Chapter I. LITERATURE REVIEW

I.1 Cystic Fibrosis

I.1.1 Cystic Fibrosis - The Disease

Cystic fibrosis (CF) is a severe, autosomal recessive disease that affects humans, especially the Caucasian population (Cystic Fibrosis Foundation, CFF, 2003; Oreinstein et al., 2002). To date, the underlying basis of the disease is not fully understood, but is known to be caused by mutations in the CFTR protein (Ko and Pedersen, 2001). CF is considered the most important genetic pediatric disorder, since it is common and fatal in childhood and young adult life with a mean survival time of 32 years in the US (Ko and Pedersen, 2001; CFF 1999). Roughly one in 3000 births in Caucasian populations are diagnosed with the disease. There are about 30,000 CF patients and nearly 1 million carriers of the disease in the United States (Welsh et al., 1995; Welsh and Ramsey, 1998). Although primarily a disease of Caucasian populations, other races have lower frequencies of CF as in the Middle East (Kabra et al., 1996; Rawashdeh et al., 2000; Banjar 1999) and Asia (Imaizumi 1995; Wagner et al., 1999). The deletion (Δ) of a single phenylalanine (F) in position 508 within the first putative nucleotide binding domain is the most common mutation in nearly all populations (CFF; Kabra et al., 1996). Other mutations, over 900, result in varying degrees of symptoms.

I.1.2 Symptoms of CF

The major clinical manifestations of CF disorder are chronic pulmonary disease, pancreatic insufficiency and increased chloride ion concentration in sweat (Ratjen and Doring, 2003). CF affects the lungs, pancreas, liver, salivary glands, skin and reproductive organs (Oreinstein et al., 2002). The epithelial cell layers of these organs are affected by reduced chloride and sodium movement across the plasma membrane. The buildup of mucus viscosity severely compromises the functions of the pancreas and liver that secrete needed enzymes for digestion (Naruse et al., 2002). This results in large appetites but low nutrient absorption leading to malnutrition and poor weight gain. In males, the blockage of the epididymis and vas deferens cause azoospermia and sterility (Dodge, 1995). Commonly CF patients die from lung complications. The mucus lining of the CF lung is thickened and viscous causing persistent cough and wheezing. This is the most common phenotype of CF patients. The reduced vitality of the lungs leads to pneumonia and chronic infections by *Staphylococcus aureus* and *Pseudomonas*

aeruginosa (Abman et al., 1991). Bacteria multiply on and interact with the CF airway cells, which are unable to facilitate the natural killing of these invaders (Knowles and Boucher, 2002).

I.1.3 Detection of CF Disease

The increased chloride (Cl^-) ion concentration of sweat has been a cornerstone to the diagnosis of CF (CFF). Traditionally, a sample of a baby's sweat was analyzed for an increased salt concentration compared to a normal baby's sweat. With the advent of molecular tools and discovery of the gene responsible for the disease, new more concise diagnostic tests can be used along with the sweat test to detect CF. Most of CFTR mutations can be detected with commercial screening panels (Rosenstein and Cutting, 1998). A sample of blood is taken and genomic DNA is isolated and analyzed for mutations in the CFTR gene. Both of these tools for diagnosis are not perfect and other indirect tests must also be performed. Detection of pancreatic insufficiency and reduced levels of fecal chymotrypsin or pancreas-specific elastase can confirm the disorder (Phillips et al., 1999). Prenatal screens can be performed and carrier testing has been recommended for individuals who have a family member diagnosed with CF. In Europe, some parental and populational screenings have been carried out for the mutation (ΔF508) to analyze the percentage of carriers, people who are at least heterozygous for CF.

I.1.4 Treatment of CF

There is not a cure for CF, but many of the symptoms can be treated. Although increased sweat Cl^- concentration has been used to diagnose CF for over forty years, it does not contribute to mortality like pancreatic insufficiency and chronic lung infection. Antibiotics are administered to combat the constant attack of the bacteria in the lungs, but the development of antibiotic-resistant bacteria in chronic infections is common especially in adult patients. Chest percussions are performed regularly to loosen up the mucus in the lungs (CFF). DNase treatments aid in pulmonary function (Fuchs et al., 1994). Pancreatic enzyme and vitamin supplements are taken to aid in proper digestion and uptake of food (Ratjen and Dorring, 2003). These modern treatment strategies have prolonged some CF patients' lives into mature adulthood, but only treat the symptoms.

To date, there is no "magic bullet" for effectively treating chronic lung infections. Following discovery of the cystic fibrosis transmembrane conductance regulator (CFTR)

gene and its most common mutation, it was hoped that CF could be cured by gene replacement therapy. The development of a therapy cure has been hampered by difficult experimental and safety problems (Welsh and Ramsey, 1998; Zeitlin, 2000). The use of viral and liposomal vectors for introduction of CFTR into airway epithelial cells has only worked transiently at best in human trials (Zabner, et al., 1997; Welsh, 1999; Harvey, et al., 1999). The interest in a more conservative approach leading to new insights into CFTR structure/function and dysfunction has arisen after attempting to " quick fix what is broken." Renewed attempts to do *in vitro* work on CFTR protein may provide insight into the chemical, molecular, and pathogenic basis of CF making possible the development of novel, rational and cost effective strategies to identify agents that correct the structural defect.

I.2 CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (CFTR)

I.2.1 CFTR Gene

In 1989, the location of the CF gene was determined by chromosome walking and jumping (Rommens, et al., 1989). The CF locus is located on chromosome 7 band q31, spans 250 kb, and contains a minimum of 24 exons. Nearly concurrently, the CFTR cDNA was cloned and characterized by the same group from cultured epithelial cells (Riordan, et al., 1989). The mRNA length is about 6500 nt comprising a 6219 nt coding region plus poly (A) tail and 3' non-translated regions. Transcripts were found in the pancreas, nasal polyps, lungs, colon, sweat glands, placenta, and liver. Transcripts of the same length were found in both normal humans and CF patients. The modeling of the CFTR gene product revealed that it contained 12 membrane domains, 2 ATP binding domains and a regulatory domain. The ΔF508 mutation is in the first ATP binding domain. In 1991, the genomic sequence was determined, but it was more complex than previously thought and contained 27 exons and 26 introns (Zielenski, et al., 1991). There are many other animal species that have homologous genes to the human CFTR. The predicted protein is 1480 amino acids with a predicted mass of 168,138 daltons.

I.2.2 CFTR Protein

From the proposed structure and based on amino acid homology, the human CFTR protein is classified into the ATP Binding Cassette (ABC) superfamily of transporter proteins (review by Sheppard and Welsh, 1999). Eukaryotes, including plants,

and prokaryotes have ABC transporters. There are conserved sequences in the ATP binding regions of all ABC transporter proteins that are known as Walker A and B motifs and ABC signature motifs (Dean et al., 2001; Theodoulou, 2001). These transporters require energy to move their target molecules (anions, cations, alkaloids, sugars, lipids, steroids or conjugated proteins) across membranes. *Arabidopsis thaliana* contain 129 open reading frames (ORFs) with the signature ABC motifs, twice the number of the ABC transporters of humans which have 51 ORFs (Sanchez-Fernandez et al., 2001). CFTR has homologues in many species especially in mammals, but not in *Arabidopsis* plants (Theodoulou, 2000). CFTR is closely related to the multi-drug resistance associated protein (MRP) type of transporter. The structure of CFTR contains a membrane spanning domain (MSD1), a nucleotide binding domain (NBD1), and a regulatory domain (R), then another membrane spanning domain (MSD2), and a nucleotide binding domain (NBD2) (Fig. I.1). The nucleotide binding domains and the regulatory domain are on the intracellular side of the plasma membrane and control the opening of the pore. Hydrolysis of cyclic AMP or ATP by the NBDs and phosphorylation of the regulatory domain are necessary for anion transport through the CFTR pore. There is accumulating evidence for the interaction of CFTR with other proteins. The epithelial sodium channel (ENaC) seems to be repressed by CFTR (Kunzelmann, 2003; Stutts et al., 1997). A renal potassium channel is also modulated by CFTR (McNicholas et al., 1996). These associations between CFTR and other proteins are not well characterized and further studies are needed.

CFTR functions as a chloride anion channel and all the domains work in concert for proper function and cell localization in the cell. The anion selectivity was determined using patch-clamp experiments where $Br^- \geq Cl^- > I^- > F^-$ (Anderson et al., 1991). The NBDs act as gatekeepers and work together to open and close the gate. The R domain and extracellular loop 1 seem to play a critical role in the selectivity of the pore (Sheppard et al., 1993). The binding of ATP to the NBD's and phosphorylation of the regulatory domain by protein kinase A or C are necessary for opening of the channel. After the ATP energy expenditure of the NBD's, the channels close. The CFTR channel moves anions outwardly from the cytosol to the apoplastic space.

CFTR is a glycoprotein with 2 N-linked glycosylation sites on the extracellular loop between membrane spans 7 and 8. The protein can be detected in three different

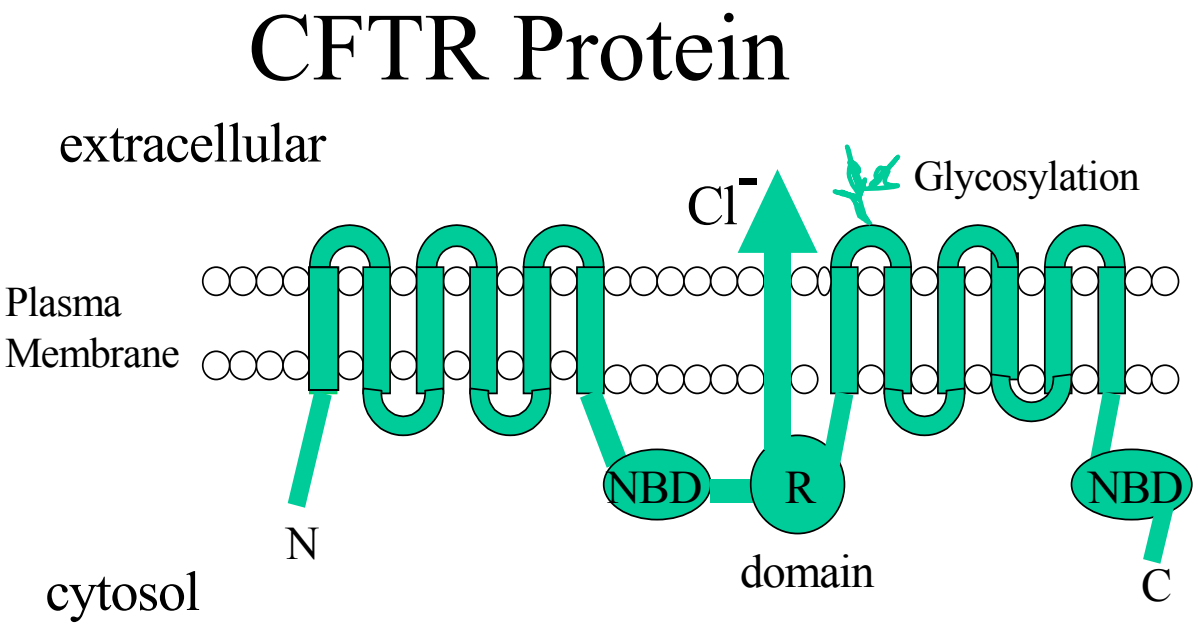

Figure I.1 Model of CFTR Structure

CFTR, an intrinsic membrane protein, has 12 transmembrane spans, 2 nucleotide binding domains (NBD), and a regulatory domain (R). CFTR protein is normally found in low abundance in the plasma membrane of epithelial cells. It is an outward rectifying anion channel with 1or 2 N-linked glycosylation sites on the extracellular loop between membrane spans 7 and 8.

glycosylated forms giving an apparent molecular size of 127 kDa (no glycosylation), 131 kDa (mannose core), and 160 kDa (fully glycosylated with polygalactosaminogylcans) (Gregory et al., 1990). Only the fully processed glycosylated protein is found in the plasma membrane after being passed through the Golgi (O'Riordan et al., 2000). Recombinant CFTR protein expressed in mammalian cells shows the same glycosylation as native CFTR, while the ΔF508-CFTR mutant had only the core glycosylated form. Core mannose glycosylation modifications of protein occur in the ER of eukaryotic organisms. Insect cell line (Sf9) expressing CFTR had only the core mannose glycosylation at 130kDa (O'Riordan et al., 1995). Even though the glycosylation appears crucial for protein localization and trafficking in human cells, the non-glycosylated protein still functions normally in chloride secretion (Morris et al., 1993). Some of the ΔF508-CFTR mutant is fully trafficked from ER to plasma membrane *in vitro* cell cultures at reduced temperatures (26 ^{o}C) and can be detected at the cell surface of cultured cells (Denning et al., 1992). However, this treatment of reduced temperature for patients with CF would not be feasible due to hypothermia.

I.2.3 CFTR Expression

CFTR is primarily expressed in the epithelium of lungs, gastro-intestinal tract, liver, pancreas, and sexual organs at low levels (Welsh et al., 1995). The CFTR promoter acts like a tissue specific "housekeeping" type gene with low levels of expression (Yoshimura et al., 1991). The CFTR protein that is found in endothelial tissue of umbilical vein and lung microvasculature has a similar low expression and plasma membrane localization as compared to the epithelial CFTR (Tousson et al., 1998). Cardiac myocytes also express CFTR, but there is altered splicing, resulting in the elimination of exon 5 and causing different channel specific activity (Hart et al., 1996). Human CFTR has been expressed in cells of other species including Chinese hamster ovary (CHO) cells (O'Riordan et al., 1995; Dho et al 1993), insect cells (Sf9) (O'Riordan et al., 1995; 2000), yeast (Kiser et al., 2001; Huang et al., 1996), and *Xenopus* oocytes (Bear et al., 1991). O'Riordan et al. (1995) reported CFTR heterologous expression in CHO cells to be 2 mg /4 x10^9 cells and in Sf9 insect cells to be 3 mg/1.5 x10^9 cells (O'Riordan et al., 1995). Expression of CFTR in prokaryotes, i. e. *E. coli*, resulted in lethality. A potential cryptic bacterial promoter in the CFTR cDNA sequence could have

caused the expression of CFTR and thus result in the death of bacteria (Gregory et al., 1990).

I.3 TOBACCO AS A MODEL SYSTEM FOR CFTR EXPRESSION

I.3.1 Limitations of Various Expression Systems

Currently, the majority of recombinant protein production is done in mammalian cell systems, bacterial fermentation tanks and yeast fermentation tanks. Mammalian cell lines do express functional CFTR, but they have low yields, are expensive to maintain, and large-scale production is challenging and expensive (Larrick and Thomas, 2001). Insect lines have higher expression levels, but have altered glycosylation patterns, and CFTR protein has been difficult to extract. In yeast cells, CFTR never reaches the plasma membrane and contains similar glycosylation patterns as insect cell-expressed CFTR (Kiser et al., 2001). CFTR expression in yeast also shows some function in *in vitro* membrane potential experiments, but is produced at much lower levels (5-10%) than insect systems (Huang et al., 1996). CFTR expression is very detrimental if not lethal to *E. coli* (Gregory et al., 1990).

I.3.2 Tobacco as a Model System

Tobacco is a good model system for transgenic expression of human proteins because of its ease of genetic manipulation, low cost of production, and ability to make posttranslational modifications (Giddings, 2001; Larrick and Thomas, 2001). Tobacco is becoming a model plant for heterologous expression of many soluble proteins, especially ones that are complex and require eukaryotic processing (Cramer et al., 1999). It may become particularly useful since it is not capable of carrying human pathogens and it is not a food source for humans. Tobacco has been used to express numerous human related antibodies (Larrick et al., 2001, Mason et al., 1992). Human collagen has been expressed and properly assembled as a homotrimeric protein in transgenic tobacco (Ruggiero et al., 2000). Human membrane proteins such as muscarine cholinergic receptor in tobacco (Mu et al., 1997) and N-acetylglucosamyl transferase in the dicot plant *Arabidopsis* (Gomez et al., 1994) have been expressed and shown to be functional. Thus, it appears that tobacco can synthesize and process many human polypeptides into functional proteins. Even though protein levels are modest in plant systems, usually 0.01%-0.4% of total soluble protein, the ability to increase the scale is almost limitless (Giddings, 2001), and production costs of the same amount of protein could be 10 to 50

times less than in *E. coli* as compared to large scale *E coli* production (Kusnadi et al., 1997).

I.3.3 Regulation of Expression Systems of Recombinant Protein in Plants

Many transgenic production systems utilize constitutive promoters to drive high expression of recombinant proteins. A commonly used promoter to drive high expression of foreign proteins in tobacco is the constitutive cauliflower mosaic virus promoter, CaMV 35S with dual enhancers (CaMV $35S^{DE}$). However, high constitutive expression has been linked with the possibility of post-transcriptional silencing. The constitutive high expression of a possible deleterious protein in tobacco could also cause unwanted harm to the plant as in the pokeweed antiviral protein (Lodge et al., 1993).

Therefore, use of a promoter that is more tightly regulated can be useful. When potential toxicity is involved, constitutive expression may be detrimental to the plant. Inducible promoters can overcome the toxicity issues. The wound-inducible promoter MeGA™ (mechanical gene activation) was derived from the tomato *Hmg 2* gene, which encodes the defense-related protein, 3-hydroxy-3-methylglutaryl CoA reductase (Weissenborn et al., 1995). This promoter is expressed at high levels 24 hours after induction by wounding and shows that it can be activated post harvest. Production of recombinant protein can be 1-10% of total protein (Weissenborn et al., 1995). Several plant, bacterial, and human genes have been expressed using the MeGA promoter system in tobacco (Cramer et al., 1999).

I.3.4 *Agrobacterium* Transformation of Plants

Transgenic tobacco is generally produced using the *Agrobacterium*-mediated leaf transformation system (Horsch et al., 1986). *A. tumefaciens* contains the machinery to insert specific DNA segments into a host plant's genome (Zhu et al., 2000). The Ti plasmid has been modified, so that foreign DNA fragments can be cloned into the transfer region between left border and right border tandem repeats. A selectable marker, usually antibiotic resistance, driven by nopaline synthase (nos) gene promoter and containing the nos 3'non-translated region, and a multiple cloning site for gene insertion are contained within the transfer region. When *A. tumefaciens* carrying this Ti plasmid is co-cultivated with a suitable plant host via wounding, the DNA between the borders is transferred into the plant genome (reviewed by Zupan et al., 2000).

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Chapter II Expression of Human CFTR in Transgenic Plants

II.1 Introduction

The molecular basis of CF disease has become clearer since the discovery and cloning of the CFTR gene. However, even 14 years after the isolation of this gene in 1989 by Rommens et al., a cure by gene replacement therapy has not come to fruition (Welsh, 1999; Davies et al., 1998). Initial replacement therapy results in cell cultures and mice looked promising, but clinical trials have been not successful due to many factors. Inefficiency of targeting cells and the transient nature of therapy systems are the two more common reasons of failure (Welsh, 1999). Because of the difficulties of current replacement therapies, an attempt to gain a greater understanding of CFTR structure, function, and interactions has been undertaken by many research groups. Normal CFTR is expressed at low levels in epithelial cells of the organs, primarily lungs and pancreas, that are affected by CF disease (Ratjen and Doring, 2003). When CFTR has been heterologously expressed in many cell lines, it behaves as an energy dependent and phosphorylation-regulated chloride channel (Sheppard and Welsh, 2001). Based on its amino acid sequence, CFTR is in the super-family of ATP-binding cassette (ABC) transporters (Riordan et al., 1989; Dean et al., 2001). Most ABC transporters have four distinct domains: transmembrane domain 1 (MSD), nucleotide binding domain 1 (NBD), transmembrane domain 2, and nucleotide binding domain 2. CFTR has an additional regulatory domain containing sites for phosphorylation between the first NBD and the second MSD. Many of the ABC transporters have multiple transport functions. Therefore, CFTR is likely to have more roles in the cell than just as an energy dependent chloride channel, and some evidence for regulation of other ions channels exists (Kunzelmann et al., 2000).

CFTR protein occurs at low levels in natural sources and cannot be purified in amounts sufficient for crystallization and large-scale drug discovery studies. The potential of crystalizing CFTR and obtaining the protein structure is high, since in recent years, intrinsic membrane proteins have been successfully crystallized (Landau and Rosenbusch, 1996; Kolbe et al., 2000). Recombinant CFTR has been expressed in several mammalian cells including Chinese hamster ovary (CHO) cells (O'Riordan et al., 1995; Dho et al., 1993), Hela cells (Zhang et al., 1998), NIH-3T3 fibroblasts (Stutts et al., 1993), and human carcinoma cells (Yoshimura et al., 1991). Due to stringent control of CFTR biosynthesis, only limited amounts of heterologous protein can accumulate in

these cell lines. CFTR has been expressed at higher levels in a baculovirus-infected insect line (Sf9) (O'Riordan et al., 1995). This cell line can produce from 0.5 mg to 3 mg CFTR per liter of cells. However, only basal oligosaccharide addition occurred (O'Riordan et al., 2000), since only core mannose-containing N-linked glycans are added to proteins in Sf9 cells (Altmann et al., 1993). In yeast, recombinant CFTR is not fully glycosylated and remains in the ER, then is degraded (Kiser et al., 2001). The expression levels are similar to CHO cells and the size of the CFTR protein is similar to insect (Sf9) expressed CFTR (Huang et al., 1996; Kiser et al., 2001).

The ability of plants to express a wide variety of human proteins has been demonstrated (reviewed by Giddings, 2001; Larrick and Thomas, 2001; Cramer et al., 1999). Three different plant-synthesized therapeutics are in human clinical trials: 1. secretory IgA plantibody directed against *Streptococcus mutans*, the major cause of dental caries, in tobacco (Planet Biotechnology, Inc.), 2. monoclonal IgG plantibody directed against specific cancer cells produced in corn (Monsanto, NeoRx) and 3. Heat labile enterotoxin β-subunit produced in potato as an edible vaccine (Lauterslager et al., 2001). Of particular interest for expression of CFTR in plants are the three mammalian intrinsic membrane proteins 3-hydroxy-3-methylglutaryl (HMG) CoA reductase (containing 8 transmembrane spans) (Chappell et al., 1995), N-acetyl glucosamyl transferase (Gomez and Chrispeels, 1994), and galactosyltransferase (Bakker et al., 2001), that were successfully produced in plants. Thus plants appear to be capable of not only synthesizing human polypeptides, but also of interpreting signals for folding, targeting, and membrane insertion.

The objective of this research is to determine the feasibility of using transgenic tobacco for the production of high levels of human CFTR for structure/function studies. It was not clear whether functionally active CFTR would adversely impact plant growth or viability, so two gene constructs containing CFTR driven by the dual enhanced (de) 35S Cauliflower mosaic virus (CaMV 35S ^{DE}) constitutive promoter or the inducible MeGA promoter were developed. We found the construct driven by the CaMV 35S ^{DE} promoter was lethal in *E. coli*, but the constructs driven by the inducible MeGA promoter were not. The MeGA promoter-driven constructs were placed into a plant binary vector and transformed into tobacco via *Agrobacterium*-mediated transformation (Horsch et al., 1986). Presence of the recombinant CFTR in transgenic plants was analyzed by PCR and

confirmed by Southern analysis. Expression of CFTR in plants was analyzed by RT-PCR. Protein expression was determined by western blot analysis using commercially available antibodies.

II.2 Materials and Methods

II.2.1 Development of Promoter: CFTR constructs

The strategy for expressing human CFTR in tobacco involved fusion of the full-length CFTR coding region (cDNA clone pBQ4.7, kindly provided by Dr. J. Rommens, Univ. of Toronto) to either a constitutive or inducible promoter (Figure II.2). The widely used strong constitutive promoter from the cauliflower mosaic virus (CaMV 35S) with dual enhancers (de) (Kay et al., 1987) was tested for production of CFTR in plants. A wound inducible promoter MeGA (provided by CropTech Corp. Blacksburg, VA) derived from the defense-related tomato *Hmg*2 gene encoding 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGR), was also used to drive human CFTR expression. The CFTR cDNA contains 4.7 kilobases and its large (Cheng et al., 1990) size and lack of unique or convenient restriction sites made subcloning into plant expression vectors a challenge. The 5' end of the gene (570 bp) was modified by polymerase chain reaction (PCR; 30 cycles: 95^{o}C, 1 minute; 51^{o}C, 1 min.; 72^{o}C, 1 min.) to provide a *Kpn*I restriction site flanking the ATG start codon. Amplification of the CFTR cDNA plasmid DNA using the CFTR specific primers W1 (5'TATGGTTACC ATGCAGAGGTCGC; start codon underlined) and W2 (5'CCATGAGGAGTGCCA CTTGCA) (Table II.1) and Stratagene's (LaJolla, Ca) Herculase enhanced DNA polymerase resulted in a PCR product of 642 base pairs which included the unique *Xba*I site (TCTAGA) 570 base pairs downstream of the start codon. The modified 5'CF PCR product was digested with *Kpn*I and *Xba*I and the 570 bp fragment was isolated using PCR Cleanup Kit from Qiagen (Valencia, CA). The 5'CF fragment was ligated into the pBC plasmid (Stratagene) as a *Kpn*I-*Xba*I fragment. *E. coli* cells (Top Ten cells; Invitrogen, Carlsbad, CA) were transformed and selected for positive clones on LB media containing 30mg/l chloramphenicol. Colonies were grown in 5ml cultures of LB with 30mg/l chloramphenicol, and plasmids were isolated with Qiagen plasmid DNA mini extraction kit. Clones were checked by restriction digestion and PCR. The 3' end of CFTR was excised from pBQ4.7 as an *Xba*I- *Sac*I fragment and ligated into the pBC5'CF

Primer name	Sequence	Position and Strand[1]
W1, 5'CFTR	5'TATGGTACCATGCAGAGGTCGC	-9 CFTR, exon 1, +
W2, 3'CFTR	5'CCATGAGGAGTGCCACTTGCA	636 CFTR, exon 6a, _
MeGA 5'	5'GGCAAGCTTGTCGACCAATACGATATTACC	-450 MeGA , +
W16, 3'MeGA	5'GGTACCTTTCACCGGTAAGTCTGC	-1 MeGA, _
CL-1, TEV -132	5'TCAACACAACATATACAAAACAAAGAA	-132 CaMV ^{35SDE}, _
T7,Bacterial primer	5'GTAATACGACTCACTATAGGGC	-1 *Kpn I* vector pBC, +
W21, NPT II rev	5'AAGGTGAGATGACAGGAGAT	-321 NPT II gene, +
W51, CF104	5'TCAGACATATACCAAATCCCTTC	104 CFTR, exon 2, +
W52, CF754r	5'CTCTCTGATCTCTGTACTTCATCAT	754 CFTR, exon 6b, _
Nt actin 5'	5'ACCCTGTACTACTCACTGAAGCAC	
Nt actin 3'	5'CATCGGAAACGTTCAGCACCGATG	
NPT 5a	5'AGTATTGAACAAGATGGATTGCA	_
NPT 3b	5'TCAGAAGAACTCGTCAAGAAGG	+
W46, 3'CF2	5'CCCATGAGGAGTGCCACTTGCA	636 , CFTR exon 6a _
W47, 5'CF 2	5'CAGAGGTCGCCTCTGGAAAAGG	3, CFTR exon 1 +

1. Position and direction are in reference to start codon of CFTR cDNA.

Table II.1 Primers Used to Clone and Analyze CFTR

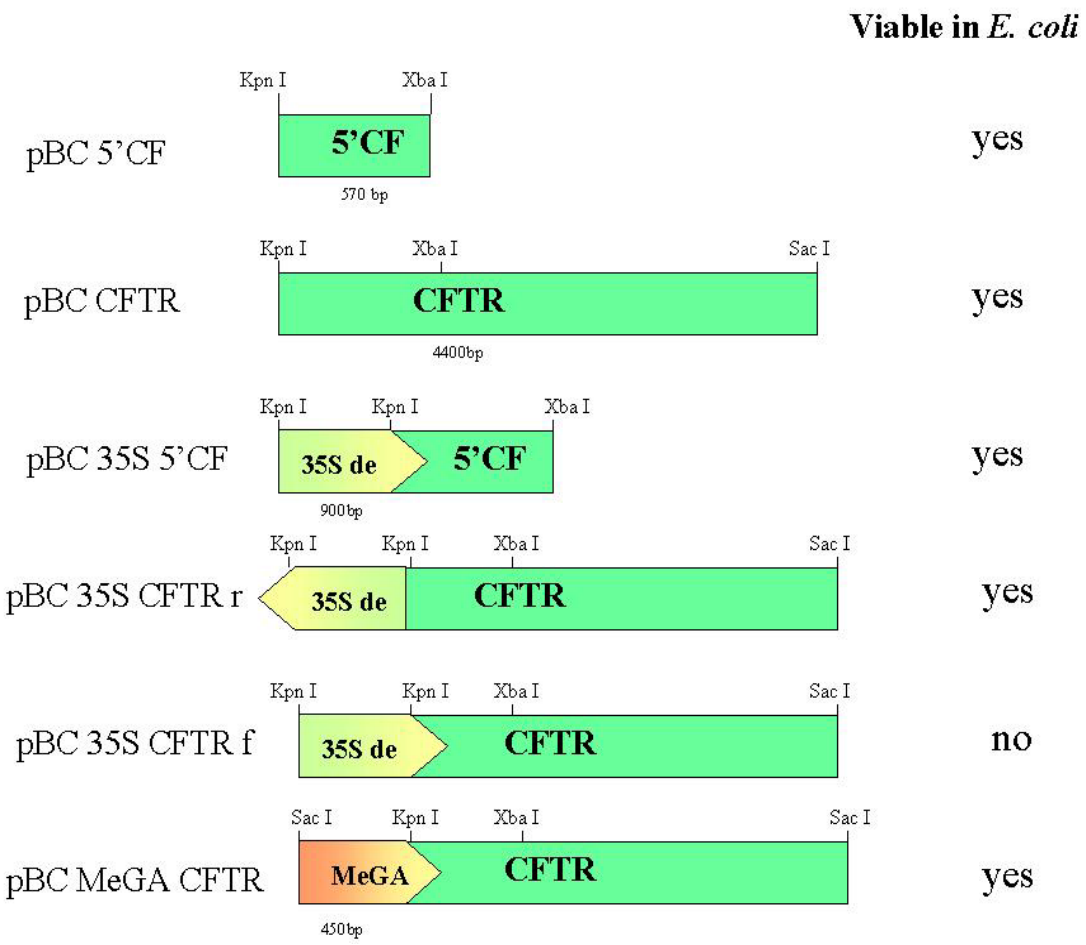


Figure II.1 Constructs Made for Expression of CFTR. The constructs were stable in *E.coli* except the $35S^{DE}$ (CaMV promoter) forward direction with respect to full-length CFTR cDNA. Constructs are not to scale. A viable construct is termed as being able to be replicated in bacteria without harming the host.

plasmid. The resulting plasmid, termed pBC CFTR (Figure II.1), was sequenced to confirm junction sites and amplified sequences.

The constitutive CaMV 35S^{DE} promoter was excised from a pBC-derived plasmid (pBC R8-2 kindly provided by Dr Fabricio Medina-Bolivar) using *Kpn*I and ligated upstream of the CFTR gene in pBC CFTR (Figure II.2). Orientation of the promoter with respect to the cDNA was checked via PCR (30 cycles: 95^{o}C, 1 minute; 51^{o}C, 1 min.; 72^{o}C, 1 min.) using CaMV 35S^{DE} specific primer CL1 (5'TCAACACAACATATACA AAACAAACGAA) and W2. There were no positive oriented full-length CaMV 35S^{DE} CFTR pBC clones found. Sequence confirmation of promoter orientation and junctions revealed only the reverse orientation amoung full-length CFTR cDNA clones.

Plasmids that fused the CFTR coding region with the wound-inducible MeGA promoter were also developed. There was not a *Kpn*I site on the 3' end of the MeGA promoter that would facilitate cloning with CFTR. PCR using *Taq* DNA polymerase (Ready-to-go beads; Amersham/Phamacia, San Francisco, CA) was used to create a *Kpn*I site at the 3'end of the MeGA promoter. The primers 5' MeGA (5'GGCAAGCTTGTCG ACCAATAC GATATTACC) and W16 (5'GGTACCTTTCACCGGTAAGTCGTC) in Table II.1 were used to create a 450 base pair PCR product, using the same conditions previously described, that was cloned into the TA cloning vector pCR2.1 (Invitrogen). The MeGA clones were digested by *Kpn*I and were checked for orientation in pCR2.1 plasmid. Positive oriented MeGA clones with respect to restriction enzyme sites were sequenced to check for errors made by the *Taq* DNA polymerase. The MeGA promoter was removed by *Kpn*I digestion and ligated into the pBC CFTR plasmid. The orientation of the MeGA promoter with respect to CFTR cDNA in the vector pBC CFTR plasmid was determined by PCR. Primer pairs, 5'MeGA/W2 and T7/W16, were used in the PCR reactions (30 cycles: 95^{o}C, 1 minute; 51^{o}C, 1 min.; 72^{o}C, 1 min. and 30 cycles: 95^{o}C, 1 min.; 49^{o}C, 1 min.; 72^{o}C, 1 min., respectively). Sequence analysis confirmed the orientation of the MeGA promoter relative to CFTR.

For *Agrobacterium*-mediated transformation of plants, the MeGA:CFTR construct was excised from pBC CFTR containing MeGA using *Sac*I and cloned into the plant binary vector pBIB-kan (Becker et al., 1990) as a SacI fragment (Figure II.3). The large pBIB-kan plasmid was isolated by alkaline lysis and isopropanol precipitation (Sambrook and Russell, 2001), and purified using the Qiagen Qiaex II protocol for cleanup and

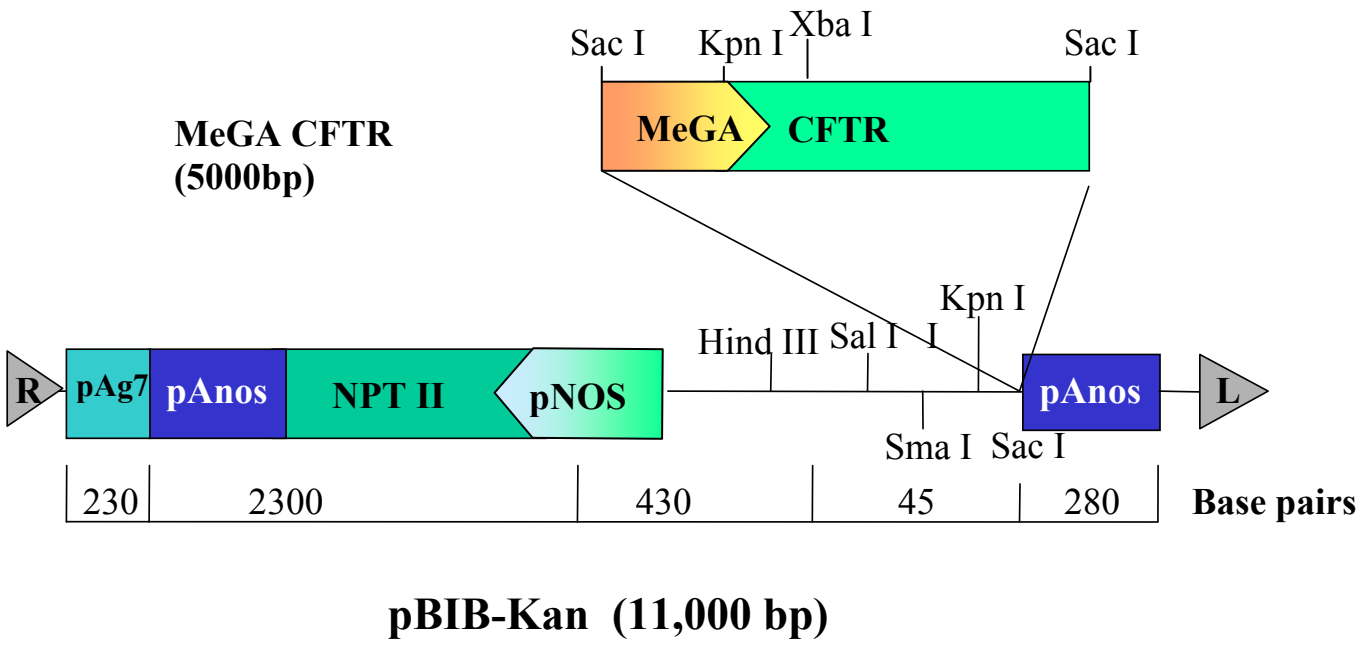


Figure II.2 Insertion of MeGA CFTR into Plant Binary Vector

The T-DNA insertion segment of pBIB is the region between the right border (R) and the left border (L). MeGA:CFTR was ligated into pBIB-Kan as a 5 kilobase *Sac*I fragment. The pBIB MeGA:CFTR construct containing both NPT II and CFTR genes and was transformed into *Agrobacterium* and T-DNA region into tobacco.

concentrating low copy high molecular weight plasmid DNA. The orientation of the MeGA:CFTR insert was determined by PCR. Primers W21 (5'AAGGTGAGATGA CAGGAGAT) and W16 (Table II.1) were used in a PCR reaction (30 cycles: 95^{o}C, 1 minute; 59^{o}C, 1 min.; 72^{o}C, 1 min.) to confirm the presence of MeGA promoter in the binary vector. The primer pair W21/W2 (Table II.1) was used in the PCR reaction (30 cycles: 95^{o}C, 1 minute; 52^{o}C, 1 min.; 72^{o}C, 1 min.) to detect CFTR in pBIB binary plasmid. The correctly-oriented pBIB MeGA:CFTR clones were isolated and purified as previousouly described above for transformation into *Agrobacterium tumefaciens* strain LBA4404 via a freeze-thaw method adapted from Gelvin and Schliperoort (1988). Transformed *A. tumefaciens* was grown in YEP media plus antibiotics.

II.2.2 *Agrobacterium*-Mediated Plant Transformation

Agrobacterium tumefaciens strain LBA4404 was used to produce stable transgenic tobacco plants containing the MeGA:CFTR sequences. The petiole scoring method (Medina-Bolivar et al., 2003) or leaf disc method (Horsch et al., 1986) of infection of tobacco was used. Small, fully developed *Nicotiana tabacum* cv. Xanthi leaves were excised from the plant with 0.5 cm of petiole remaining on the leaf. A scalpel tip was dipped into a two-day old *A. tumefaciens* colony and then used to score the petiole parallel to the veins. Alternatively for leaf dip method, Xanthi leaves were cut into 5 mm by 10 mm strips and incubated for 30 minutes in *A. tumefaciens* that had been resuspended into Murashige and Skoog (MS) (Murashige and Skoog, 1962) liquid media. The leaf strips were then blotted dry on sterile Whatman paper. The scored plant leaves and cut leaves were placed on MS media for 2-3 days until a halo of bacteria was seen growing out of the petiole. The leaves were placed on selection MS shooting media with: 1-naphthaleneacetic acid (NAA), 6-benzylaminopurine, 200 mg/l kanamycin to select for transgenic plants, and 500 mg/l carbenicillin to kill the bacteria. After two to three weeks, shoots and calli formed at the site of wounding. The shoots were placed on MS rooting media (no hormones) containing 200 mg/l kanamycin and 250 mg/l carbenicillin to select against any escaped untransformed plantlets. Roots appeared about two weeks after placement on the media. The rooting plants were analyzed for the presence of the transgenes, CFTR and neomycin phosphotransferase (NPT II).

II.2.3 Analysis of MeGA:CFTR Transgenic Tobacco Plants

PCR was used to confirm the integration of the transgene into the genome of the plant. Genomic DNA from non-transformed Xanthi and transformed plant material was isolated via DNeasy plant extraction kit (Qiagen, Valencia, Ca). One hundred mg of leaf tissue were pulverized in the presence of liquid nitrogen. Then buffers were added to separate the genomic DNA from RNA and proteins according to the manufacturer's specifications. The genomic DNA was eluted from the DNeasy column in 200µl of elution buffer. Gene-specific primers W1 and W2 (Table II.1) were used for specific amplification of the 570 bp target CFTR DNA from tobacco genomic DNA. PCR amplification using NPT II primers NPT5a (5'AGTATTGAACAAGATGGATTGCA) and NPT3b (5'TCAGAAGAACTCGTCAAGAAGG) (Table II.1) were used to determine the presence of NPT II DNA in plants.

Southern blot analysis was used to confirm the data generated by the genomic PCR screening. Twenty micrograms of genomic DNA was digested with *Sac*I, *Kpn*I or *Spe*I, then separated on 0.8% agarose gel at 30 volts for 16 hours. Digested DNA was neutrally transferred to uncharged nylon membrane (Nytran, Keene, NH) by capillary transfer (Sambrook and Russell, 2001). The DNA was cross-linked to the nylon membrane with UV irradiation. The membrane was incubated in Church pre-hybridization buffer (0.5M phosphate buffer, 1% BSA, 1 mM EDTA and 7% SDS) for 4 hours at 55^{o}C. Then the membrane was incubated in hybridization buffer (Church buffer with boiled, sheared salmon sperm DNA and ^{32}P-labeled CFTR probe) for 48 hours at 55^{o}C. The probe was made with a 1.5 kb fragment (exon 1-10 in cDNA) of CFTR, αdCTP ^{32}P and Prime-It kit (Stratagene). The membrane was washed two times in 1x SSC, 0.1% SDS for 30 minutes at 55^{o}C. Then it was washed two times in 0.1x SSC, 0.1% SDS at 55^{o}C for 30 minutes. The membrane was wrapped in Saran wrap and exposed to Kodak X-OMAT film for seven days at –80^{o}C.

II.2.4 Analysis of RNA Expression in CFTR Transformed Plants

Transcript levels were analyzed by reverse-transcriptase polymerase chain reaction (RT-PCR). A pasta maker with 2 mm teeth was used to wound mature leaves. Wounded tissue was collected at 0, 12, and 24 hour after storage in sealed containers in a high moisture environment at 23^{o}C. Tissue was frozen using liquid nitrogen and stored at

–80^{o}C until further analysis. Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) along with phenol-chloroform-isoamyl alcohol mixture (25:24:1) was used to extract and purify total RNA from the frozen plant tissues. Two grams of induced leaf tissue were ground in 8 ml of Tri-Reagent with a polytron homogenizer (Fisher, Suwanee, GA). Eight milliliters of phenol/chloroform were added to the homogenate, then mixed vigorously and centrifuged in an Eppendorf 5810 centrifuge in a fixed angle roter at 10,000x g for 10 minutes. Equal volume of phenol/chloroform mixture was added to the aqueous layer, mixed and spun at 10,000x g for 10 minutes. The aqueous layer was re-extracted with an equal volume of chloroform and centrifuged at 10,000x g for 10 minutes. The total RNA was precipitated with 3 volumes of isopropanol and pelleted at 10,000x g for 15 min. The dried pellet was resuspended in 250 µl diethylpyrocarbonate (DEPC)-treated water. The ProSTAR first-stand RT-PCR kit (Stratagene) was used to create first strand cDNAs as per the manufacturer's protocol using oligo dT (15mer; Stratagene). Identification of CFTR-expressing transformants was based on PCR amplification of CFTR using primers W1 and W2 or W51 and W52 (Table II.1). Parameters for PCR using the W51/W52 primer pair were thirty cycles: 1minute denaturation at 95 ^{o}C, 1 minute annealing at 53 ^{o}C, and 1 minute elongation at 72^{o}C. Tobacco actin primers, 5' Nt actin (5'ACCCTGTACTACT CACTGAAGCAC) and 3' Nt actin (5'CATCGGAAACGTTCAGCACCGATG), that flank an intron were used to discriminate between genomic DNA and synthesized cDNA. Any contaminating genomic PCR actin product would be 157 bp larger that the RNA RT-PCR product. PCR reaction conditions for actin primers were thirty cycles: 1 minute denaturation at 95 ^{o}C, 1 minute annealing at 54 ^{o}C, and 1 minute elongation at 72 ^{o}C.

The RNA isolation was also performed on a small scale using 100 mg of tissue. The above protocol was proportionally reduced. The leaf tissue was ground under liquid nitrogen in a 1.5 ml eppendorf tube using an eppendorf pestle. Total RNA was resuspended in 50 microliters of DEPC-treated water.

II.2.5 Membrane Protein Isolation

Crude membrane proteins were isolated by differential centrifugation. One to two grams of plant leaves were wounded using the 2 mm teeth on a pasta maker. The wounded tissue was stored in sealed 50 ml conical tubes (Falcon) with a water moistened Kimwipe at 22 ^{o}C for 0, 12, 24, or 48 hours. Tissue was frozen in liquid nitrogen and

stored at –80 ^{o}C. Tissues were ground using a mortar and pestle in the presence of liquid nitrogen. Six milliliters of membrane extraction buffer containing 100 mM potassium phosphate pH 7.4, 250 mM sucrose, 5 mM DTT, 5 mM EDTA, 0.6% Polyvinyl-pyrrolidone (PVP-40) and Complete protease inhibitor cocktail (Roche, Indianapolis, IN) were added to ground tissue. The plant extract was filtered through Miracloth (Calbiochem, LaJolla, Ca) and centrifuged in a Eppendorf centrifuge at 8,000x g for 10 minutes to remove large cellular debris. The supernatant was further centrifuged at 25,000x g for 20 minutes in a Sorval super-speed centrifuge to pellet the chloroplasts, nucleus, and mitochondria. Approximately 3 ml of the supernatant was centrifuged at 100,000x g for 1 hour, 4 ^{o}C in a Beckman ultracentrifuge. The resulting microsomal pellet was resuspended in membrane suspension buffer containing 100 mM potassium phosphate, pH 7.4, 5mM DTT, 5mM EDTA and +/- 0.1% Triton X-100. Protein concentration was determined using Advanced Protein Assay Reagent (Cytoskeleton, Denver, CO) and bovine serum albumin (BSA) standards. Proteins were stored at –20 ^{o}C prior to western blot analysis.

II.2.6 Antibodies for CFTR Detection

Protein expression of CFTR in the transformed plants was analyzed by immuno-detection using commercially available antibodies. There were several commercially available CFTR antibodies for the use in immunodetection of human CFTR protein. These mouse antibodies were made to react to synthetic peptide sequences of human CFTR. Three antibody groups were chosen for their unique sequence recognition (see Figure II.2). Affinity Bioreagents' (Golden, CO) clone CF3 (mouse monoclonal, MAI-935, and polyclonal) antibodies recognize residues 103-117, the first extracellular loop of CFTR. Another antibody, anti-R-domain, was raised against a peptide from the regulatory domain of CFTR (Affinity Bioreagents). The C terminus domain antibody, anti-mouse monoclonal CF 24-1, was made by R&D Systems (Minneapolis, MN) and recognizes residues 1377-1480.

II.2.7 Western Blotting and Stained Protein Gels

Microsomal proteins were analyzed by SDS polyacrylamide gel electrophoresis. The mild detergent lysophosphotidylglycerol (palmitoyl), LPG (Avanti Lipids, Alabaster, AL) was added at 12 mg/ml and incubated on ice for 1 hour or longer before adding 6x SDS protein loading dye (Sambook and Russell, 2001) to create micelles and lower the

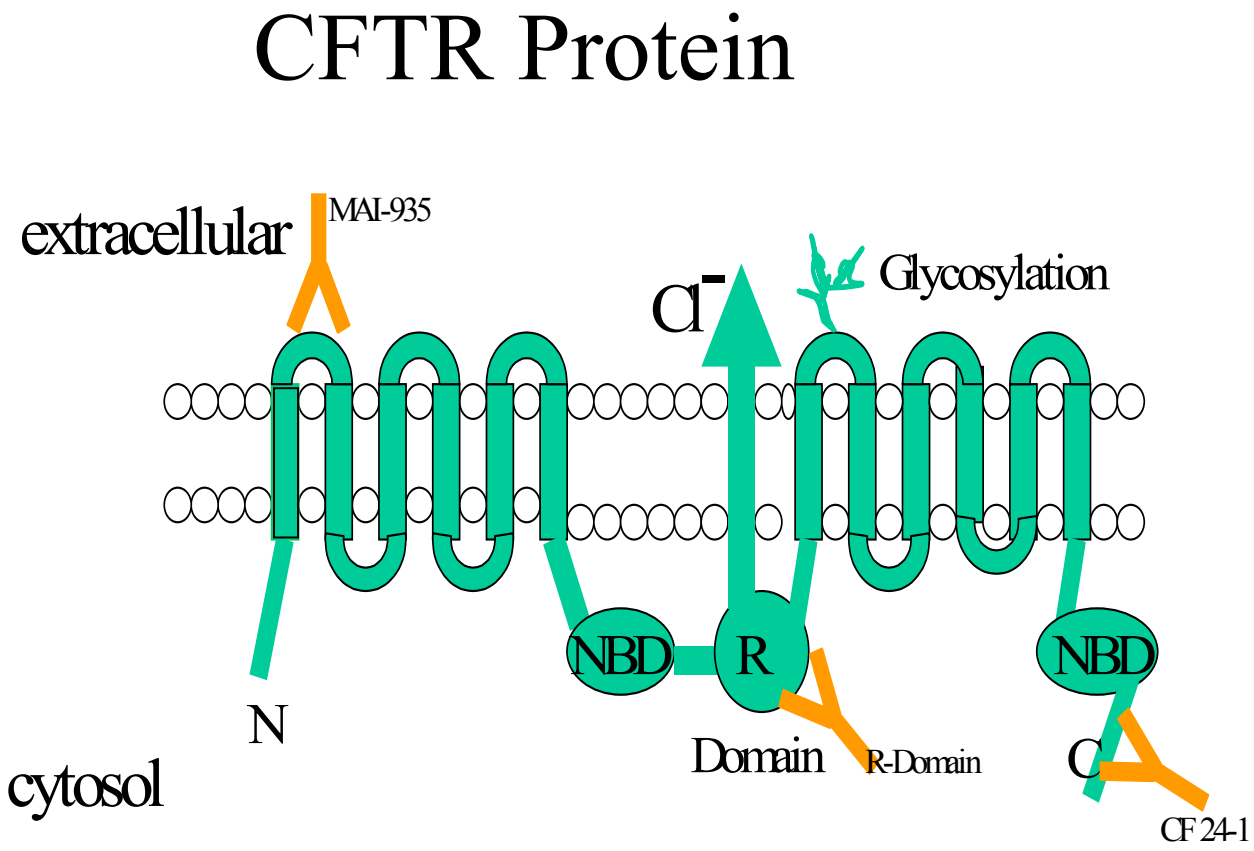


Figure II.3 Position of Antibody Recognition to CFTR Protein

The monoclonal antibodies' recognition sites to CFTR protein are illustrated. MAI-935 (CF-3) was made to recognize the first extracellular loop, R-domain antibody recognized the regulatory domain of CFTR, and CF24-1 binds to the carboxy terminus of CFTR protein.

aggregation of CFTR proteins. Ten to twenty micrograms of total protein were boiled in SDS loading buffer for 10 minutes. Samples were loaded into 6%, 12%, or 8-16% Tris-glycine precast gels (Novex, San Diego, CA). Gels were run at 100 volts until dye front was run off the gel. Benchmark prestained protein ladder (Invitrogen, Carlsbad, CA) or New England Biolabs' (Beverly, MA) broad range prestained protein ladder was used to approximate size of proteins in the extracts. Gels were stained with Coomassie protein staining dye. Proteins from other gels were electro-transferred overnight to nitrocellulose membranes (Biorad, Melville, NY). The nitrocellulose membranes were incubated in blocking buffer, 1x PBS 0.1% Tween-20, 3% BSA fraction V (Roche or Sigma, St. Louis, MO), for 2 hours slowly mixed. Primary antibody, anti-CFTR antibody, at 1:10,000 dilution each in blocking buffer was added onto the membrane and shaken slowly for 1.5 hours. The membrane was washed three times for 20 minutes in 1x PBS, 0.1% Tween-20. Then the secondary antibody, goat anti-mouse antibody conjugated to alkaline phosphatase (Biorad), at 1:5000 dilution in blocking buffer was added to the membrane and gently mixed for 45 minutes. The membrane was washed three times for 15 minutes in 1x PBS, 0.1% Tween-20. The membrane was washed two times in CDP-Star Detection buffer, for two minutes. The membrane was incubated for five minutes in 5ml of detection buffer containing 250 μl Nitroblock II solution (Tropix, Bedford, MA) and 50 μl CDP-Star (Roche). Excess liquid was drained off and the membrane was wrapped in cling wrap. Membrane was exposed to Kodak film (X-OMAT) for 1-10 minutes and processed in a Konica automatic film developer.

II.3 Results

II.3.1 Cloning of CFTR Constructs

Cloning of the full length CFTR gene driven by the constitutive CaMV 35S^{DE} promoter was problematic. The promoter was introduced into the full-length or 5'end of CFTR in both forward and reverse orientations (Figure II.1). PCR and sequencing confirmed the orientation of the promoter with respect to the CFTR cDNA. However, when the constructs were introduced into bacteria during subsequent cloning steps, no colonies were ever formed when the full-length CFTR cDNA was driven by the CaMV 35S^{DE} promoter in the forward direction. Other constructs with the promoter in the reverse orientation and/or with partial cDNA sequence did result in viable colonies. Because the ligation of promoter and gene was successful as checked by restriction digest and PCR using gene specific primers, it was concluded that the CaMV 35S^{DE} promoter was leaky in *E. coli*. It was known that CFTR expression in bacteria was lethal (Gregory et al., 1990), but not until this experiment that CaMV 35S^{DE} promoter might be leaky. Studies undertaken concurrently showed that translational fusion of CaMV 35S^{DE} promoter to β–glucuronidase (GUS) and green flourescent prtein (GFP) in *Agrobacterium* resulted in expression of the protein (personal communication Fabricio Medina-Bolivar, Virginia Tech and DebWiessenborn, Croptech Corp.). The constitutive CaMV 35S^{DE} promoter may have prokaryotic transcription factor binding sites.

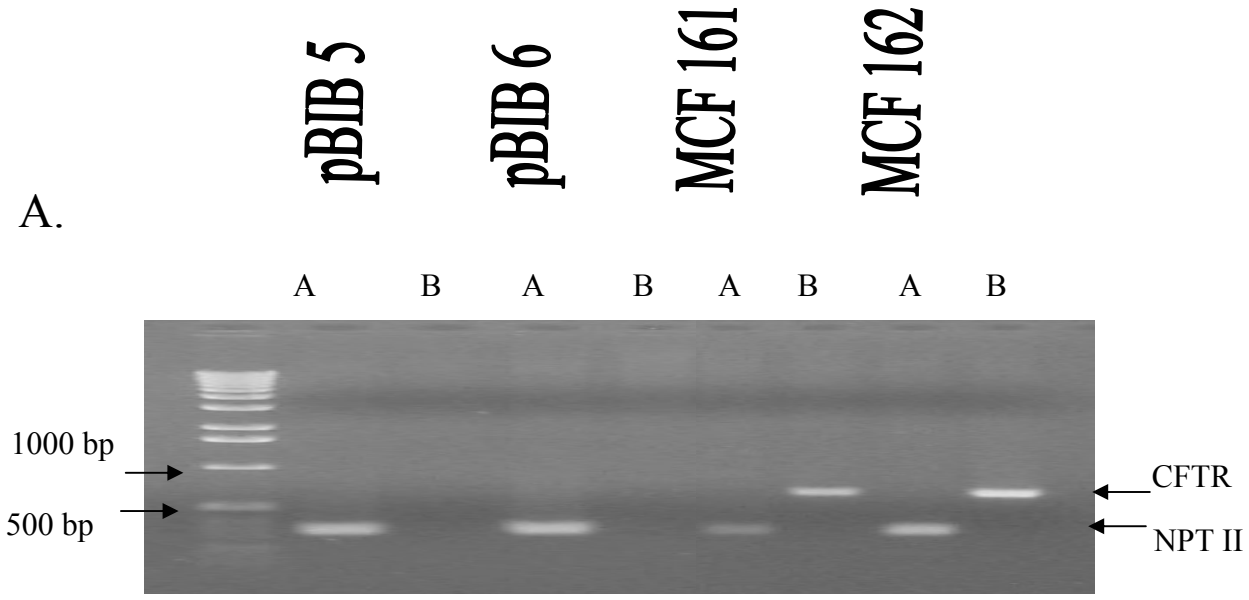
In contrast to CaMV 35S^{DE}, these researchers (Medina-Bolivar and Wiessenborn) found that the wound inducible MeGA promoter fusions did limit the expression of these reporter proteins in bacteria. Thus, it was possible that the MeGA promoter would allow successful cloning steps and expression of CFTR in plants. The MeGA promoter was fused to the full-length CFTR and was successfully propagated in bacteria and used to transform tobacco plants. Twenty-five plantlets out of 100 chosen formed roots during the kanamycin resistance selection process. These resistant plants were used for subsequent DNA and RNA analysis.

II.3.2 Analysis of Transgenic Plants for CFTR

Kanamycin resistant plants were analyzed for the insertion of CFTR into their genomes. Genomic DNA from these positive plants was extracted for Southern blot

analysis and for PCR using CFTR gene specific or NPT II specific primers. As seen in Figure II.4, a 650 bp CFTR PCR product was detected in representative plants transformed with MeGA:CFTR. These plants also contained NPT II based on detection of a 290 bp NPT II product. Of the 25 plants screened, only two of the plants, MCF 111 (Figure II.4.B) and MCF 131(not shown), were not positive as analyzed by PCR. These two plants did grow slower and more aberrantly than other plants on selection media. There were no apparent foliar differences in CFTR-containing plants compared to non-transformed plants or empty vector transformants, which was expected since MeGA is inducible and not constitutive. However, some plants, MCF 161 and MCF 162, did have greater numbers of roots and more elongated root hairs initially, but grew more normally as the plants matured. Southern hybridization analysis was also used to confirm genomic integration (see Figure II.5). Digested DNA (*Sac*I) of transgenic plant lines MCF 162 and MCF 161 (lanes 4 and 5) contained a cross-hybridizing fragment of 5 kb. The genomic DNA fragment was slightly larger than control CFTR fragments (4.4 kb in lanes 2 and 3) because of the addition of the MeGA promoter. Southern blot analysis using *Kpn*I and *Spe*I restriction enzymes shown that plant line MCF161 had 2 copies of CFTR and plant line MCF 162 had 3 copies of CFTR. There was no cross reactivity of the CFTR-specific probe with non-transformed Xanthi, indicating no closely related homologous gene exists in tobacco as also reported for *Arabidopsis* (Theodoulou, 2000).

A.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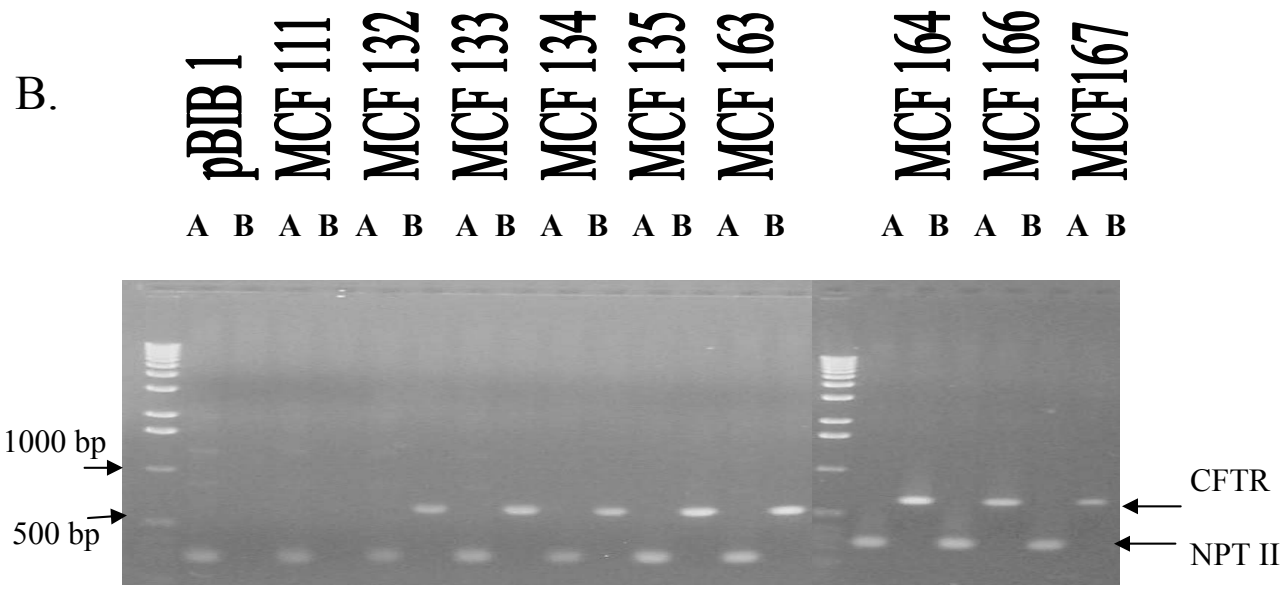
B.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NPT II

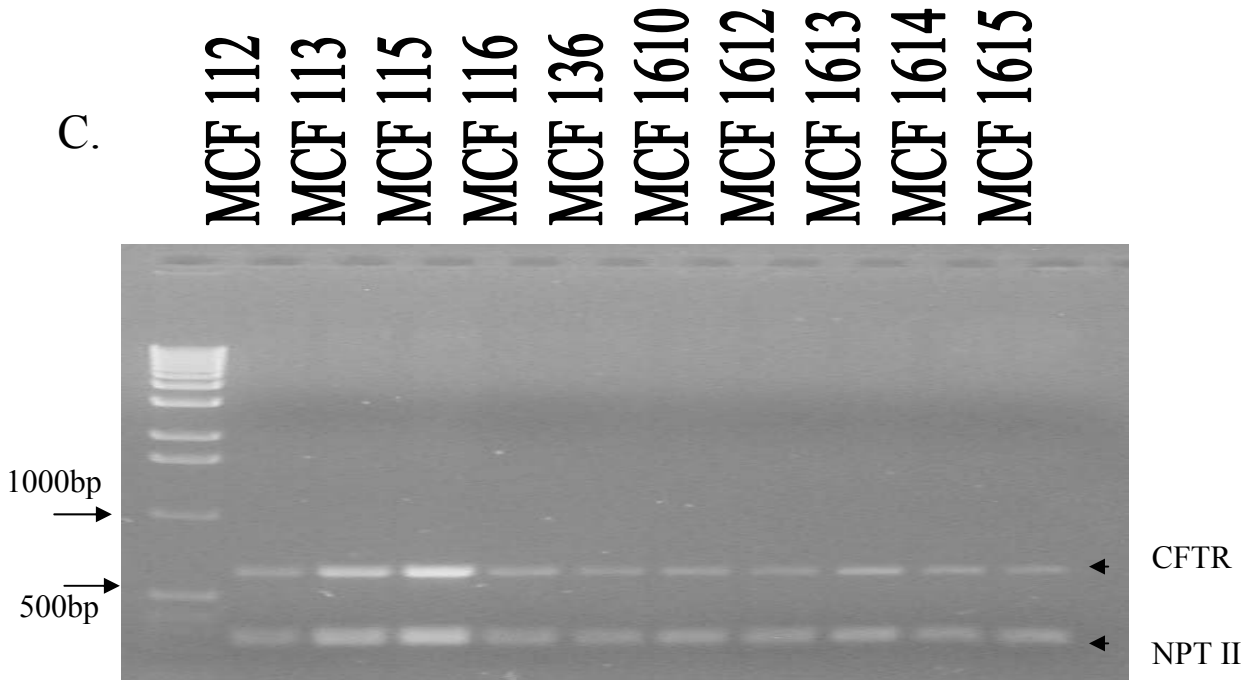

Figure II.4. Genomic Screening of Transgenic Tobacco Plants by PCR. Panel A and B show plant lines from MeGA:CFTR transformation. Genomic DNA extracted from transgenic plants containing the CFTR gene was PCR amplified with specific primers for NPT II (lanes A, primers NPT 5a and W21) and CFTR (lanes B, primers W51 and W52) to create 290 bp and 650 bp products, respectively. pBIB plants are empty vector controls that only contain NPT II gene. MCF plants contain CFTR driven by MeGA promoter. Primer sequences are shown in Table II.1. Panel C shows more genomic screening of MeGA:CFTR transgenic plants using dual primer sets for NPT II (Kan^R) and CFTR in PCR reactions. The primers were the same as used in Figure II.4A and II.4B. Non-transformed Xanthi gave no PCR products with either primer sets used.

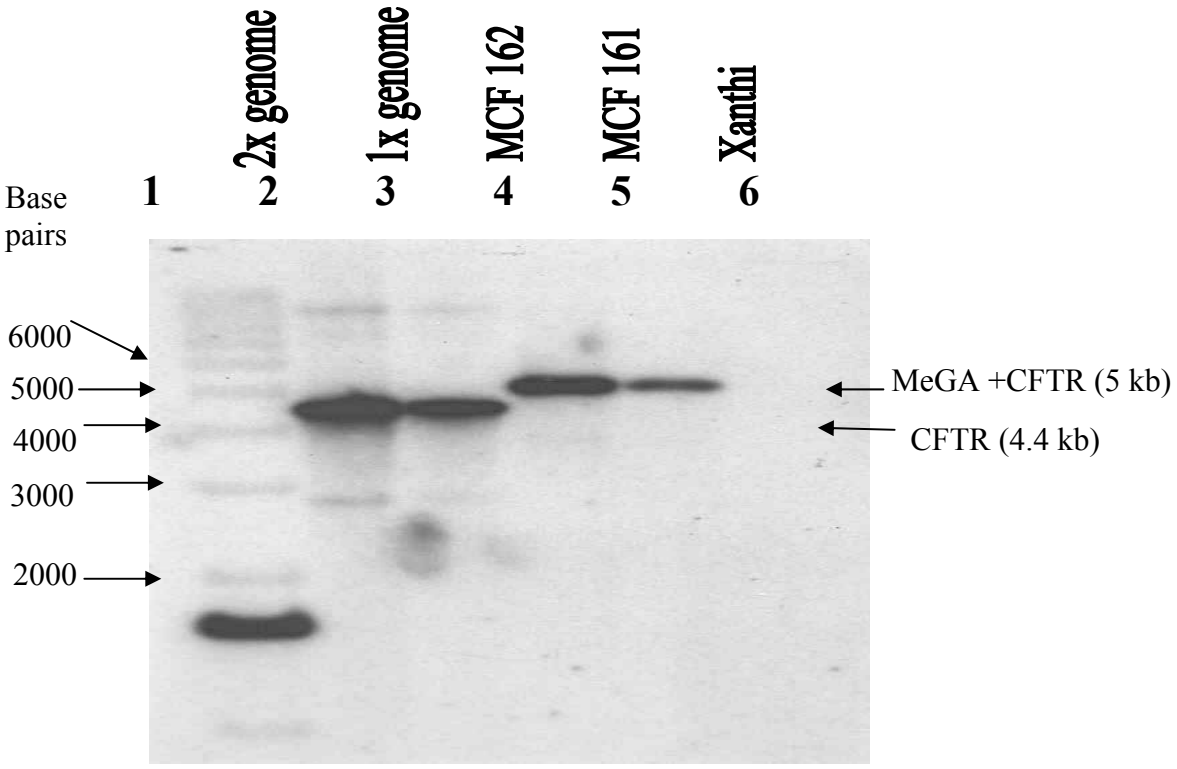

Figure II.5 Southern Blot Analysis of Transgenic MeGA:CFTR Plants. Southern hybridization of plant genomic DNA, 20 μg, after digestion with *Sac 1* enzyme. The probe was a 1.5kb fragment of CFTR cDNA labeled with ^{32}P including exons 1-10 of cDNA. Lane 1 is 1kb ladder (Invitrogen). Lane 2 is 20 pg and lane 3 is 10 pg of CFTR cDNA (4.4 kb). Lane 4 is 20 μg genomic DNA of plant line MCF 162 and lane 5 is 20 μg genomic DNA of plant line MCF161. Lane 6 contains 20 μg genomic DNA from Xanthi non-transformed.

II.3.3 RNA Expression of MeGA:CFTR

In order to study RNA expression of the CFTR transgene, excised leaves were wounded and a time course performed based on previous studies with MeGA induction (Weissenborn et al., 1995). Nine to twelve hours are reported to be the peak range of transcript levels of MeGA-driven genes (Park et al., 1992). CFTR- specific RT-PCR products were detected using RNA from MeGA:CFTR leaves 12 hours after wound induction (Figure II.6). Every transformed plant except MCF 168 and MCF 134 showed expression at 0 hours as well as 12 hours, which was slightly unexpected. This result indicated that MeGA was more constitutive in very young plant tissue. Plant line MCF134 showed the expected inducible pattern for MeGA expression (Figure II.6.D). This RT-PCR method was not quantitative for expression of CFTR directly, but it is a qualitative measurement of the presence for low levels of transcript. The leaf tissue used was very young, not fully expanded, and the plantlets were still being maintained on agar medium when initially analyzed. MeGA-driven expression of genes is developmentally regulated as well as wound inducible, so expression of MeGA-driven CFTR could be occurring in these tissues. Several plant lines were tested by three independent induction experiments and shown to have similar expression patterns (Figure II.6 panels A, B, C, and D). In all samples there was little to no genomic DNA contamination in the total RNA extracts as shown by using tobacco actin primers that span an intron, allowing differentiation between genomic DNA and mRNA. Tissues expressing CFTR mRNAs were subsequently analyzed for CFTR protein production.

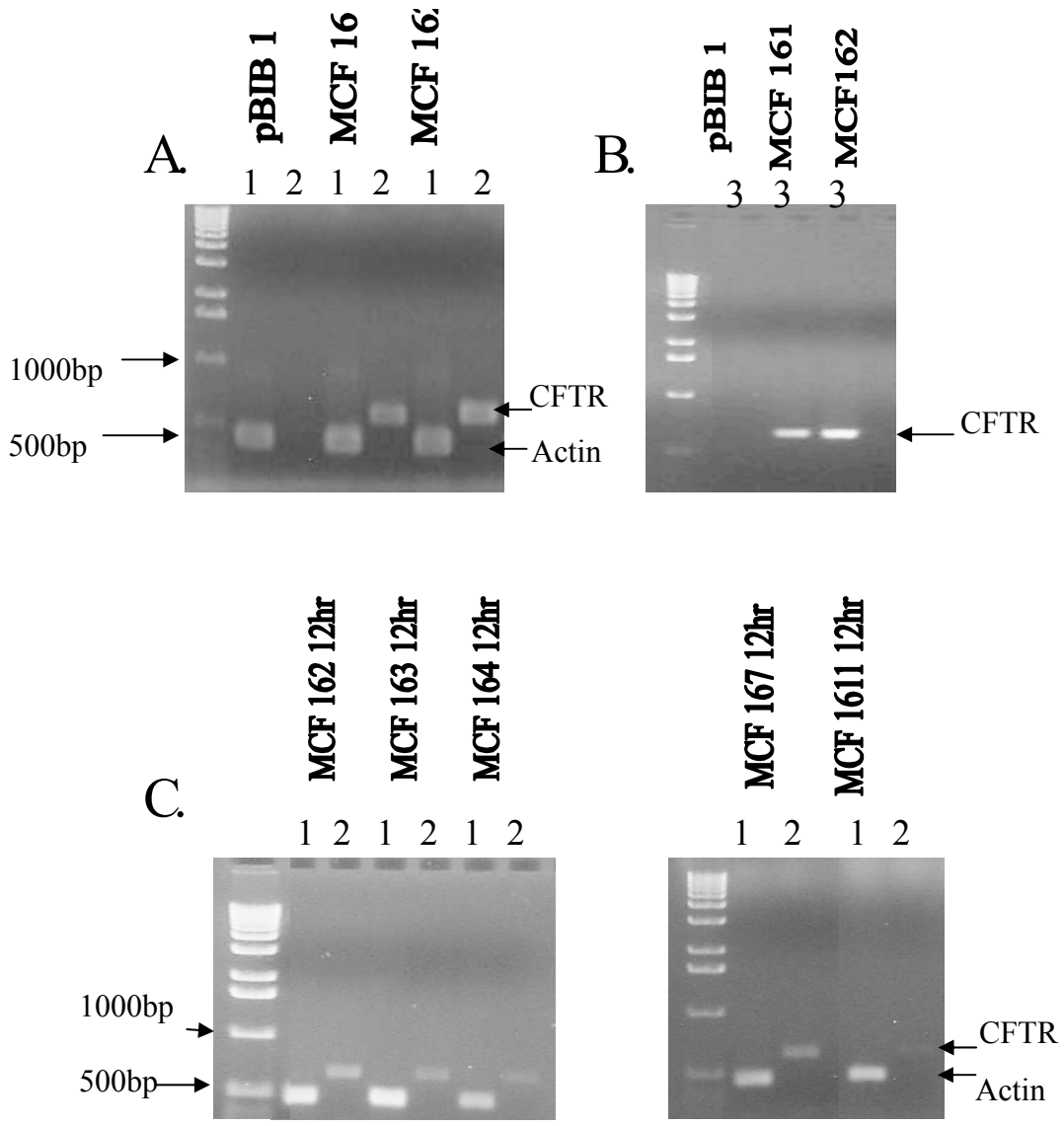

Figure II.6 RT-PCR of MeGA:CFTR Plants. A-C are RT-PCRs of RNA from leaf tissue that had been induced for 12 hours. Tobacco actin primers generating a 450 bp RT-PCR product, were used to serve as a control for message RNA quality. Figures II.4.A and B are one set of tissue induction, while block C is a separate induction event using different leaf tissue. In each panel lane 1 represents the RT-PCR product using actin primers, lane 2 represents the RT-PCR product using CFTR primers, W46 and W47, lane 3 represents the RT-PCR product using CFTR primers W51 and W52.

D.

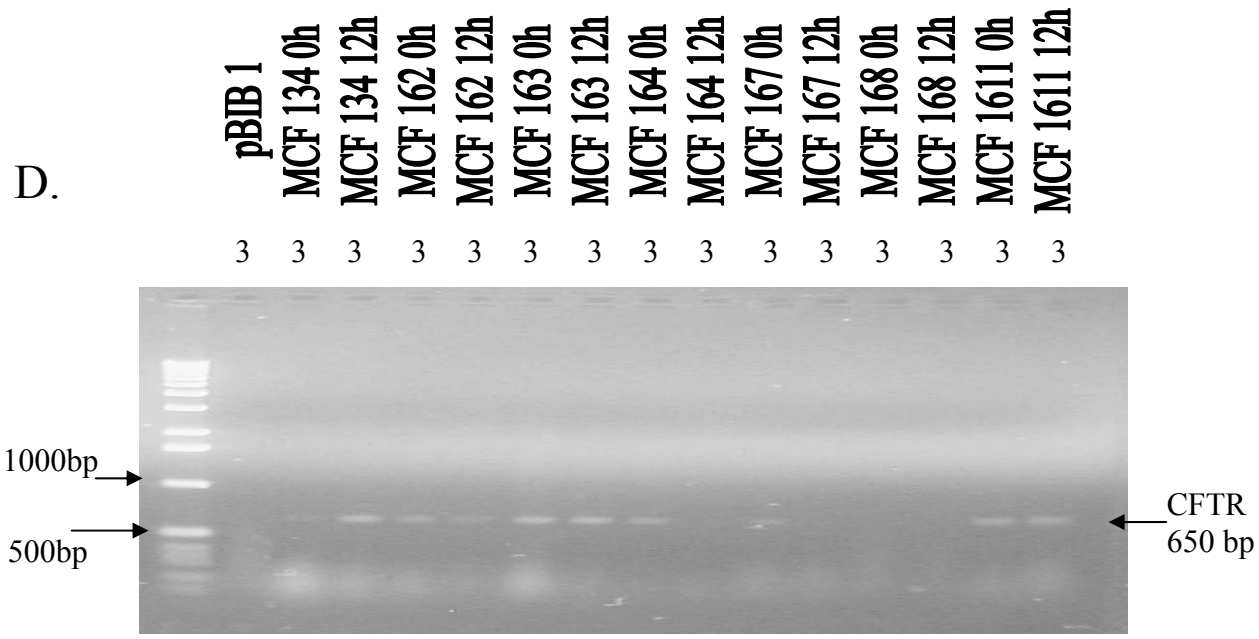

Figure II.6 RT-PCR of MeGA:CFTR Plants. (Continued) Figure II.4.D is a time course of induction for 0 and 12 hours for leaf tissues of transgenic plants. Lane 3 represents RT-PCR product of 650 bp using primers W51 and W52.

II.3.4 Protein Expression Analysis of MeGA:CFTR

For transgenic tobacco having MeGA:gene fusions, transgene product is generally recovered 24-48 hours after wound induction (Cramer et al., 1999). Protein was isolated from MeGA:CFTR leaf material at 0, 24, and 48 hours post induction. Membrane proteins were enriched by differential centrifugation and detected by western blot analysis. For initial analysis of membrane proteins, Coomassie stained gels and western analysis revealed a protein aggregation at the top of the gel for samples from the MeGA:CFTR plants, but not in the Xanthi plants. Mild detergents, Triton X-100 and LPG, were used to solubilize the CFTR aggregates (Huang et al., 1998). After detergent treatment, the aggregation disappeared and unique protein bands appeared in the transformed plant microsome extracts. Two sources of native mammalian CFTR, vero cells (monkey kidney cells) and HT29 human colon carcinoma cells, were used as positive controls since no purified CFTR protein or other positive controls were available. Both of these animal cells showed a 170-180 kDa protein band on western immunoblots using microsomal protein extracts (Figure II.7 and 8). The anti-CFTR antibodies cross-react with several Xanthi tobacco proteins including the Rubisco large subunit (55kDa) and proteins near 110 kDa and 210 kDa size. Each of the antibodies used individually gave the similar banding patterns, so the use of all three together could give a more specific recognition of CFTR (Figure II.7 and 8).

A 130-140 kDa protein unique to the transformed plants was detected only in the microsome fraction (Figures II.7 and 8). At 24 hours of induction, the unique band was faint and the monoclonal antibodies cross-reacted with many proteins in the plant microsome extract. Since CFTR protein is complex and large, the 48 hour induction time was used to allow for protein processing. Plant MCF161 showed a weak 140 kDa band at 0 hours and very strong band at 48 hours (Figures II.7 and II.B). For other plant lines, MCF162, MCF 134, and MCF 1611, the 48 hour induction of the 130-140 kDa protein band was subtle as compared to expression in non-wounded extracts. Also, these plant lines expressed this protein at time zero. This 140 kDA band is similar in size to the CFTR expressed in the insect and yeast production systems. Also, there was a protein band of larger molecular weight (about 300 kDa) that was unique to the microsomal fraction of induced transgenic plants, especially in plant line MCF161 at 48 hours.

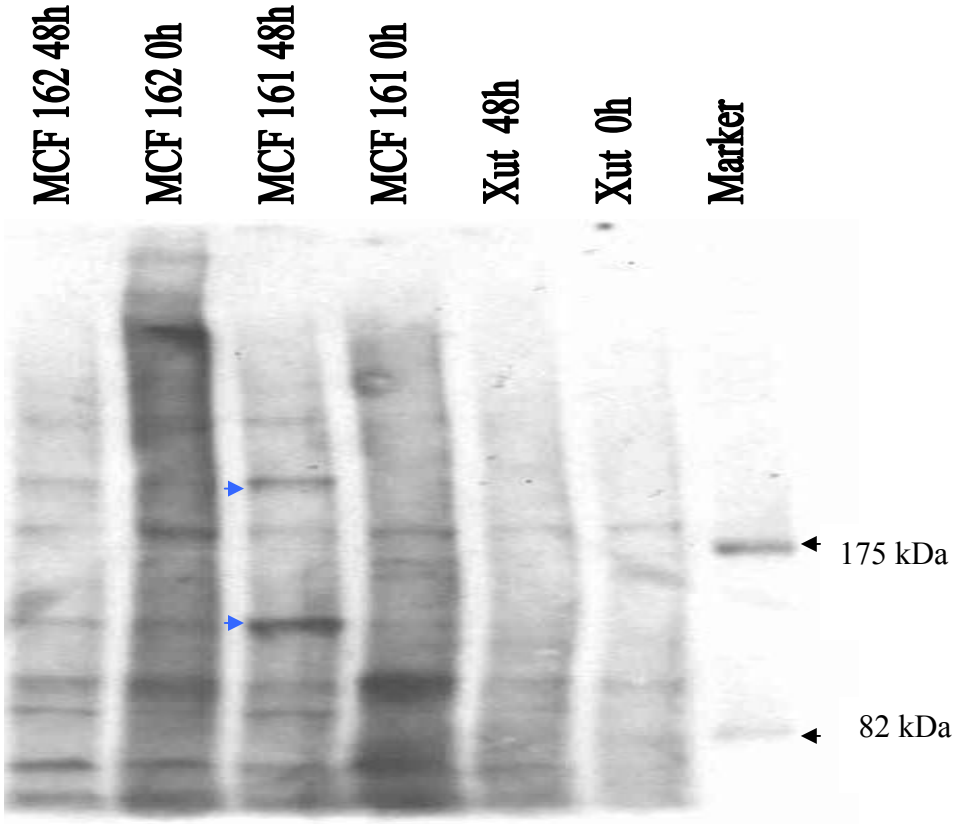

Figure II.7 Western Analysis of MeGA:CFTR Plants. Immunodetection of CFTR was done using MAI-935, CF3, antibody. The wound induction time is 0 and 48 hours for each plant line. Proteins were resolved on 6% tris-glycine gels. Blue arrows indicate novel protein bands in CFTR transgenic plants.

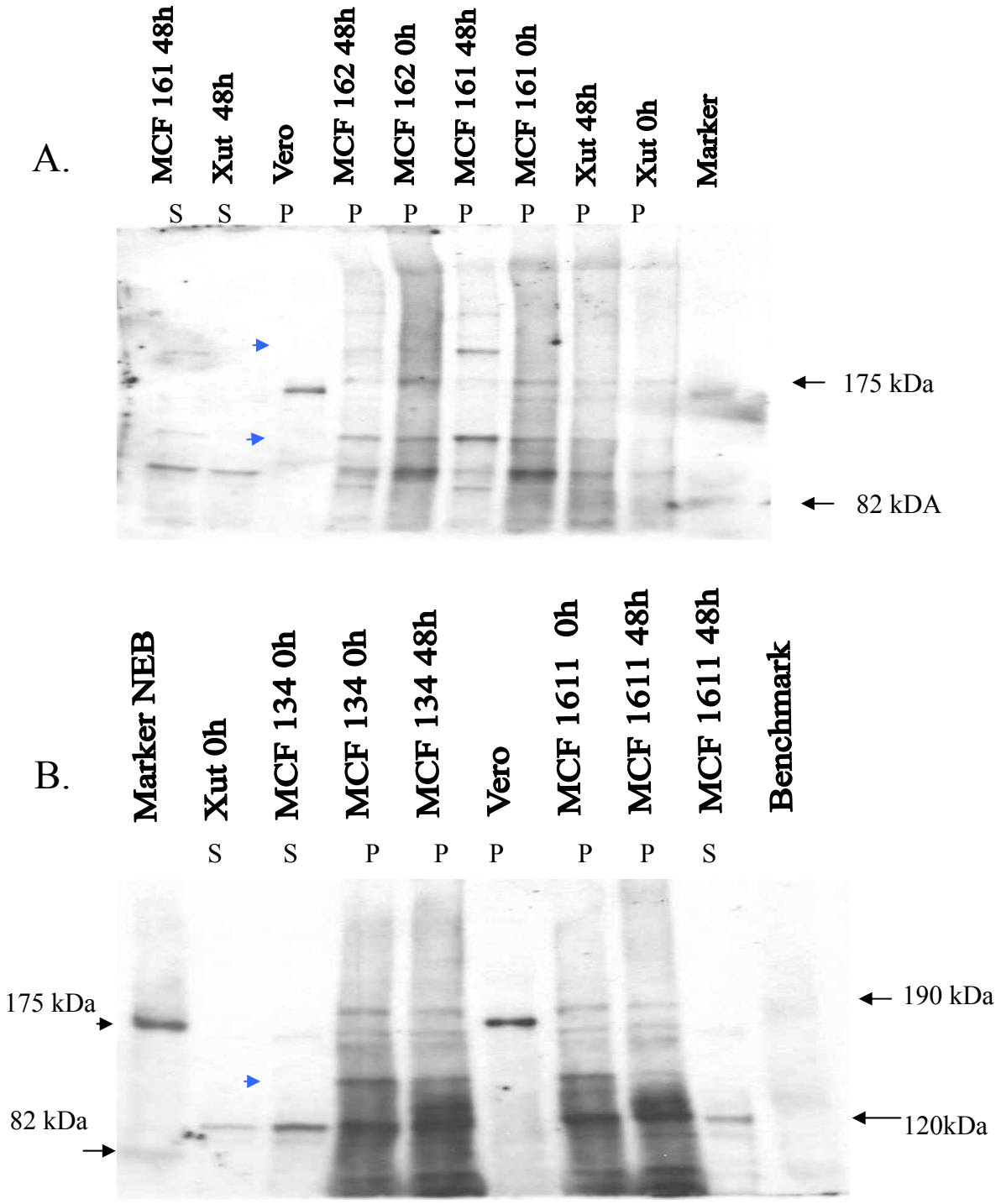

Figure II.8 Western Analysis of MeGA:CFTR Expression. Figure II.8.A and B are detection of CFTR by the combination of antibodies of MAI-935, R domain, and CF24-1 (C-terminus) of plant lines before and after 48 hours of induction. The lanes that are indicated by "S" represent the supernantant of the 100,000xg centrifugation, while lanes "P" represent the resuspended microsomal pellet of 100,000xg centrifugation. Blue arrows indicate unique protein band detection by CFTR antibodies.

II.4 Discussion

To express human CFTR in plants, two plant promoters were fused to the cDNA of CFTR. The cauliflower mosaic virus promoter with dual enhancers (CaMV 35S ^{DE}) was initially chosen to drive constitutive expression of CFTR protein in plants, because it has been used successfully to express many heterologous proteins, including human proteins. Due to the lethality of CFTR expression in *E. coli* (Gregory et al., 1990; Boyd et al., 2000) and the leaky nature of the CaMV 35S ^{DE} promoter in bacteria, CFTR constructs driven by the CaMV 35S ^{DE} promoter were not viable. A more specific and controlled expression system was needed to successfully express CFTR in plants. Therefore we tested the utility of the wound inducible promoter (MeGA) to drive expression of this protein in the plant. The MeGA promoter was derived from the *Hmg*2 gene, which encodes the defense related HMG CoA reductase (HMGR). MeGA is a truncated version (450 bp) of the 2.3 kb tomato *Hmg*2 promoter region originally characterized by promoter:GUS fusion (Yu, 1995) and appears to retain all the defense-related regulation of the larger fragment (Cramer et al., 1999). Of significance for this project, MeGA has minimal expression in bacteria unlike the caulimovirus promoters. Plant binary constructs containing MeGA-driven CFTR were introduced into *A. tumefaciens* and used to transform tobacco. Successful integration of MeGA:CFTR in transgenic tobacco was shown by PCR and Southern analysis. Twenty-five plants were found to contain the transgenes CFTR and NPT II.

In order to identify lines expressing MeGA:CFTR, young plant were initially screened at the RNA level. The *Hmg*2 gene is expressed in unstressed plants in trichomes, pollen, during cotyledon expansion, lateral root initiation (Weissenborn et al., 1995) and fruit ripening (Gillaspy et al., 1993). *Hmg*2 and MeGA promoters respond strongly to stresses like wounding (Weissenborn et al., 1995), pathogen attack (Westwood et al., 1998), and elicitors (Cramer et al., 1999; Rodriguez-Concepcion and Gruissem, 1999). Peak RNA levels generally occur 9-14 hours after a wound event (Cramer et al., 1985) and protein expression peaking later at 24-72 hours (Rodriguez-Concepcion and Gruissem, 1999; Cramer et al., 1999) depending on the product produced. However, the CFTR transcript is large (4.4 kb) and is generally not accumulated to high levels in mammalian cells or in heterologous systems (Gregory et al., 1990; O'Riordan et al., 1995). Reverse-transcription PCR was therefore selected to

detect the presence of CFTR transcript in tobacco, because this method is very sensitive and can detect transcripts using small amounts of CFTR RNA. Transcripts were detected 12 hours post-wounding of young tissue in most of the plants shown to contain the CFTR gene (see Figure II.6A-C). However, transcript was also detected in non-induced tissue (time = 0hr) which was not expected (Figure II6.D). The plant line MCF 134 showed a more typical induction pattern, while lines MCF 161 and MCF 162 showed equal or greater expression at time zero than at 12 hours. This may be due to the developmental expression or environmental conditions. Very young tissue growing *in vitro* was used in the experiments, and MeGA may be active under these conditions. The growth conditions in phyta boxes (Sigma, St. Louis, MO), a physical hindrance on growth, could have played a role in causing MeGA induction by creating stress for plants. The RT-PCR method used was mainly qualitative. The tissues were taken at different times and different levels of leaf maturity. This variability between sampling along with the young tissues that were used could explain the possible constitutive nature of the RNA expression results. Allowing the plants to develop and increase in biomass may furnish transgenic material providing more consistent inducibility and product yield. The sampling could be done on one leaf for the entire time course of RNA expression.

The goal of the project was to test the feasibility to produce CFTR protein in tobacco plants. CFTR is a large membrane-associated glycoprotein and found in low abundance in epithelial tissues of animals. Its predicted mass is 168 kDa based on amino acid sequence, but its apparent size is greatly affected by glycan composition (Gregory et al., 1990). Typically, MeGA driven proteins show peak expression 24 to 72 hours after wounding of mature tissues. While the plant samples used for the 0 and 48 hour induction experiments were more mature than the previous 24 hour experiment, and expression was similar to predicted MeGA expressed proteins especially in plant line MCF 161. The unique band in the induced MCF plants, approximately 135 kDa, was near to the reported CFTR protein size that was produced in insect and yeast cells (O'Riordan et al., 1995; Kiser et al., 2001). The control cell lines vero and HT 29 gave a CFTR antibody reactive band at ~170 kDa. The 135 kDa band has been reported to comprise of CFTR having only core glycosylation. Cells possessing the most common disease-causing mutation in CFTR, ΔF508, create this size of CFTR protein, and the CFTR protein resides in the ER (Lukacs et al., 1993). The unique and inducible high molecular weight band (~300kDa)

could be a CFTR dimer protein or altered glycosylation forms. Attempts to assay the glycosylation status of the plant-produced CFTR by enzymatic digestion were not successful. After treatment with the glycosidases, endoH or endoF, the mobility of putative CFTR protein remained the same in either vero cell- or plant-derived CFTR extracts. The protein mixture of the microsomal pellet was complex, and the glycosidase enzymes probably need more purified protein to effectively digest glycans. Definitive identification based on electrophoretic behavior or cross-reactivity with available antibodies is difficult due to the unusual mobility of this highly hydrophobic glycoprotein and the limitations of current peptide-based anti-CFTR antibodies. As more biomass becomes available from plant line MCF 161, it maybe possible to purify sufficient tobacco-derived CFTR for N-terminal sequence analysis or MADLI-TOF mass spectrometry of tryptic digests.

Our data suggests that our putative CFTR product is found exclusively in membrane enriched fragments. The microsomal preparation contains the plasma membrane, endoplasmic reticulum (ER), tonoplast membranes and Golgi membranes, so the exact location of the CFTR protein was not determined. The use of sucrose gradients, commonly used to separate these membranes, could further characterize the protein location and purify the plant expressed CFTR protein. Immuno-localization by EM or confocal microscopy could be used to assay the destination of expressed CFTR in the cells of plants. However, immunofluorescence analysis may be challenging in tobacco due to autofluoresence of tobacco. With larger amounts of starting material, plant expressed CFTR protein localization, purification, and further characterization should become more feasible.

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Chapter III. Introduction of a Plant Intron into CFTR cDNA

III.1 Introduction

As described in Chapter II, the production of CFTR protein in transgenic tobacco was successful using the inducible promoter MeGA™, while the engineering of constitutive expression of CFTR was futile. However, the development of bioproduction systems involving strong constitutive promoters for CFTR expression is still desirable. The physiological effects of constant expression of CFTR in tobacco could prove insightful on structure and function of this important human protein. The inability to propagate CaMV $35S^{DE}$:CFTR plasmids in *E. coli* was presumably due to expression of the 35S promoter in bacteria leading to synthesis of CFTR, which is detrimental. The insertion of an intron into the cDNA of CFTR could alleviate the detrimental production of CFTR protein in bacteria.

Most of eukaryotic genes (80-90%) contain exonic sequences that are interrupted by intervening sequences (introns). These introns need to be removed from transcripts so that proper translation into functional proteins occurs. Introns can allow multiple products from single genes. The removal or processing of introns is called splicing and occurs in the nucleus. Splicing begins with the pre-message RNA transcription from genomic DNA and assembly of a large ribonucleoprotein complex termed the spliceosome (Lorkovic et al., 2000). This complex catalyzes the excision of intronic sequences as a loop structure, termed a lariat, and ligates the exons together (Brown and Simpson, 1998). This process is conserved throughout higher organisms and some of the splicing signals are common to all eukaryotes (Lim and Burge, 2001).

Introns of most eukaryotes contain a basic design consisting of 5'splice site, branch point and 3'splice site (Table III.1), but vary greatly in sequence. The general consensus sequence for the intron/exon junctions in *Arabidopsis* is 5'-AG:**GT**AA…. ….GC**AG:**GT-3' with the GT and AG (in bold) the only nucleotides that are consistent in all organisms (Lim and Burge, 2001). The branchpoint in higher animals is located 20 to 22 nt upstream of the 3'splice site. In plants, the range is significantly larger with branchpoints 20 to more than 100 nt upstream of the 3'splice site. The consensus branchpoint sequence for most animals and plants is cytosine, uracil, purine, adenine, pyrimidine, (CURAY) with the uracil and adenine being the essential nucleotides. In plants, unlike animals, only a few of the branchpoint sites have been experimentally confirmed (Lui and Filipowicz, 1996). Vertebrates have polypyrimidine tracts in the

introns between the branchpoint and 3'splice site, while plants require AU- and U-richness as compared to flanking exonic sequences (Carle-Urioste et al., 1997; Ko et al., 1998). There is even divergence among plant groups. Dicots are more stringent than monocots with respect to the transitions from GC-rich exon to AU-rich intron sequence (Simpson and Filipowicz, 1996). Intron position and sequence do play a role in the translational and splicing efficiency of the mRNA in plants (Bourdan et al. 2001).

Expression of certain eukaryotic and viral genes in bacteria causes detrimental effects to the host. This fact makes the cloning and expression more difficult. Several commonly used plant-based promoters, for example CaMV 35S, are recognized by bacterial transcription factors and cause expression of the target gene. Most cloning steps use bacteria to create large amounts of desired construct plasmids. When cloning genes of a detrimental nature into bacteria, expression of the gene needs to be suppressed.

The detrimental effects of bacterial expression of harmful genes can be overcome by insertion of an intron into the coding region. Nearly all introns have stop codons thus creating truncated non-functional proteins in bacteria. Introns can change the protein that is translated in bacteria by having a longer transcript than genes without an intron. This technology has been successfully used in plant virus cloning and in CFTR viral vector plasmid in bacteria. Plant viral cDNAs, plum pox virus (Lopez-Moya and Garcia, 2000) and lettuce mosaic virus (Yang et al., 1998) are lethal to bacteria when fused to the CaMV 35S promoter. With plant intron insertion into the cDNA, both of these viral constructs were stable in *E. coli* and their ability to be infectious to their hosts was not compromised (Lopez-Moya and Garcia, 2000; Yang et al,. 1998). For development of mammalian expression vectors, insertion of intron 6a-6b of human CFTR back into the cDNA of CFTR was required for plasmid stability in *E. coli* (Boyd et al., 1999).

The objective of this research was to place a plant intron into CFTR cDNA in order to stabilize CFTR in plant-based expression vectors in bacteria. A plant intron was chosen and inserted into the cDNA of CFTR. The tomato *Hmg 2* intron 2 was selected as a good candidate for insertion into the CFTR exon 3/4 junction. The criteria were: few restriction sites in the intron, small size of the intron (197 bp), presence of an inframe stop codon 12 bp into the intron sequence, and similarity of junction region of CFTR exon 3/4 junctions in both plants and humans. Intronic sequences were inserted into the cDNA of CFTR by

Table III.1 Crucial Features of Intron Anatomy

Intron	**5' Splice Site**	**Branch point[2]**	**3' Splice Site**
Plant/Human[1] consensus	AG:**GU**AAG	C**U**U/C**A**A/G	C**AG**:GU
CFTR intron 3	GGG:GUAAG	CUCAG -60	UGUAG:GAA
*Hmg*2 intron 2[a]	CTG:GUAUU	CUAAA -20	GACAG:GGA
*Hmg*2 intron 2[b]	CTG:GUAUU	UUGAU -46	GACAG:GGA
*Hmg*2 intron 2[c]	CTG:GUAUU	UUGAU -55	GACAG:GGA
CFH2-2	GGG:GUAUU	CACUA -20	GACAG:GAA
CFΔH2	GGG:GUAUU	CUAAA -20	GACAG:GAA

Bold letters are crucial for splicing recognition

[1]Human and higher plants are highly conserved in the regions except for the branch point location.

[2]Branch points are proposed and base pair distance is from 3'splice site.

[a,b,c] Possible putative branchpoint sequences of *Hmg*2 intron 2

PCR mutagenesis. The intron-containing CFTR was fused to the CaMV $35S^{DE}$ promoter or MeGA promoter. Bacterial clones of both promoter fusion constructs were stable and plants were transformed via *Agrobacterium*-mediated transformation. Intergration of transgene was confirmed by PCR and Southern analysis. Expression of the CFTR gene and splicing of the intron was determined by RT-PCR.

III.2 Materials and Methods

III.2.1 Construction of Intron-Containing CFTR Gene

PCR mutagenesis was used to create a unique splice site in the cDNA of CFTR between exon 3 and exon 4. PCR-based mutagenesis was used to create both 3'and 5' intron-exon junction sites and to introduce a unique *Spe*I site. The rest of the *Hmg*2 intron 2 was amplified by PCR with primers that provided flanking *Spe*I sites and then introduced into the modified CFTR sequence as a 168 bp *Spe*I fragment to recreate the entire *Hmg*2 intron 2 at the exon 3/4 junction site.

Creation of 5' Splice Site. PCR was performed to create the splice sites of *Hmg*2 intron 2 in the cDNA of CFTR. The plasmid pBC:CFTR and primers W1 (Table II.1) and W14 (5'ACTAGTTAA GGAAAAACTATTCCCCTAAATATAAAAAGATTCC) were used to introduce a *Spe*I site (underlined) and the 5'splice site of *Hmg*2 intron 2 at the 3' end of exon 3 of CFTR. The PCR product, CF3-H2, was 310 bp and was flanked by *Kpn*I and *Spe*I. Parameters for PCR using the primer pairs were thirty cycles: 1 minute denaturation at 95°C, 1 minute annealing at 53°C, and 1 minute elongation at 72°C.

Creation of 3' Splice Site. The primers W15 (5'ACTAGTTATCTATAACT GTGTACAGGAAGTCACACCAAAGCAGTAACA) and W2 (Table II.1) were used in PCR to introduce the *Hmg*2 intron 2 3'splice site and a *Spe*I site (underlined) adjacent to the beginning of exon 4 of CFTR. The PCR product, CF4-H2, was 350 base pairs and was flanked by *Spe*I on one end and contained the *Xba*I site of CFTR cDNA. Parameters for PCR using the primer pairs were thirty cycles: 1 minute denaturation at 95°C, 1 minute annealing at 53°C, and 1 minute elongation at 72°C. The CF3-H2 product was digested by *Kpn*I and *Spe*I, and the CF4-H2 product was digested by *Spe*I and *Xba*I. A trimolecular ligation was used to reconstruct the CFTR coding sequence but with the "ends" of the *Hmg*2 intron 2 inserted between exon 3 and 4 (Figure III.1). These digested pieces were ligated into pBC CFTR that was cut with *Kpn*I and *Xba*I. The original *Kpn*I/*Xba*I piece was removed from the pBC CFTR construct by digestion and gel

purification. The vector pBC 3'CFTR portion (8 kb) was gel extracted and purified using a Gel Extraction Kit (Qiagen). The modified 5'CFTR containing splice sites and the 3'CFTR were ligated. The new splice site-containing pBC CF-H2 vector was transformed into *E. coli* cells (Top Ten, Invitrogen). The colonies that grew on selection media were checked for insertion of the splice site by restriction digestion with *Kpn*I, *Xba*I and *Spe*I. The plasmids from selected colonies were sequenced to confirm insertion and fidelity of the splice site.

Introduction of Remaining Portion of *Hmg*2 Intron2. The internal portion of the *Hmg*2 intron 2 was PCR amplified using primers W10 (5'ACTAGTCGTCTATGCAT GGTCTGT) and W11 (5'ACTAGTGGAGACAATATT ATCTAAAAG) with the template of pTH295 (genomic clone of tomato *Hmg*2, Park et al., 1992) to introduce a *Spe*I site (undelined). Parameters for PCR were 30 cycles: 1 minute denaturation at 95^{o}C, 1 minute annealing at 53^{o}C, and 1 minute elongation at 72^{o}C. The 190 base pair product was digested by *Spe*I and inserted into the *Spe*I site in pBS (Invitrogen) as a *Spe*I fragment. This pBS intron 2 plasmid was sequenced for DNA polymerse fidelity. The *Spe*I internal fragment of intron 2 was ligated into pBC CF-H2 vector. Plasmid DNA from antibiotic-resistant colonies was isolated. Digestion of the pBC CF-H2 intron 2 plasmid by *Spe*I was used to identify isolates that contained the internal intron fragment. PCRs using primer sets W10/W2 and W1/W11 were performed to identify clones having the desired orientation of the *Spe*I fragment. DNA sequencing was used to confirm the clones that were correct with respect to the PCR reactions. Constructs that contained the desired intronic sequence were called pBC CFH2-2.

Promoter Fusions and Plant Transformation Vectors. The MeGA promoter (see Chapter II) was fused to this construct via the *Kpn*I site adjacent to the CFTR start codon. Orientation of the MeGA promoter with respect to the CFH2-2 was determined by PCR and sequencing. The MeGA:CFH2-2 construct was inserted into pBIB-kan binary vector (Becker et al., 1990) as a *Sac*I/*Sac*I fragment. Insertion and orientation was monitored as described for pBIB MeGA:CFTR in Chapter II.2.1. CFH2-2 was inserted into pBIB-kan as a *Kpn*I/*Sac*I fragment. The CaMV 35S^{DE} promoter was inserted as a *Kpn*I fragment into the pBIB CFH2-2. Orientation of CaMV 35S^{DE} promoter in the pBIBCFH2-2 was determined by PCR and sequencing. The constructs, pBIBMeGA:CFH2-2 (named CHM)

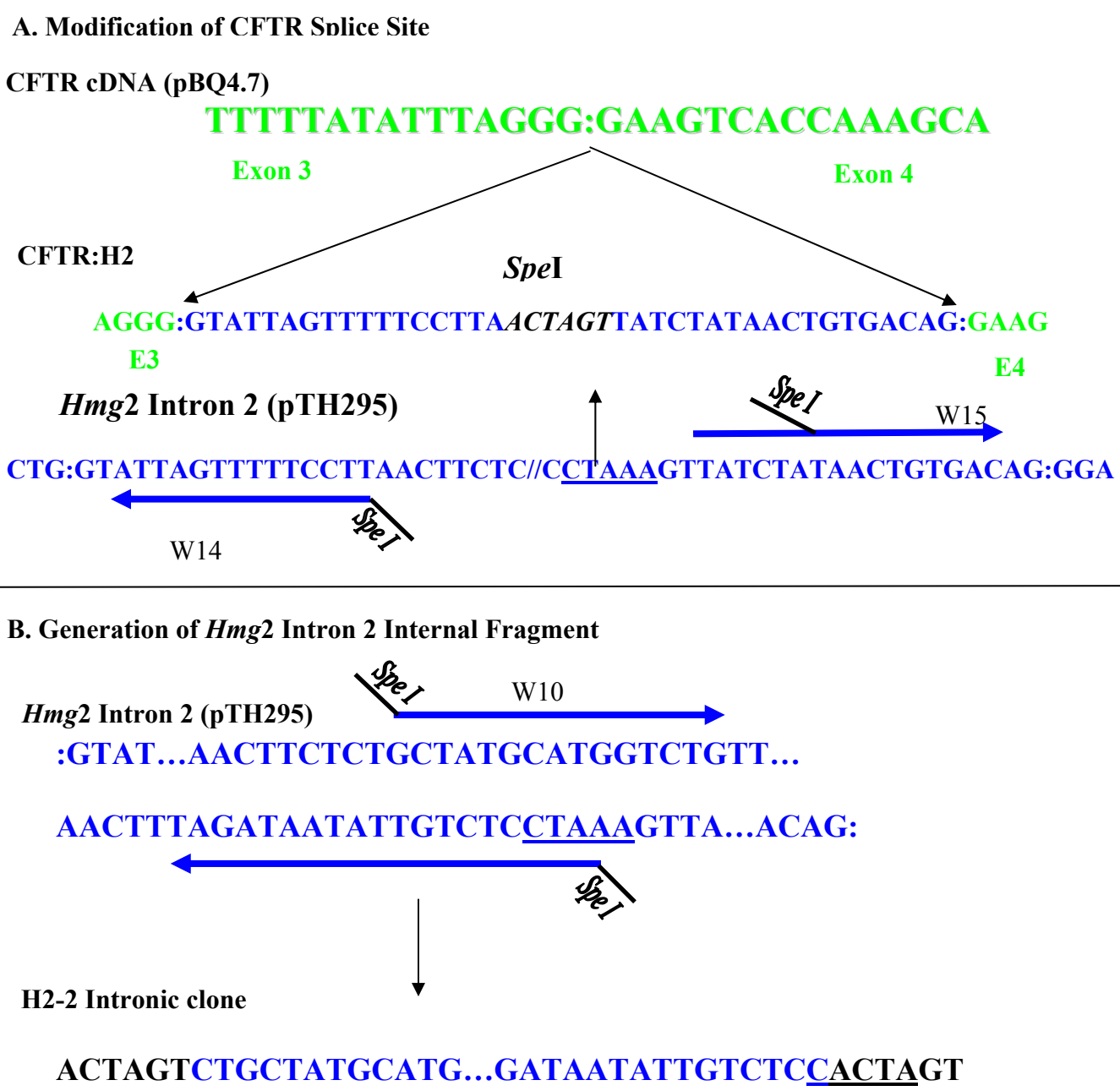

Figure III.1 Intron Insertion into cDNA of CFTR. **A.** The tomato *Hmg*2 intron 2 (from pTH295 genomic clone) was inserted between exon 3 (E3) and exon 4 (E4) of the CFTR cDNA. PCR mutageneses were used to introduce the *Hmg* intron splice sites individually to the E3 and E4 sequences of the cDNA of CFTR. The use of a unique restriction site, *Spe*I, enabled the incorporation of intronic sequence (H2-2) into the CFTR gene. The putative branchpoint of *Hmg*2 intron 2 is underlined. **B**. Cloning of H2-2 internal section of the intron with the addition of *Spe*I site.

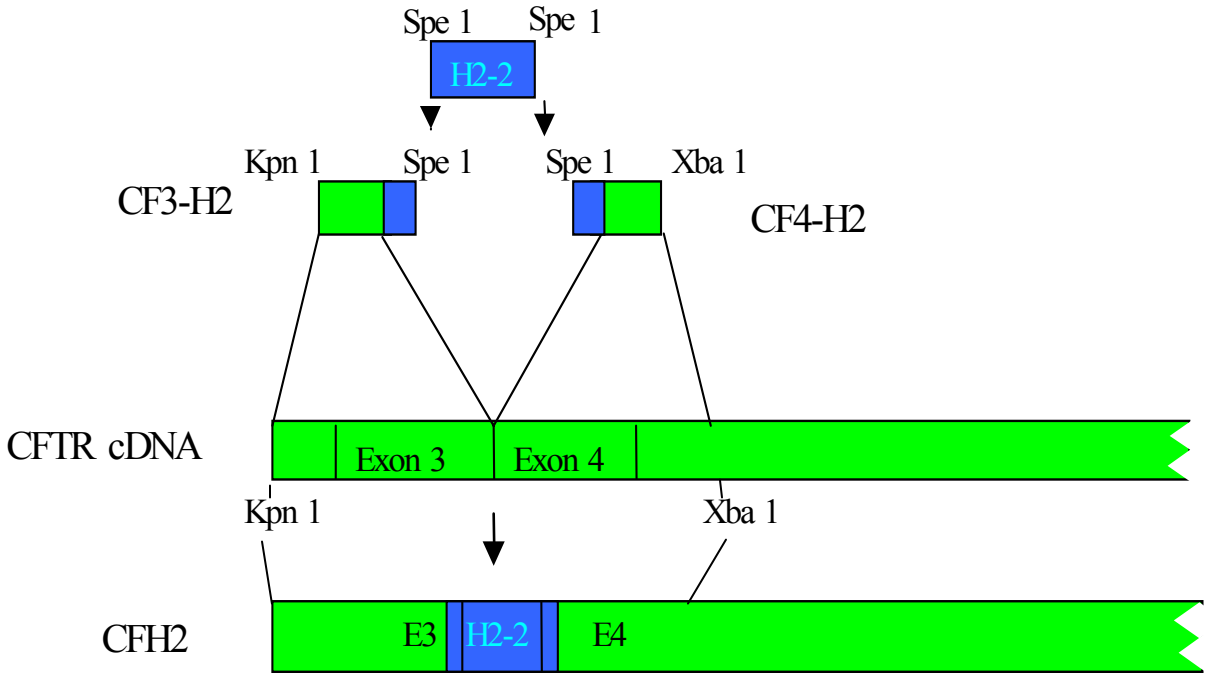

Figure III.1.C. Diagram of Intron Introduction into cDNA. The large view of the construction of intron insertion process shows the insertion of 197 bp intron into the exon3/4 junction site of CFTR. Diagram is not to scale.

and pBIB CaMV 35S^{DE}:CFH2-2 (named CHC) were then transformed into *Agrobacterium tumefaciens* and plants as described below (Figure III.2).

Site Directed Mutagenesis of pBC CF:H2-2. The original construction of the *Hmg*2 intron 2 insertion (described above) had modified the possible branchpoint from CTAAA to CACTA by incorporating the *Spe*I site, which may compromise splicing. Site-directed mutagenesis (X-cite Mutagenesis, Stratagene) was used to revert the branchpoint in the pBC CFH2-2 plasmid back to the original sequence of *Hmg*2 intron2. The PCR-based mutagenesis was performed using primers W47 (5'ACAATATTATCT AAGTTCAAGAGATC) and W48 (5'CTCCTAAAGTTATCTATAACTGTGAC) on the pBC CFH2-2 plasmid. The protocol for mutagenesis was 10 cycles of 1 minute at 95^{o}C, 1.5 minutes at 55^{o}C, and 3.5 minutes at 72^{o}C. The primers were oriented in opposite directions on different stands of DNA, creating a new linear plasmid. The template plasmid was digested away using *Dpn*I which digested methylated DNA. Plasmid DNA isolated from most bacteria including *E. coli* is methylated when plasmids replicate *in vivo*. The remaining uncut linear plasmid DNA was ligated and transformed into *E. coli* Top Ten cells. Colonies that grew on selection media were grown in liquid cultures and plasmids were isolated. The mutated plasmids were digested by *Spe*I. PCR was performed using W1 and W2 primers (Table II.1) on the templates pBC CFH2-2 and new pBC CFΔH2-2. The PCR products were digested by *Spe*I and checked for band size differences. Successful mutagenesis will eliminate the 3' *Spe*I site. The correctly mutated construct was sequenced for confirmation of the restoration of branch point back to original tomato *Hmg*2 intron 2. The promoters CaMV 35S^{DE} and MeGA were inserted into the pBC CFΔH2-2 plasmid as *Kpn*I fragments. Insertion of promoters was confirmed by restriction digestion with *Kpn*I and orientation of promoters was confirmed by PCR (Figure III.2).

III.2.2. Insertion of Promoter:CFTR:Intron Constructs into a Plant Binary Vector

The binary vector pBIB-KAN was used in these experiments (Becker et al., 1992). This vector contains the NOS promoter driving expression of the neophosphotransferase II (NPT II) gene, conferring kanamycin resistance, and a multiple cloning site with a polyA

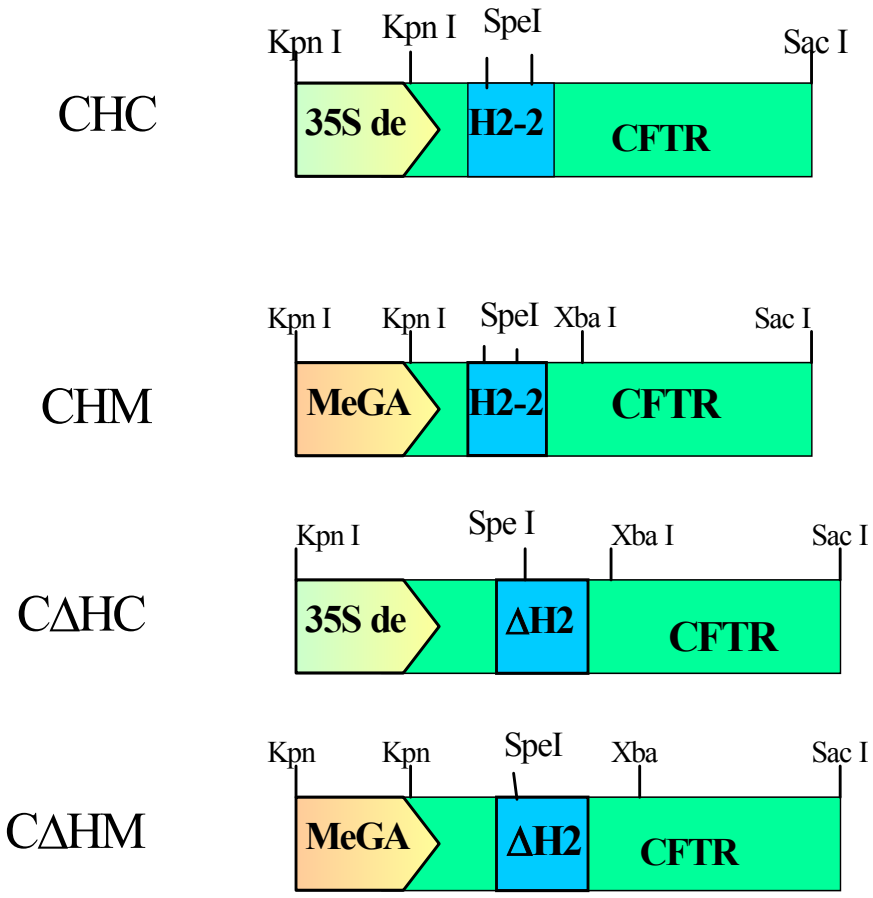

Figure III.2 CFTR-Intron Constructs Used for *Agrobacterium*-Mediated Transformation of Tobacco. Introns were placed into the exon 3 and exon 4 junction in the cDNA of CFTR. Intron H2 has two *Spe*I sites and the modified intron ΔH2 has only the 5' *Spe*I site and the restored putative branchpoint signal sequence. It is not to scale.

signal of the NOS gene. The CFH2-2 and CFΔH2-2 were inserted as a *Kpn*I/*Sac*I fragment. The CaMV $35S^{DE}$ promoter was placed as a *Kpn*I fragment into the pBIBCFH2-2 and pBIB CFΔH2-2 plasmids. The new constructs were called pBIB CHC and pBIB CΔHC. Orientation of the promoter was analyzed by PCR, using primers W21/W2, and sequencing. The MeGA CFH2-2 (CHM) and MeGA CFΔH2-2 (CΔHM) constructs were inserted as a *Sac*I fragment into pBIB plasmid. PCR, using W21/W16 primers, and sequencing were used to confirm the orientation of gene insertion.

III.2.3 *Agrobacterium tumefaciens* Transformed with CFTR Plus Introns

Agrobacterium tumefaciens strain LBA4404 was used to produce stable transgenic tobacco plants. This strain is widely used to transform solanaceous plants including tobacco and contains all the necessary components to infect tobacco and transfer DNA into the plant genome. *A. tumefaciens* was transformed with pBIB, pBIBCHC, pBIB CΔHC, pBIB CHM, and pBIB CΔHM by the freeze/thaw method (Chapter II.2.1). Selection for transformed colonies was performed on YEP media containing 60 mg/l streptomycin and 100 mg/l kanamycin. Colonies were grown in 5 ml cultures of YEP medium and plasmids were isolated. Restriction digests with *Sac*I were performed and PCR using W21 and W2 primers were used for confirmation of transformation.

III.2.4 Transformation of Tobacco Using *Agrobacterium tumefaciens*

The petiole scoring method of infection of tobacco with the *A. tumefaciens* LBA4404 was used (Medina-Bolivar et al., 2003). Small, fully developed Xanthi leaves were excised from plants grown *in vitro* with about 0.5cm of petiole on the leaf. A sterilized scalpel was dipped into an *Agrobacterium* colony and then used to score the petiole parallel to the veins. The scored plant leaves were placed on MS medium for 2-3 days until a halo of bacteria was seen growing out of the petiole. The leaves were then placed on selection MS shooting medium, including hormones α-napthalene acetic acid (NAA) and 6-benzylaminopurine, with 200 mg/l kanamycin to select for transgenic plants, and 500 mg/l carbenicillin to kill the bacteria. After two to three weeks, shoots formed. The shoots were excised and placed on MS rooting medium (no hormones) containing 200 mg/l kanamycin and 250 mg/l carbenicillin to select against any escaped non-transformed plantlets and bacteria. Roots usually formed about two weeks after

placement on the rooting media. The rooted plants were analyzed for the presence of the transgenes, CFTR and NPT II.

III.2.5 Analysis of Transformed Tobacco Plants

PCR was used to confirm the integration of the transgene into the genome of the plant. Specific amplification of a target DNA using specific primers for CFTR was used to confirm putative transformants. Genomic DNA was obtained from the Xanthi plants using the Qiagen DNeasy plant extraction kit (Qiagen). One hundred mg of leaf tissue were pulverized in the presence of liquid nitrogen. Then buffers were added to separate the genomic DNA from RNA and proteins. The genomic DNA was eluted from the DNeasy column in 200ul of elution buffer. Gene specific primers W1, 5'CFTR, (5'TA TGGTACCATGCAGAGGTCGC) and W2, 3'CFTR, (5'CCATGAGGAGTGCCAC TTGCA) were used for specific amplification of the CFTR DNA from tobacco genomic DNA. The expected product size was 825 base pairs. PCR amplification using NPT II primers (5'AGTATTGAACAAGATGGATTGCA) and (5'TCAGAAGAACTCGTC AAGAAGG) was used to determine the insertion of transfer DNA. Transformed plants were propagated and some plants were transferred to soil in 4 inch pots and grown in a greenhouse.

Southern blot hybridization analysis was used to confirm the data generated by the genomic PCR screening. Genomic DNA (20μg) was digested with *Sac*I, then separated in a 0.8% agarose gel at 30 volts for 16 hours. Digested DNA was neutrally transferred to uncharged nylon membrane (Nytran) by capillary transfer (Sambrook and Russell, 2001). The DNA was cross-linked to the nylon membrane with UV irradiation. The membrane was incubated in Church pre-hybridization buffer (0.5M phosphate buffer, 1% BSA, 1 mM EDTA and 7% SDS) for 4 hours at 55^{o}C. The membrane was incubated in hybridization buffer (Church buffer with sheared salmon sperm DNA and ^{32}P labeled CFTR probe) for 48 hours at 55^{o}C. The probe was made by using 1.5 kb fragment (exon 1-10 in cDNA) of CFTR, αdCTP ^{32}P and Prime-It kit (Stratagene). The membrane was washed two times in 1x SSC, 0.1% SDS for 30 minutes at 55^{o}C. Then it was washed two times in 0.1x SSC, 0.1% SDS at 55^{o}C for 30 minutes. The membrane was wrapped in Saran wrap and exposed to Kodak X-OMAT film for seven days at -80^{o}C.

III.2.6 RNA Expression and Splicing Detection Using RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) was used to analyze transgene expression as in Chapter II and to assess intron splicing. For the MeGA:CFH2-2 (CHM) and MeGA:CFΔH (CΔHM) construct-containing plants, a time course of wounding was performed, while the CHC and CΔHC plant leaves were only collected at one time point. Mature leaves (2 grams) were wounded using a 2 mm wide teeth wheel of a pasta maker and stored at room temperature, 23^{o}C. Tissues were collected at 0 hrs, 12 hrs and 24 hrs after wounding and quick frozen in liquid nitrogen and stored at –80^{o}C. Total RNA was extracted to analyze transgene expression and splicing of inserted intron. Tri-Reagent (Molecular Research Center, Inc) was used along with phenol-chloroform mixture to extract and purify total RNA from the plant tissues. Two grams of leaf tissue were ground in 8 ml of Tri-Reagent by a polytron homogenizer (Fisher). Eight milliliters of phenol/chloroform were added to the homogenate, then mixed vigorously and centrifuged at 10,000x g. The aqueous phase was taken and nucleic acids were precipitated by iso-propanol. The dried pellet was resuspended in 250 µl diethylpyrocarbonate (DEPC)-treated water. The ProSTAR first-stand RT-PCR kit (Stratagene) was used to create message and gene specific cDNA's as per manufacturer's protocol. The oligo dT (15mer) primer was used to amplify all mRNA transcripts into first strand cDNAs. A PCR step using CFTR primers, W1 and W2 or W 51and W 52, was used to elucidate the expression of genes of interest and splicing of intron sequences. In order to assess splicing of the introduced intron, primers that flank the intronic sequence were used. The mRNA RT-PCR product should be smaller than that of the genomic DNA product. As a control of potential genomic DNA contamination in the RNA samples, tobacco actin primers that flank an intron were used (see Chapter II). The genomic PCR product of the actin primers is 157 bp larger that the RNA RT-PCR product.

The RNA isolation was also performed on a small scale using 100 mg of tissue. The above protocol was scaled down proportionately. The leaf tissue was ground under liquid nitrogen in a 1.5 ml eppendorf tube using an eppendorf pestle. Total RNA was resuspended in 50 microliters of DEPC-treated water as done in Chapter II.

III.3 Results

III.3.1 CFTR with Intron Construction and Tobacco Transformation

Transformation of tobacco with CFTR constructs containing the plant *Hmg*2 second intron resulted in stable transformed plants. The bacterial cloning steps were not inhibited by CFTR with intron constructs driven by either constitutive CaMV $35S^{DE}$ or inducible MeGA promoter. Further study of the design used to introduce the intron in CF:H2-2 revealed that the introduced *Spe*I site may alter the potential branchpoint site. Therefore, an additional site-specific mutagenesis was performed to restore the altered site and the resulting intron sequence was named ΔH2. *Agrobacterium*-mediated transformation resulted in twenty transformed plants with constructs $35S^{DE}$:CFTR:H2 (CHC) and 10 transformants with constructs MeGA: CFTR:H2 (CHM) contained the initial *Hmg*2 intron, and only three plants of each constitutive (CΔHC) and inducible promoter (CΔHM) with the intron containing the original putative branchpoint sequence restored (ΔH). The transgenic plants were tested for insertion of intron containing CFTR into the plant genome via PCR. Representative transformants are shown in Figures III.A and B. Amplification using CFTR gene specific primers W51 and W52 that span the intronic sequences resulted in a PCR fragment of 848 base pairs indicating the presence of the intron within CFTR. The primers for the selectable marker NPT II showed the presence in control plasmid (pBIB CHC 1) and transformed plants (CHC 111, CHC 116, CHC 117) but not in untransformed Xanthi plants (Figure III.3A). Plant CHC 116 did not show the presence of CFTR, but did show the NPT II gene. ODC primers were plant specific primers to the ornithine decarboxylase (ODC) gene, and they were used as an endogenous control to confirm plant genomic DNA quality (Figure III.3.A). Genomic integration of one plant, CHC111, was also confirmed by Southern blot analysis (Figure III.4). The plant CHC 111 had two cross-hybridizing fragments, 9kb and 13kb, that suggested that there are two separate copies of the T-DNA in the plant genome. The plants containing the ΔH2 intron construct grew slower than non-transformed plants and had some lesions on some of the initial leaves. The plants carrying the CFTR:H2 intron grew normally with respect to non-transgenic Xanthi.

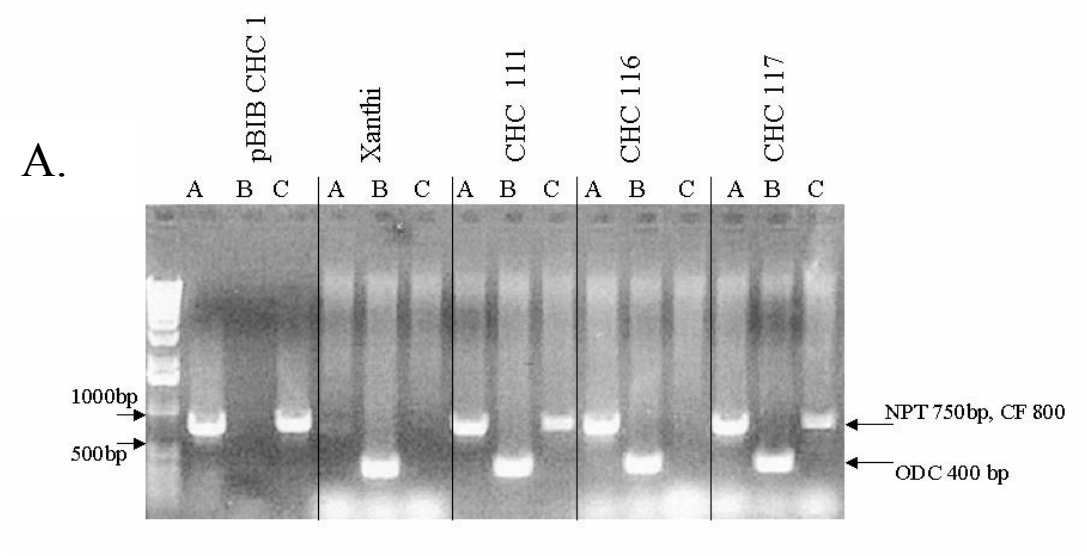

Figure III.3.A PCR Analysis of Transformed Plants. Lanes A represent the PCR 750 bp reaction using NPT 5a and NPT3b primers. Lanes B represent the PCR 0.4 kb reaction using the primers of ODC5 and ODC3. Lanes C represent the PCR 0.8 kb reaction using primers W22 and W2. CFTR and ODC primers were specific, while NPT primers were not, see lane Xanthi A.

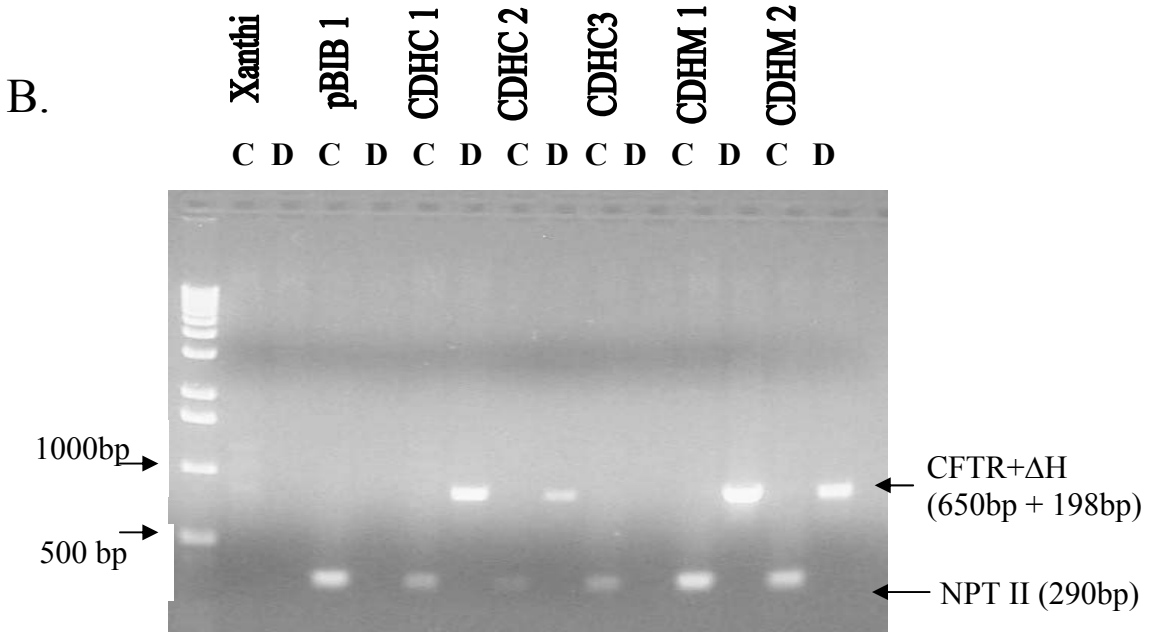

Figure III.3.B PCR Analysis of Transformed Plants. Lane C represents NPT II bands (Primed with W21, NPT 5a). NPT II was found in all transformed plants, but not in Xanthi (untransformed). Lane D represents the CFTR:ΔH amplified band 848 bp (primed with W51, W52). CFTR was found only in plants CΔHC 1, CΔHC 2, CΔHM 1, and CΔHM 2.

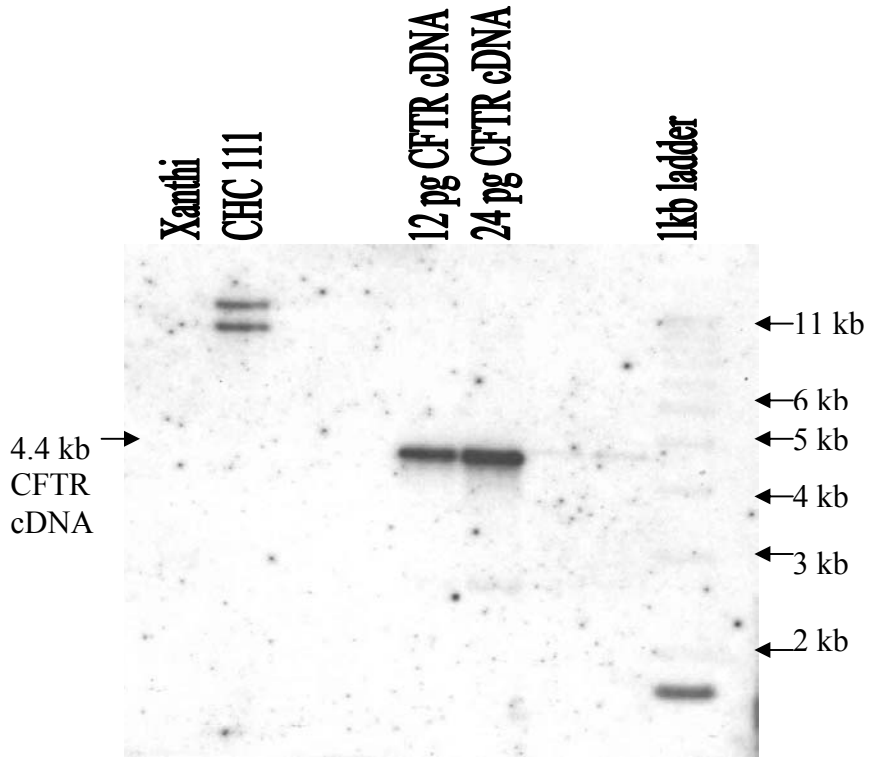

Figure III.4 Southern Analysis of Intron Containing CFTR Plants. Twenty micrograms of plant genomic DNA was digested with *Sac*I, a unique restriction site in T-DNA, and probed with a 1.5 kb 5' CFTR (exons 1-10) ^{32}P-labeled product. Plant CHC 111 revealed two bands of approximately 9 kb and 13 kb. There were no bands in the non-transformed tobacco lane. The CFTR cDNA lanes represent an estimation of 1 and 2 gene copies per genome.

III.3.2 Transcript Expression and Splicing Analysis of CFTR:Intron Plants

To determine whether the transgenic plants spliced the intron of transcripts from the intron-containing CFTR transgene, total RNA was extracted from leaf tissue and used for RT-PCR. For transgenic plants containing constructs driven by the MeGA promoter (CHM, CΔHM), RNA was isolated from leaves that had been wound induced for 0, 12 or 24 hours. For plants containing constructs driven by the constitutive promoter (CHC, CΔHC), untreated leaves were used. PCR primers were designed to span the introduced intron; thus, the products generated from unspliced CFTR transcripts would be 197 bp larger than spliced transcripts. For RT-PCR reactions using primers W46 and W47, CFTR products of 640 bp (spliced) or 838 bp (unspliced) are expected. Products of representative RT-PCR reactions from plants containing CFTR with the H2 intron are shown in Figure III.5. RNA from five CHC plant lines was assayed (results for CHC lines 117 and 118 are shown in Figure III.5). All plants showed significant CFTR product, but only of the higher molecular size (~840 bp). The presence of the intron was further confirmed by submitting the gel-purified CHC117 RT-PCR product to digestion with *Spe*I; three DNA fragments resulted indicting that both *Spe*I sites within the intron remained (dat not shown). There was little to no CFTR transcript detected from the MeGA-driven CFTR plants containing the H2 intron (CHM lines). Six CHM plant lines were analyzed and none showed significant CFTR product from RNA isolated 0, 12, or 24 hours post wounding (CHM line 2132 is shown in Figure III.5). The data of only the higher molecular weight RT-PCR product from the CHC lines suggest that the H2 intron was not successfully spliced from the CFTR gene.

RT-PCR analyses were also performed on RNA isolated from plants containing CFTR with the altered ΔH intronic sequence. As shown in Figure III.6A, CFTR-specific products were amplified from RNA isolated from plants containing the MeGA promoter-driven constructs (CΔHM) following 12 hours induction. Comparisons of these PCR products with products generated from plants expressing an intron-less CFTR (see Chapter II) demonstrated that the intron was not successfully spliced from the CΔHC transcripts (Figure III.6B). The three CΔHC plants that were positive for CFTR at the DNA level did not show any CFTR expression. The same results were obtained using a different set of PCR primers (W51 and W52 yielding products of 650 bp or 838 bp for

spliced or unspliced transcript, respectively) that may be more selective for CFTR sequences (Figure III.6.C). The expected sizes of the reaction product for primer set W51/W52 are 650 bp for unspliced transcript and 838 bp for spliced CFTR transcript. PCR amplification using actin-specific primers that flank an intron were used as an internal control to ensure RNA quality and confirm the absence of contaminating DNA. The actin RT-PCR product is 450 bp (Figures III.5 and III.6); genomic DNA would yield a PCR product 650 bp. These data suggest that insertion of a plant intron into the CFTR gene did facilitate the cloning and bacterial propagation steps required to produce transgenic plants expressing the full-length CFTR product driven by a strong constitutive promoter. However, the intron derived from the tomato *Hmg*2 gene does not appear to be effectively spliced when placed in the context of the human CFTR gene.

III.4 Discussion

The expression of the human CFTR protein in heterologous systems has presented many difficulties. Bacterial systems cannot accommodate the expression of CFTR and attempts to express CFTR in bacteria result in death of host (Gregory et al., 1990). When attempting to express deleterious proteins in eukaryotic systems, modification of bacterial expression during cloning steps is crucial. Two ways of reducing undesirable bacterial expression are modifying the promoter for expression as reported in Chapter II and inserting intronic sequences to prevent the translation of the transgene in bacteria. In the current study, a plant intron was placed in the exon 3 and exon 4 junction of CFTR cDNA. This permitted propagation of sequences in bacteria, but did not result in proper splicing in tobacco. Intron splicing requirements in plants are not fully understood, therefore several reasons for the lack of splicing could exist (Lorkovic et al., 2000). The original H2 intron may have resulted in altering the putative branchpoint due to the placement of the *Spe*I site (Table III.1). This was not anticipated as a problem in the initial intron design strategy because in other well-characterized systems the branchpoint is CURAY as it is in most plant introns. Introduction of the *Spe*I site modified 3 nucleotides 21-19 bases upstream of the 3'splice site. However, branchpoints in plants range from 20 to hundreds of nucleotides away from the 3'splice site, and the region created by the insertion of the *Spe*I site (CACUA) is not similar to the plant branchpoint

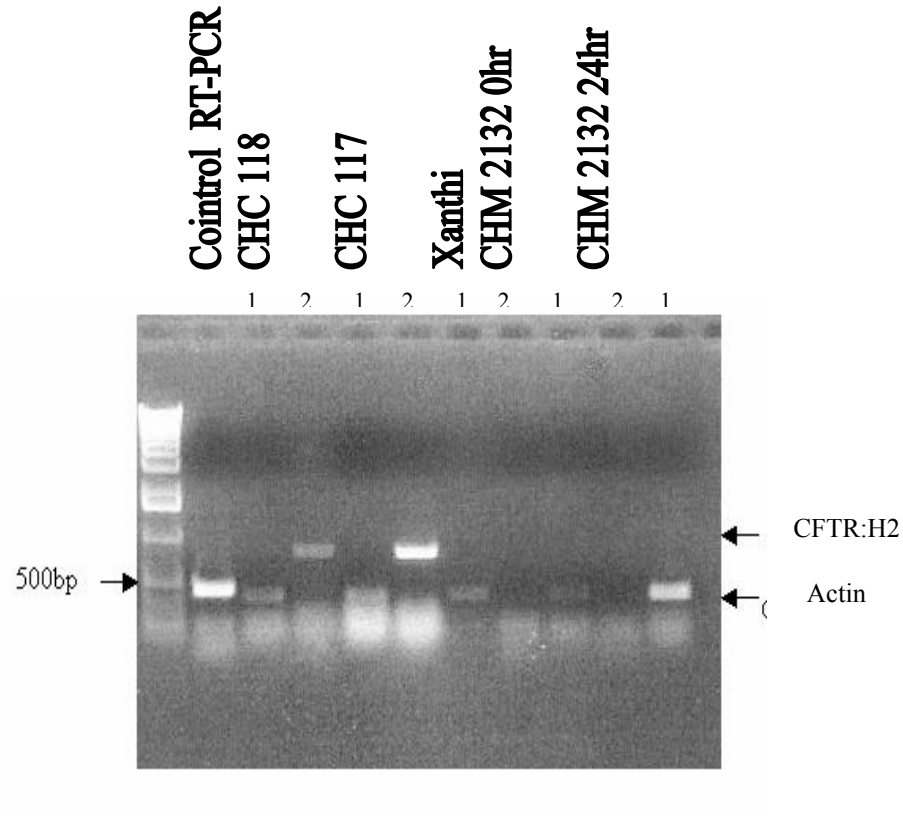

Figure III.5 RT-PCR of H2-2 Intron-Containing CFTR Plants. The RT-PCR reaction in lane 1 was performed with the primers for tobacco actin. RT-PCR reaction in lane 2 used CFTR primers W46 and W47 (1-650 CFTR cDNA).

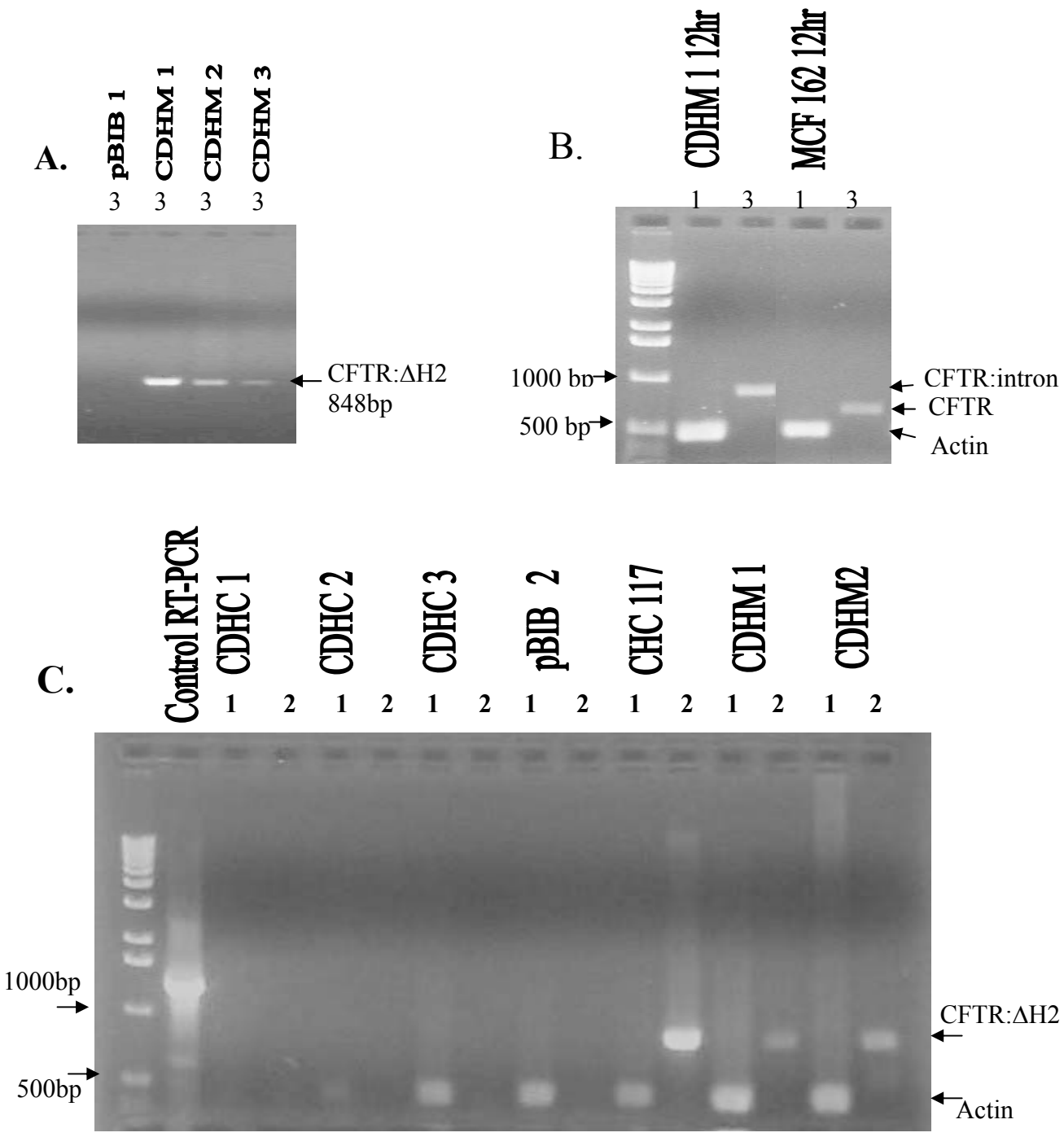

Figure III.6 RT-PCR of CFTR+Intron Plants. RT-PCR reactions in lanes 1 used tobacco actin primers. RT-PCR reactions in lanes 2 used CFTR primers W46 and W47 (1-650 CFTR cDNA). RT-PCR reaction product in lanes 3 were made using primers W51 and W52 (104-754 CFTR cDNA). Intron H2 or modified H (ΔH) sequence is 197 bp insert into CFTR cDNA at position 290. CHC 117, CHC 118, CΔHM 1, CΔHM 2, and CΔHM 3 show CFTR expression but no splicing occurred and only gave 848 bp product from RT-PCR.

consensus sequence (CUG/CAT/A). However, to ensure that this modification was not the basis for lack of splicing, site directed mutagensis was used to eliminate this *Spe*I site and restore the region to the native *Hmg*2 intron 2 sequence (ΔH). However, the "native" *Hmg*2 intron was also not spliced from CFTR.

Even though plants and animals share many common splicing elements, normally animal introns are not spliced in plants or even monocot introns in dicot plants (Baynton et al., 1996). The one reported animal gene spliced in plants is the SV40 small t intron, and it is AU rich (Hunt et al., 1991). In contrast, animals can splice plant introns (Hartmuth et al., 1986; Brown et al., 1986). This fact poses an added problem to expressing a human gene containing a plant intron. Special consideration is needed on the placement of the intronic sequence with respect to the exonic sequence that surrounds the intron. Apparently all plant intron-splicing rules are required to maintain proper splicing. The placement of an assumed splicible intron, ΔH2, in a non-conforming plant exonic junction, CFTR exon 3/exon 4, resulted in no splicing. The 5'splice site, 3'splice site, and branchpoint sequence fall into the parameters of proper plant intron splicing. Thus, exon context may be what is limiting splicing in tobacco. The uracil (U) and adenosine/uracil (AU) richness of the intron sequence as compared to flanking exon sequence is apparently critical for the recognition of intron splicing machinery (Bendel et al., 1998). In dicot plants, there is an average 15% enrichment for U and AU in the intronic sequence (Brendel et al., 1998). The AU content of the H2-2 and ΔH2 is 66 % while the flanking regions (50 bases) of CFTR exon 3 and exon 4 are 72 % and 60 %, respectively. In contrast, the flanking *Hmg*2 regions in its native context exon 2 and exon 3 are 58 % and 50 % AU, respectively. With the 5'splice site being the first to be cleaved, the AU percentage ratio is not favorable for splicing. The exon 1/exon 2 junction is the only splice site junction in the first 10 exons of CFTR that fits the plant's requirements with the AU content for these exons being 50 %, exon 1, and 52 %, exon 2.

The strategy developed for the introduction of a plant intron into a human cDNA involved a three-step process that theoretically should be universally applicable for the insertion at known exon junctions. Although issues surrounding junction and branchpoint requirements were considered, designing introns to accommodate context is significantly more challenging. The future insertion of intronic sequence in the CFTR exon 1/exon 2

junction could fill the requirements of proper splicing signals in plants. The modification of the intron sequence to boost the AU content (Rose, 2002) could aid the recognition of the intron/exon sequences. The expression of the complex and important CFTR protein in the heterologous system of tobacco can be challenging. Attention to detail and understanding the biology of many different species will allow the production of such an important human protein in tobacco possible.

III.5 References

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Chapter IV Summary and Future Diections

Cystic fibrosis (CF) is a severe human disease without a cure. CF is the result of defects in the gene known as cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a chloride channel and a member of the ABC transporters super family. Different mutations in the CFTR gene cause varying severity of CF. Replacement therapy for CF has not been successful and bioproduction of the CFTR protein may aid in finding a cure for CF. Large amounts of protein are needed for drug discovery and further characterization of protein function. Other recombinant protein production systems have low yields and are expensive or produce altered states of CFTR.

Tobacco has been successfully used as a bioreactor for complex recombinant proteins. To express CFTR in tobacco, the cDNA of CFTR was fused to either a constitutive promoter or an inducible plant promoter. Knowing that CFTR is not stably expressed in bacteria, a plant intron was introduced into the cDNA of CFTR to prevent lethalty. Both of these strategies could have aided in the expression of human CFTR in plants. The intronic sequence was not spliced from the CFTR cDNA. The inducible promoter allowed production of CFTR protein similar in size to that produced in other non-mammalian recombinant expression systems to be produced in plants.

Future experiments could continue to characterize the plant-produced CFTR. Purification of the plant-produced CFTR would allow study of the function of the protein in synthetic lipid bilayers or patch clamp experiments. Localization experiments would help determine placement of the plant made CFTR in the plant cell. Modifications of the intron containing CFTR could allow contitutive expression of the protein, and functional roles of CFTR in plants could be studied. The putative protein product could be purified, and amino acid sequencing would confirm that CFTR was produced in tobacco. The cost effective, large-scale production system of tobacco could allow enough CFTR to be made to increase the knowledge of this important protein. Expression of this low abundance but crucial protein in tobacco may help in finding a cure for cystic fibrosis.

Vita

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William Witt was born on November 25th, 1975 in Mt. Vernon, Kentucky. He graduated from Rockcastle County Highschool in May 1994. He graduated from the University of Kentucky with a B. S. degree in Agriculture Biotechnology. He worked at the Tobacco and Health Research Institute as lab technician from June 1998 to July 2000 under Dr. Indu Maiti. In August 2000, he began graduate studies in the Department of Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA., to pursue a M. S. degree in plant physiology. His graduate work was under the direction of Drs. Cynthia Denbow and Carole Cramer.