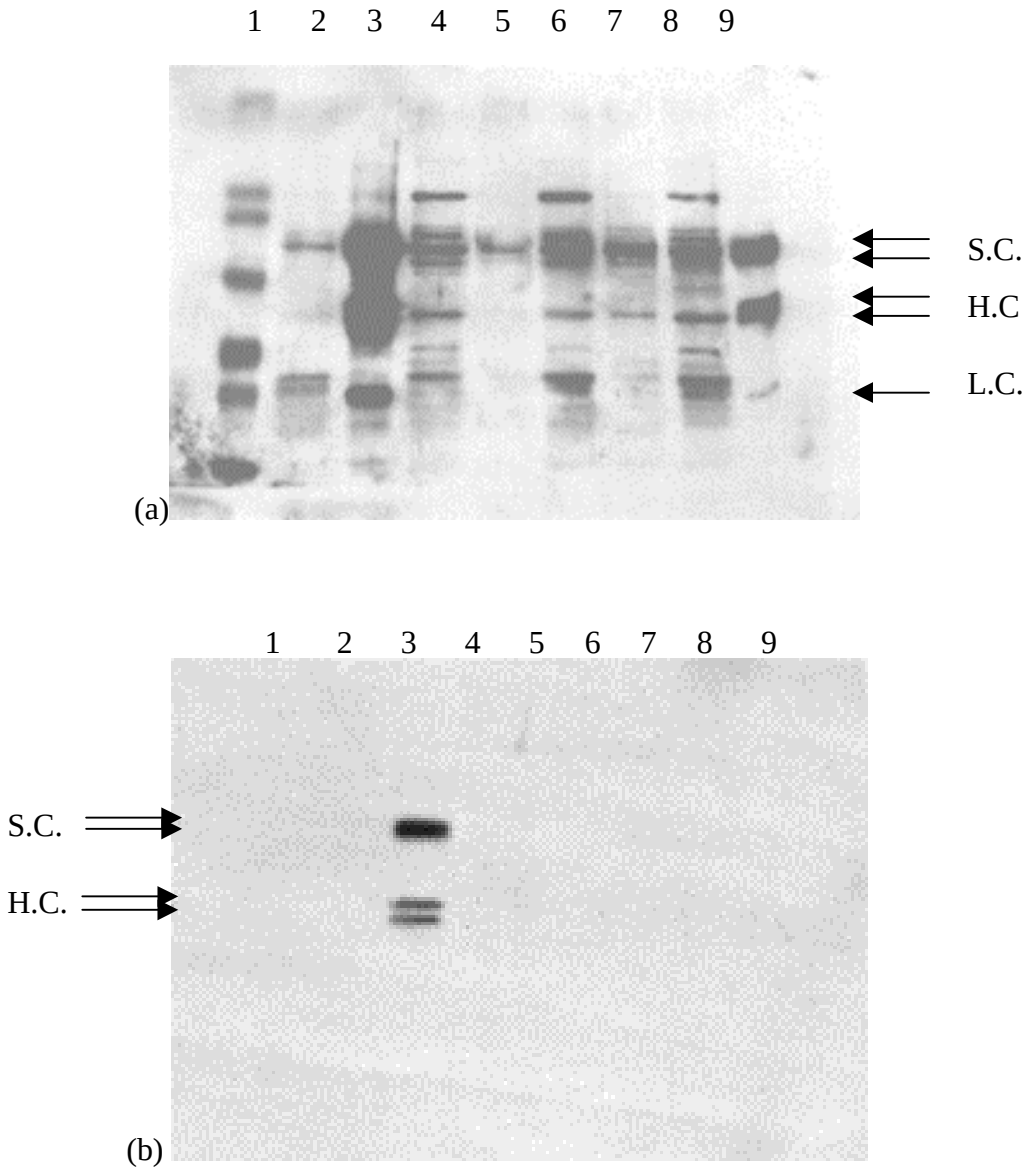

Figure III.6 Western immunoblot analysis using polyclonal antisera to hPC serum to detect recombinant hPC in transgenic plants. Plant protein samples were extracted from leaf tissue in hPC extraction buffer (see methods), followed by a 45% ammonium sulfate precipitation. Thirty micrograms of remaining soluble plant proteins were loaded for each sample. (a): Western blot using the primary polyclonal antisera (Sigma rabbit IgG to serum hPC) at a dilution of 1:500. (b): Western blot of membrane in (a) using primary polyclonal antisera that was pre-absorbed to protein extracted from non-transformed *Nicotiana tabacum* var. Xanthi. Lane 1: Prestained SDS-PAGE standards (Bio-Rad Laboratories Inc., Hercules, CA); Lane 2: non-transformed *Nicotiana tabacum* var. Xanthi; Lane 3: rhPC from pig milk (500 ng); Lane 4: XC2-17T1-005; Lane 5: phn0-1; Lane 6: phn0-5; Lane7: phn0-9; Lane 8: phn0-13; Lane 9: rhPC from pig milk (50 ng). S.C.: hPC single chain; H.C.: hPC heavy chain; L.C.: hPC light chain.

heavy chains and light chain. As shown in Fig.III.6a, although 50 ng of recombinant hPC from pig (lane 9) was detected easily, there was also a lot of cross-reaction to plant proteins (e.g. from non-transformed *Nicotiana tabacum* var. Xanthi) and molecular marker proteins.

In order to reduce the non-specific cross-reactivity, the primary polyclonal antibodies were pre-absorbed against a large amount of non-transformed *Nicotiana tabacum* var. Xanthi proteins (fixed to nitrocellulose membrane) before being used in hPC detection on western blots. As shown in Fig.III.6b, the signals from plant protein samples were eliminated by this treatment and the titer to hPC standard was reduced.

In summary, non-pre-absorbed polyclonal antibodies showed significant non-specific binding, although the patterns for transgenic plants were different from wild-type plants. No positive results were achieved after the antibody was pre-absorbed against non-transgenic plant extracts.

III.3.2.2 Monoclonal antibodies for hPC light chain and heavy chain

Two commercially-available mouse monoclonal antibodies raised against serum hPC, the anti-hPC CH-2 for heavy chain (Sigma) and anti-hPC CH-4 for light chain (Sigma, Appendix D.a & D.b), were used to test for detection of rhPC from pig. Compared to the rabbit polyclonal antibody and chicken anti-hPC serum (see below), both monoclonal antibodies showed lower sensitivity. While the rabbit polyclonal and chicken anti-hPC heavy chain could easily detect 50 ng of pig rhPC, the monoclonal antibodies barely detected 100 ng of hPC and always had background problems (data not shown). These antibodies were not used in further analyses.

III.3.2.3 Deglycosylation of hPC and antibodies

The cross-reactivity of these hPC antibodies may be highly dependent on the glycosylation form of the target protein. The commercially available hPC antibodies

were raised against the serum glycoprotein and glycan components can often be highly immunogenic (Chrispeels et al., 1996). Previous comparisons of native and deglycosylated hPC on western immunoblots using the rabbit polyclonal antisera suggested that a significant proportion of the cross-reactivity was directed against the glycan (Weissenborn, Oishi and Cramer, unpublished data).

Since the glycans produced in plant- versus mammalian-systems are likely to differ (Chrispeels et al., 1996, Figure III.5), this may limit the usefulness of these antibodies. To avoid this problem, sequences encoding the hPC light or heavy chain were expressed in *E.coli* and the resulting non-glycosylated proteins were purified and used to immunize chickens (Pelletier, Grabau and Cramer, unpublished results).

Briefly, two DNA fragments encoding partial sequences of hPC heavy chain and light chain were synthesized by PCR. The PCR products were digested with *EcoRI* and *Hin*dIII and ligated into the corresponding sites of expression vector pET32a and transferred to *E. coli* strain BL21DE3. The recombinant proteins synthesized from the bacterial system were purified as thioredoxin-His_6-hPC fusions by nickle-affinity chromatography. Purified peptides comprising by the partial heavy chain protein sequence and partial light chain sequence were injected into chickens to produce antisera specifically targeted to heavy chain and light chain of hPC. IgY was purified from the yolks of eggs taken from immunized chickens.

The detection of glycosylated rhPC and deglycosylated rhPC using the chicken anti-heavy chain IgY is shown in Fig.III.7a. The results demonstrated the anti-heavy chain antibodies derived from chicken were specific to the hPC protein sequences (single chain and heavy chain) and showed strong cross-reactivity to non-glycosylated forms. As shown in Fig.III.7b, antibodies raised against *E. coli*-synthesized hPC light chain showed cross-reactivity to the hPC light chain and single chain forms.

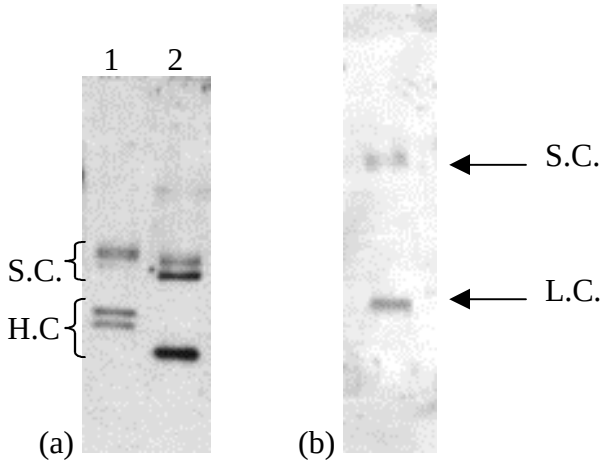

Figure III.7 Western immunoblots using chicken anti hPC antibodies to test the sensitivity of detection to rhPC. (a): Detection of glycosylated rhPC and deglycosylated rhPC using the chicken anti-heavy chain IgY. Lane 1: 50 ng of recombinant hPC from transgenic pig milk. Lane 2: 50 ng of rhPC after treatment with the endoglycosidase F, and N-Glycosidase F (Boehringer Mannheim Corporation, Indianapolis, IN). (b): Detection of glycosylated rhPC using the chicken anti-light chain bleed #2. 100ng of rhPC was loaded. S.C.: hPC single chain; H.C.: hPC heavy chain; L.C.: hPC light chain.

III.3.2.4 Western blot analysis using antibodies derived from chicken

Based on previous estimations of hPC in plant XC2-17T1 (Cramer et al., 1996), detection of hPC in crude extracts is unlikely. One would need to load at least 2.5 mg of crude leaf extract to detect 50 ng hPC (<0.002% of total soluble protein). Hence, more concentrated protein samples may be required to detect hPC from leaf tissue.

Fig.III.8 shows the western blot of partially purified plant protein samples. The results suggested that the 45% salt precipitation and Con A column purification (binds high-mannose glycoproteins) increased the amount of cross-reacting material detected in the transgenic plants compared to control plants, including proteins in the size range of hPC heavy chain. Although the amount of Con A purified proteins loaded was much less than the 45% ammonium sulfate-soluble samples, the signals were much stronger. However, non-specific cross-reactivity still remained a problem (Figure III.8).

In order to reduce non-specific cross-reactivity of the chicken IgY antibodies, additional blocking steps were tested. It was found that inclusion of 5% non-fat dry milk in all blocking and antibody incubation steps decreased background problems. Fig.III.9 shows a western blot performed using crude plant protein samples and detected by chicken anti-heavy chain IgY using these blocking conditions. The results showed the presence of novel proteins in transgenic plants in the size range of the deglycosylated form of rhPC (Figure III.9). Consistent with a putative identification of these proteins as hPC heavy chain, cross-reacting peptides of this size were not evident in preliminary experiments of plants proteins isolated under non-reducing conditions (data not shown). Native hPC is a heterodimer with the light chain and heavy chain linked by a sulfhydryl bond such that the protein migrates with hPC single chain.

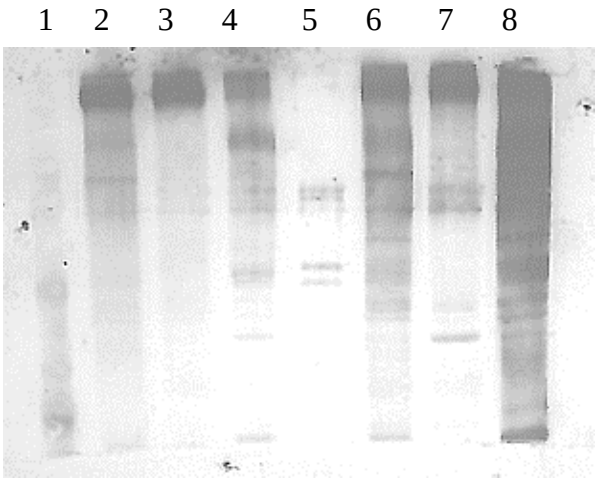

Figure III.8 Western blot of partially purified plant protein samples. Plant protein samples (45% ammonium sulfate soluble proteins) were purified by Con A column chromatography. Chicken anti-heavy chain IgY was used as primary antibody. Lane 1: Prestained SDS-PAGE standards (Bio-Rad); Lane 2: 20µg of salt-cut purified non-transformed *Nicotiana tabacum* var. Xanthi protein; Lane 3: 20µg of Con A flow-through sample of non-transformed *Nicotiana tabacum* var. Xanthi protein; Lane 4: 5µg of salt-cut and con A purified non-transformed *Nicotiana tabacum* var. Xanthi protein; Lane 5: 50 ng of rhPC from pig milk; Lane 6: 20µg of salt-cut purified phn0-13 protein; Lane 7: 20µg of Con A flow-through sample of phn0-13 protein; Lane 8: 5µg of salt-cut and con A purified phn0-13 protein.

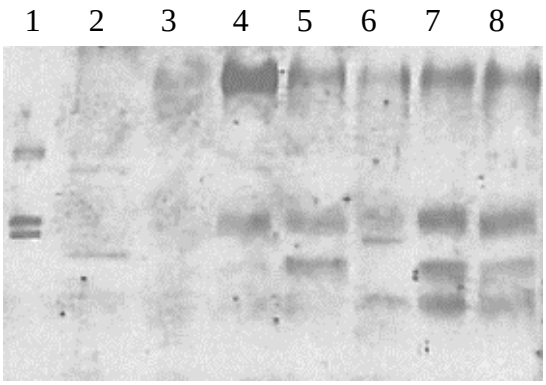

Figure III.9 Western blot of crude protein extracts from transgenic and non-transformed *Nicotiana tabacum* var. Xanthi plants. 50μg of crude plant protein samples were loaded per lane. Plant proteins were treated with β-mercaptoethanol. Chicken anti-heavy chain IgY was used as primary antibody. Lane 3-5 are the protein samples extracted one week before the proteins shown in lane 6-8. 1) 50ng of rhPC from pig milk; 2) 50ng of pig rhPC treated with endoglycosidase F and N-Glycosidase F; 3) non-transformed *Nicotiana tabacum* var. Xanthi protein; 4) XC2-17T1-005 protein; 5) phn0-13 protein; 6) non-transformed *Nicotiana tabacum* var. Xanthi protein; 7) XC2-17T1-005 protein; 8) phn0-13 protein.

III.4 CONCLUSION

The hPC expression for 11 newly generated $35S^{DE}$:hPC plants and the highests of the 35S:hPC plants (XC2-17T1-005) were examined at both RNA and protein levels. As expected, the expression levels varied significantly among the different transgenic lines, presumably due to position effects. The position of the transgene with respect to neighboring genes may affect functional transcription of transgene, in which the transgene expression may be reduced or enhanced. A stronger plant constitutive promoter CaMV $35S^{DE}$ was utilized to increase the expression of the hPC transgene. However, the hPC RNA expression levels in CaMV 35S:hPC-containing transgenic plants were higher or equal to the RNA expression levels in the CaMV $35S^{DE}$:hPC fusion transgenic plants generated in this study. These results suggest that the limitation in hPC accumulation in tobacco plants was not due to the promoter.

RNA analyses indicated that several of the transgenic lines were producing hPC transcript at significant levels. Failure to detect hPC at the protein level may therefore be due to limitations in the immunodetection system based on commercially available antibodies to hPC. Antibodies raised against the serum hPC glycoprotein appeared generally ineffective in detecting hPC synthesized in plants. In contrast, the chicken IgY raised against non-glycosylated hPC detected novel cross-reacting proteins in crude protein extracts of transgenic tobacco compared to non-transformed *Nicotiana tabacum*, suggesting that hPC peptides accumulate in transgenic leaves and undergo cleavage to yield heavy (and presumably light) chain hPC. In order to confirm the identify of this material as hPC, future analyses of the tobacco-synthesized hPC will involve cross-reaction with the light-chain specific antisera, further analysis of hPC mobility under reducing versus non-reducing conditions, and purification of the tobacco-synthesized protein for confirmation by N-terminal sequencing. Once the identification is confirmed, subsequent analyses addressing activity and processing (especially γ`-carboxylation and N-linked glycosylation) can be performed.

III.5 REFERENCES

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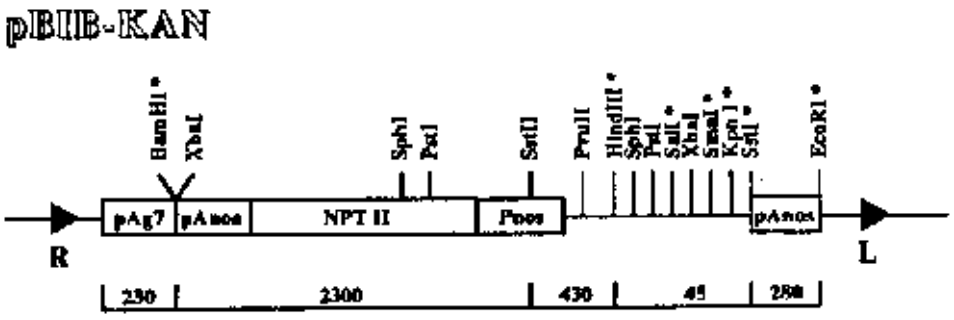
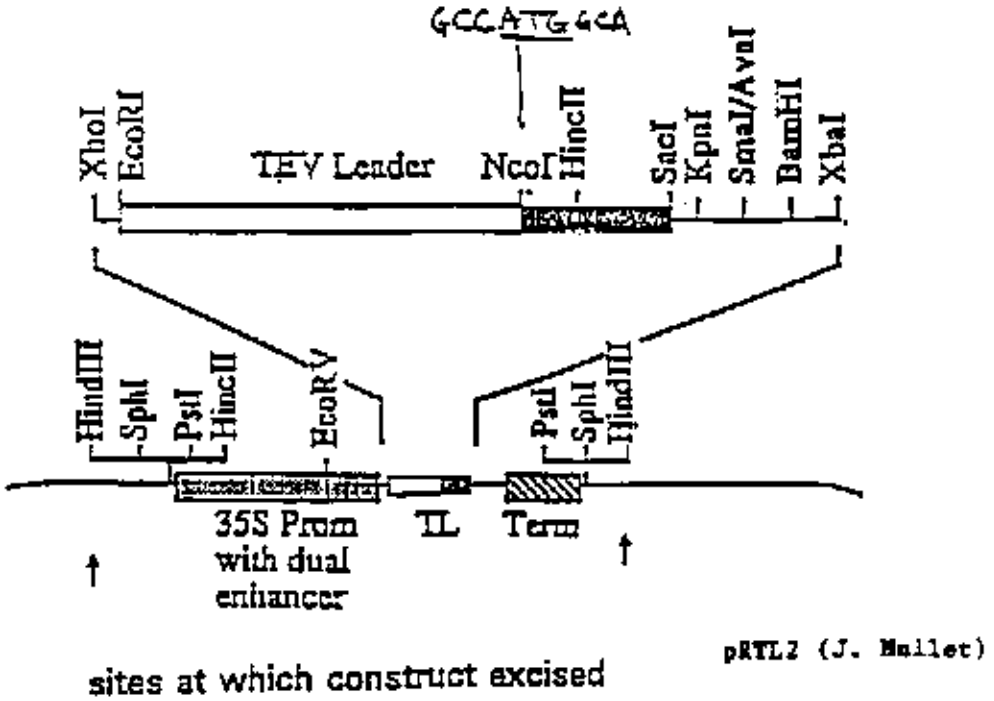
Appendix A

The binary vector pBIB-Kan (Becker, 1990)



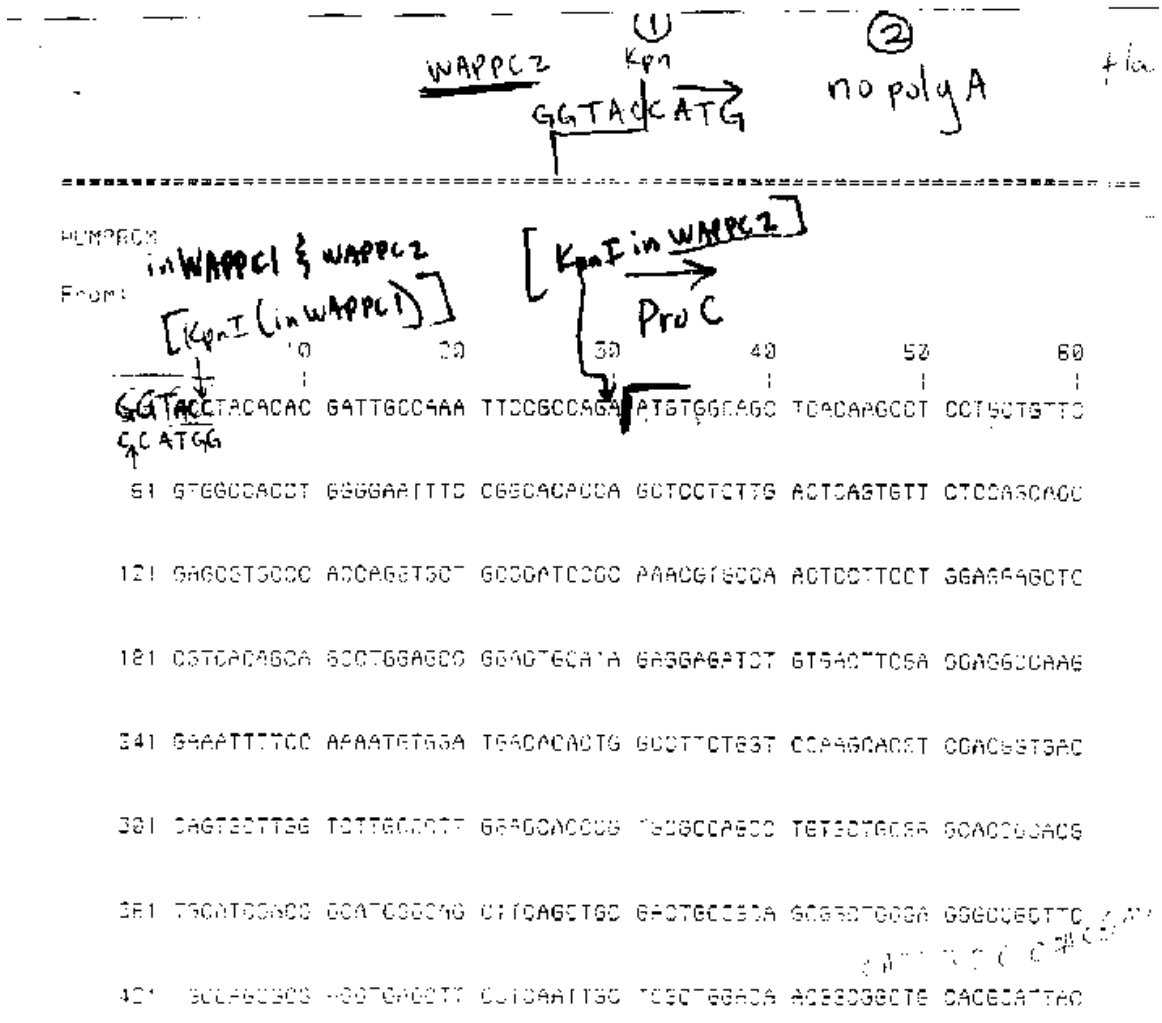
Appendix B

Vector pRTL2 containing dual-enhanced CaMV 35S promoter (provided by Dr. J. Mullet, Texas A&M).

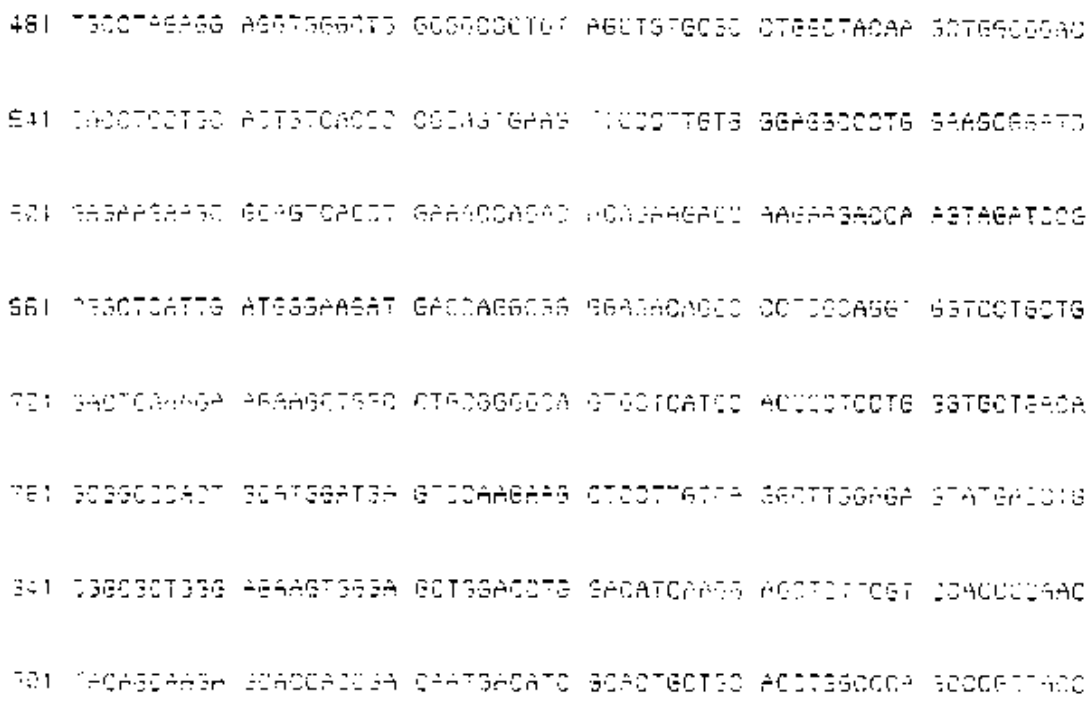


Appendix C

Vector WAPPC-1 provided by Dr. W. Velander, Virginia Tech.



Appendix C (continued)



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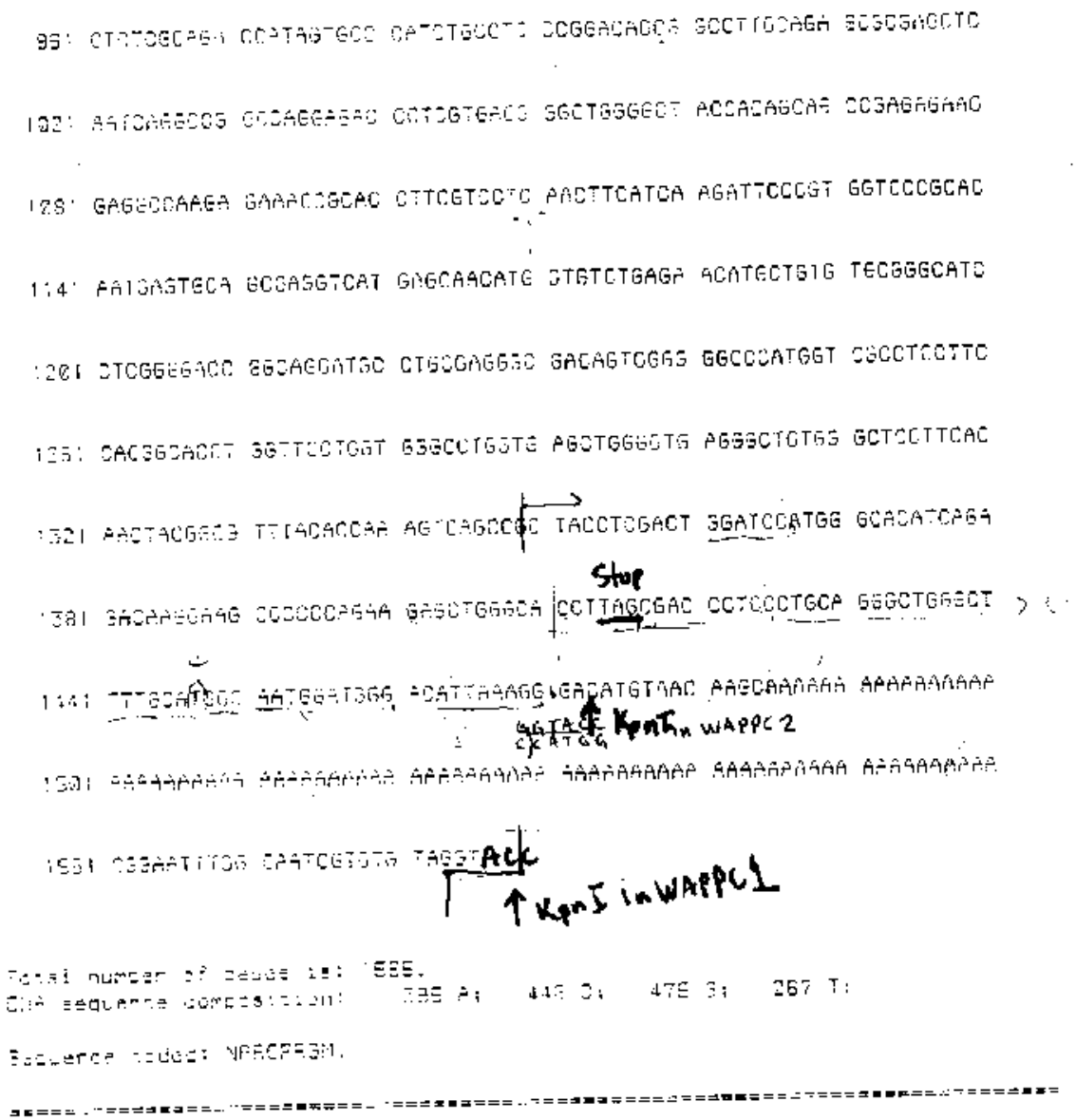

Appendix D.a

Commercially-available mouse monoclonal antibodies raised against serum hPC, the anti-hPC CH-2 for heavy chain (Sigma).

SIGMA
CHEMICAL COMPANY

Product No. P-5305
Lot 086H4820

Monoclonal Anti-Human Protein C
Purified Mouse IgG1
Clone HC-2

Monoclonal Anti-Human Protein C (mouse IgG1 isotype) is derived from the hybridoma produced by the fusion of mouse myeloma cells (cell line Sp 2/O-Ag-14) and splenocytes from an immunized mouse. Protein C purified from human plasma was used as the immunogen. The antibody is purified by HPLC using a protein A column. The isotype is determined using Sigma ImmunoType™ Kit (Sigma Stock No. ISO-1) and by a double diffusion immunoassay using Mouse Monoclonal Antibody Isotyping Reagents (Sigma Stock No. ISO-2). The product is supplied as a liquid in 10mM HEPES, 140mM NaCl, pH 7.4, containing 0.05% sodium azide (see MSDS) as a preservative.

Specificity

Monoclonal Anti-Human Protein C, a divalent cation independent antibody, recognizes an epitope on the heavy chain of protein C and binds to protein C zymogen. The antibody strongly inhibits the activation of protein C but has no effect on the function of activated protein C. No reaction is observed with the activation peptide itself or with the heavy chain after removal of the activation peptide

Description

approximately 20,000 fold[5]. Activated protein C cleaves essential peptide bonds in the heavy chains of factors Va and VIIIa which result in their in-activation and consequently in inhibition of the coagulation cascade[6,7,8]. Free plasma protein S serves as a cofactor for activated protein C's inhibitory functions probably by enabling the reactions to take place on platelet and endothelial cell membranes[9]. Activated protein C also enhances fibrinolysis by forming a complex with plasminogen activator inhibitor, thus allowing enhanced activity of plasminogen activator[9]. Inactivation of activated protein C in plasma requires at least two "serpin" inhibitors. One inhibitor's activity is enhanced by heparin[10] while the other (α-1-antitrypsin) is heparin independent[11].

Hereditary and acquired protein C deficiency states have been recognized to be associated with thrombosis. Homozygous severe protein C deficiency manifests in the newborn by massive thrombosis[12] and purpura fulminans[13]. Heterozygotes for this entity usually do not manifest thrombosis[14,15]. However, patients affected by a different heterozygous (partial) protein C deficiency frequently present a thrombotic tendency during young adulthood[16]. Acquired deficiency has been observed in patients with disseminated intravascular coagulation, liver diseases, complications following surgery and in those taking coumarin drugs[17].

Appendix D.a (continued)

Protein C is a vitamin K dependent plasma zymogen which plays an essential role in the regulation of blood coagulation. The nucleotide sequence of the gene that codes for protein C has been determined[1]. Protein C is synthesized by liver parenchymal cells as a single chain polypeptide[2], but in plasma it consists mainly of a heavy chain (41 kD) linked by a disulfide bond to a light chain (21 kD)[3]. The plasma concentration of protein C is approximately 4 µg/ml with a half-life of about 15 hours[4]. Activation of human protein C involves the release of a dodeca-peptide from the C-terminal domain of the heavy chain[1]. This is accomplished inefficiently by thrombin which cleaves an Arg-Leu bond, but when thrombin forms a 1:1 high affinity complex with the endothelial membrane protein thrombo-modulin, activation of protein C is accelerated following surgery and in those taking coumarin drugs[5].

Uses

Anti-Protein C may be used for immunochemical determination of protein C levels in normal and pathogenic human plasma. Determination of protein C levels can be used in the study of regulation of blood coagulation and fibrinolysis.

Protein Concentration: 4 mg/ml

Working Dilution

A dilution of 1:400 was determined by indirect immunoblotting using denatured and reduced pooled human plasma.

Appendix D.b

Commercially-available mouse monoclonal antibodies raised against serum hPC, and anti-hPC CH-4 for light chain.

SIGMA BIOSCIENCES™

Product No. P-7058
Lot 031H4822

Monoclonal Anti-Human Protein C
Purified Mouse Immunoglobulin
Clone HC-4

Monoclonal Anti-Human Protein C (mouse IgG2a isotype) is derived from the HC-4 hybridoma produced by the fusion of mouse Sp2/0-Ag14 myeloma cells and splenocytes from BALB/c mice immunized with protein C purified from human plasma.[1,2] The isotype is determined using Sigma ImmunoType Kit (Sigma Stock No. ISO-1) and by a double diffusion immunoassay using Mouse Monoclonal Antibody Isotyping Reagents (Sigma Stock No. ISO-2). The product is provided as purified antibody in 10 mM HEPES, 140 mM NaCl, pH 7.4, containing 0.05% sodium azide (see MSDS)* as a preservative.

Specificity

Monoclonal Anti-Protein C, a divalent cation-independent antibody, recognizes an epitope on the protein C light chain and binds with equal affinity to protein C zymogen or activated protein C. The antibody inhibits the function of activated protein C.

Description

ognized in the last decade to be associated with thrombosis.

Uses

Assays of plasma protein C levels are useful for the detection of hereditary and acquired deficiency states as well as for studies of the control mechanisms of blood coagulation and fibrinolysis. Clone HC-4 may be used in ELISA[1,2] and immunoblotting under denaturing conditions.

Protein Concentration: 4.0 mg/ml

Antibody Performance:

By immunoblotting: The antibody detects a closely spaced doublet at molecular weight of 62 kD using SDS-denatured, non-reduced, barium citrate adsorbed human plasma.

Storage

Protein C is a vitamin K-dependent plasma zymogen which plays an essential role in the regulation of blood coagulation. Human protein C was first purified by Kisiel et. al.,[3] with the nucleotide sequence of the gene having been recently determined.[4] Protein C is synthesized by liver parenchymal cells as a single chain-polypeptide, but in plasma it consists mainly of a heavy chain (41kD) linked by a disulfide bond to a light chain (21 kD).[3] The plasma concentration of protein C is about 4 μg/ml with a half life of about 15 hours. Activation of human protein C involves the release of a dodecapeptide from the N-terminal domain of the heavy chain.[3] This is accomplished inefficiently by thrombin which cleaves an Arg-Leu bond, however, when thrombin forms a 1:1 high affinity complex with the endothelial membrane protein-thrombomodulin, activation of protein C is accelerated about 20,000 fold. Activated protein C (APC) cleaves essential peptide bonds in the heavy chains of factors Va and VIIIa which result in their inactivation and consequently the inhibition of the coagulation cascade. Free plasma protein S serves as a cofactor for APC's inhibitory functions probably by enabling the reactions to take place on platelet and endothelial cell membranes. Hereditary and acquired protein C deficiency states have been rec

For continuous use store, at 0-5°C. For extended storage, freeze in working aliquots. Repeated freezing and thawing is not recommended. Storage in "frost-free" freezers is not recommended. If slight turbidity occurs upon prolonged storage, clarify the solution by centrifugation before use.

* Due to the sodium azide content a material safety data sheet (MSDS) for this product has been sent to the attention of the safety officer of your institution. Consult the MSDS for information regarding hazardous and safe handling practices.

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VITA

Hao Ni was born on June 17, 1973 in Tianjin, China. He spent six years in Kun Ming Lu elementary school, then another six years in Yao Hua high school. In summer of 1991, he began his college life in Nankai University majoring in biochemistry. In June of 1995, he graduated in honor with a B.S. degree in biochemistry. From August of the same year, he began his graduate study in the Department of Plant Pathology, Physiology and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA., to pursue a M.S. degree in plant physiology with specialty in plant biotechnology. He was under the direction of Dr. Carole Cramer, and worked in her laboratory located in Fralin biotechnology center.

Hao Ni