EXPRESSION OF RECOMBINANT PORCINE PREPRORELAXIN IN *NICOTIANA TABACUM*

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Abstract

Relaxin is a small peptide hormone that has demonstrated potential therapeutic actions for cardiovascular disease and fibrosis. Additionally, relaxin has demonstrated the ability to protect the heart from injuries caused by ischemia and reperfusion, promote the healing of ischemic ulcers, and counteract allergic responses. The objective of this research was to express fully processed porcine relaxin in transgenic tobacco plants, as an alternative to current methods of producing relaxin.

Two recombinant relaxin genes were constructed that contained the patatin signal peptide cDNA fused in frame to prorelaxin cDNA, which was codon-optimized for expression in *Nicotiana tabacum*, under the control of either the "super" promoter or the dual enhanced cauliflower mosaic virus 35S promoter. Eighteen transgenic tobacco plants were generated that were transformed with the above recombinant genes. Preprorelaxin, mRNA was detected in 12 of the transgenic plants. Fully processed relaxin protein was not found in any tobacco plants that had demonstrated gene expression by northern blot analysis. Preprorelaxin was only identified in extracts from transgenic plants that contained the insoluble protein fraction, as determined by western blot analysis. Additionally, an increased yield of preprorelaxin was identified after incubation of tobacco leaves in an ubiquitin inhibitor.

DEDICATION

This thesis is dedicated to my wife, Carol**.** This work is truly a testament of your loving and (much needed) support throughout my graduate studies. I would not have gone this far without you by my side.

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Finally, I want to thank my family and friends for all the support and encouragement you have giving me.

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CHAPTER 1

INTRODUCTION

Previous studies have established that relaxin, an endogenous mammalian hormone, could be an efficacious treatment for several chronic medical conditions such as cardiovascular disease, and fibrosis in the kidney, liver, and lung. Furthermore, relaxin may protect the heart from injuries caused by ischemia and reperfusion, promote the healing of wounds, and counteract allergic responses.

The isolation of naturally-occurring relaxin would not be sufficient to meet the potential future demand of this therapeutic protein. Attempts to efficiently produce recombinant relaxin have been severely hindered because all tested expression systems are incapable of correctly processing the protein.

Previous research has suggested that *Nicotiana tabacum* contains the essential modifying enzymes necessary for the correct processing of recombinant relaxin. Therefore, it may be advantageous to utilize *N. tabacum* as an expression system to produce recombinant relaxin.

1.1 Processing of relaxin

Preprorelaxin is the primary translational product of the relaxin gene and is a single-chain protein consisting of four regions: the signal peptide, B chain, C chain, and A chain. There are two disulfide bonds connecting the B and A chains as well as a single disulfide bond within the A chain. Preprorelaxin is converted to prorelaxin by the cleavage of the signal peptide. Finally, the C chain is cleaved yielding relaxin, a 6-kDa, two-chain, peptide hormone. This process is schematically represented in Figure 3.1.

1.2 Therapeutic applications of relaxin

The protective effects of relaxin on the cardiovascular system have been corroborated by multiple studies. Relaxin induces a prompt and concentration-dependant dilation of arterioles, capillaries, and venules in the heart (Bani, 1997), liver (Bigazzi, Bani, and Sacchi, 2001), and kidney (Danielson, Sherwood, and Conrad, 1999), with more potency than two well known vasodilatory agents, acetylcholine and sodium nitroprusside (Bani, 1997). Relaxin also induces a concentration-dependant inhibition of platelet aggregation (Bani et al., 1995). These studies illustrate the therapeutic actions of relaxin to counteract vasoconstriction and thrombosis, the main pathogenic steps of cardiovascular disease. In addition, porcine 1 (P1) relaxin has protective effects against myocardial injury during ischemia and reperfusion in the hearts of rats (Bani et al., 1998) and guinea pigs (Masini et al., 1997). Relaxin's cardiovascular protective effects are further supported by the significantly increased left ventricular (LV) end diastolic pressures, chamber stiffness, and LV collagen levels observed in the hearts of the relaxin 1 knockout (M1RKO) mice (Du et al., 2003).

Multiple studies have established the antifibrotic effects of relaxin in various organs. Infusion of recombinant human 2 (rH2) relaxin demonstrated efficacy in treatment of lung fibrosis through studies utilizing human lung fibroblasts *in vitro* and bleomycin-induced fibrosis in mice *in vivo* models (Unemori et al., 1996). Similarly, rH2 relaxin infusions significantly inhibited the accumulation of collagen in the airways of ovalbumin-sensitized mice exposed to ovalbumin aerosols (Kenyon, Ward, and Last, 2003). Relaxin treatments had therapeutic effects in studies utilizing three different *in vitro* models of renal fibrosis and an *in vivo* mouse model of

a rapidly progressing renal fibrosis (McDonald et al., 2003). Additional, infusions with rH2 relaxin decreased interstitial fibrosis and inhibited the progression of renal disease in separate studies utilizing rats, which were chemically-induced with severe renal interstitial fibrosis (Garber et al., 2001). Furthermore, relaxin treatments effectively modulated collagen deposition in an *in vivo* rat model of chemically-induced liver fibrosis (Williams et al., 2001). These results have been corroborated by the observation of progressive lung fibrosis in M1RKO mice and that subcutaneous infusion of rH2 relaxin significantly decreased lung wet weight, collagen content and concentration during the early and developed stages of lung fibrosis (Samuel et al., 2003).

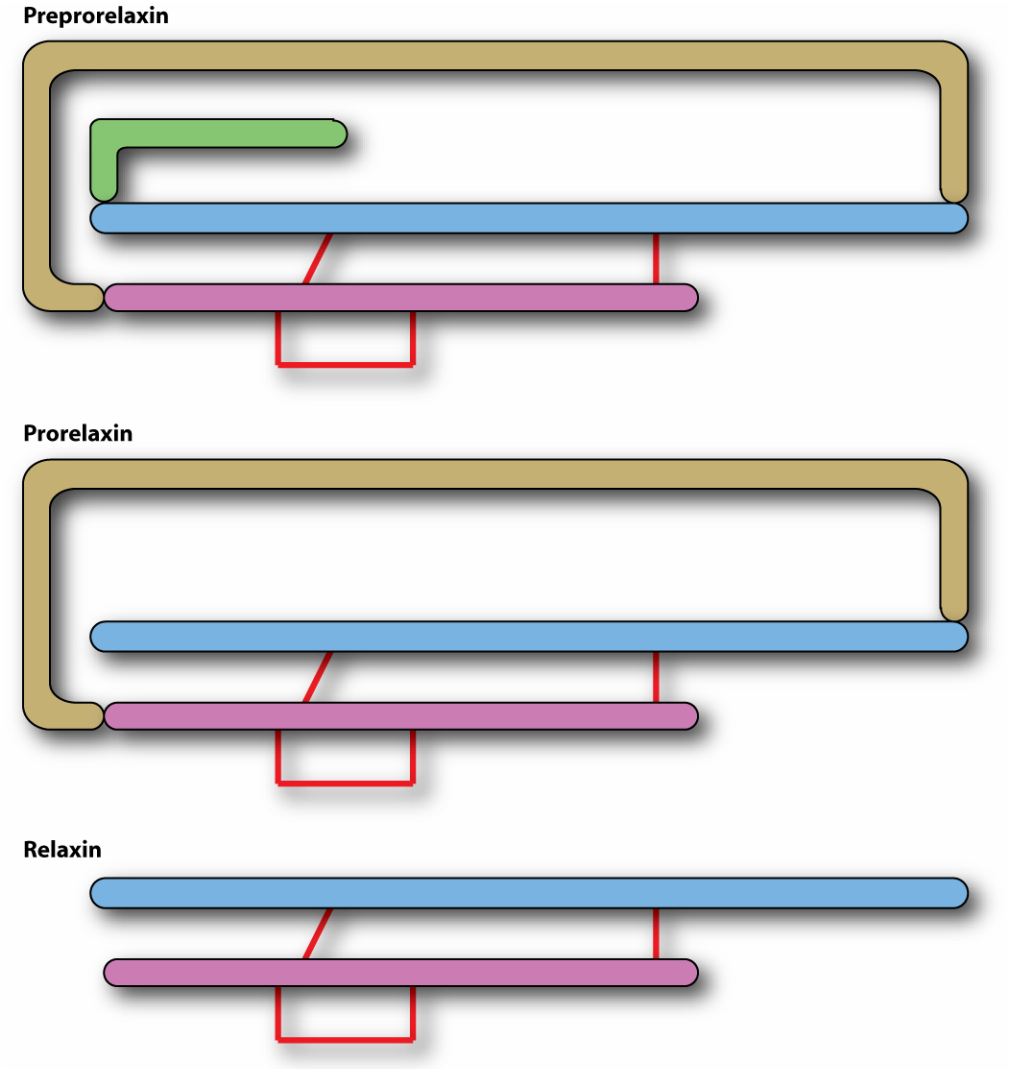

Figure 1.1: Schematic representation of the processing of preprorelaxin to relaxin by consecutive removal of signal peptide (green region) and the C chain (tan region). Light blue and lavender regions represent the B and A chains, respectively. The red lines indicate disulfide bond formation.

Infusions of rH2 relaxin selectively induced angiogenesis at wound sites in rodent models (Unemori et al., 2000). These results indicate that relaxin may be an effective treatment to promote the healing of wounds, since the mechanisms of wound healing are highly dependant upon the availability of ample local blood vessels (Yamaguchi and Yoshikawa, 2001). This

conclusion was further supported by the observation that wound sizes were smaller in diabetic mice that received relaxin treatments versus non-treated mice (Huang et al., 2001).

Additional studies highlight the potential therapeutic value of relaxin in treating allergic reactions and inflammations. Porcine 1 relaxin significantly inhibited the histamine release in isolated mast cells from rats and guinea pigs (Masini et al., 1994), a process that initiates an allergic response. Furthermore, P1 relaxin reduced the severity of the pathologies of allergic asthma through an *in vivo* guinea pig model (Bani et al., 1997).

1.3 Endogenous and recombinant relaxin production/purification

Porcine relaxin (P1) can be readily purified from sow ovaries, the only abundant source for endogenous relaxin purification, with yields of approximately 200 mg of relaxin per kg of ovaries (Sherwood and O'Byrne, 1974). However, sow ovaries may not be a reliable feed stock for industrial scale purifications since the relaxin content of ovaries varies greatly depending upon the stage of pregnancy (Doczi, 1960). Endogenous human relaxin 2 (H2) has been purified from human fetal membranes (Bigazzi, 1981). However, this procedure was designed to purify sufficient quantities of H2 relaxin to develop a homologous radioimmunoassay for quantitative measurement of relaxin in human tissues and body fluids and not for industrial scale purifications of H2 relaxin.

Recombinant relaxin genes have been expressed in *Escherichia coli* (Reddy et al., 1992; Soloff et al., 1992), Chinese hamster ovary (CHO) cells (Vu et al., 1993), African green monkey kidney COS-1 cells (Soloff et al., 1992), and human kidney 293 cells (Marriott, Gillece-Castro, and Gorman, 1992). These expression systems were capable of producing prorelaxin but did not have the necessary endogenous metabolic pathways to process prorelaxin into relaxin. Further investigation of the recombinant relaxin expression in human kidney 293 cells demonstrated that the conversion of prorelaxin into relaxin appears to be limited to cells that express certain converting enzymes. Two enzymes were identified that can process prorelaxin into relaxin: Kex2, a prohormone processing enzyme from *Saccharomyces cerevisiae* and mPC1, prohormone convertase 1 from *Mus musculus*. These researchers concluded that the expression of a recombinant relaxin gene will yield fully processed relaxin only in expression systems that contain a regulated secretory pathway containing the appropriate prohormone-processing enzymes.

A slightly modified approach of recombinant relaxin expression was attempted in *S. cerevisiae* (Yang et al., 1993) attempting to circumvent the relaxin processing problem. A relaxin transgene was constructed that utilized a hexapeptide in lieu of the C chain of prorelaxin. This approach yielded a recombinant protein very similar to relaxin. However, 20-fold more of the recombinant relaxin was needed to elicit a similar bioassay response as produced from P1 or H2 relaxin. The decreased bioactivity may be caused by incorrect formation of the disulfide bonds connecting the B and A chains of the recombinant relaxin.

The only successful and reproducible method to produce relaxin has been through the chemical combination of the B and A chains of relaxin (Burnier and Johnston, 1998). This method yielded properly processed relaxin using chemically synthesized B and A chains (Canova-Davis, Baldonado, and Teshima, 1990) and with recombinant B and A chains of relaxin produced by transgenic *E. coli* strains (Canova-Davis et al., 1991). However, there are two inherent inefficiencies associated with this process. First, this approach requires the utilization of two separate processes to produce and purify the B and A chains. Second, yields of less than

20% have been reported (Burnier and Johnston, 1998) for the additional processing steps necessary to correctly combine the B and A chains.

1.4 Kex2 orthologue in *Nicotiana tabacum*

Nicotiana tabacum has the metabolic pathways necessary to produce many recombinant mammalian proteins that require complex post-translational modifications, such as human protein C (Cramer et al., 1996), human homotrimeric collagen I (Ruggiero et al., 2000), human hemoglobin (Dieryck et al., 1997), and various antibodies (Ma et al., 1995). In addition, an orthologue to the *S. cerevisiae* Kex2 enzyme, which can process prorelaxin into relaxin (Marriott, Gillece-Castro, and Gorman, 1992), has been identified in *N. tabacum*. The existence of the Kex2 orthologue has been elucidated because recombinant KP6 was properly processed into the α and β polypeptide components in transgenic tobacco (Kinal et al., 1995), which requires a proteolytic enzyme with the same substrate specificity as Kex2 (Tao et al., 1990). Additionally, the Kex2 orthologue was further substantiated and identified as a Golgi-resident enzyme (Jiang and Rogers, 1999). These results implicate that recombinant preprorelaxin targeted to the default secretory pathway in tobacco plants may be properly processed into relaxin.

1.5 Objectives

The overall goal of this research is to verify the expression of recombinant porcine relaxin in transgenic tobacco plants. The following objectives will be pursued to achieve the aforementioned goal: (1) construct a vector that drives the expression of relaxin in tobacco plants, (2) generate transgenic tobacco plants containing recombinant relaxin gene, (3) verify recombinant relaxin gene expression at the transcriptional level, and (4) verify recombinant relaxin gene expression at the translational level.

CHAPTER 2

LITERATURE REVIEW

2.1 Biochemical properties of relaxin

Relaxin is a member of the insulin/relaxin superfamily of peptide hormones that also includes insulin, insulin-like growth factors (IGF-I and IGF-II), and the various designated insulin-like peptides. These peptide hormones are initially synthesized as a prohormone comprising of a signal peptide, followed by a B-C-A chain domain. Additionally, each of these polypeptides have two disulfide bonds between the B and A chains and a disulfide bond within the A chain. The C chain is cleaved during post-translational processing for the following member of the protein superfamily: insulin, insulin-like factor 3, and relaxin. A schematic of the covalent structure of fully processed relaxin is presented in Figure 2.1 (Sherwood, 2004).

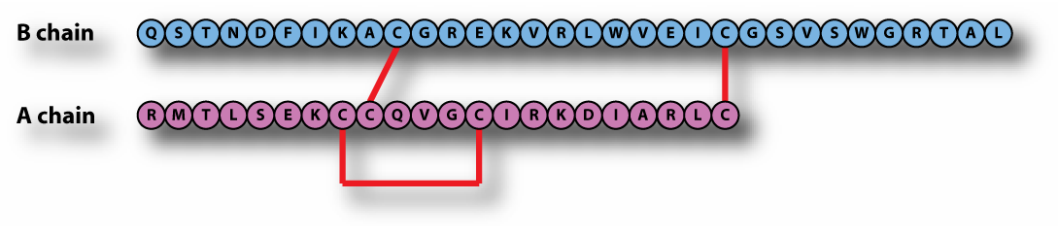

Figure 2.1: Schematic representation of the covalent structure of pig 1 relaxin (Haley et al., 1987). Red lines represent disulfide bonds between cysteine residues. Light blue and lavender circles represent amino acids in the B and A chains, respectively.

Evidence of relaxin has been established in a multitude of species, including pig, rat, shark, skate, whale, porpoise, rabbit, horse, dog, cow, guinea pig, human, and marsupials (Sherwood, 1994). Three variants of relaxin have been identified in humans and two variants of relaxin have been identified in mouse, pig, and rat. The first variant, human 1 (H1) relaxin is not known to be secreted in the blood stream and the expression of H1 relaxin gene is limited to only the decidua and placental trophoblast. Additionally, there are no homologous relaxin variants in the aforementioned species. Human 2 (H2) relaxin is homologous to the pig 1 (P1), mouse 1 (M1), and rat 1 (R1) relaxin and all the additional forms of relaxin identified in the aforementioned species. Human 3 (H3) relaxin, and its recently identified orthologues, mouse 3 (M3) and pig 3 (P3), are predicted to be neuropeptides (Sherwood, 2004).

However, nearly all of the potential therapeutic actions of relaxin have been elucidated from M1, P1, and H2 relaxin, which are three of the four only known forms secreted into the blood stream. Studies investigating the therapeutic actions of relaxin were conducted by the administration of pure P1 or recombinant H2 (rH2) relaxin for *in vivo* or *in vitro* studies or deduced by the analysis of phenotypes of M1 relaxin knockout mice (Sherwood, 2004).

An alignment of the amino acid sequences of the B and A chains of M1, P1, and H2 relaxin is presented in Figure 2.2. All the cysteine residues and the three adjacent glycine residues, which provide flexibility for disulfide bonds, are invariant in all known relaxin species. The Arg-X-X-X-Arg-X-X-Ile motif in the B chain, which is required for bioactivity (Bullesbach and Schwabe, 2000), is highly conserved in all relaxin species. Despite these highly conserved regions, only 30 to 60% amino acid sequence identity of relaxin exists between species.

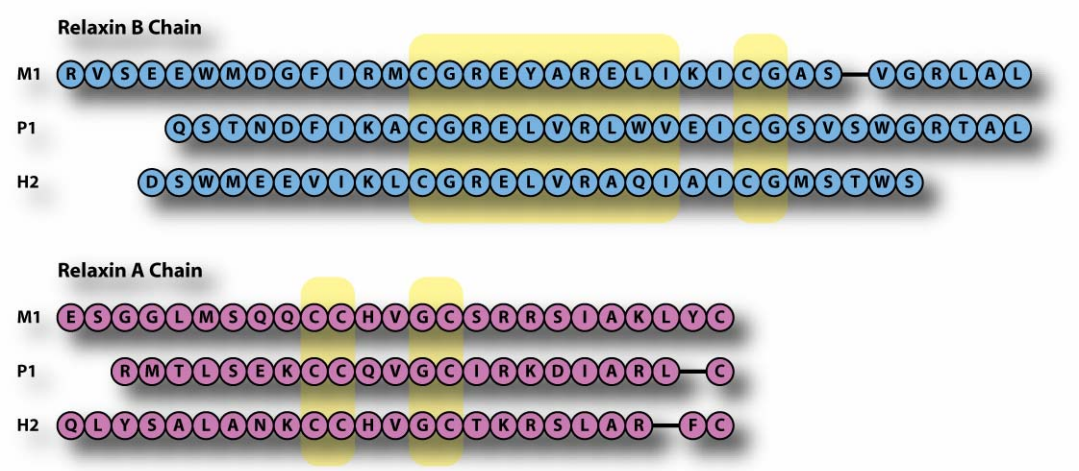

Figure 2.2: Amino acid sequence alignment of M1 (Evans et al., 1993), P1 (Haley et al., 1987), and H2 (Hudson et al., 1984) relaxin. The residues of the B and A chains are represented by light blue and lavender circles, respectively. Regions highlighted in pale yellow indicate highly conserved regions that contribute to the bioactivity of relaxin.

It was discovered that leucine-rich G protein coupled receptor 7 (LGR7) is a receptor for relaxin and that leucine-rich G protein coupled receptor 8 (LGR8) may be a receptor for relaxin. This study demonstrated that relaxin elicits bioactivity through a signaling pathway initiated by the binding and activation of LGR7. Consistent with these findings, transcripts of LGR7 have been found in all tissues where activity of relaxin of has been reported. Relaxin also binds, albeit with low affinity, to cells that contain only LGR8 receptors. However, it has yet to be determined if relaxin elicits any physiological action *in vivo* through LGR8 (Hsu et al., 2002).

There is significant evidence that supports the theory that relaxin can elicit physiological and other therapeutic actions for different cells by initiating multiple different pathways, even though the intracellular signal pathways are not completely understood for any specific cell. For example, studies have suggested that relaxin uses the protein kinase A, receptor tyrosine kinase, mitogen-activated protein kinase, and extracellular signal regulated kinase 1 and 2 signaling pathways in different cells. Relaxin has also been demonstrated to increase nitric oxide generation and subsequent rise of intracellular cGMP levels in various tissues. It has been suggested that various pathways for different target cells enables relaxin to have a broad range of therapeutic and physiological effects on numerous tissues and organs (Sherwood, 2004).

2.2 Stability and degradation analysis of relaxin

The stability of rH2 relaxin is maximized at pH 5.0, as determined through degradation analysis of rH2 relaxin in aqueous solutions ranging from pH 3.0 to pH 9.0 at 35°C and constant ionic strength. The breaking and interchanging of disulfide bonds were the cause of rH2 relaxin degradation at a pH value greater than 7.5. In comparison, the oxidation of Met (B4, fourth residue from the N-terminal of the B chain), and Met (B25) and the cleavage of Asp (B1) were the dominant degradation pathways at a pH value less than 4.0 (Nguyen and Shire, 1996). The stability of P1 relaxin may be greater than rH2 relaxin in acid solutions since P1 relaxin lacks an acidic residue at the N-terminus of the B chain and lacks methionine residues on the B chain. This assumption is supported by previous research describing the extraction of P1 relaxin from porcine ovaries in 1.6 N HCl (Bullesbach and Schwabe, 1985).

The degradation rate of rH2 relaxin did not correspond to Arrhenius kinetics throughout the temperature range of 5 to 35°C. Instead, two sections of linearity were represented in the Arrhenius plot of rH2 relaxin degradation rates. It was proposed that two degradation pathways of different activation energies exist, to explain these results. This hypothesis was corroborated with chromatograms of samples that exhibited degradation profiles shifting from the oxidation of Met (B4) and Met (B25) residues to the cleavage of Asp (B1) residue as the storage temperature was increased from 5 to 35°C (Nguyen and Shire, 1996).

The Met (B4) and Met (B25) residues of rH2 were demonstrated to readily oxidize in the presence of trace amounts of hydrogen-peroxide. This reaction followed first-order kinetics and yielded up to three different relaxin variants with either one or both methionine residues being oxidized to methionine sulfoxide with Met (B25) oxidizing at a rate 2.5 times faster than Met (B4). Additionally, it was demonstrated that the oxidation reaction was independent of $[H^+]$, ionic strength, and buffer concentration. However, the oxidation of the relaxin by peroxide did not significantly affect bioactivity, as measured by the upregulation of cAMP by human endometrial cells *in vitro* (Nguyen, Burnier, and Meng, 1993).

The Met (B4) and Met (B25) residues of rH2 were also demonstrated to readily oxidize in the presence of intense light. Greater than 91% of rH2 relaxin was altered by oxidation of one or both methionine residues after 17 days of exposure to approximately 3600 foot-candles of light, as determined by reverse phase HPLC. However, the oxidation of rH2 by intense light did not significantly affect bioactivity, as measured by the upregulation of cAMP by human endometrial cell *in vitro* (Cipolla and Shire, 1991).

It was demonstrated that rH2 relaxin can be rapidly oxidized by trace amounts of transitional metal ions, such as Cu^{2+} and Fe^{3+}, in the presence of a pro-oxidant, such as ascorbic acid. It was demonstrated that His (A12), Met (B4), and Met (B25) were readily oxidized, with His (A12) being the most susceptible. The oxidation of these residues caused an irreversible aggregation and precipitation of rH2 relaxin at neutral and basic pH ranges. Over 75% of a 0.2 mg/mL solution of rH2 relaxin precipitated within 25 minutes, at pH 7 and 8. In comparison, there was minimal precipitation after 150 minutes, at pH 5. The metal-catalyzed oxidation was inhibited by the addition of EDTA, a chelating agent. In addition, it was proposed that the oxidation of His (A12) was critical for causing the changes in the secondary and tertiary structure leading the aggregation of rH2 relaxin, since oxidation of Met (B4) and Met (B25) by light or hydrogen peroxide does not cause a change in bioactivity (Li et al., 1995).

2.3 Proteolytic post-translational modifications in plants

Protein modifications, such as proteolytic cleavage, typically occur along the secretory pathway in plants. Initially, an amino terminal signal peptide directs proteins requiring post-translational modifications to the endoplasmic reticulum (ER). The signal peptide is cleaved and the remainder of the protein is released in the ER lumen where protein assembly and folding can occur and then transferred to the Golgi apparatus where most protein modifications occur. The processed protein is transported to the extracellular space (Faye et al., 2005) or is directed to the vacuolar system by the presence of a sorting signal (Neumann, Brandizzi, and Hawes, 2003). For example, 12 amino acids on the amino-terminus of proaleurain have been demonstrated to target the protein to the lytic vacuole (Holwerda, Padgett, and Rogers, 1992), and in contrast the carboxyl terminal region of pro2S albumin has been demonstrated to target a protein to the protein storage vacuole (Shimada et al., 2002).

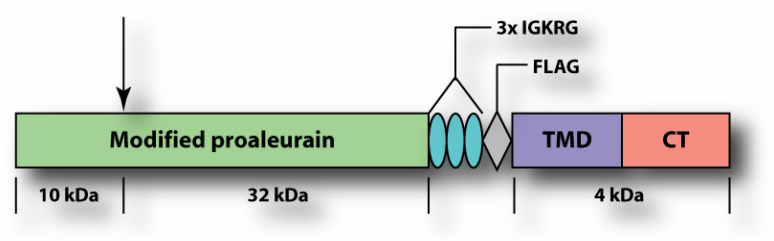

Figure 2.3: Schematic of chimeric proaleurain reporter protein. Pale green rectangle represents proaleurain modified such that it does not contain the lytic vacuole sorting signal, with the arrow indicating proteolytic processing site. Cyan ovals represent the triplet of the Kex2 cleavage site (IGKRG). Gray diamond represents FLAG peptide. Lavender and mauve rectangles represent the transmembrane domain and cytoplasmic tail from BP-80, respectively (Jiang and Rogers, 1999).

The existence of a proteolytic processing pathway in tobacco cells was established that exhibited similar substrate specificity to the yeast converting enzyme, Kex2 (Jiang and Rogers, 1999). This was demonstrated by utilizing a chimeric reporter protein, represented in Figure 2.3, consisting of the following regions: modified proaleurain, triplet of the Kex2 substrate site, FLAG peptide, single transmembrane domain (TMD) of BP-80, followed by the cytoplasmic tail (CT) of BP-80. The proaleurain was mutated such that the sorting signal, which is the four residue motif Asn-Pro-Ile-Arg, was deleted thereby enabling secretion and simultaneously preventing targeting to the lytic vacuole. The TMD and CT regions served two distinct roles. First, these regions targeted the reporter protein to the lytic prevacuole, which is where the proteolytic processing of proaleurain to aleurain occurs. The second role was to maintain the full length reporter protein as an integral membrane protein (Jiang and Rogers, 1998). Therefore, the modified proaleurain would be released and secreted into the medium, if the reporter protein was proteolytically digested at the Kex2 substrate sites. Endogenous Kex2-like protease could be localized to the Golgi if the released proaleurain was not processed to aleurain, because the proaleurain was mutated by deleting the sorting signal so the mutated proaleurain could not target itself to the lytic prevacuole. In contrast, the presence of aleurain would suggest that the Kex2-like protease would be localized within the lytic prevacuole. Cleavage of the Kex2 substrate site could also be verified because the reporter protein would divide in a fashion that would lead to the separation of epitopes recognized by antibodies raised against aleurain and FLAG. Expression of this chimeric reporter gene in tobacco protoplast cultures led to the cleavage of the protein at the Kex2 substrate site and the modified proaleurain was secreted. These results demonstrated that a Kex2-like protease existed and is localized within the Golgi. In addition, it was demonstrated that the tobacco Kex2-like protease has the same requirements for substrate specificity as the yeast Kex2, through amino acid substitutions of the Kex2 substrate site. More specifically, a hydrophobic-X-basic-basic four residue motif (where X was any residue) was required for cleavage by the Kex2-like protease.

However, the existence of a Kex2-like protein has not been identified in plants, even though it was demonstrated that tobacco cells contain an enzyme with comparable catalytic activity. In fact, no protein with overwhelming sequence identity to Kex2 is present in the *Arabidopsis thaliana* proteome. The most similar protein was subtilase, which only had 38% sequence similarity along the peptidase S8 region of Kex2, spanning from residues 149 through 445 with a score of 43.9, as determined by the BLOSUM62 matrix. Nonetheless, this analysis does not refute the aforementioned research that established endogenous Kex2-like proteolytic activity of tobacco cells.

2.4 Expression of recombinant proteins in plants

Several proteins requiring specific proteolytic processing and/or specific disulfide bond formation have been successfully produced by recombinant DNA technologies utilizing tobacco or other plants as an expression system. In addition, a couple of peptide hormones in the same protein family as relaxin have been successfully produced in tobacco.

Transgenic tobacco plants have demonstrated the capability to properly process the KP6 toxin from *Ustilago maydis*. This toxin is produced from proteolytic processing of the KP6 preprotoxin. The signal peptide of KP6 preprotoxin must be removed and then the polypeptide must be endoproteolytically digested at three different locations by an enzyme with the same substrate specificity as the Kex2 enzyme. These processes yield mature KP6 toxin, which consists of two distinct peptide chains, designated α and β polypeptides. A diagram of the KP6 preprotoxin and its components are presented in Figure 2.4. Killer activity of the toxin requires the presence of both the α and β polypeptides as separate subunits since the precursor protein is inactive (Peery et al., 1987).

The recombinant KP6 toxin was produced from tobacco plants that contained KP6 preprotoxin cDNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter. These plants correctly processed the recombinant KP6 preprotoxin demonstrated by the KP6 killer activity with the same activity and specificity as endogenous KP6 toxin in concentrated extracellular extracts of the transgenic plants. The correct processing of the KP6 preprotoxin was additionally verified by electrospray mass spectroscopy, amino-terminal sequencing of the β polypeptide and by the size exclusion chromatography elution profile of the α polypeptide (Kinal et al., 1995).

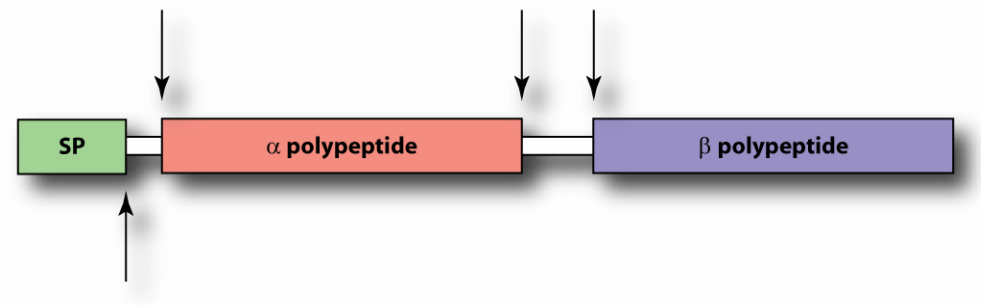

Figure 2.4: Schematic of the KP6 preprotoxin and its processing. Pale green, mauve, and lavender rectangles represent the signal peptide, the α polypeptide, and β polypeptide of the precursor protein, respectively. The upward and downward facing arrows indicate cleavage sites recognized by a signal peptidase and the tobacco homolog of Kex2 protein, respectively (Kinal et al., 1995).

Transgenic tobacco plants have demonstrated the capability to properly process a homotrimeric variant of human collagen I. This protein is initially synthesized from three individual proα1(I) chains, which consists of an N-propeptide, a helix forming domain, and a C-propeptide, and is represented in Figure 2.5. Each proα1(I) chain undergoes significant post-translational modification prior to the assembly of procollagen, which includes the formation of disulfide bonds within the C- and N-propeptides, glycosylation of residues in the C-propeptide, and hydroxylation of proline residues within the helix forming domain. Three proα1(I) chains are recruited by noncovalent interactions between the C-propeptides and stabilized by interchain disulfide bonds between all three C-propeptides. Procollagen assembly is complete after the

triple helix is formed in a zipper-like fashion initiating from the C-terminus. Then, collagen is yielded after the removal of all propeptide regions of procollagen (Lamande and Bateman, 1999).

Recombinant homotrimeric human collagen I was produced from tobacco plants that contained human proα1(I) chain cDNA under the control of the dual enhanced CaMV 35S promoter. The recombinant collagen I had an identical structure as the endogenous collagen I except the proline residues in the helix forming domain were not hydroxylated. This caused the recombinant protein to have lower thermal stability in comparison to endogenous bovine collagen, since the hydroxylation of proline residues allow for hydrogen bonding within the triple helix thereby increasing the stability of this region. It was proposed that co-expression of prolyl 4-hydroxylase, the enzyme responsible of proline hydroxylation, may enable tobacco plants to produce bona-fide homotrimeric collagen I (Ruggiero et al., 2000).

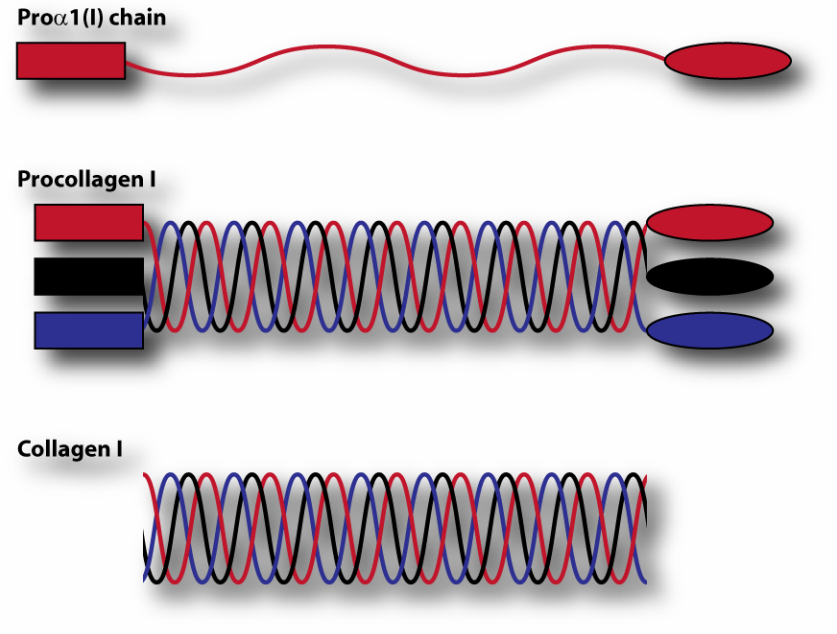

Figure 2.5: Schematic illustration of the assembly and processing of three Proα1(I) chains to yield homotrimeric human collagen I. Individual Proα1(I) chains are presented in different colors. Rectangles, curved lines, and ovals represent the amino propeptide, the triple helix forming domain, and the carboxyl propeptide, respectively. Three carboxyl propeptides from individual Proα1(I) chains associate and the triple helix forms from the carboxyl to amino terminal, yielding procollagen I. Then all propeptide regions are removed yielding collagen I (Lamande and Bateman, 1999).

Tobacco plants have demonstrated the capacity to perform several post-translational modifications necessary to produce functional human protein C (hPC), a complex serine protease. This protein undergoes extensive modifications including proteolytic digestion, glycosylation, disulfide bond formation, β-hydroxylation, γ-carboxylation. The proteolytic cleavage removes the pre- and pro-signal sequences, removal of an internal dipeptide to yield a light chain (21 kDa) and a heavy chain (42 kDa), and the removal of a dodecapeptide from the larger chain to activate the protein (Cramer et al., 1996).

Recombinant hPC was produced from tobacco plants that contained the cDNA of hPC under the control of the dual enhanced CaMV 35S promoter. Proteolytic digestion yielding the heavy and light chains was confirmed by western blot immunodetection with antibodies raised

against the heavy chain of hPC. Further experiments also confirmed the glycosylation and disulfide bond formation of recombinant hPC. However, the extremely low expression levels of recombinant hPC prevented further analysis confirming the complete processing and activity of the protein (Ni, 1997).

Plants have demonstrated the capability to properly process two peptide hormones in the same family as relaxin, insulin-like growth factor-1 (IGF-1) and insulin. Both of these proteins are comprised of a signal sequence and B-C-A chain domain configuration. Furthermore, there are two disulfide bonds that link the B and A chains and there is a single disulfide bond within in the A chain. Insulin, just like relaxin, is further processed by the removal of the C chain (Sherwood, 2004).

Recombinant human IGF-1 was produced from tobacco plants that contained a chimeric IGF-1 transgene which consisted of either the *E. coli* Lam B or rice prolamin signal sequence fused to the coding sequence of the B-C-A domain of human IGF-1 under the control of the maize ubiquitin promoter. The recombinant IGF-1 was detected in unpurified protein extracts obtained from transgenic tobacco leaves in ELISA analysis. Additionally, western blot analysis confirmed the ELISA results and established that the protein was of the expected molecule weight. Finally, the recombinant IGF-1 showed indistinguishable bioactivity from IGF-1 standards as determined by SH-SY5Y cell proliferation assay. These results establish that tobacco plants can produce and accumulate functional and biologically active recombinant human IGF-1 (Panahi et al., 2004).

Recombinant human insulin was produced from *Arabidopsis thaliana* plants that contained a chimeric insulin transgene. The transgene consisted of the tobacco pathogenesis related protein signal sequence (PR-S) fused to the coding sequence of human insulin under the control of the phaseolin promoter, a seed-preferred promoter. The fidelity of the recombinant insulin was verified by SDS-PAGE analysis, elution profiles from a C18 column, and mass spectral analysis in comparison to human insulin standards (Moloney et al., 2005).

2.5 Proteasomal degradation pathway of proteins

Two major systems of protein degradation exist in eukaryotic cells, which are lyosomal and proteasomal pathways. Degradation by the lyosomal apparatus is primary associated with membrane-associated proteins or extracellular proteins that have entered the cell by endocytosis. However, the majority protein degradation occurs by the proteasome pathway. In this pathway, proteins are covalently linked to multiple molecules of ubiquitin. The tagged protein is rapidly hydrolyzed by the 26S proteasome into small peptides typically 3 to 20 residues in length which are further hydrolyzed to individual amino acids by other peptidases. The 26S proteasome consists of a 20S subunit and two 19S subunits. The 19S complexes recognize ubiquitinated proteins and denature the proteins in order to facilitate the entry of the proteins to the 20S subunit, where the hydrolysis of the protein into small peptides occurs.

Several low molecular weight compounds have been identified that can readily enter cells and selectively inhibit the proteasomal degradation pathway. If these inhibitors cause an increase in the level of a protein, it is a strong indication that the identified protein is targeted to the proteasomal degradation pathway. It has been demonstrated that one such inhibitor, Z-Leu-Leu-Leu-al (MG132), has the ability to effectively inhibit the proteasomal pathway in plant cell cultures at a level of 100 μM. This conclusion was established through the observation that treatment of *Nicotiana tabacum* cv Bright Yellow 2 (BY2) cells with MG132 caused a reversible

inhibition of the degradation of a cyclin-chloramphenicol acetyltransferase fusion protein which hydrolyzed by the proteasomal pathway (Genschik et al., 1998).

2.6 Conclusions

Relaxin has promising potential to become a pharmaceutical to treat several medical conditions. To meet this future need, a new method of producing relaxin that is economically viable should be developed. Cell culture recombinant expression systems have been incapable of converting prorelaxin into relaxin. Tobacco, on the other hand, has been shown to possess an endogenous proteolytic pathway that is capable to convert prorelaxin into relaxin. Therefore, tobacco may be an ideal expression system for the production of recombinant relaxin.

CHAPTER 3

EXPERIMENTAL

3.1 Materials

Agarose, 6-benzylaminopurine (BA), calcium chloride ($CaCl_2$), chloroform, diethyl pyrocarbonate (DEPC), ethidium bromide (ETBR), formaldehyde, Guanidine HCl, lithium chloride (LiCl), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 3-[N-morpholino] propanesulfonic acid (MOPS), 1-naphthaleneacetic acid (NAA), Phytagel, potassium hydroxide (KOH), RNA loading buffer, sodium acetate, sodium dodecyl sulfate (SDS), streptomycin, Tris(hydroxymethyl)aminomethane (Tris), urea, and Z-Leu-Leu-Leu-al (MG132) were obtained from Sigma-Aldrich Company (St. Louis, MO). Ethylenediaminetetraacetic acid (EDTA), citric acid, peptone, phenol, sodium chloride (NaCl), sodium citrate, PCR-grade water, and yeast extract were obtained from Fisher Scientific (Pittsburg, PA). NuPAGE antioxidant, 4x LDS sample loading buffer (424 mM Tris HCl, 564 mM Tris, 8% (w/v) lithium dodecyl sulfate, 40% (w/v) glycerol, 2.04 mM EDTA, 0.88 SERVA Blue G250, 0.7 mM Phenol Red), 20x MES SDS running buffer (1 M 4-Morpholineethanesulfonic acid, 1 M Tris, 2% (w/v) SDS, 20 mM EDTA), and 20x NuPAGE transfer buffer (500 mM Bicine, 500 mM Bis-Tris, 20 mM EDTA) were obtained from Invitrogen Life Technologies (Carlsbad, CA). Concentrated protein assay reagent, goat anti-rabbit alkaline phosphatase (AP) conjugate, Immun-Star AP chemiluminescent substrate conjugate, non-fat dry milk and Tween 20 were obtained from Bio-Rad Laboratories, Incorporated (Hercules, CA). Carbenicillin, dithiothreitol (DTT), kanamycin, Murashige and Skoog basal salts and vitamins, and sucrose were obtained from Bioworld (Dublin, OH). Bovine serum albumin (BSA), hybridization buffer, the North2South biotin random prime kit (containing nuclease-free water, heptanucleiotide mix, dNTP mix, Biotin-N^4-dCTP, reaction buffer, Klenow fragment and 0.5 M EDTA; pH 8.0), and the North2South chemiluminescent kit (containing blocking buffer, streptavidin-horseradish peroxidase (HRP) conjugate, 4x wash buffer, substrate equilibration buffer, luminol/enhancer solution, and stable peroxide solution) were obtained from Pierce Biotechnology, Incorporated (Rockford, IL). All restriction endonuclease, T4 DNA ligase and 40x TAE buffer (1.6 M Tris-acetate, 40 mM EDTA) were obtained from Promega Corporation (Madison, WI). QIAEXII gel extraction and Qiaprep spin miniprep kits were obtained from Qiagen Incorporated (Valencia, CA). Agar, ethanol, methanol, Mastr Mix, pBC phagemid vector, and 6% sodium hypochlorite were obtained from Becton, Dickinson and Company (Sparks, MD), Aaper Alcohol and Chemical Company (Shelbyville, KY), EMD Chemicals, Incorporated (Gibbstown, NJ), Eppendorf (Westbury, NY), Stratagene (La Jolla, CA), and The Clorox Company (Oakland, CA), respectively.

Phytatray II tissue culture boxes, scalpel blades and handles were obtained from Sigma-Aldrich Company (St. Louis, MO). Blotting pads and 4-12% Bis-Tris NuPAGE gels were obtained from Invitrogen Life Technologies (Carlsbad, CA). Filter paper, 10DG disposable chromatography columns, and 0.2 μm polyvinylidene difluoride (PVDF) membranes were obtained from Bio-Rad Laboratories, Incorporated (Hercules, CA). 3MM chromatography paper and 0.45 μm PVDF syringe filters were obtained from Whatman Incorporated (Sanford, ME). Miracloth, Hybond-N+ positively-charged nylon membranes, deep Petri dishes, Microcon YM-3 centrifugal filter units, and 50-mL polystyrene tubes were obtained from EMD Biosciences, Incorporated (La Jolla, CA), Amersham Pharmacia Biotech (Piscataway, NJ), Fisher Scientific

(Pittsburg, PA), Millipore Corporation (Billerica, MA), and Becton Dickinson and Company (Franklin Lakes, NJ), respectively.

3.2 Methods

The research project was separated into four different objectives: (1) construction of preprorelaxin expression vectors, (2) generation of transgenic plants, (3) analysis of gene expression, and (4) analysis of protein expression. Detailed procedures of the steps involved in completing these objectives are discussed in the subsequent subsections.

3.2.1 Construction of preprorelaxin expression vectors

Prorelaxin cDNA, which was codon-optimized for expression in *Nicotiana tabacum*, was cloned downstream from the patatin signal peptide cDNA. The codon-optimized sequence is presented in Appendix I. This modified preprorelaxin cDNA was cloned into two different *Agrobacterium* binary vectors which differed by the type of promoter driving the expression of the preprorelaxin cDNA.

DNA cloning techniques were performed by standard recombinant DNA methods (Sambrook, 2001). Qiaprep spin miniprep and QIAEXII gel extraction kits were utilized to purify DNA from *Escherichia coli* cultures and agarose gels, respectively. DNA electrophoresis was performed on 1% (w/v) agarose gels in 1x TAE buffer using the Mini-Sub Cell GT cell electrophoresis apparatus (Bio-Rad Laboratories, Incorporated; Hercules, CA).

3.2.1A Sources of DNA

The codon-optimized prorelaxin cDNA was received from Genscript Corporation (Piscataway, NJ) cloned into a pUC-57 plasmid at the *Xba*I and *Sac*I restriction sites on the 5' and 3' ends of the cDNA, respectively. This plasmid was designated as pUC57-RLX. The patatin signal peptide cDNA (Bevan et al., 1986) was obtained from the R8-2 plasmid (Medina-Bolivar and Cramer, 2004), which was kindly provided by Dr. Luis Fabricio Medina-Bolivar (Arkansas State University). The X3 and X4 vectors (Buswell et al., 2005) were utilized as the *Agrobacterium* binary vectors. The X3 vector was a modified pGPTV vector (Becker et al., 1992) that contained the "super promoter" (Ni et al., 1995). This promoter consisted of a triplet of the octopine synthase activator followed by the mannopine synthase activator, the mannopine sythase promoter and the translation leader sequence of the 5' untranslated region from the tobacco etch virus (TEV leader) (Carrington and Freed, 1990). The X4 vector was a modified pBIB-Kan vector (Becker, 1990), which contained the dual enhanced cauliflower mosaic virus (CaMV) 35S promoter (Kay et al., 1987) followed by a modified TEV leader, such that the ATG initiation site at the end of the sequence was deleted (Li et al., 1997). Vector maps of plasmids used for DNA sources in the construction of preprorelaxin expression vectors are presented in Figure 3.1.

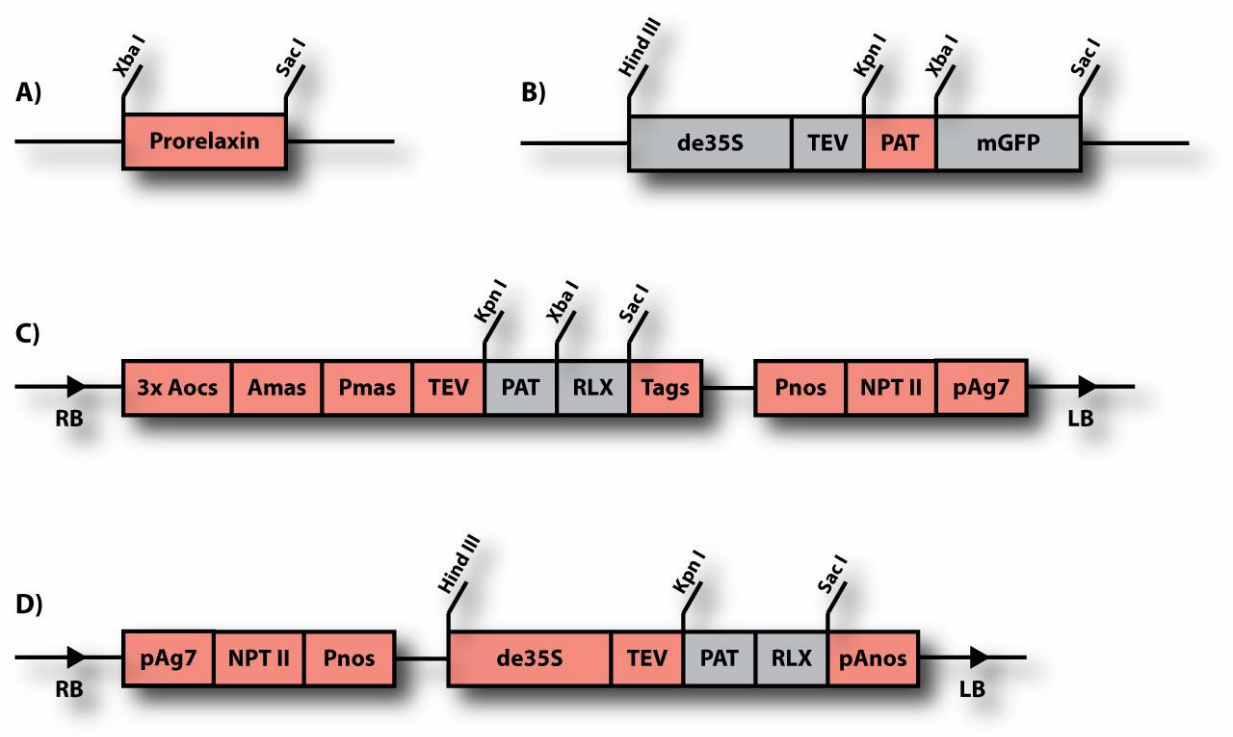

Figure 3.1: Vector maps of plasmids utilized for DNA source material. Mauve-colored regions represents DNA utilized for further cloning, grey-colored regions do not. (A) Map of the codon-optimizied prorelaxin cDNA cloned into the pUC57 plasmid. (B) Map of the R8-2 insert cloned into the pBC plasmid. (C) Map of the T-DNA region of X3 vector. (D) Map of the T-DNA region of the X4 vector. Legend: Prorelaxin: codon-optimized prorelaxin cDNA; de35S: dual enhanced CaMV 35S promoter; TEV: TEV leader; PAT: patatin signal peptide cDNA; mGFP: modified green fluorescent protein cDNA; RB: right border of T-DNA; 3x Aocs: triplet of the octopine synthase activator; Amas: mannopine synthase activator; Pmas: mannopine synthase promoter; RLX: porcine relaxin cDNA; Tags: agropine synthase terminator; Pnos: nopaline synthase promoter; NPT II: neomycin phosphotransferase II gene (kanamycin resistance); pAg7: agropine synthase polyadenylation sequence; LB: left border of T-DNA; pAnos: nopaline synthase polyadenylation sequence.

3.2.1B DNA cloning

The patatin signal peptide cDNA was excised from R8-2 by digesting with *Kpn*I and *Xba*I restriction endonucleases. The 72 bp fragment was separated by DNA electrophoresis, gel purified, and ligated into a pBC phagemid vector at the *Kpn*I and *Xba*I restriction sites, at the 5' and 3' ends of the patatin cDNA, respectively. The resulting plasmid was designated as pBC-patatin.

The prorelaxin cDNA was excised from the pUC57-RLX plasmid by digesting with the *Xba*I and *Sac*I restriction endonucleases. The 477 bp fragment was separated by DNA electrophoresis, gel purified, and ligated into pBC-patatin downstream of the patatin signal peptide cDNA. The resulting plasmid was designated as pBC-RLX2. The fidelity and alignment of the modified preprorelaxin cDNA were verified by DNA sequencing using the T7 (5'-GTAATACGACTCACTATAGGG C-3') and T3 (5'-AATTAACCCTCACTAAAGGG-3') primers at the Virginia Bioinformatics Institute (Blacksburg, VA).

The modified preprorelaxin cDNA was excised from the pBC-RLX2 plasmid with the *Kpn*I and *Sac*I restriction endonucleases. The 555 bp fragment was separated by DNA electrophoresis, gel purified, and ligated into the X3 and X4 vectors downstream of the TEV

leader. The resulting plasmids were designated RLX3 and RLX4, respectively. The fidelity of the constructs was verified by DNA sequencing with 3RelExtRev (5'-GCTGGAGCTCTTAACA CAAC-3') and 5PatExtFwd (5'-GCCGGGTACCAATGGCAACT-3') primers (Sigma-Genosys; The Woodlands, TX) at the Virginia Bioinformatics Institute. Vector maps of all constructed plasmids are presented in Figure 3.2.

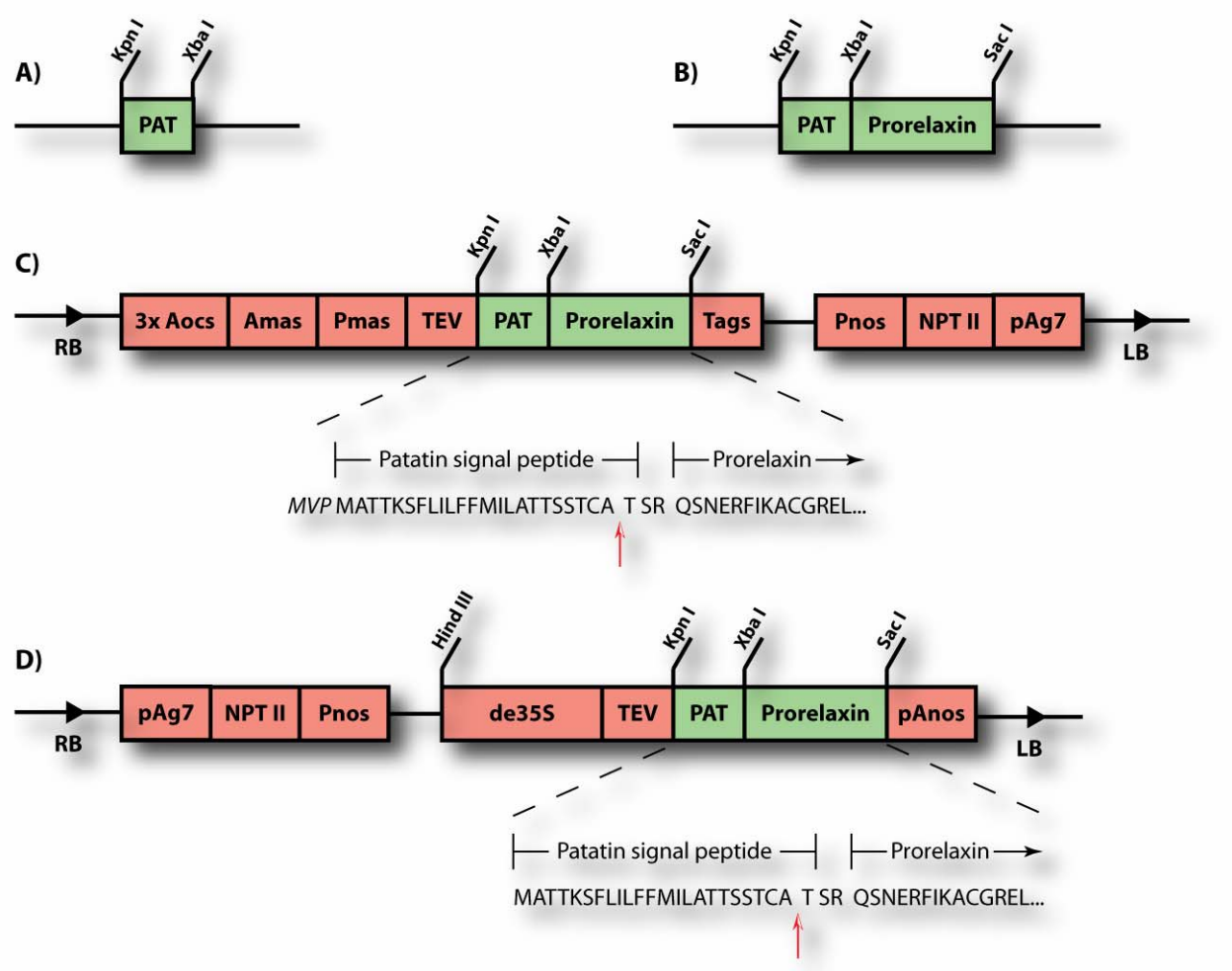

Figure 3.2: Vector maps of constructed plasmids. Pale green-colored regions represent subcloned insert DNA, mauve-colored regions represent vector segments. (A) Map of the insert region of pBC-patatin. (B) Map of the insert region of pBC-RLX2. (C) Map of the T-DNA region of RLX3 vector. (D) Map of the T-DNA region of the RLX4 vector. Legend: PAT: patatin signal peptide cDNA; Prorelaxin: codon-optimized prorelaxin cDNA; RB: right border of T-DNA; 3x Aocs: triplet of the octopine synthase activator; Amas: mannopine synthase activator; Pmas: mannopine synthase promoter; TEV: TEV leader; Tags: agropine synthase terminator; Pnos: nopaline synthase promoter; NPT II: neomycin phosphotransferase II gene (kanamycin resistance); pAg7: agropine synthase polyadenylation sequence; LB: left border of T-DNA; de35S: dual enhanced CaMV 35S promoter; pAnos: nopaline synthase polyadenylation sequence. Zoomed region shows predicted initial translation of expression vectors. Red arrows indicate predicted cleavage site of the patatin signal peptide.

3.2.2 Generation of transgenic tobacco plants

The RLX3 and RLX4 vectors were mobilized into *Agrobacterium tumefaciens* by a freeze thaw method (Holsters et al., 1978). Transgenic plants containing the T-DNA region of

these vectors were developed by direct *Agrobacterium*-mediated transformation (Medina-Bolivar and Cramer, 2004).

All seeds, explants, and plants were grown aseptically in a CU-32L growth chamber (Percival Scientific; Boone, IA) with a 16-hour light, 8-hour dark photoperiod at 24°C in a modified MS media (mMS) which consists of Murashige and Skoog basal salts and vitamins (Murashige and Skoog, 1962), 3% (w/v) sucrose, 0.04% (w/v) $MgSO_4 \cdot 7H_2O$, 0.4% (w/v) Phytagel, and adjusted to pH 5.7 with KOH. All tissue culture work was performed in a laminar flow hood (NuAire Corporation; Plymouth, MN) to maintain the sterility of samples.

3.2.2A Establishing sterile *Nicotiana tabacum* plants

Nicotiana tabacum cv. Xanthi seeds, kindly provided by Dr. Carol Wilkinson (Southern Piedmont Agricultural Research and Extension Center), were surface sterilized by a two-minute wash in 70% ethanol followed by an eight-minute wash in 6% sodium hypochlorite. The disinfectants were removed by rinsing the seeds four times in sterilized water (De Neve et al., 1998). The seeds were transferred to deep petri plates containing approximately 50 mL of mMS media. After the seeds germinated, the seedlings were transferred to Phytatray II boxes containing approximately 100 mL of mMS media and were maintained by excising and transplanting the apical shoot into fresh mMS media, every four to five weeks.

3.2.2B Transforming *Agrobacterium tumefaciens*

An individual colony of *A. tumefaciens* strain LBA4404, kindly provided by Dr. Luis Fabricio Medina-Bolivar (Arkansas State University), was used to inoculate 5 mL of YEP broth (10 g/L peptone, 10 g/L yeast extract, and 5 g/L sodium chloride) supplemented with 30 mg/L of streptomycin. The culture was incubated at 28°C with shaking at 250 RPM in a Queue Radial Shaker (Queue Systems; Parkersburg, WV) until a white precipitate formed. The cells were collected by centrifuging the cultures at 10,000 x g, for 10 minutes at 4°C in a 5810 R centrifuge (Eppendorf AG; Westbury, NY) and the supernatant was discarded. The cells were suspended in 100 μL of ice-cold 20 mM $CaCl_2$ and 1 μg of RLX3 or RLX4 was added to each suspension. The cells were frozen in liquid nitrogen and were immediately thawed in a 37°C water bath (Precision; Winchester, VA) for two minutes. One milliliter of YEP broth media was added to the cells and the suspension was incubated at 28°C and shaking at 250 RPM for 4 hours. The culture was spread on YEP media supplemented with 15 g/L of agar, 30 mg/L streptomycin, and 100 mg/L kanamycin and incubated for 48 hours at 28°C.

Polymerase chain reaction (PCR) amplification was employed to confirm the presence of the RLX3 or RLX4 plasmids in transformed colonies. Each PCR reaction contained 5 pmol of 5PatExtFwd, 5 pmol of 3RelExtRev, 10 μL of MasterMix, and PCR-grade water to bring the reaction volume to 25 μL. A Robocycler Gradient 40 (Stratagene; La Jolla, CA) was used to perform the PCR amplification using the following thermocycling conditions: one cycle of 95°C, 5 minutes; then 30 cycles of 95°C, 1 minute, 51°C, 1 min, 72°C, 1 minute; and a final extension at 72°C, 10 minutes. Additionally, PCR amplification was employed to confirm the presence of the disarmed Ti plasmid in transformed colonies. Each PCR reaction contained 5 pmol of VirD2A (5'-ATGCCCGATCGAGCTCAAGT-3'), 5 pmol of VirD2E (5'-CCTGACC CAAACATCTCGGCT-3'), 10 μL of Eppendorf MasterMix, and PCR-grade water to bring the reaction volume to 25 μL. The reaction was performed as described above except an annealing temperature of 53°C was used.

3.2.2C Generating transgenic *Nicotiana tabacum* plants

Leaves from sterile *N. tabacum* plants were aseptically excised at the petiole, halfway between the stem and the base of the leaf lamina. The leaves were cut along the petiole with a number 11 scalpel blade that was inoculated with the transformed *A. tumefaciens*. The procedure was performed on 30 leaves; 15 leaves were inoculated with *A. tumefaciens* containing the RLX3 plasmid and the other 15 leaves were inoculated with *A. tumefaciens* containing the RLX4 plasmid.

The explants were placed on mMS media in deep Petri plates, for 48 hours. The explants were then transferred to mMS media supplemented with 0.1 mg/L NAA, 1.0 mg/L BA, 250 mg/L kanamycin, and 500 mg/L carbenicillin and were incubated until shoots started to form from the wound site. The shoots were transferred to mMS supplemented with 250 mg/L kanamycin and 500 mg/L carbenicillin and the shoots that developed into plants were maintained by excising and transplanting the apical shoot into fresh mMS media supplemented with 100 mg/L of kanamycin, every four to five weeks. Each plant that developed came from a unique explant.

3.2.3 Analysis of gene expression

RNA was extracted from tobacco leaf samples using phenol/SDS extraction and selective precipitations (Ausubel, 2002). The expression of relaxin transgene was verified by northern blot analysis using a biotin-labeled modified preprorelaxin DNA probe and a streptavidin-HRP conjugate via chemiluminescent detection.

3.2.3A Purification of RNA from plant tissue

Plant tissue was harvested and 1.50 g of leaf tissue was ground under liquid nitrogen with a mortar and pestle. The ground tissue was transferred to a 50 mL polystyrene tube containing 5 mL of phenol and 15 mL of RNA grinding buffer (180 mM Tris, 90 mM LiCl, 1% (w/v) SDS, and 4.5 mM EDTA; pH 8.2). The slurry was homogenized with a 10 mm x 195 mm sawtooth probe attached to a PowerGen 700 homogenizer (Fisher Scientific; Pittsburg, PA) at setting 5 for 60 seconds. Five milliliters of chloroform was added and the slurry was homogenized again for 15 seconds at setting 2. The slurry was incubated at 50°C for 20 minutes and then the liquid phases were separated by centrifugation at 7,500 x g and 4°C for 15 minutes. The aqueous phase was extracted two more times in 10 mL of equal parts phenol and chloroform and then was extracted two addition times in 5 mL of chloroform to remove trace amounts of phenol.

The aqueous phase was brought to 2 M LiCl, by the addition of DEPC-treated (Sambrook, 2001) 8 M LiCl, and the solution was incubated overnight at 4°C. The precipitate was collected by centrifugation for 20 minutes at 15,300 x g and 4°C. The supernatant was discarded and the pellet was rinsed in DEPC-treated 2 M LiCl and was solubilized with DEPC-treated water. The solution was brought to 2 M LiCl and was incubated on ice for 2 hours. The precipitate was collected as described above and the pellet was suspended in DEPC-treated water. The solution was brought to 0.3 M sodium acetate, by the addition of DEPC-treated 3M sodium acetate; pH 5.2 and 2.5 volumes of ice-cold 100% ethanol were added. The solution was incubated overnight at -20°C and the RNA precipitate was collected by centrifugation for 15 minutes at 17,700 x g and 4°C. The pellet was solubilized in 100 μL of DEPC-treated water and the absorbance at 260 nm was measured using a UV-1601 spectrophotometer (Shimadzu Corporation; Kyoto, Japan) to quantify the RNA concentration.

3.2.3B DNA probe generation

The DNA sequence encoding for the modified preprorelaxin was amplified by PCR using the 5PatExtFwd and 3RelExtRev primers, as previously described in section 3.2.2, using pBC-RLX2 plasmid as a template. The amplification product was separated from residual template and ogilonucleotides by DNA electrophoresis. The DNA band migrating around 550 bp was excised from the gel and was purified using the QIAEX II gel extraction kit.

The biotin-labeled preprorelaxin probe was generated using the North2South biotin random prime kit. Twenty microliters of purified PCR product was mixed with 4 μL of nuclease-free water and 10 μL of heptanucleotide mix. The DNA was denatured by heating the sample for 5 minutes at 100°C, and then was immediately frozen in an ethanol/dry ice bath for 5 minutes. The sample was thawed on ice, and the following was added: 8 μL of dNTP mix, 2 μL of Biotin-N^4-dCTP, 5 μL of reaction buffer, and 1 μL of Klenow fragment. The sample was incubated in a 37°C water bath for 60 minutes and the reaction was stopped by the addition of 2 μL of 0.5 M EDTA; pH 8.0. The unincorporated nucleotides were removed using the Qiaprep spin miniprep kit.

3.2.3C Northern blot analysis

Twenty micrograms of each RNA sample was concentrated to less than 2 μL in a Vacufuge vacuum centrifuge (Eppendorf AG; Westbury, NY). Ten microliters of RNA loading buffer was added to each concentrated sample and the RNA was denatured by heating in a 65°C water bath for 10 minutes followed by a short incubation on ice. During the sample preparation, a 1% (w/v) agarose gel in 1x MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 20 mM EDTA; pH 7.0), containing 2% (v/v) formaldehyde was cast. The denatured RNA samples were loaded onto the prepared formaldehyde/agarose gel. The electrophoresis was performed for three hours at 50 V, using 1x MOPS buffer as the running buffer. The quality of the RNA was assessed by staining the gel in 0.25 μg/mL ETBR.

The RNA was transferred overnight to a Hybond-N+ membrane via capillary transfer method (Reed and Mann, 1985) using 20x SSC (3 M NaCl, 300 mM sodium citrate; pH 7.0) as the transfer buffer. The transferred RNA was fixed to the membrane in the Spectrolinker UV Crosslinker (Spectronics Corporation; Westbury, NY) using the optimal crosslink setting. The membrane was allowed to dry on 3MM chromatography paper after crosslinking.

The blot was incubated at 50°C in a Hybridiser HB-1D (Techne, Incorporated; Burlington, NJ) for 30 minutes in 10 mL of preheated hybridization buffer. Twenty microliters of the probe was denatured by heating for 10 minutes at 100°C followed by chilling for five minutes in an ice bath. The denatured probe was added to 10 mL of preheated hybridization buffer and the membrane was incubated in this solution overnight at 50°C. The blot was washed twice in low stringency buffer (2x SSC, 0.1% (w/v) SDS) at room temperature, followed by two washes in high stringency buffer (0.1x SSC, 0.1% (w/v) SDS) preheated to 50°C, with wash times of 5 and 15 minutes, respectively.

The blot was detected for modified preprorelaxin mRNA using the North2South chemiluminescent kit. The membrane was incubated in 20 mL of blocking buffer for 15 minutes. Sixty-seven microliters of Streptavidin-HRP conjugate was added to the blocking buffer and the membrane was incubated in this solution for an additional 15 minutes. The blot was washed four times in 20 mL of 1x wash buffer, with each wash lasting five minutes. This was followed by a five-minute incubation in 20 mL of substrate equilibration buffer. The blot was incubated in equal volumes of luminol/enhancer and stable peroxide solutions for five

minutes and was exposed in a ChemiDoc XRS molecular imager (Bio-Rad Laboratories, Incorporated; Hercules, CA).

3.2.4 Analysis of protein expression

Protein samples were extracted from tobacco leaf tissue in aqueous or chaotropic buffers and were analyzed by western blot analysis. Purified porcine relaxin (Sherwood and O'Byrne, 1974), kindly provided by Dr. O. David Sherwood (University of Illinois at Urbana-Champaign), was used for a positive control throughout all protein expression analysis.

3.2.4A Protein extraction

Fresh leaf tissue was harvested and homogenized under liquid nitrogen in a mortar and pestle. The ground tissue was added to three volume of citrate buffer (33 mM citric acid, 17 mM sodium citrate, 1 mM EDTA; pH 4.0). The samples were incubated for 15 minutes with intermittent mixing using a Vortex Genie 2 mixer (Fisher Scientific; Pittsburg, PA). The extracts were clarified by centrifuging at 15,000 x g, and 4°C for 15 minutes followed by filtering through miracloth. The extracts were concentrated by ultrafiltration using YM-3 centrifugal filters for 100 minutes at 14,000 x g.

Extraction of insoluble and soluble proteins was performed as above with the following modifications. First, 6 M Guanidine HCl buffered in 50 mM Tris·HCl; pH 8.0 was used as the extraction buffer. Second, the ground tissue was incubated in the chaotropic buffer for 60 minutes with intermittent vortexing. Third, the extracts were filtered using a 0.45 μm PVDF syringe filter after centrifugation. Three milliliters of each sample was desalted and eluted in 4 mL of 8 M urea buffered in 50 mM Tris·HCl; pH 8.0 using 10 DG disposable chromatography columns.

Incubation of leaf tissue in a proteasome inhibitor prior to extraction was performed as follows. The stem was cut such that the three uppermost leaves were harvested. The explant was placed in 25 mL of 100 μM of MG 132 and was incubated in this solution for three hours at room temperature. The leaves were excised along the petiole and insoluble and soluble proteins were extracted from the leaves as previously described.

3.2.4B Quantification of protein concentration

The protein concentration of tobacco extracts were measured using the Bio-Rad protein assay (Bradford, 1976). The dye reagent was prepared by diluting one part of the protein assay dye concentrate with four parts of deionized water. Twenty microliters of sample was mixed with 1 mL of the protein assay reagent and incubated at room temperature for 10 minutes. The absorbance was measured at 595 nm. The protein concentrations were determined using a standard curve calculated from known bovine serum albumin samples, ranging from 250 ng/μL to 850 ng/μL.

3.2.4C Western blot analysis

Protein samples were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions. The protein samples were diluted with 4x LDS sample buffer and 500 mM DTT to 1x and 50 mM concentrations, respectively. The samples were heated for 10 minutes at 70°C and were loaded onto a 4-12% Bis-Tris NuPAGE gel in an XCell SureLock Mini-Cell (Invitrogen Life Technologies; Carlsbad, CA). The outer chamber of the unit was filled with 600 mL of 1x MES SDS running buffer and 200 mL of the running buffer supplemented with

500 μL of NuPAGE antioxidant was added to the inner chamber. The proteins were separated by applying 200 V for 40 minutes.

The resolved protein samples were transferred to a 0.2 μm Immun-Blot PVDF membrane by electrophoretic blotting (Towbin, Staehelin, and Gordon, 1979). The membrane was wetted in methanol, rinsed in deionized water, and equilibrated in 1x NuPAGE transfer buffer containing 10% methanol for several minutes. The protein gel was removed from the cassette and a piece of filter paper was placed on the gel. The equilibrated membrane was placed on the other side of the gel and was covered with another piece of filter paper. This assembly was placed on top of two blotting pads, presoaked in transfer buffer, inside of the cathode core of the XCell II Blot Module (Invitrogen Life Technologies; Carlsbad, CA). Two more presoaked blotting pads were placed on top the membrane assembly and the blot module was secured in the XCell SureLock Mini-Cell. Transfer buffer was added to the module to cover the blotting pads and approximately 650 mL of deionized water was added to the outer chamber. The proteins were transferred to the membrane by applying 20 V for 60 minutes.

The membrane was allowed to dry on a sheet of 3MM chromatography paper. The blot was rewetted in 100% methanol and was washed in two changes of TBS buffer (20 mM Tris, 500 mM NaCl; pH 7.5). The unoccupied membrane binding sites were blocked by incubating with 0.2% (w/v) nonfat dry milk in TBS buffer for 60 minutes, followed by a 10-minute wash in TTBS (0.1% (v/v) Tween 20 in TBS). The membrane was incubated overnight with a 1:20,000 dilution of rabbit anti-porcine relaxin, kindly provided by Dr. Kevin Van Cott (University of Nebraska Lincoln), in TTBS containing 0.2% (w/v) non-fat dry milk. The blot was washed in two times in TTBS, with the first wash lasting 10 minutes and the final wash lasting 30 minutes. The membrane was incubated with a 1:3,000 dilution of goat anti-rabbit AP conjugate in TTBS containing 0.2% (w/v) non-fat dry milk for two hours. The blot was washed four times in TTBS, with the first three washes lasting 10 minutes and the last wash lasting 60 minutes. The membrane was incubated in Immun-Star AP chemiluminescent substrate for five minutes and was exposed in a ChemiDoc XRS molecular imager.

CHAPTER 4

RESULTS

4.1 Construction of preprorelaxin expression vectors

Agrobacterium binary vectors were constructed to transform tobacco for the expression of recombinant porcine relaxin. First, codon-optimized porcine prorelaxin cDNA was cloned in frame with the patatin signal sequence and the resulting plasmid was designated as pBC-RLX2. Incorporation of the prorelaxin cDNA in pBC-RLX2 was verified with PCR amplification using relaxin specific primers and by restriction enzyme digestion (Figure 4.1). The PCR reaction amplified a DNA fragment of approximately 500 bp that corresponded to the DNA fragment resulting from PCR amplification of the pUC57-RLX plasmid. Digestion of the pBC-RLX2 plasmid with either *Xba*I or *Sac*I restriction enzyme yielded a fragment of the expected size, approximately 4,000 bp. Additionally, digestion with both *Xba*I and *Sac*I yielded two fragments, approximately 3,500 and 500 bp, which corresponded to the predicted sizes of the pBC fragment and prorelaxin cDNA, respectively.

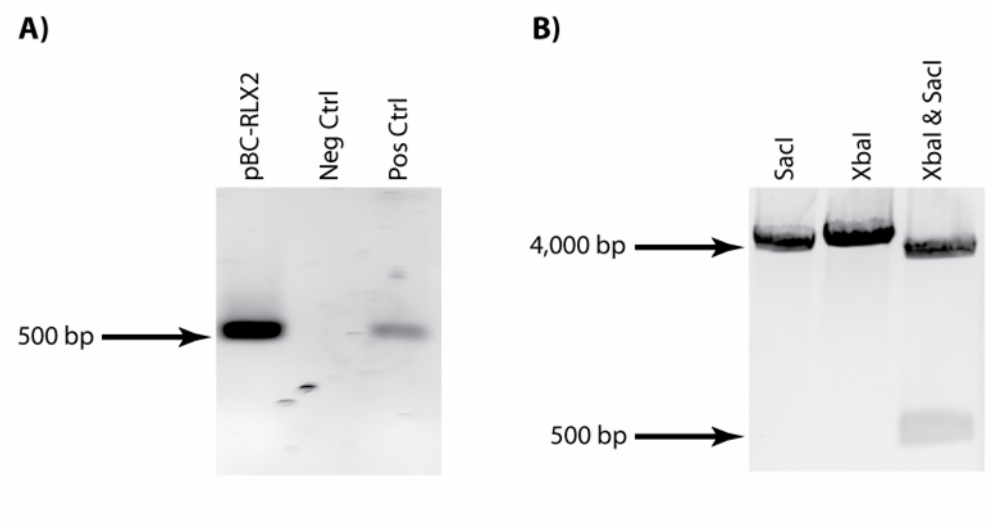

Figure 4.1: PCR amplification and restriction enzyme digestion of pBC-RLX2 plasmid resolved on a 1% agarose gel. Panel A: PCR amplification with 5RelExtFwd and 3RelExtRev primers of pBC-RLX2 plamsid, pUC-57 plasmid containing codon-optimized prorelaxin cDNA (Pos Ctrl), and no DNA (Neg Ctrl). Panel B: Digestion of pBC-RLX2 plasmid with XbaI only (XbaI), SacI only (SacI), and XbaI and SacI (XabI & SacI) restriction enzymes. Arrows indicate DNA fragment length as determined by DNA ladders, which are not shown.

The fidelity of the preprorelaxin insert of pBC-RLX2 plasmid was verified by DNA sequencing, with the T7 and T3 primers. The results of the sequencing reaction showed perfect alignment except for the first several residues closest to the sequencing primer which corresponded to a region of low resolution on the chromatogram. However, sequencing with a primer from the opposing direction showed the proper sequence which also corresponded to a region of high resolution on the chromatogram. Therefore, it was concluded that the sequence fidelity of the preprorelaxin cDNA insert in pBC-RLX2 was correct. An alignment of the sequencing reactions against the expected pBC-RLX2 insert is presented in Figure 4.2.

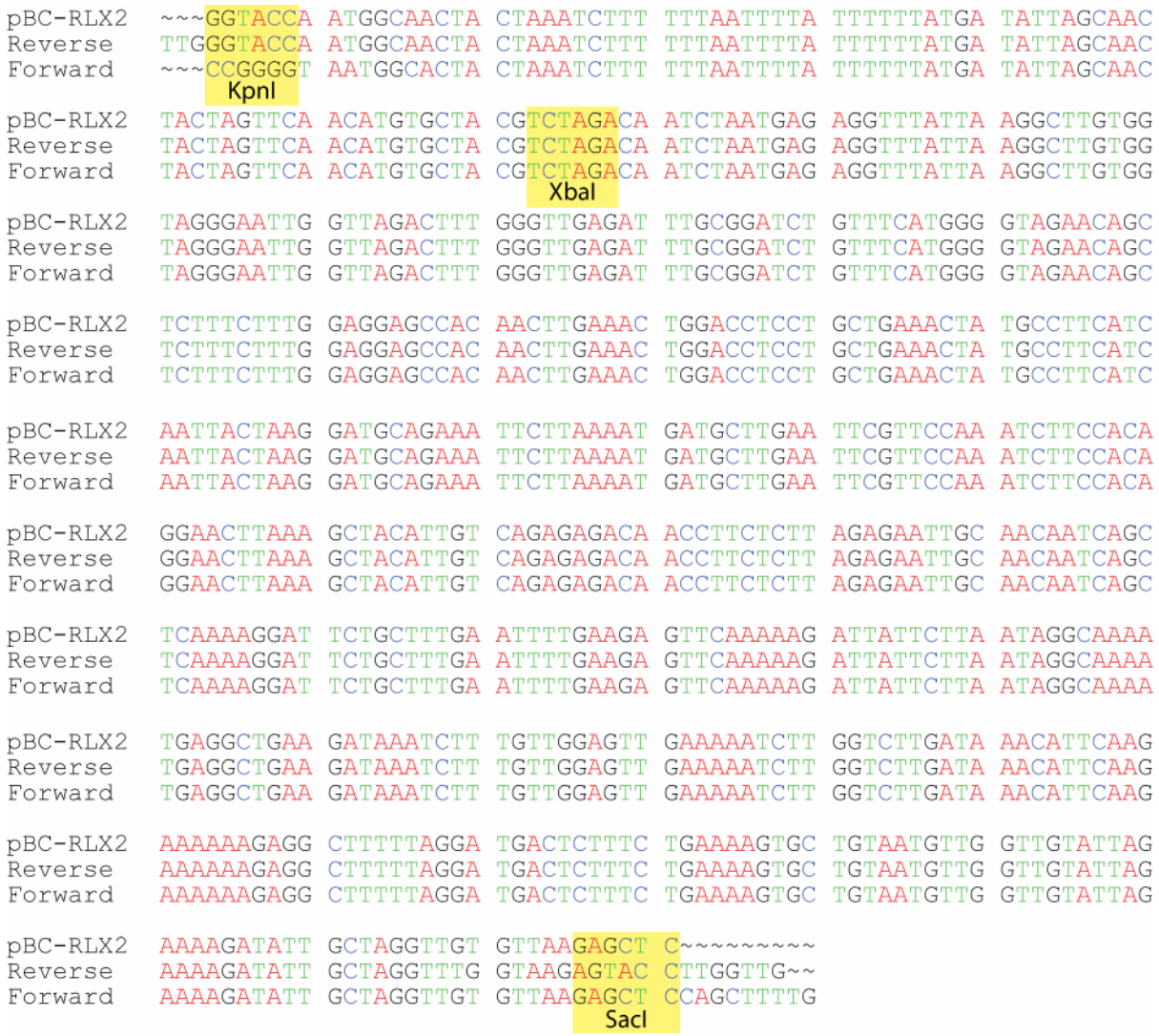

Figure 4.2: DNA sequence alignments of the pBC-RLX2 insert, DNA sequence obtained from sequencing with the T7 primer, and the reverse complement of the sequence obtained from sequencing with the T3 primer. Highlighted regions indicate restriction enzyme sites used during cloning. The cDNA for the patatin signal peptide is located between the *Kpn*I and XbaI restriction sites and the codon-optimized prorelaxin cDNA is located between the XbaI and SacI restriction sites.

The pBC-RLX2 insert was subcloned into two binary vectors, RLX3 and RLX4, downstream of the TEV leader under the control of the super promoter or dual enhanced CaMV 35S promoter, respectively. The RLX3 and RLX4 ligation reactions were used to transform chemically competent GC10 *E. coli* cells. The presence of the preprorelaxin cDNA in the individual colonies resulting from the transformation reaction was verified by PCR amplification with the 5PatExtFwd and 3RelExtRev primers. The PCR reaction amplified a DNA fragment of approximately 550 bp that corresponded to the DNA fragment resulting from PCR amplification of the pBC-RLX2 plasmid (Figure 4.3).

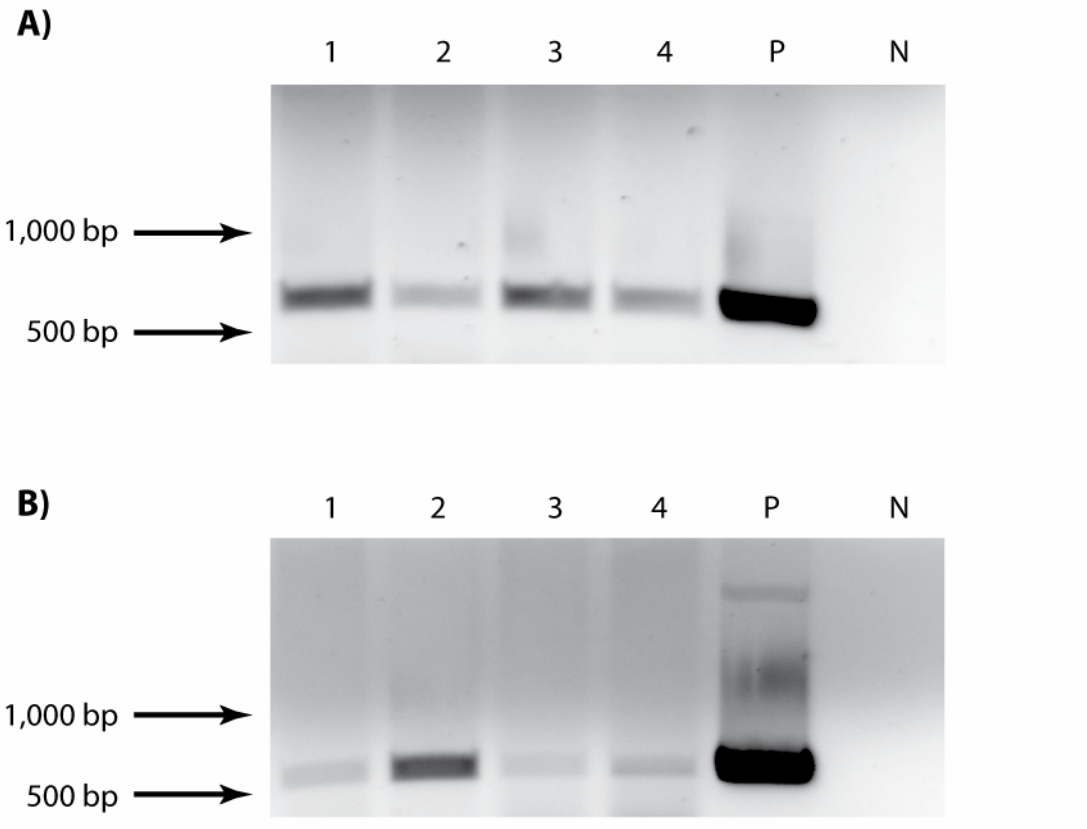

Figure 4.3: PCR reactions using 5PatExtFwd and 3RelExtRev primers of individual colonies of GC10 competent cells obtained after transformation with the RLX3 and RLX4 plasmids resolved on a 1% agarose gel. Panel A: PCR amplification using four individual RLX3 transformants (lanes 1, 2, 3, and 4), pBC-RLX2 plasmid DNA (lane P), and no DNA (lane N). Panel B: PCR amplification using four individual RLX4 transformants (lanes 1, 2, 3, and 4), pBC-RLX2 plasmid DNA (lane P), and no DNA (lane N).

The fidelity of the preprorelaxin insert of RLX3 plasmid was verified by DNA sequencing, with the 5PatExtFwd and 3RelExtRev primers. The results of the sequencing reaction showed perfect alignment except for the first several residues closest to the sequencing primer which corresponded to a region of low resolution on the chromatogram. However, sequencing with a primer from the opposing direction showed the proper sequence which also corresponded to a region of high resolution on the chromatogram. Additionally, the predicted 5' untranslated region contained an ATG start codon nine base pairs upstream of the translation start site of the patatin signal peptide which originated from the NcoI cloning site in the plasmid. However, this additional start codon does not cause a frame shift in the preprorelaxin but does add an additional methionine, valine, and proline residues on the amino terminal of the patatin signal peptide. Therefore, it was concluded that the sequence fidelity of the preprorelaxin cDNA was acceptable. An alignment of the sequencing reactions against the expected RLX3 plasmid is presented in Figure 4.4.

The fidelity of the preprorelaxin insert of RLX4 plasmid was also verified by DNA sequencing, with the 5PatExtFwd and 3RelExtRev primers. The results of the sequencing reaction showed perfect alignment except for the first several residues closest to the sequencing primer which corresponded to a region of low resolution on the chromatogram. However, sequencing with a primer from the opposing direction showed the proper sequence which also

corresponded to a region of high resolution on the chromatogram. Additionally, the predicted 5' untranslated region contained no identified ATG start codons. Therefore, it was concluded that the sequence fidelity of the preprorelaxin cDNA was acceptable. An alignment of the sequencing reactions against the expected RLX4 plasmid is presented in Figure 4.5.

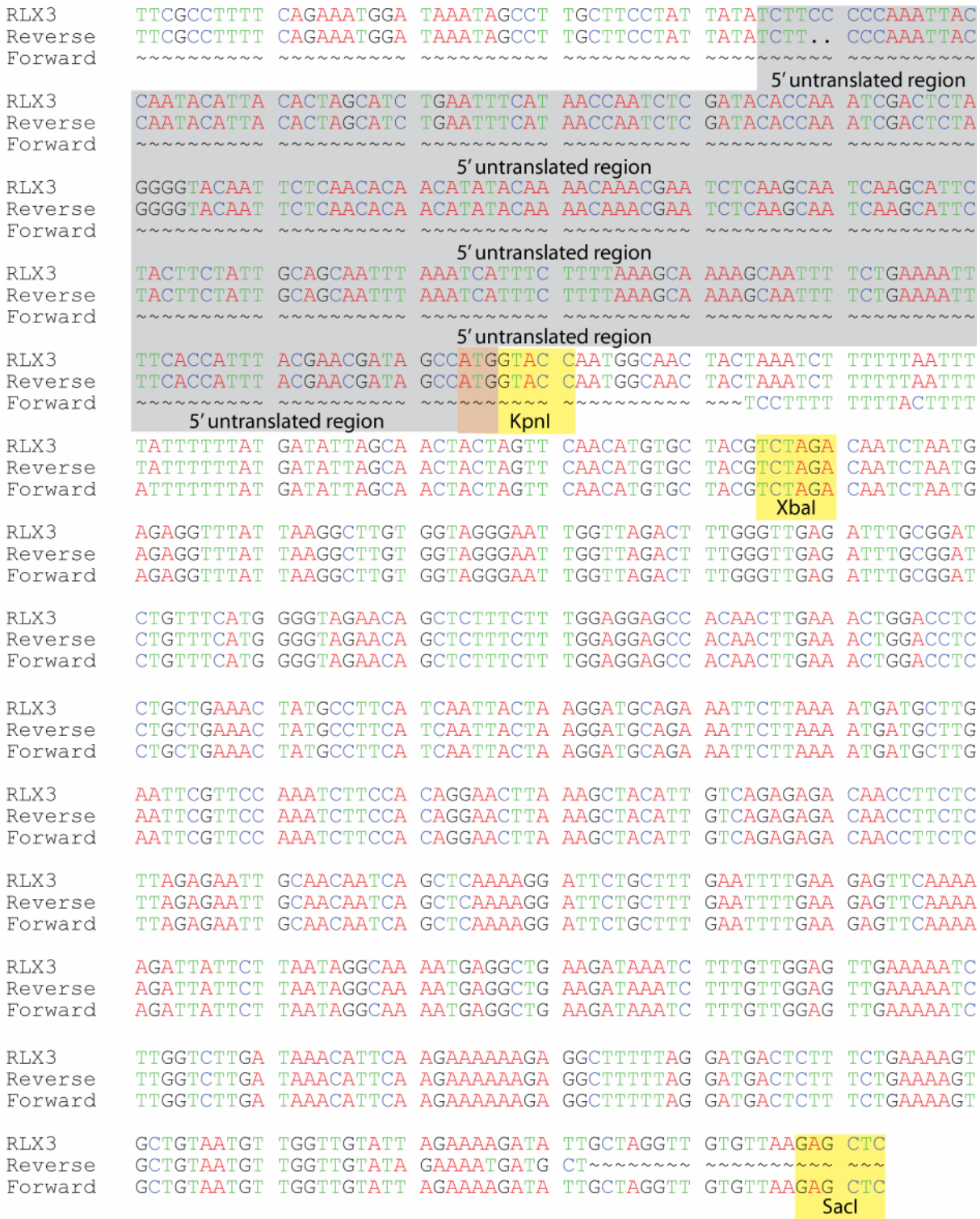

Figure 4.4: DNA sequence alignment of RLX3 vector, and DNA sequence obtained by sequencing with the 5PatExtFwd primer and the reverse complement of the DNA sequence obtained by sequencing with the 3RelExtRev primer. Yellow highlighted regions indicate restriction enzyme sites and gray highlighted region indicated the 5' untranslated region. Patatin signal sequence and prorelaxin cDNA are located between the *Kpn*I and XbaI sites and *Xba*I and SacI sites, respectively. Brown region highlights extra start codon.

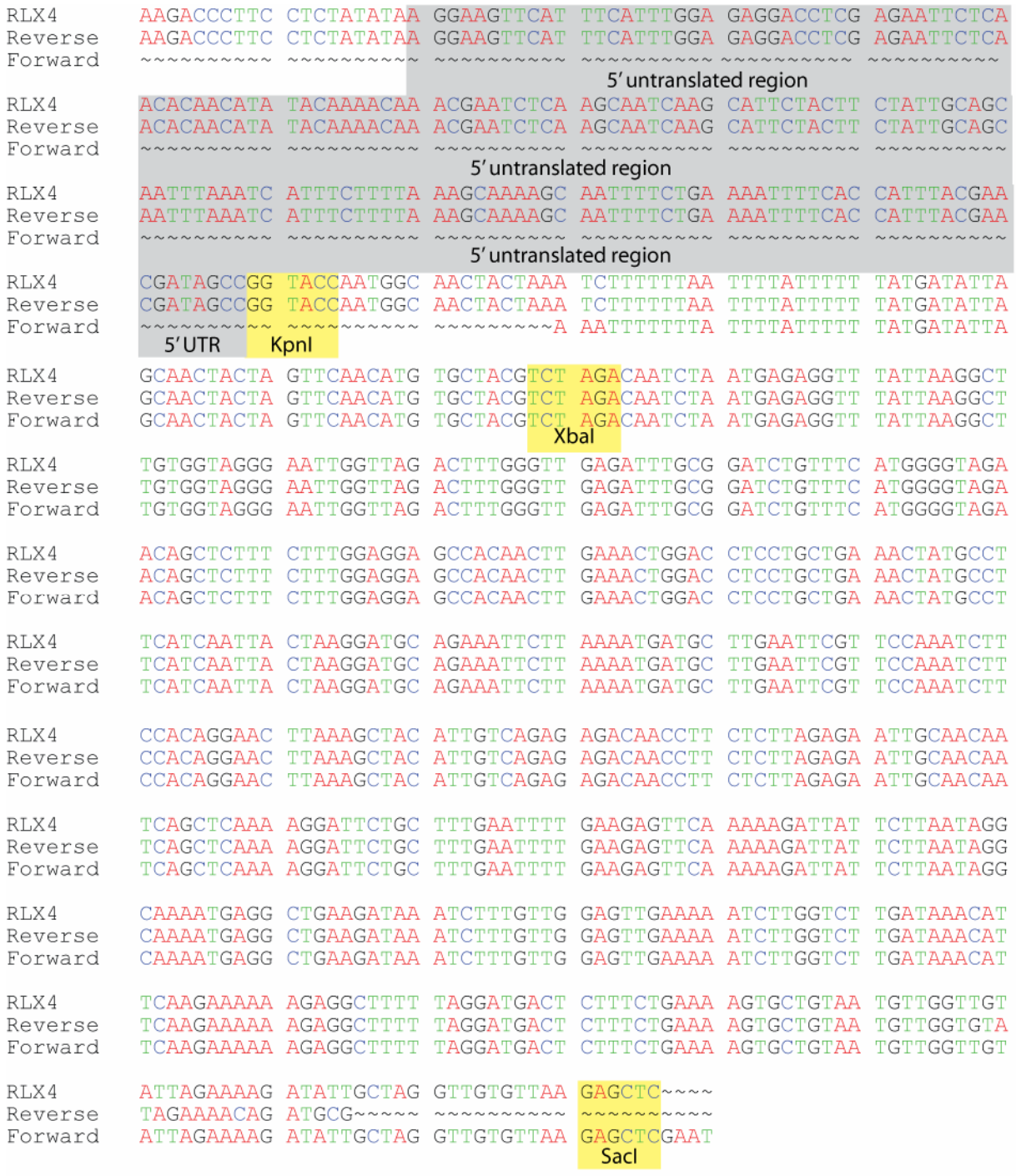

Figure 4.5: DNA sequence alignment of RLX4 vector, and DNA sequence obtained by sequencing with the 5PatExtFwd primer and the reverse complement of the DNA sequence obtained by sequencing with the 3RelExtRev primer. Yellow highlighted regions indicate restriction enzyme sites used during cloning and gray highlighted region indicated the 5' untranslated region. Patatin signal sequence is located between the *Kpn*I and *Xba*I sites and the codon-optimized prorelaxin cDNA is located between the *Xba*I and *Sac*I sites.

4.2 Generation of transgenic tobacco plants

RLX3 and RLX4 plasmids were used to transform *Agrobacterium tumefaciens* strain LBA4404 by a freeze thaw method. Five colonies of LBA4404 transformed with the RLX3 plasmid and five colonies transformed with RLX4 plasmid were PCR amplified with VirD2 primers and with relaxin specific primers. The reactions were resolved on a 1% agarose gel and are presented in Figure 4.6. Colony RLX3-5 and RLX4-1 were used to transform *Nicotiana tabacum* plants. Eight transgenic plants were generated that were transformed with the RLX3 plasmid and ten transgenic plants were generated that were transformed with the RLX4 plasmid.

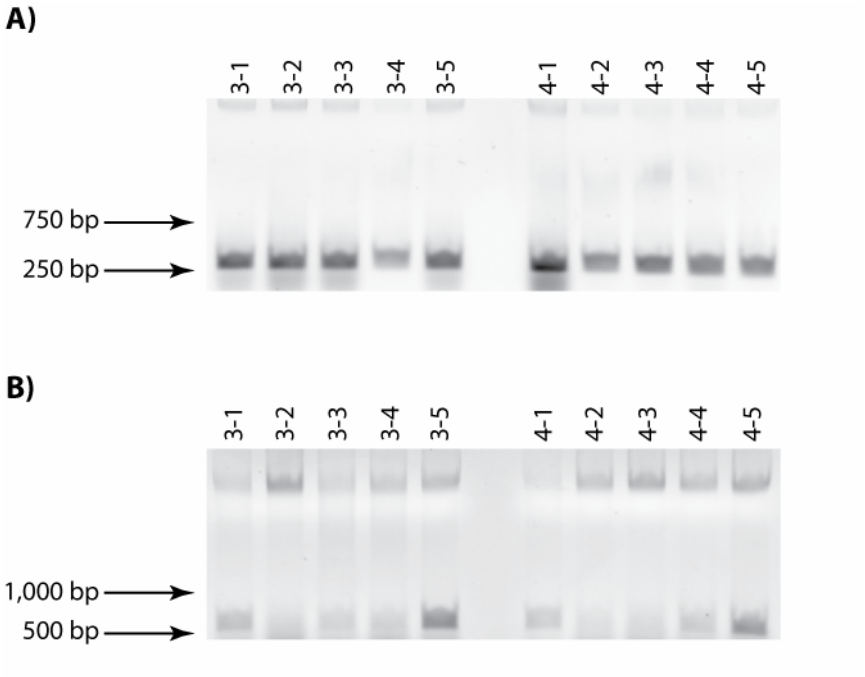

Figure 4.6: PCR reactions using individual colonies LBA4404 transformed with the RLX3 and RLX4 plasmids resolved on a 1% agarose gel. Panel A: PCR amplification using VirD2A and VirD2E primers. Panel B: PCR amplification using 5PatExtFwd and 3RelExtRev primers. Five colonies of LBA4404 transformed with the RLX3 plasmid were tested (3-1 thru 3-5) and five colonies of LBA4404 transformed with the RLX4 plasmid (4-1 thru 4-5) were tested.

Fifteen sterile *Nicotiana tabacum* cv. Xanthi leaves were inoculated with transformed *A. tumefaciens* colony RLX3-5 and an additional 15 leaves were inoculated with colony RLX4-1. Shoots that regenerated from the wound site were transferred to fresh media containing selective antibiotics. Eight shoots transformed with the T-DNA region of RLX3 plasmid and ten shoots transformed the T-DNA region of the RLX4 plasmid rooted and developed into plants. An illustration of the various stages of the plant transformation procedure is presented in Figure 4.7.

4.3 Analysis of gene expression

RNA was purified from tobacco leaves using phenol/SDS extraction followed by selective precipitation. Total RNA concentration of each sample was determined by UV spectrophotometry.

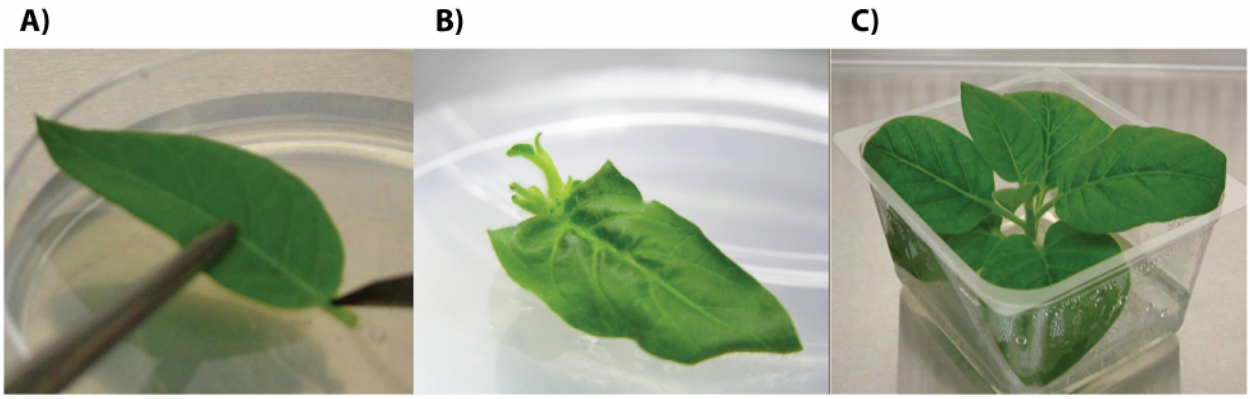

Figure 4.7: Photographs of three stages of the plant transformation process. Panel A: Wounding of the petiole of sterile *Nicotinia tabaccum* leaves with a scalpel inoculated with transformed *A. tumefaciens*. Panel B: Regeneration of shoots from the wound site after approximately three week incubation in media containing plant growth regulators promoting the formation of shoots and with selective antibiotics. Panel C: Developed transgenic plants after incubating shoots in media containing selective antibiotics.

The template DNA for preprorelaxin-specific, biotin-labeled, DNA probe was generated by PCR amplification of pBC-RLX2 with the 5PatExtFwd and 3RelExtRev primers. The amplified DNA was separated by DNA electrophoresis then purified from the agarose gel. The purified preprorelaxin DNA was labeled with biotin using the North2South biotin random prime kit.

Twenty micrograms of total RNA was resolved by electrophoresis on a formaldehyde and agarose gel and transferred to a positively-charged nylon membrane. The membrane was incubated with the preprorelaxin-specific, biotin-labeled DNA probe and the hybridized probe was detected using a streptavidin-HRP conjugate with chemiluminescent substrate.

Twelve transgenic plants, RLX3 plant lines P2, P4, P5, and P8 and RLX4 plant lines P1, P2, P3, P6, P7, P8, P9 P10, had detectable preprorelaxin mRNA levels as demonstrated in the northern blots presented in Figure 4.8. Additionally, preprorelaxin mRNA was not detected in RNA isolated from untransformed tobacco leaves. The RNA isolated from RLX4 P3 and RLX4 P6 had the highest amount of preprorelaxin mRNA.

4.4 Analysis of protein expression

Total soluble proteins of transgenic lines with the highest detectable amounts of preprorelaxin mRNA were extracted in a pH 4.0 citrate buffer supplemented with 1 mM EDTA. Five hundred microliters of each protein extract were concentrated by ultrafiltration and the protein concentrations of each concentrated sample were determined by the Bio-Rad protein assay, using BSA as a standard protein. Equal amounts of total soluble protein were resolved by SDS-PAGE electrophoresis and the samples were detected for the presence of relaxin by western blot analysis, presented in Figure 4.9.

Neither relaxin nor unique bands at a molecular weight corresponding to the predicted sizes of prorelaxin or preprorelaxin were detected in any of the transgenic lines. Additionally, the positive control demonstrated that relaxin was effectively retained during concentration, because 100% retention would yield approximately 6 ng of relaxin in the positive control which corresponds well to the western blot.

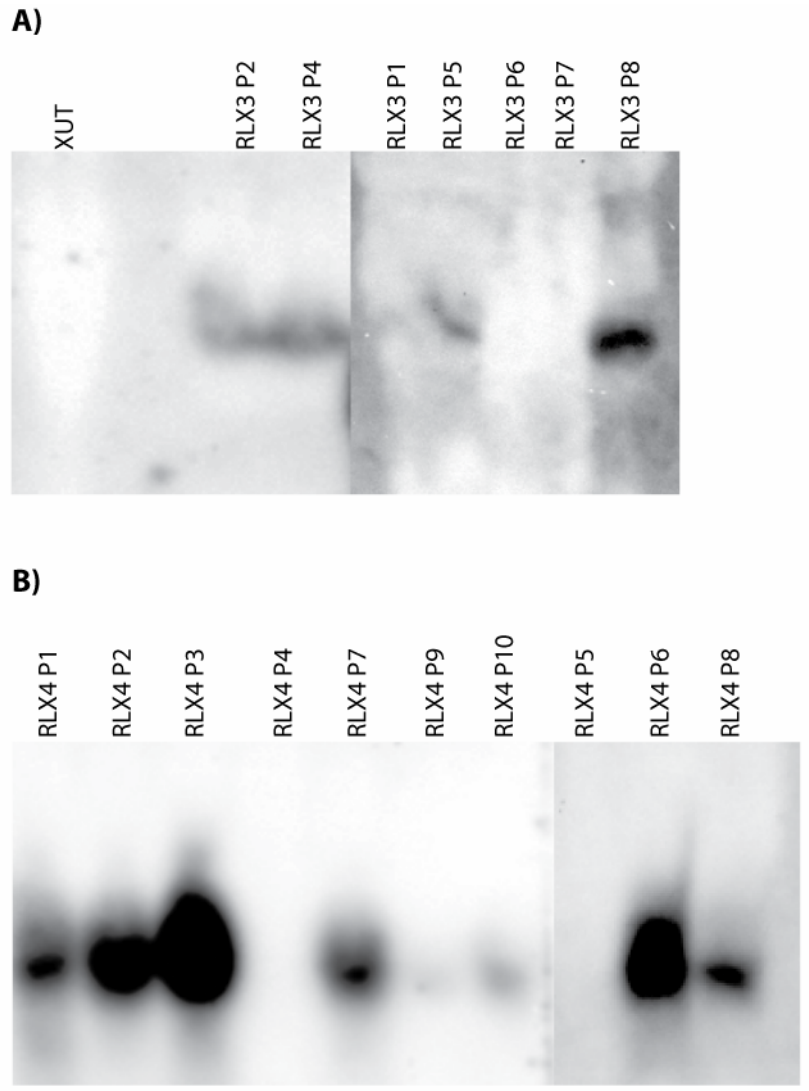

Figure 4-8: Northern blot analysis of extracted RNA from transgenic tobacco plants using a relaxin-specific, biotin-labeled, DNA probe. RNA isolated from the leaves of transgenic plant lines RLX3 P2, P4 P5 and P8 and RLX4 P1, P2, P3, P6, P7, P8, P9, and P10 had detectable amounts of preprorelaxin cDNA. Additionally, preprorelaxin mRNA was not detected in RNA isolated from Xanthi untransformed tobacco (XUT) leaves. Panel A: Northern blot analysis of RLX3 lines. Panel B: Northern blot analysis of RLX4 lines.

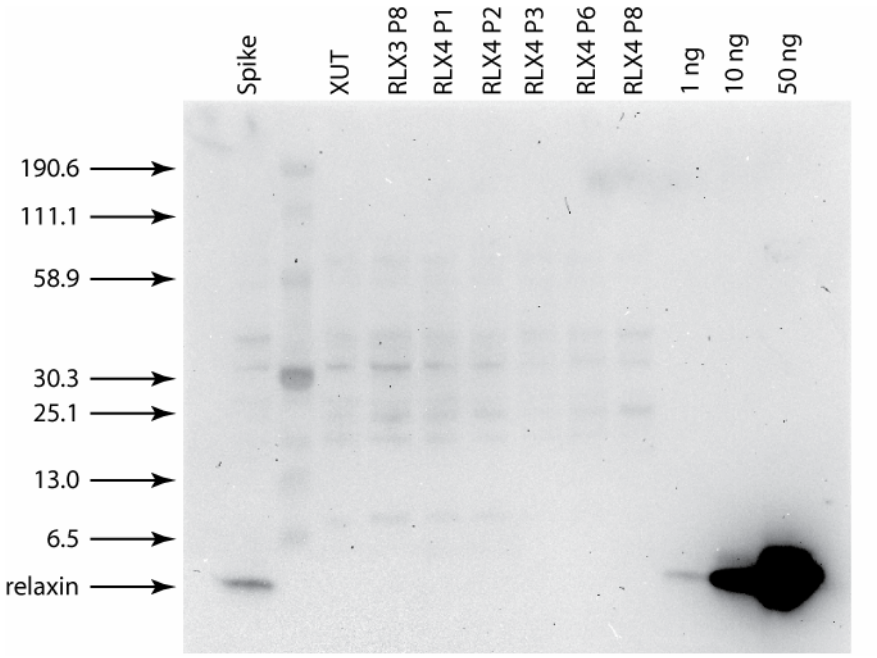

Figure 4-9: Western blot of concentrated total soluble protein extracts. Resolved proteins were transferred to 0.2 μm PVDF membrane and were probed with rabbit anti-relaxin followed by goat anti-rabbit-AP conjugate. Signal was captured using the ChemiDoc XRS. No unique proteins were detected in the transgenic lines that were not present in the untransformed tobacco sample (XUT). The positive control, which is untransformed tobacco extract spiked with relaxin during extraction (Spike), demonstrated that relaxin was retained during extraction and concentration. The last three lanes are serial dilution of porcine relaxin standard. Molecular weights (kDa) are shown on the right side of the gel.

Soluble and insoluble proteins of transgenic lines with the highest detectable amounts of preprorelaxin mRNA were extracted in 6M Guanidine-HCl. The samples were desalted and eluted in 8M urea by size exclusion chromatography. The protein concentrations of each sample were determined by the Bio-Rad protein assay, using BSA as a standard protein. Equal amounts of total protein were resolved by SDS-PAGE electrophoresis and the samples were detected for the presence of relaxin, or relaxin precursors by western blot analysis, presented in Figure 4.10.

A unique band was detected in the transgenic samples that migrated at 20.5 kDa, which is the predicted size of the recombinant preprorelaxin. Additionally, the positive control demonstrated that relaxin was effectively retained during extraction and buffer exchange.

Transgenic plant RLX4 P2 was analyzed to determine if incubation in a ubiquitin inhibitor would increase the amount of the unique protein that migrates at the predicted molecular weight of recombinant preprorelaxin. The transgenic plant RLX4 P2 and an untransformed tobacco plant were incubated in 100 μM MG132, prior to extraction with 6M Guanidine-HCl and buffer exchange into 8M urea. Soluble and insoluble proteins were extracted from RLX4 P2 and the same untransformed tobacco plant to be utilized as additional controls for the experiment. The protein concentration of each sample was determined by the Bio-Rad protein assay, using BSA as a standard protein. Equal amounts of total protein, 12.7 μg, were resolved by SDS-PAGE electrophoresis and the samples were detected for the presence of relaxin, or relaxin precursors by western blot analysis, presented in Figure 4.11.

A unique band was detected in the both transgenic samples that migrated at 20.5 kDa, which is the predicted size of the recombinant preprorelaxin. Additionally, the RLX4 P2 sample that was incubated in MG132 had 3.0 times greater amount of the unique protein.

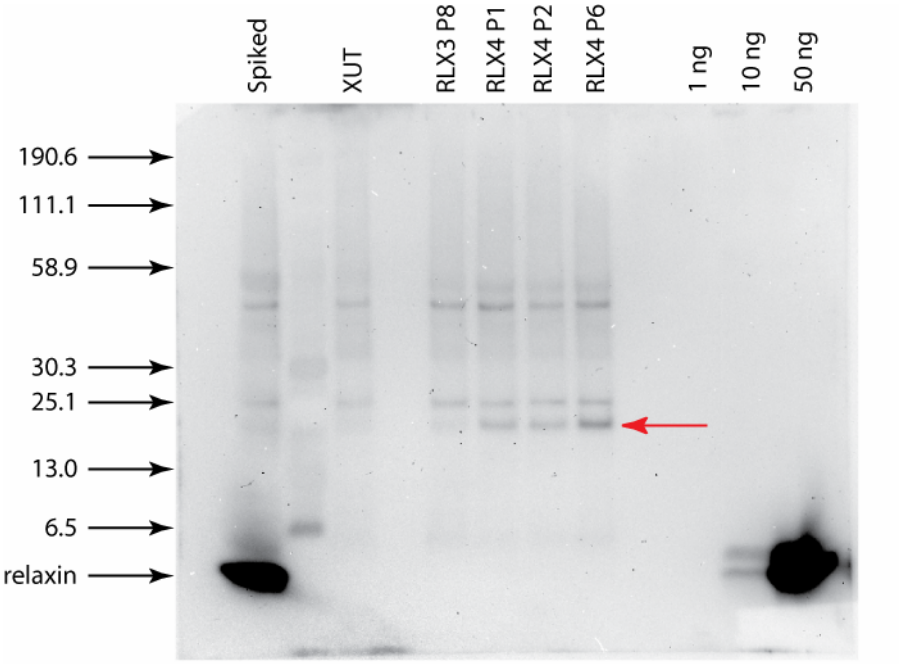

Figure 4.10: Western blot of extracted soluble and insoluble proteins from tobacco leaves. Resolved proteins were transferred to 0.2 µm PVDF membrane and were probed with rabbit anti-relaxin followed by goat anti-rabbit-AP conjugate. Signal was captured using the ChemiDoc XRS. A unique protein was detected in the transgenic lines, shown by the red arrow, that were not present in the untransformed tobacco sample (XUT). The positive control, which is untransformed tobacco extract spiked with relaxin during extraction (Spike), demonstrated that relaxin was retained during extraction and buffer exchange. The last three lanes are serial dilution of porcine relaxin standard. Molecular weights (kDa) are shown on the right side of the gel.

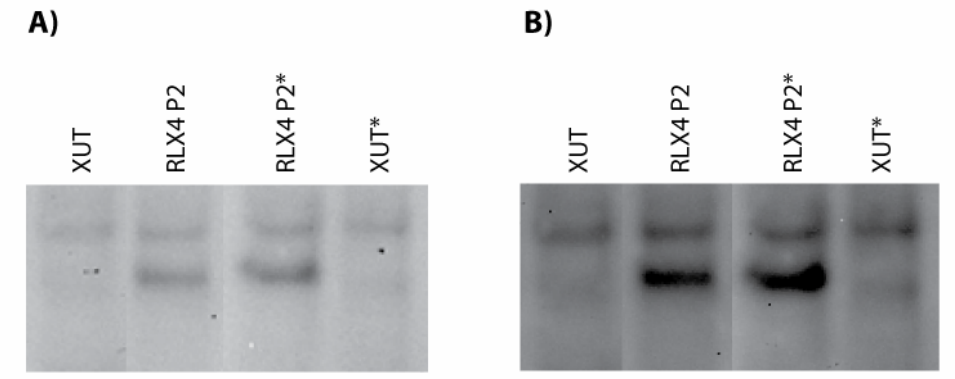

Figure 4.11: Western blot of extracted soluble and insoluble proteins from tobacco leaves. Resolved proteins were transferred to 0.2 µm PVDF membrane and were probed with rabbit anti-relaxin followed by goat anti-rabbit-AP conjugate. Signal was captured using the ChemiDoc XRS. A unique protein was detected in the transgenic line that was not present in the untransformed tobacco sample (XUT). (*) indicates samples incubated in MG132 for three hours prior to extraction. Panel A: Twenty minute exposure of membrane. Panel B: Sixty minute exposure of membrane.

CHAPTER 5

DISCUSSION

The results presented in the previous chapter demonstrated that multiple and independent transgenic tobacco plants were generated that expressed the recombinant preprorelaxin gene. Additionally, it was demonstrated that a protein of 20.5 kDa, which is the predicted size of preprorelaxin, was present in some transgenic plants but not in untransformed tobacco plants.

5.1 Construction of preprorelaxin expression vectors

DNA sequencing of the vectors used to transform the tobacco plants from the 5' and 3' ends of preprorelaxin cDNA corresponding perfectly to the preprorelaxin cDNA except for the first several base pairs adjacent to the sequencing primers. However, those residues not resolved on the chromatogram were well resolved by sequencing from the opposite direction. Figure 5.1 demonstrates this fact along the 5' end of preprorelaxin cDNA in the pBC-RLX2, which is indicative of all alignment regions that did not have a consensus along the several base pairs closest to a sequencing primer. Therefore, it was concluded that the preprorelaxin cDNA had no insertions, deletions, or mutations that would cause a change in the predicted translated protein.

Additionally, sequencing of the RLX4 plasmid demonstrated that there were no start codons (ATG) in the 5' untranslated region. Therefore, translation of the preprorelaxin mRNA would occur at the predicted start codon of the patatin signal peptide. Sequencing of the RLX3 plasmid demonstrated that there was a single start codon nine base pairs before the start codon of the patatin signal peptide, but there were no additional start codons in the 5' untranslated region. This will cause an addition of a methionine, valine, and proline residues to the N-terminus of preprorelaxin but does not cause a shift in the open reading frame of the preprorelaxin transgene.

5.2 Generation of transgenic tobacco plants

Eighteen plants were regenerated from shoots infected with *A. tumefaciens*, which contained the RLX3 or RLX4 plasmid. Each of these transgenic plants were independently transformed, since only a single shoot from each inoculated tobacco petiole was propagated into rooting media. Therefore, the integration of the relaxin transgene in the genome of each transgenic plant should be unique. Additionally, each transgenic line should have variable expression levels of the relaxin transgene, since the location of a transgene within the genome is a main source of variability of expression.

The transformation process was also performed under the conditions outlined in Section 3.2.2C, except the petiole was wounded and inoculated with *A. tumefaciens* that did not contain a binary vector, such as the RLX3 or RLX4 plasmid. This was employed as a negative control to verify that a recombinant transgene that contained the NPTII gene, which is present in the RLX3 and RLX4 plasmids, is essential for the transformation of tobacco plants under the selective regeneration conditions. In these samples, there was a significant reduction in the number of shoots that regenerated. The majority of the regenerated shoots were located on the wound site furthest away from the media. None of these shoots developed into plants, when they were transferred to rooting media. The control experiment supported the conclusion that the eighteen transgenic plants contained at least the NPTII gene of either the RLX3 or RLX4 plasmids.

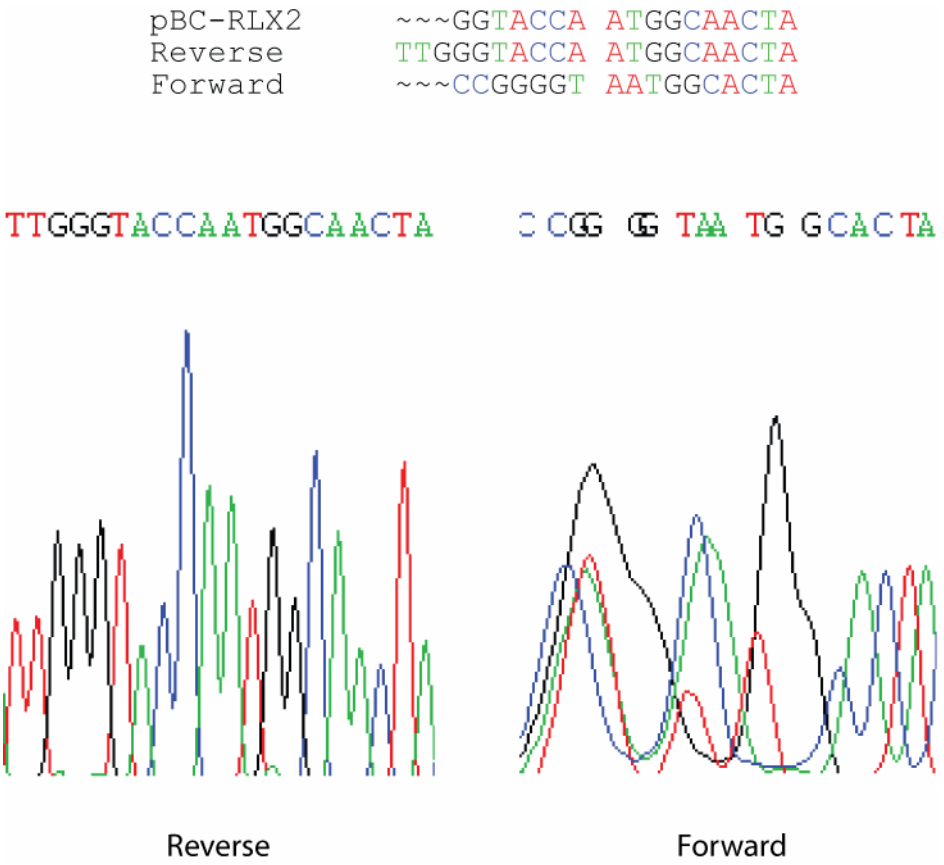

Figure 5.1: Sequence alignments and corresponding chromatograms of the 5' end of the preprorelaxin cDNA. The DNA sequenced was the pBC-RLX2 plasmid. Forward and reverse indicates alignment generated by sequencing with the T3 primer and the reverse complement of the T3 primer, respectively. Only the 5' region that did not have consensus is shown to demonstrate that the first several sequenced base pairs are not resolved (Forward) but the corresponding residues sequenced from the opposite direction are well resolved (Reverse).

The transgenic shoots and plants were cultured, for two propagations, under carbenicillin, a selective antibiotic used to eliminate the *A. tumefaciens*. It was deduced that all transgenic plants were free of *A. tumefaciens* contamination after the antibiotic selection, since none of the plants became contaminated with *A. tumefaciens* for the 12 months the plants were maintained after removal of the carbenicillin. This fact is important because the presence of preprorelaxin mRNA or relaxin protein or precursors could be detected during gene expression or protein expression analysis as a result of *A. tumefaciens* expressing the RLX3 or RLX4 transgene.

5.3 Analysis of gene expression

Twelve of the eighteen transgenic plants had detectable and variable amounts of preprorelaxin mRNA. These plant lines were: RLX3 P2, RLX3 P4, RLX3 P5, RLX3 P8, RLX4 P1, RLX4 P2, RLX4 P3, RLX4 P6, RLX4 P7, RLX4 P8, RLX4 P9, and RLX4 P10. Two northern blots were performed and the highest expressing plant lines for each blot were RLX4 P3 and RLX4 P6. Furthermore, the RLX4 transgenic plants had a generally higher expression level of the relaxin transgene than the RLX3 plants. Additionally, preprorelaxin mRNA was not detected in untransformed tobacco plants.

Four hundred picograms of digested pBC-RLX2 plasmid DNA were analyzed on both northern blots as a positive control and as a method to qualitatively compare results from both northern blots. This control was detected in both northern blots. The following plant lines generated a larger signal than the pBC-RLX2 control: RLX4 P1, RLX4 P2, RLX4 P3, RLX4 P6, and RLX4 P7. The following plant lines generated a signal comparable to the pBC-RLX2 control: RLX3 P8 and RLX4 P8. The rest of the lines that had detectable amounts of preprorelaxin cDNA generated less of a signal than the pBC-RLX2 control.

The T-DNA regions of the RLX3 and RLX4 plasmids contained two genes, the relaxin transgene and the NPTII gene. Therefore, the signal generated by transgenic plants, but was not detected in untransformed tobacco, could be attributed to either the detection of NPTII or preprorelaxin mRNA. In order to account for this fact the high stringency washes were performed such that the melting temperature (t_m) of the probe-target hybrid would be 70°C, which was determined from the T_m calculation of a DNA-RNA hybrid shown below.

$$T_m = 79.8^\circ C + 18.5\left(\log_{10}\left[Na^+\right]\right) + 58.4\left(X[GC]\right) + 11.8\left(X[GC]\right)^2 - 0.50\left(\text{formamide}\%\right) - \frac{820}{n}$$

Where: $[Na^+]$ is the molar concentration of Na, 0.0195 M.
X[GC] is the mole fraction of G & C content, 0.3661.
formamide % is the percentage of formamide, 0%.
n is length of the probe, 575 bp.

Therefore, the T_m of a probe-target hybrid would drop below the wash temperature, 50°C, if homology of the target mRNA and the probe dropped below 86%, assuming that there is a 1.4°C drop in T_m for each 1% mismatch in the probe-target hybrid. This would rule out the possibility of probe hybridizing to the NPTII mRNA, since they only share approximately 35% sequence identity to the preprorelaxin probe. The probability that the mRNA in the transgenic samples was preprorelaxin mRNA was further corroborated since the pBC-RLX2 control was also detected during the northern blot analysis.

5.4 Analysis of protein expression

Western blot analysis of the total soluble proteins extracted from the highest expressing transgenic tobacco plants, as determined by northern blot analysis, was unable to identify a unique band in any of the transgenic plant samples. However, a unique band at 20.5 kDa was detected in western blot analysis of extracted insoluble and soluble protein from the transgenic samples. This band corresponded to the predicted molecular weight of preprorelaxin. These results support the following three claims. First, the relaxin transgene is being translated, since a unique band the size of preprorelaxin was detected. Second, the preprorelaxin is not being properly processed, since a unique band at 18.5 kDa (the size of prorelaxin) or at the size of relaxin standard were not detected in the western blot analysis. Third, the recombinant preprorelaxin is not soluble in aqueous buffer and is probably not properly folded by the transgenic tobacco, since the protein was only identified in the extracts of insoluble and soluble proteins.

Further protein analysis demonstrated that a three fold greater yield of recombinant preprorelaxin could be extracted if the transgenic plants were incubated in MG132, an ubiquitin inhibitor, for three hours. This result may indicate that the recombinant protein is being tagged by ubiquitin to target the preprorelaxin for proteasomal degradation.

These results indicate that the relaxin gene is being translated, but there is no evidence suggesting that relaxin is being processed. Additionally, the results suggest that the preprorelaxin is being targeted for proteasomal degradation. The following two circumstances may explain these results. First, tobacco may not have the biochemical pathways necessary for the proteolytic processing of porcine preprorelaxin. Therefore, the recombinant preprorelaxin is targeted for proteasomal degradation. Second, the recombinant preprorelaxin may be targeted for degradation before it has a chance to be properly processed. This circumstance would prevent any conclusion pertaining to the capability of tobacco plants to properly process relaxin.

5.5 Suggestions on future research of relaxin production

The results suggest that the recombinant porcine preprorelaxin is not being properly processed into relaxin. While fully processed relaxin was not identified in transgenic plant protein extracts, the experiments do not completely refute the possibility that some of the preprorelaxin is being processed into relaxin. This is because the recombinant preprorelaxin was barely detected in the western blot analysis. If a small fraction of the preprorelaxin was processed into relaxin, it may not be detected during the protein analysis.

The hypothesis that tobacco does not have the endogenous pathways to process preprorelaxin could also be corroborated or refuted by generating new transgenic tobacco plants with a new porcine preprorelaxin transgene that contained a FLAG epitope in the surface accessible region of the C chain. This would provide an additional and more sensitive method of detecting the recombinant relaxin. For example, if western blot analysis of protein extracts from these new transgenic plants with a FLAG antibody identified only one unique protein migrating at a 20.5 kDa, it would confirm that tobacco plants cannot process the preprorelaxin. In contrast, if a unique band was detected at 12 kDa, the size of the C chain, it would suggest that the preprorelaxin is being proteolytically cleaved. The reasoning behind placing the FLAG epitope in the C chain is that proper proteolytic processing of the modified preprorelaxin would yield bona fide relaxin. This would be ideal for comparing the bioactivity of the recombinant relaxin to purified porcine relaxin standards.

The initial assumption that tobacco plants contained the appropriate biochemical pathways to properly process porcine preprorelaxin was based on the following two premises. First, that the Kex2 enzyme can proteolytically process prorelaxin into mature relaxin. This premise was supported by research by Marriott, Gillece-Castro, and Gorman (1992) that demonstrated that recombinant H2 prorelaxin is properly processed in mammalian cell cultures if recombinant Kex2 enzyme is also expressed. Second, that tobacco plants contain proteolytic enzyme that has the same substrate specificity and processing capabilities as the Kex2 enzyme. This premise was supported by research by Jiang and Rogers (1999). They demonstrated that tobacco plants contain a processing enzyme with the same proteolytic specificity as the Kex2 enzyme that was localized in the Golgi, by using a chimeric reporter protein. However, this argument could be invalidated if porcine relaxin is processed by a different biochemical pathway than its human counterpart. There was no available research that has identified the processing pathway of porcine relaxin and therefore it was assumed that the two relaxins were processed in the same manner. If more conclusive evidence is obtained that demonstrates that tobacco plants cannot process porcine relaxin it may support the possibility that porcine and human relaxins are processed differently. Additionally, this would not refute the possibility of tobacco plants being able to properly process recombinant H2 relaxin, which has greater therapeutic applications than porcine relaxin.

Therefore, it is suggested that future research concerning the expression of recombinant relaxin in plants should focus on the expression of H2 relaxin and not its porcine counterpart. I would suggest generating two different transgenic tobacco lines, both under the control of the de35S promoter with the TEV leader. The first construct would just contain codon-optimized H2 preprorelaxin cDNA. The second construct would contain codon-optimized H2 preprorelaxin cDNA with a FLAG epitope integrated in the C chain. This construct would aid in the identification of the H2 relaxin in protein extracts.

As an alternative to plant based expression of recombinant relaxin, the expression of H2 relaxin should be further investigated in *S. cerevisiae*. This expression system should yield fully processed H2 relaxin, since it has been demonstrated that the Kex2 enzyme, which is an endogenous *S. cerevisae* protein, is capable of the appropriate proteolytic processing of H2 prorelaxin. Yang *et al.* (1993) documented an attempt at producing recombinant H2 relaxin in yeast. However, they used a modified relaxin transgene, which used a DNA sequence encoding for a hexapeptide linker in lieu of the C chain, which is 104 residues in length. The expressed protein had 5% of the bioactivity in comparison to recombinant H2 relaxin standards. The hexapeptide linker may have prevented the proper association of the A and B chains of relaxin, thereby causing the decreased bioactivity of the expressed protein. Therefore, it may be worthwhile to reinvestigate the expression of recombinant H2 relaxin in *S. cerevisae*, using the original C chain instead of a peptide linker.

CHAPTER 6

CONCLUSIONS

1. Two recombinant preprorelaxin genes were constructed that contained prorelaxin cDNA, codon-optimized for expression in tobacco, fused to the patatin signal peptide cDNA under the control of either the “super” promoter or the dual enhanced CaMV 35S promoter.
2. Eighteen transgenic tobacco plants were generated that contained on of the transgenes, eight with the “super” promoter and ten with dual enhanced CaMV 35S promoter.
3. Preprorelaxin mRNA was detected in 12 of the transgenic plants.
4. The dual enhanced CaMV 35S promoter generally had higher expression levels in leaf tissue, in comparison to the “super promoter.”
5. Fully processed relaxin protein was not found in the tobacco plants that had detectable levels of preprorelaxin mRNA.
6. Preprorelaxin was identified in extracted insoluble and soluble proteins from the transgenic plants.
7. Incubation of a proteasomal inhibitor prior to extraction of insoluble and soluble proteins led to increase of preprorelaxin.

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APPENDIX A

CONSTRUCTION OF pBC-RLX2 PLASMID

The pBC-patatin and pUC57-RLX plasmids were purified from 5-mL cultures of *E. coli* containing the plasmids. The concentration of DNA in each purified preparation was determined by UV spectrophotometry and the results are presented in Table A.1. Two micrograms of each plasmid was digested with the *Xba*I and *Sac*I restriction enzymes for two hours at 37°C. The digest reaction compositions are listed in Table A.2. The DNA fragments resulting from the digestions were separated by DNA electrophoresis, presented in Figure A.1.

Table A.1: UV spectrophotometry readings of pBC-patatin and pUC57-RLX.

Sample (Name)	**Sample (μL)**	**ddH_2O (μL)**	**Abs (260 nm)**	**Abs (280 nm)**	**Abs ratio (260/280)**	**Conc (ng/μL)**
pBC-patatin	2	98	0.070	0.400	1.75	175
pUC57-RLX	2	98	0.019	0.120	1.58	48

Table A.2: Reaction compositions for digestion of pBC-patatin and pUC57-RLX. The buffer used was 10X Multicore buffer.

Sample (Name)	**Sample (μL)**	**ddH_2O (μL)**	**Buffer (μL)**	**10X BSA (μL)**	***Sac*I (μL)**	***Xba*I (μL)**
pBC-patatin	11	35	6	6	1	1
pUC57-RLX	42	4	6	6	1	1

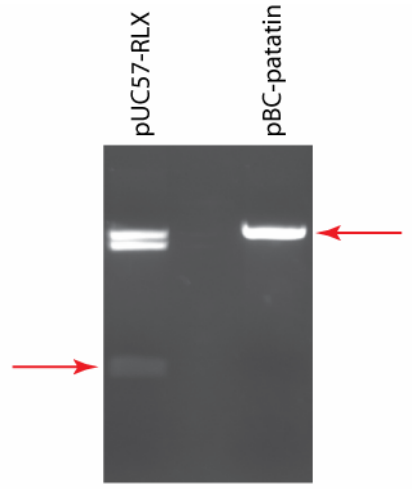

Figure A.1: Digest of pBC-patatin and pUC57-RLX with XbaI and SacI. The red arrows indicate DNA fragments excised and purified from the agarose gel. Each lane contains 20 µL of the digest reaction.

The 500 bp fragment from the pUC57-RLX digest and the 3,500 bp fragment from the pBC-patatin digest were purified from the agarose gel. The purified DNA fragments were ligated overnight at 4°C. The ligation reaction composition is listed in Table A.3. Five microliters of the ligation reaction was used to transform GC10 competent cells.

Table A.3: Reaction compositions for pBC-RLX2 ligation. Insert and vector refers to purified pUC57-RLX fragment and purified pBC-patatin fragment, respectively. Buffer is 10X T4 ligase buffer and ATP concentration was 10 mM.

Sample (Name)	Insert (µL)	Vector (µL)	Buffer (µL)	ATP (µL)	T4 ligase (µL)
pBC-RLX2	5	2.5	1	1	1

A transformed colony was used to inoculate a 5-mL culture from which the pBC-RLX2 plasmid was purified. The concentration of pBC-RLX2 DNA was determined by UV spectrophotometry, results presented in Table A.4.

Table A.4: UV spectrophotometry readings of pBC-RLX2.

Sample (Name)	Sample (µL)	ddH_2O (µL)	Abs (260 nm)	Abs (280 nm)	Abs ratio (260/280)	Conc (ng/µL)
pBC-RLX2	4	96	0.019	0.141	1.81	319

The presence of the codon-optimized relaxin cDNA in the purified plasmid was verified by PCR amplification and restriction digests, results in Figure 4.1 and Figure 4.2, respectively. The composition of the PCR reaction and the restriction digests are presented in Table A.5 and Table A.6, respectively.

Table A.5: Reaction compositions for PCR verification of pBC-RLX2. Template DNA for pBC-RLX2 and positive control samples were 3.2 ng/μL pBC-RLX2 plasmid, 4.8 ng/μL pUC57-RLX plasmid, respectively. The stock solution of primers was 10 μM.

Sample Name	**5RelExtFwd (μL)**	**3RelExtRev (μL)**	**Master Mix (μL)**	**ddH_2O (μL)**	**Template DNA (μL)**
pBC-RLX2	0.5	0.5	10	12	2
pos. ctrl.	0.5	0.5	10	12	2
neg. ctrl.	0.5	0.5	10	14	0

Table A.6: Reaction compositions for digest verification of pBC-RLX2. Three digest verifications were performed; one the XbaI only, one with SacI only, and one with both enzymes. The column sample refers to which digest was performed. The Buffer column refers to 10X Multicore Buffer.

Sample (Name)	**Sample (μL)**	**ddH_2O (μL)**	**Buffer (μL)**	**10X BSA (μL)**	***Sac*I (μL)**	***Xba*I (μL)**
*Xba*I	3.1	12.4	2	2	0.0	0.5
*Sac*I	3.1	12.4	2	2	0.5	0.0
*Xba*I & *Sac*I	3.1	11.9	2	2	0.5	0.5

APPENDIX B

CONSTRUCTION OF RLX3 AND RLX4 PLASMIDS

The pBC-RLX2 plasmid was purified from a 5-mL culture of *E. coli* containing the plasmid. The X3 and X4 plasmids were purified from four 5-mL cultures of *E. coli* containing the plasmids then were ethanol precipitated to concentrate the plasmid DNA. The concentration of DNA in each purified preparation was determined by UV spectrophotometry and the results are presented in Table B.1. Two micrograms of each plasmid was digested with the *Kpn*I and *Sac*I restriction enzymes for 90 minutes at 37°C. The digest reaction compositions are listed in Table B.2. The DNA fragments resulting from the digestions were separated by DNA electrophoresis, presented in Figure B.1. The 550 bp fragment from the pBC-RLX2 digest and the 12 kbp fragment from the RLX3 and RLX4 digests were purified from the agarose gel. The purified DNA fragments were ligated overnight at 4°C. The ligation reaction compositions are listed in Table B.3. Five microliters of the ligation reaction was used to transform GC10 competent cells.

Table B.1: UV spectrophotometry readings of pBC-RLX2, X3 and X4.

Sample (Name)	**Sample (μL)**	**ddH_2O (μL)**	**Abs (260 nm)**	**Abs (280 nm)**	**Abs ratio (260/280)**	**Conc (ng/μL)**
X3	2	98	0.086	0.047	1.83	215
X4	2	98	0.077	0.044	1.75	193
pBC-RLX2	2	98	0.101	0.058	1.74	253

Table B.2: Reaction compositions for digestion of pBC-RLX2, X3, and X4. The buffer used was 10X Multicore buffer.

Sample (Name)	**Sample (μL)**	**ddH_2O (μL)**	**Buffer (μL)**	**10X BSA (μL)**	***Sac*I (μL)**	***Kpn*I (μL)**
X3	9.3	5.7	2	2	0.5	0.5
X4	10.4	4.6	2	2	0.5	0.5
pBC-RLX2	7.9	7.1	2	2	0.5	0.5

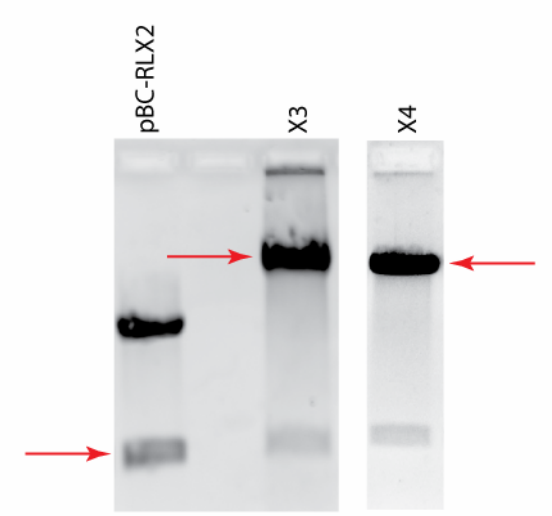

Figure B.1: Digest of pBC-RLX2, X3 and X4 with *Kpn*I and SacI. Red arrows indicate DNA bands excised and purified from the gel. The X3 and X4 samples contained 20 µL of the digest reaction and pBC-RLX2 sample contained 10 µL of the digest reaction.

The 550 bp fragment from the pBC-RLX2 digest and the 12 kbp fragment from the RLX3 and RLX4 digests were purified from the agarose gel. The purified DNA fragments were ligated overnight at 4°C. The ligation reaction compositions are listed in Table B.3. Five microliters of the ligation reaction was used to transform GC10 competent cells.

Table B.3: Reaction compositions for RLX3 and RLX4 ligations. Insert and vector refers to purified pBC-RLX2 fragment and purified X3 or X4 fragments, respectively. Buffer is 10X T4 ligase buffer and ATP concentration was 10 mM.

Sample (Name)	Insert (µL)	Vector (µL)	Buffer (µL)	ATP (µL)	T4 ligase (µL)
RLX3	3	5	1	0.5	0.5
RLX4	2.8	5.2	1	0.5	0.5

The presence of preprorelaxin cDNA in the transformed colonies was verified by PCR amplifications of individual colonies, results in Figure 4.3. The compositions of PCR verification reactions are presented in Table B.4.

Table B.4: Reaction compositions for PCR verification of RLX3 and RLX4. Template DNA for RLX3, RLX4 and positive control samples were provided from individual colonies containing the plasmids. The stock solution of primers was 10 µM.

Sample (Name)	5PatExtFwd (µL)	3RelExtRev (µL)	Master Mix (µL)	ddH_2O (µL)
RLX3	0.5	0.5	10	14
RLX4	0.5	0.5	10	14
pos. ctrl.	0.5	0.5	10	12
neg. ctrl.	0.5	0.5	10	14

Isolated colonies, RLX3-1 and RLX4-2, were used to inoculate four 5-mL cultures. The plasmids were purified from the cultures and concentrated by ethanol precipitation. The concentrations of plasmid DNA are presented in Table B.5.

Table B.5: UV spectrophotometry readings of RLX3 and RLX4.

Sample (Name)	**Sample (μL)**	**ddH_2O (μL)**	**Abs (260 nm)**	**Abs (280 nm)**	**Abs ratio (260/280)**	**Conc (ng/μL)**
RLX3	2	98	0.116	0.065	1.78	116
RLX4	2	98	0.187	0.108	1.73	187

APPENDIX C

TRANSFORMATION OF *AGROBACTERIUM TUMEFACIENS*

Agrobacterium tumefaciens strain LBA4404 were transformed with one microgram of the RLX3 (8.62 μL) and the RLX4 (5.34 μL) plasmid, as described in section 3.2.2B. The presence of the RLX3 or RLX4 plasmids and the "disarmed" virulence plasmid in the transformed *A. tumefaciens* were verified by PCR amplification, results presented in Figure 4.6. The compositions of the PCR amplification reactions are presented in Table C.1 and C.2. Individual colonies RLX3-5 and RLX4-1 were used to inoculate tobacco petioles.

Table C.1: Reaction compositions for PCR verification of "disarmed" virulence plasmid in transformed *A. tumefaciens*. Template DNA was individual colonies of *A. tumefaciens* transformed with the RLX3 or the RLX4 plasmid. The stock solution of primers was 10 μM.

Sample (Name)	**VirD2A (μL)**	**VirD2E (μL)**	**Master Mix (μL)**	**ddH_2O (μL)**
RLX3	0.5	0.5	10	14
RLX4	0.5	0.5	10	14

Table C.2: Reaction compositions for PCR verification of RLX3 or RLX4 plasmid in transformed *A. tumefaciens*. Template DNA was individual colonies of *A. tumefaciens* transformed with the RLX3 or the RLX4 plasmid. The stock solution of primers was 10 μM.

Sample (Name)	**5PatExtFwd (μL)**	**3RelExtRev (μL)**	**Master Mix (μL)**	**ddH_2O (μL)**
RLX3	0.5	0.5	10	14
RLX4	0.5	0.5	10	14

APPENDIX D

NORTHERN BLOT ANALYSIS

The pBC-RLX2 plasmid was PCR amplified with the 5PatExtFwd and 3RelExtRev primers. The composition of the PCR reaction is presented in Table D.1. The amplified DNA was separated from the oligonucleotides by DNA electrophoresis, results presented in Figure D.1.

Table D.1: Compositions of PCR reaction for template synthesis. DNA used for pBC-RLX2 sample was a solution of 4 ng/μL pBC-RLX2. The stock concentrations of the primers was 10 μM.

Sample (Name)	5PatExtFwd (μL)	3RelExtRev (μL)	Master Mix (μL)	ddH_2O (μL)	DNA (μL)
pBC-RLX2	1	1	20	26	2
neg. ctrl.	1	1	20	28	0

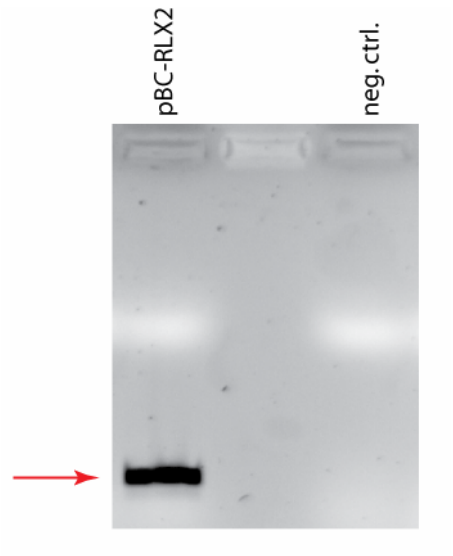

Figure D.1: Generation of template DNA for probe synthesis. Red arrow indicates DNA excised and purifed from the gel. Each sample contains 25 μL of the corresponding PCR reaction.

The 550 bp DNA fragment was purified from the agarose gel. The relaxin-specific, biotin-labeled DNA probe was generated using the North2South biotin random prime kit, as described in Section 3.2.3B, with the purified DNA.

Total RNA was purified from tobacco leaves as described in Section 3.2.3A. The concentration of RNA in each sample is presented in Table D.2. The northern blot was performed as described in Section 3.2.3C.

Table D.2: UV spectrophotometry readings of RNA samples. Total RNA concentration (ng/μL) was determined by multiplying the absorbance reading at 260 nm by 40 and dividing by the dilution of the sample. XUT is RNA purified from untransformed xanthi tobacco leaves.

Sample (Name)	**Sample (μL)**	**ddH_2O (μL)**	**Abs (260 nm)**	**Conc (mg/mL)**
XUT	1	99	0.859	3.4
RLX3 P1	1	99	0.478	1.9
RLX3 P2	1	99	0.855	3.4
RLX3 P4	1	99	1.129	4.5
RLX3 P5	1	99	0.500	2.0
RLX3 P6	1	99	0.539	2.2
RLX3 P7	1	99	0.560	2.2
RLX3 P8	1	99	0.475	1.9
RLX4 P1	1	99	1.430	5.7
RLX4 P2	1	99	1.111	4.4
RLX4 P3	1	99	1.037	4.1
RLX4 P4	1	99	1.378	5.5
RLX4 P5	1	99	0.545	2.2
RLX4 P6	1	99	0.491	2.0
RLX4 P7	1	99	1.078	4.3
RLX4 P8	1	99	0.530	2.1
RLX4 P9	1	99	0.904	3.6
RLX4 P10	1	99	0.779	3.1

APPENDIX E

TOTAL SOLUBLE PROTEIN ANALYSIS

Total soluble protein was extracted from the highest expressing lines, as determined by northern blot analysis, and then concentrated, as described in Section 3.2.4A. The procedure was repeated for untransformed tobacco leaves, as a negative control, and with untransformed tobacco leaves spiked at a concentration of 0.33 ng/μL, as a positive control. The final predicted concentration of relaxin in the positive control is predicted to be 2.56 ng/μL, assuming 100% recovery during concentration. The protein concentration of each extract was determined by Bio-Rad Protein Assay as described in Section 3.2.4B. The absorbance reading of the BSA standards and the corresponding BSA standard curve are presented in Table E.1 and Figure E.1, respectively. The absorbance readings and calculated concentrations for the extracts are presented in Table E.2.

Table E.1: Protein assay absorbance readings of BSA standards. Standards were diluted in pH 4.0 extraction buffer.

Sample (Name)	BSA Conc (ng/μL)	Abs (595 nm)	Abs (595 nm)	Abs (Average)
A	850	1.343	1.331	1.337
B	700	1.219	1.219	1.219
C	550	1.099	1.111	1.105
D	400	0.952	0.926	0.939
E	250	0.783	0.791	0.787

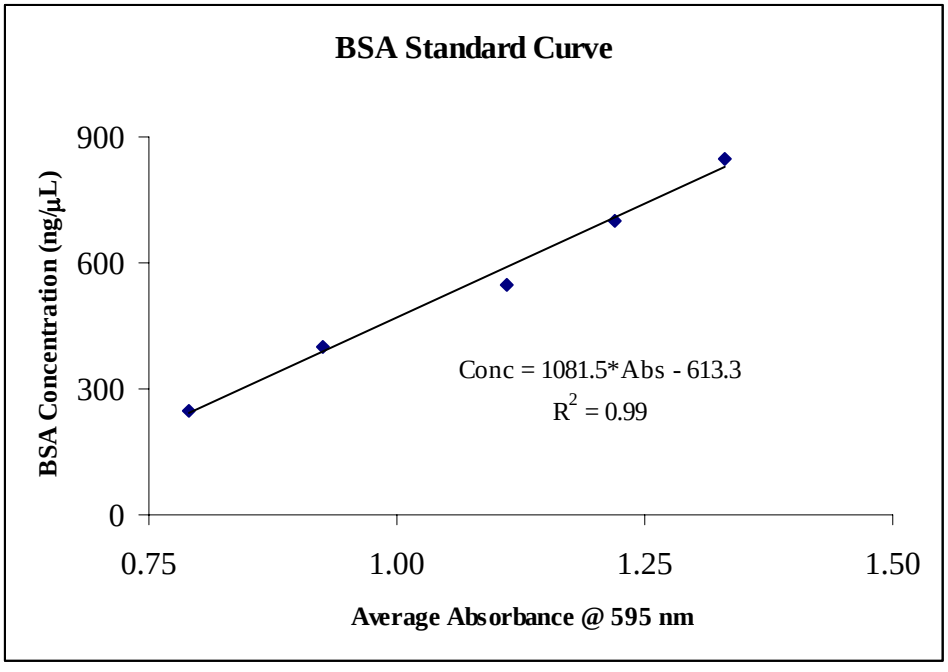

Figure E.1: BSA standard curve. BSA standards were diluted in pH 4.0 extraction buffer.

Table E.2: Protein assay absorbance readings of total soluble protein extracts. Concentrations calculated based on linear regression of BSA standards. XUT sample is untransformed xanthi extract and Spiked sample is XUT spiked with relaxin at an initial concentration of 0.33 ng/mL and a predicted final concentration of 2.56 ng/μL, assuming 100% recovery during concentration.

Sample (Name)	Sample (μL)	Dilutant (μL)	Abs (595 nm)	Abs (595 nm)	Abs (Average)	Conc (ng/μL)
XUT	10	10	0.710	0.708	0.709	305
Spiked	10	10	0.775	0.758	0.767	412
RLX3 P8	10	10	0.736	0.711	0.724	311
RLX4 P1	10	10	0.608	0.606	0.607	85
RLX4 P2	10	10	0.625	0.625	0.625	126
RLX4 P3	10	10	0.657	0.644	0.6505	166
RLX4 P6	10	10	0.772	0.764	0.768	423
RLX4 P8	10	10	0.686	0.674	0.68	231

Equal amounts of protein, 1 μg per sample, was resolved by SDS-PAGE analysis and detected for relaxin by western blot analysis, as described in Section 3.2.4C and with results presented in Figure 4.9. The composition of each sample loaded onto the SDS-PAGE gel is presented in Table E.3.

Table E.3: Compositions of total soluble protein extracts for SDS-PAGE electrophoresis. DTT was from a 500 mM stock solution. Relaxin stock of 100 pg/μL, 1 ng/μL, and 10 ng/μL were sources of relaxin for the 1 ng, 10 ng, and 50 ng relaxin samples, respectively.

Sample (Name)	Sample (μL)	ddH_2O (μL)	4X LDS (μL)	DTT (μL)
XUT	3.28	9.72	5	2
Spiked	2.43	10.57	5	2
RLX3 P8	3.22	9.78	5	2
RLX4 P1	11.75	1.25	5	2
RLX4 P2	7.95	5.05	5	2
RLX4 P3	6.01	6.99	5	2
RLX4 P6	2.36	10.64	5	2
RLX4 P8	4.34	8.66	5	2
1 ng relaxin	10	3	5	2
10 ng relaxin	10	3	5	2
50 ng relaxin	5	8	5	2

APPENDIX F

SOLUBLE AND INSOLUBLE PROTEIN ANALYSIS

Soluble and insoluble proteins were extracted from transgenic plant leaves and desalted and eluted into 8M urea, as described in Section 3.2.4A. The procedure was repeated for untransformed tobacco leaves, as a negative control, and with untransformed tobacco leaves spiked at a concentration of 2 ng/μL, as a positive control. The predicted final concentration of relaxin in the positive control is 1.5 ng/μL, assuming 100% recovery during the buffer exchange procedure. The protein concentration of each extract, after buffer exchange, was determined by the Bio-Rad Protein Assay, as described in Section 3.2.4B. The absorbance readings of the BSA standards and the corresponding BSA standard curve are presented in Table F.1 and Figure F.1, respectively. The absorbance readings and calculated concentrations for the extracts are presented in Table F.2

Table F.1: Protein assay absorbance readings of BSA standards. Standards were diluted in 8M urea.

Sample (Name)	BSA Conc (ng/μL)	Abs (595 nm)	Abs (595 nm)	Abs (Average)
A	850	0.973	0.946	0.960
B	700	0.796	0.839	0.818
C	550	0.684	0.675	0.680
D	400	0.557	0.537	0.547
E	250	0.384	0.364	0.374

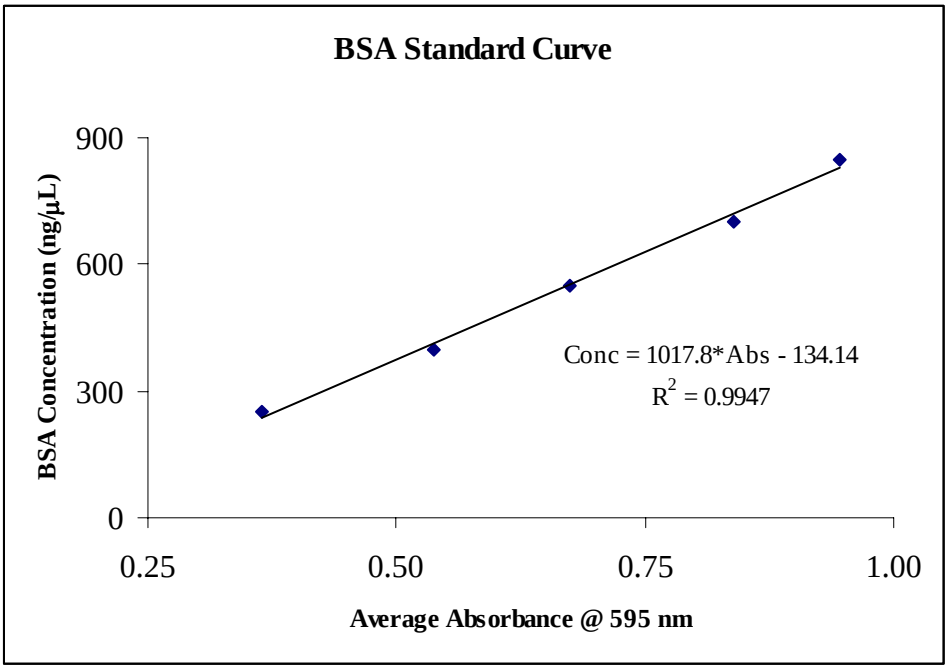

Figure F.1: BSA standard curve. BSA standards were diluted in 8M urea.

Table F.2: Protein assay absorbance readings of extractions of insoluble and soluble proteins from tobacco leaves. Concentrations calculated based on linear regression of BSA standards. XUT sample is untransformed xanthi extract and Spiked sample is XUT spiked with relaxin at an initial concentration of 2 ng/mL and a predicted final concentration of 1.5 ng/μL, assuming 100% recovery during buffer exchange.

Sample (Name)	Sample (μL)	Dilutant (μL)	Abs (595 nm)	Abs (595 nm)	Abs (Average)	Conc (ng/μL)
XUT	5	15	0.533	0.527	0.530	1596
Spiked	5	15	0.589	0.554	0.572	1768
RLX3 P8	5	15	0.837	0.836	0.837	2869
RLX4 P1	5	15	0.517	0.516	0.517	1540
RLX4 P2	5	15	0.508	0.507	0.508	1502
RLX4 P6	5	15	0.633	0.633	0.633	2023

Equal amounts of protein, 12.7 μg per sample, was resolved by SDS-PAGE analysis and detected for relaxin by western blot analysis, as described in Section 3.2.4C with results presented in Figure 4.10. The composition of each sample loaded onto the SDS-PAGE gel is presented in Table F.3.

Table F.3: Compositions of extracted soluble and insoluble proteins from tobacco leaves for SDS-PAGE electrophoresis. DTT was from a 500 mM stock solution. Relaxin stock of 100 pg/μL, 1 ng/μL, and 10 ng/μL were sources of relaxin for the 1 ng, 10 ng, and 50 ng relaxin samples, respectively.

Sample (Name)	Sample (μL)	ddH_2O (μL)	4X LDS (μL)	DTT (μL)
XUT	7.97	5.81	5	2
Spiked	7.19	5.03	5	2
RLX3 P8	4.43	8.57	5	2
RLX4 P1	8.26	4.74	5	2
RLX4 P2	8.47	4.53	5	2
RLX4 P6	6.29	6.71	5	2
1 ng relaxin	10	3	5	2
10 ng relaxin	10	3	5	2
50 ng relaxin	5	8	5	2

APPENDIX G

UBIQUITIN INHIBITOR ASSAY

Leaves from RLX4 P2 and untransformed tobacco were incubated in 100 mM MG132 for three hours. Soluble and insoluble proteins were extracted from RLX4 P2 and untransformed tobacco leaves that were incubated in the ubiquitin inhibitor and desalted and eluted into 8M urea. Additionally, soluble and insoluble proteins were extracted from RLX4 P2 and untransformed tobacco leaves that were not incubated in the ubiquitin inhibitor in the same manner. The protein concentration of each extract, after buffer exchange, was determined by the Bio-Rad Protein Assay, as described in Section 3.2.4B. The absorbance readings of the BSA standards and the corresponding BSA standard curve are presented in Table F.1 and Figure F.1, respectively. The absorbance readings and calculated concentrations for the extracts are presented in Table G.1.

Table G.1: Protein assay absorbance readings of samples for ubiquitin inhibitor assay. Concentrations calculated based on linear regression of BSA standards. XUT sample is untransformed xanthi extract and (MG132) indicates samples incubated in MG132 prior to extraction.

Sample (Name)	Sample (μL)	Dilutant (μL)	Abs (595 nm)	Abs (595 nm)	Abs (Average)	Conc (ng/μL)
XUT	5	15	0.533	0.527	0.530	1596
RLX4 P2	5	15	0.508	0.507	0.508	1502
XUT (MG132)	10	10	0.653	0.685	0.669	1087
RLX4 P2 (MG132)	10	10	0.605	0.629	0.617	979

Equal amounts of protein, 12.7 μg per sample, was resolved by SDS-PAGE analysis and detected for relaxin by western blot analysis, as described in Section 3.2.4C with results presented in Figure 4.11. The composition of each sample loaded onto the SDS-PAGE gel is presented in Table G.2. Volume analysis comparing the density difference of the unique bands in RLX4 P2 samples that were or were not incubated in MG132 are presented in Table G.3.

Table G.2: Compositions of samples for ubiquitin inhibitor assay for SDS-PAGE electrophoresis. DTT was from a 500 mM stock solution and (MG132) indicates samples incubated with MG132 prior to extraction.

Sample (Name)	Sample (μL)	ddH_2O (μL)	4X LDS (μL)	DTT (μL)
XUT	7.97	5.81	5	2
RLX4 P2	8.47	4.53	5	2
XUT (MG132)	11.71	1.29	5	2
RLX4 P2 (MG132)	13.00	0.00	5	2

Table G.3: Volume analysis of unique bands in RLX4 P2 sample. The analysis was repeated five times to get a more accurate analysis. (MG132) indicates sample incubated with MG132 prior to extraction and ratio refers to the density of the unique band in the RLX4 P2 sample that was incubated in MG132 to the density of the unique band in the RLX4 P2 sample that was not incubated in MG132.

Sample (Name)	**Density (INT*mm^2)**	**Density (INT*mm^2)**	**Density (INT*mm^2)**	**Density (INT*mm^2)**	**Density (INT*mm^2)**	**Density (Average)**
RLX4 P2	637	793	1351	637	793	842.2
RLX4 P2 (MG132)	1930	2621	3532	1930	2621	2526.8
Ratio	3.0	3.3	2.6	3.0	3.3	3.0

APPENDIX H

WHEAT GERM CELL-FREE PROTEIN SYNTHESIS

The ability of plants to process preprorelaxin could be corroborated by the utilization of a wheat germ cell-free protein synthesis system to express the recombinant preprorelaxin transgene. Cell-free protein synthesis systems are an alternative to produce proteins by chemical synthesis or by *in vivo* expression of recombinant transgenes. An efficient and stable plant-based, cell-free protein system has been developed from wheat germ. Wheat embryos are washed extensively to remove the endosperm which contains several inhibitors of protein synthesis such as ribosome-inactivating proteins, thionins, and ribonucleases. The washed embryos are extracted in an aqueous buffer supplemented with additives necessary for translation, such as all 20 amino acids, protease inhibitors, creatine phosphate, ATP, GTP, and mRNA encoding for the protein of interest. Properly folded and bioactive dihydrofolate reductase, green fluorescent protein, and luciferase have been successfully produced using the wheat germ cell-free protein synthesis system with yields as high as 4 mg of protein per 1-mL reaction (Madin et al., 2000).

This system has the benefit of higher accumulation levels of recombinant protein, in comparison to *in vivo* expression in tobacco plants. The low expression of the recombinant protein in tobacco may be hindering the identification of fully processed relaxin. Therefore, if relaxin is produced using the wheat germ system it could be extrapolated that other plant systems, such as tobacco, should contain the appropriate biochemical pathways to process porcine preprorelaxin.

APPENDIX I

CODON-OPTIMIZED PRORELAXIN DNA SEQUENCE

Porcine prorelaxin cDNA and codon-optimized prorelaxin DNA sequences are presented in Figure I.1. The codon-optimized DNA sequence was cloned in the pUC-57 plasmid at the *Xba*I and *Sac*I restriction enzyme sites.

```
Prorelaxin cDNA       CAGA GTAACGAACG ATTTATTAAG GCATGCGGCC GAGAATTAGT
Codon optimized TCTAGACAAT CTAATGAGAG GTTTATTAAG GCTTGTGGTA GGGAATTGGT

Prorelaxin cDNA CCGTCTGTGG GTGGAGATCT GTGGCTCCGT CTCCTGGGGA AGAACTGCTC
Codon optimized TAGACTTTGG GTTGAGATTT GCGGATCTGT TTCATGGGGT AGAACAGCTC

Prorelaxin cDNA TCAGCCTGGA AGAGCCTCAG CTGGAAACTG GACCCCCGGC AGAAACCATG
Codon optimized TTTCTTTGGA GGAGCCACAA CTTGAAACTG GACCTCCTGC TGAAACTATG

Prorelaxin cDNA CCATCCTCCA TCACCAAAGA TGCAGAAATC TTAAAGATGA TGTTGGAATT
Codon optimized CCTTCATCAA TTACTAAGGA TGCAGAAATT CTTAAAATGA TGCTTGAATT

Prorelaxin cDNA TGTTCCTAAT TTGCCACAGG AGCTGAAGGC AACATTGTCT GAGAGGCAAC
Codon optimized CGTTCCAAAT CTTCCACAGG AACTTAAAGC TACATTGTCA GAGAGACAAC

Prorelaxin cDNA CATCACTGAG AGAGCTACAA CAATCTGCAT CAAAGGATTC GAATCTTAAC
Codon optimized CTTCTCTTAG AGAATTGCAA CAATCAGCTC AAAAGGATTC TGCTTTGAAT

Prorelaxin cDNA TTTGAAGAAT TTAAGAAAAT TATTCTTAAC AGACAAAATG AAGCAGAAGA
Codon optimized TTTGAAGAGT TCAAAAAGAT TATTCTTAAT AGGCAAAATG AGGCTGAAGA

Prorelaxin cDNA CAAAAGTCTT TTAGAATTAA AAAACTTAGG TTTAGATAAA CATTCCAGAA
Codon optimized TAAATCTTTG TTGGAGTTGA AAAATCTTGG TCTTGATAAA CATTCAAGAA

Prorelaxin cDNA AAAAGAGACT GTTCCGTATG ACACTGAGCG AGAAATGTTG TCAAGTAGGT
Codon optimized AAAAGAGGCT TTTTAGGATG ACTCTTTCTG AAAAGTGCTG TAATGTTGGT

Prorelaxin cDNA TGTATCAGAA AAGATATTGC TAGATTATGC TGA
Codon optimized TGTATTAGAA AAGATATTGC TAGGTTGTGT TAAGAGCTC
```

Figure I.1: Sequences of porcine prorelaxin and codon-optimized prorelaxin cDNA. The *Xba*I and *Sac*I restriction enzyme sites are underlined and are located at the 5' and 3' ends of the sequence, respectively. Base pairs in red were changed to generate codon-optimized prorelaxin cDNA.

VITA

W. Scott Buswell was born in Cheverly, Maryland on May 22, 1978. He received a Bachelor of Science in Biological Systems Engineering with the bioprocess engineering option from Virginia Tech in 2004. Scott completed his Master of Science degree in Biological Systems Engineering in May 2006, under the guidance and direction of Dr. Chenming Zhang.