PLANT PRODUCTION AND FUNCTIONAL CHARACTERIZATION OF CATFISH INTERLEUKIN 22 AS A NATURAL IMMUNE STIMULANT FOR AQUACULTURE

Lana Elkins[1] and Maureen Dolan[1]

[1]Arkansas State University

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Abstract

As the world population increases and wild caught fisheries decline, aquaculture offers an important sustainable solution in addressing this global challenge. However, disease management remains difficult. With limited options, there is a need for innovative solutions. The cytokine interleukin-22 (IL-22) has emerged as a possible therapeutic target for fish and has been correlated with protection under pathogen challenge. Plant-based production systems have the potential to effectively manufacture and bring unique efficacy-enhancing features to the aquaculture industry; namely, the advantages of low cost for this commodity market, ready scalability, and reduced environmental impact. Plant-expressed recombinant cfIL-22 yield and purity were adequate for in vitro activity assessment. This study serves as the first report supporting the use of plants to express therapeutic proteins with application for the aquaculture industry. Bioactivity assays showed cfIL-22 notably increased the proliferation of catfish cells, highlighting the tissue preservation capabilities of this protein. Recombinant cfIL-22 also upregulated expression of genes encoding a tissue repair protein, fibronectin, an antimicrobial peptide, Natural killer lysin-1, and a common innate immune protein, interferon. These findings support plant-made recombinant catfish interleukin-22 as a potential therapeutic for the aquaculture industry and supports further analysis of this protein for therapeutic use.

INTRODUCTION

Globally, aquaculture is the fastest growing sector of the food animal production industries, providing around 47% of food fish (FAO, 2018). With the plateau and projected decrease of wild catch fisheries as well as increased global demand for high value protein, there is significant opportunity for aquaculture to meet this demand. Disease prevention and health maintenance measures have not kept pace with industry growth, leaving fish farmers vulnerable to major economic losses. Currently the primary therapeutics used are antibiotics for remediation of bacterial diseases and a limited number of vaccines that are specific to fish species and disease pathogen (USDA, 2016; FDA, 2018). The propagation and spread of antibiotic resistant bacteria are of concern both in terms of contaminating human food products, and their release into the environment that expose wild populations to these resistant pathogens (Defoirdt, Sorgeloos, & Bossier, 2011). Antibiotic resistance caused by aquaculture practices is even more concerning than with terrestrial agriculture as water is a constant and inevitable mechanism for dispersal of drug residues, pathogens, and resistance genes. Currently 14 antibiotics listed by the World Health Organization as important for human health, are also used in aquaculture (Done, Venkatesan, & Halden, 2015). This highlights the growing and immediate need for innovative solutions to ensure safe food fish production to consumers and sustainable practices that protect the environment.

One strategy for limiting disease outbreaks and reducing antibiotic use in aquaculture is the concept of boosting the natural immune system of the fish to counter pathogen infection. Cytokines are an important

class of immune proteins in all animals that are secreted by immune cells in response to a stimulus and orchestrate key steps of the immune response (Male, Brostoff, Roth, & Roitt, 2011). Interleukin 22 (IL-22) is a cytokine first identified in mammals 20 years ago (Dumoutier, Van Roost, Ameye, Michaux, & Renauld, 2000; Xie et al, 2000). Since that time several homologs have been identified in other species (Figure 1.) with wide sequence divergence particularly among fish. This ˜22kDa protein is thought to function as a monomer and mediates its function when binding its heterodimeric receptor (IL-10βR/IL-22R) on target cells. The role of IL-22 was described in fish by Corripio-Myar, Zou, Richmond, & Secombes (2009), where they correlated IL-22 gene expression with protection of cod and haddock subjected to bacterial challenge. Vaccinated fish that survived the bacterial challenge were shown to have a particularly high level of IL-22 gene expression in the gills. These results suggest that fish IL-22 functions as its mammalian homolog in triggering protective innate immunity and may provide a therapeutic target in controlling fish disease (Monte, Zou, Wang, Carrington, & Secombes, 2011; Secombes, 2011).

IL-22 is expressed by a select number of cells, primarily immune, in response to pathogens and is a key player in mediating the innate immune system (Zenewicz and Flavell, 2011; Dudakov, Hanash, & van den Brink, 2015; Sabat, Ouyang, & Wolk, 2014; Hernandez, Gronke, & Diefenbach, 2018). Unlike most cytokines which regulate immune cells, IL-22 receptors are primarily located on non-hematopoietic cells, most notably epithelial cells that line animal mucosa. When IL-22 binds to the canonical receptor it can signal increased production of antimicrobial peptides (AMPs), tissue repair proteins and mucous proteins, protecting the host animal from pathogens. IL-22 has recently gained notable attention as a human therapeutic agent both for treatment of infectious or inflammatory diseases (Stefanich et al, 2018; Lekkerkerker et al, 2017; Gao and Xiang, 2018; Tang et al, 2018; Lin, Krogh-Andersen, Hammarström, & Marcotte, 2017) as well as tissue preservation and wound repair (Kolumam et al, 2017). While IL-22 has been expressed successfully in a number of heterologous expression systems (e.g. HEK-293 cells, CHO cells, barley grain, *E. coli* ; R&D Systems), relatively low levels of fish IL-22 expression in *E. coli* have been reported (Monte et al, 2011; Costa et al, 2013, Siupka et al, 2014, Qi et al, 2015).

Incorporating plant biotechnology as a production platform may offer an environmentally sustainable and innovative platform for producing a functional IL-22 therapeutant. Plants provide an alternative to the more traditional recombinant protein production platforms with several significant advantages (Xu, Towler, & Weathers, 2016; Topp et al, 2016). The major benefits include improved safety profiles, as this host is incapable of harboring animal pathogens, and significant cost reduction in some cases up to 1000-fold compared to mammalian systems (Xu Dolan, Medrano, Cramer, & Weathers, 2012). This makes the plant platform attractive for veterinary biologics and is currently being used for veterinary medicine (Metzler, 2006; Pelosi, Shepherd, & Walmsley, 2012) as well as human therapeutics (Aviezer et al, 2009a; 2009b). To avoid the long time input necessary to establish stable transgenic plants for protein product expression (Schillberg, Twyman, & Fischer, 2005; Fischer, Stoger, Schillberg, Christou, & Twyman, 2004), the plant transient system can be used for production of recombinant protein within days and without stable gene integration (Krenek et al, 2015). In this way, protein targets can be quickly assessed before a large scale-up to stable production (Lacroix and Citovsky, 2013; Sheludko, 2008).

Herein we explore a sustainable and cost-sensitive production platform, plants, to produce a functional recombinant IL-22 therapeutic for promoting food catfish health and disease management. As an alternative to antibiotics or chemicals, this approach aims to trigger the fishes own immune system to produce a customized cocktail of AMPs and tissue repair proteins providing an innovative approach for addressing the issue of bacterial resistance and reduce unnatural residues deposited into the environment (Levy, 2002; Romero, Feijoo, & Navarrete, 2012).

MATERIALS AND METHODS

cfIL-22 Cloning and Construct Design

Catfish IL-22 cDNA sequence was obtained from Dr. Sylvie Quiniou (USDA-ARS-Catfish Genetics Research Unit, Stoneville MS; Accession #MK956102). Two gene constructs, one codon-optimized for expression in *N.*

benthamiana (cfIL-22 opt) and a second encoding native catfish IL-22 sequence (cfIL-22), were synthesized (GeneArt®, Thermo Fisher Scientific, Waltham, MA) with the native catfish signal peptide, a 3' 6X-histidine tag and cloned into an ampicillin resistant pMA vector. Gene cassettes were cloned into a pBIB-Kan plant expression vector (Becker, 1990) downstream of the constitutive dual-enhanced 35S Cauliflower Mosaic Virus promoter (35S; Lam, Benfey, & Gilmartin, 1989), and a translational enhancer from the tobacco etch virus (TEV; Carrington and Freed, 1990) and upstream of the Tnos terminator. Gene constructs were confirmed by Sanger sequencing (CRC DNA Sequencing Facility, University of Chicago). Coding sequence for native cfIL-22 is provided in Supporting material Figure S1.

Protein expression using an Agrobacterium-mediated Transient Plant Production system

All expression constructs were mobilized into *Agrobacterium tumefaciens* strain LBA4404 using a freeze/thaw method (Holsters et al., 1978). Transformed *A. tumefaciens* (*Agro*) was grown in 5 ml of YEP medium [10 g/L Bacto-peptone (Difco), 10 g/L yeast extract (Difco), 5 g/L NaCl (Sigma-Aldrich), pH 7.0] containing 0.1 g/L of kanamycin (Sigma-Aldrich) for antibiotic selection of the expression construct and 0.06 g/L of streptomycin (Sigma-Aldrich) for selection of the T-DNA plasmid. *A. tumefaciens* cultures were grown at 28°C on an orbital shaker at 225 rpm for 2 days. The 5 ml bacterial suspensions were used to inoculate 50 ml of YEP medium containing the antibiotics and cultured for an additional 24 h. These *A. tumefaciens*cultures were diluted into induction medium (IM; 10mM MES pH 5.5) at an OD_{600} of 0.2.

Transient expression for all constructs in *Nicotianabenthamiana* plants was carried out using an*Agrobacterium* -mediated vacuum infiltration method (*Agro* -infiltration) previously described by Medrano et al (2009). Briefly, 4-6-week-old *N. benthamiana* plants were grown and maintained under controlled temperature, light and humidity. Plants were vacuum infiltrated with *A . tumefaciens* cultures containing the cfIL-22 gene or pBK (empty vector; plant background control). The*A. tumefaciens* -infiltrated tobacco plants were returned to the environmental growth chamber and leaf tissues were collected at 2-7 days post infiltration to determine harvest time providing maximum protein accumulation. All leaf tissue was frozen in liquid nitrogen and stored at -80°C until further analysis.

Protein Purification

Detailed methods included in supplemental material. Briefly, leaf tissues infiltrated with cfIL-22 or pBK empty vector (negative control) were subject to a three-step purification process. Total soluble protein was extracted from the tissues, then taken through a two-step ammonium sulfate precipitation. This was followed by nickel affinity chromatography, and finally separation on a size exclusion column. The final fraction was then concentrated and sterile filtered for use in bioassays

Protein Characterization

Qualitative and quantitative assessment of the purified protein included SDS-PAGE (NuPage 12% Bis-Tris; Invitrogen) resolution and detection by either anti-His (Genscript) western immunoblotting, silver staining (Pierce) or Coomassie Simply Blue Safe Staining (Invitrogen). Purified protein was also subject to a bioanalyzer (Experion, Pro260 chip, Bio-Rad) in providing an additional measure of purity and concentration. Spectrophotometry using a Nanodrop 1000 and the protein extinction coefficient (14,940 $M^{-1}cm^{-1}$) was also used to confirm protein concentration. Endotoxin levels of SEC-purified cfIL-22 and negative control (pBK equivalent) were determined by the Limulus Amoebocyte Assay chromogenic method in accordance with manufacturer's instructions (Charles River, Endosafe Kinetic Chromogenic Limulus Amebocyte Lysate Endochrome-K kit). Purified protein was further analyzed by MS-MS analysis at the University of Arkansas Medical Sciences (UAMS) Proteomics Core Facility and N-terminal sequencing by the Protein Facility (Office of Biotechnology, Iowa State University). N-glycan digest was performed using PNGase F (New England Biolabs) according to manufacturer's guidelines. Amino acid sequence was submitted both to I-TASSER and Raptor X modeling programs for structure prediction.

Cell Culture

Channel catfish ovary fibroblasts (CCO, ATCC CRL-2772) were used for all*in vitro* assays. CCO cells were

grown in L-15 medium (Sigma) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals). For general maintenance and growth, CCO cells were cultured in T75 plug sealed flasks (Corning, Fisher Scientific) in a humidified incubator at 28°C. Typical passage ratio at 1:3 was approximately once a week and media changed every 3 days.

Proliferation Assay

CCO cells were seeded at $0.8x10^4$ cells per well on a black 96 well plate with optical bottom (Thermo Scientific) in L-15 with 1% FBS 100µl. Following adherence, cells were treated with varying concentrations of cfIL-22 or pBK empty vector control (negative) for 24 hours. Resazurin sodium salt (0.15mg/mL; Sigma) was dissolved into DPBS (pH 7.4, Corning, Fisher Scientific), sterilized through a 0.2µm filter and stored at 4°C for up to two weeks in a light protected container. Resazurin (20 µl) was added to each well and incubated for 3 hours at 28°C. Fluorescence was read using a Biotek Synergy 2 plate reader (Excitation 560nm/emission 590nm). Data are analyzed in Microsoft Excel and reported as relative fluorescent units.

Gene Expression Analysis

CCO cells were seeded on 100mm plates (Corning, Fisher Scientific) at ˜$2.5x10^6$ cells per plate for 24 hours. Following a media exchange to L-15, 1% FBS for 24 hours, cells were treated with different amounts of cfIL-22 or pBK plant background control for varying time periods. Cells were collected by media removal, enzyme treatment (TrypLE; Thermo Scientific) and cell scraper (Fisher Scientific). Cells were resuspended in treatment media, centrifuged at 250xg, and supernatant decanted. Cell pellet was rinsed in PBS pH 7.5, collected by centrifugation and cell pellet subjected to quick freeze with liquid nitrogen and stored at -80°C.

Total RNA was extracted using Ambion Purelink RNA mini kit (Thermo Scientific) and cDNA synthesized using Superscript IV Reverse Transcriptase kit with oligo dT primer (Thermo Scientific). Quantitative PCR (qPCR) primers were designed using PerlPrimer open-access software (Marshall, 2004) and commercially synthesized Custom DNA Oligos (Thermo Fisher Scientific). qPCR was carried out using Ssofast Eva Green Supermix (Bio-Rad) and samples and reagents robotically dispensed (Ep-motion 5075, Eppendorf, Hauppauge, NY) to 384 well white plates (USA Scientific). Reaction volumes of 10µl, primer concentration of 400 nM and cDNA concentration of 10ng was used. All qPCR was carried out on Bio-Rad CFX 384. Primer efficiencies were established using a 4-fold serial dilution of cDNA sample with 80ng DNA set as an upper limit. qBase+ software (Biogazelle) was used for reference gene selection, M-value, and gene expression analysis.

Statistics

All data met assumptions of normality. Error bars represent ± standard error mean. For proliferation assay, significance between control and treated samples was determined using students T-test assuming unequal variance. For gene expression analysis, significance was determined using one way ANOVA and Tukey's post hoc analysis on GraphPad prism.

RESULTS

Cloning and Production of cfIL-22

The cfIL-22 gene sequence (generously provided by Dr. Sylvie Quiniou) was synthesized as the native sequence as well as codon optimized for enhanced production based on codon preference in the plant host system,*Nicotiana benthamiana* . Plant expression constructs were created by transferring the gene cassettes into a pBIBKan (pBK) expression vector under the control of a constitutive promoter (35S duel enhanced CaMV), tobacco etch virus (TEV) translational enhancer and Tnos terminator (Figure 2.). Both constructs used sequences encoding the native catfish IL-22 signal peptide for trafficking through the endomembrane system.

As determining expression kinetics *in planta* is protein specific and important for maximizing protein yield, leaf tissue from infiltrated plants collected at 48, 72, 96, and 120 hours post-infiltration was analyzed. Relative comparison of protein quality and yield were compared by anti-His western blot analysis with

maximum cfIL-22 protein accumulation at 96 hours post infiltration (Supporting material Figure S2). The cfIL-22 product corresponds to a 20 and 25kDa product on western blot. As plant transient expression of both native and codon-optimized cfIL-22 constructs were qualitatively and quantitatively comparable (Supporting material Figure S3), all further studies used the construct expressing the native coding sequence (cfIL-22). As determining optimal extraction buffer is also protein dependent, several buffers were tested; acidic (sodium citrate buffer, pH 5), neutral (plant phosphate buffer, pH 7), and basic (Tris buffer, pH 8.5) ranges. Plant expressed cfIL-22 protein preferentially extracted in the higher pH buffer (Supporting material Figure S4) and was adopted for all analyses in this study.

Protein Purification

To establish cfIL-22 protein bioactivity, a 3-step purification process was developed that included ammonium sulfate precipitation, nickel affinity chromatography, and size exclusion chromatography. A representative western immunoblot tracks the protein through this purification scheme (Fig. 2.2A) to the final purified product (Fig. 2.2C). A corresponding Coomassie stained gel (Fig. 2.2B) shows the enrichment of this target protein applying this purification scheme. A representative Coomassie stain and silver stain of the final product (Fig. 2.2C) along with bioanalyzer assessment resulted in greater than a 90% purity for plant produced cfIL-22. While Table 2.1 shows cfIL-22 recovery is low (21%) indicating that optimization of the SEC step is needed, this purification scheme generated high quality protein at sufficient yield (5.4 mg/kg fresh tobacco leaf) for validating cfIL-22 bioactivity.

Protein Characterization

Resolution of cfIL-22 protein on 12% SDS-PAGE generated three major bands. The ˜20 kDa and 24 kDa (Fig. 2.2C) bands were predicted to be associated with differential post-translational processing of the cfIL-22 monomer and the ˜44kDa band corresponded to the predicted size of a cfIL-22 dimer. All three products were excised from a Coomassie-stained gel and confirmed by MS/MS analysis to be cfIL-22. N-terminal sequencing of the 20 and 24kDa bands confirmed that the signal peptide was fully processed in both monomeric forms of cfIL-22 expressed in plants (supporting material Figure S5). PNGase F removal of N-glycans resulted in a single band product at 20kDa (supporting material Figure S6). This confirms the prediction that the 24kDa band corresponds to cfIL-22 protein with a single N-glycan.

Proliferation Assay

Cell proliferation assay is a simple *in vitro* assay used in establishing the activity of a variety of proteins including IL-22 (Cai et al, 2010). Interleukin-22 is known to initiate tissue preservation and repair. Therefore cell proliferation is expected to be enhanced in the presence of cfIL-22. Normalized to the no treatment control, Figure 3 demonstrates cfIL-22 concentrations from 100 to 1500 ng/ml increased CCO cell proliferation in comparison to pBK plant background control.

Gene expression analysis

In order to use CCO cells in bioassays, IL22Rα1 expression in these cells was confirmed using gene expression analysis (supporting material Figure S7). As a measure of mammalian IL-22 activity, upregulation in the expression of select response genes linked with antimicrobial activity and tissue repair function are typically monitored (Veas and Dubois, 2011, Wozniak, Hole, Yano, Fidel, & Wormley, 2014). Three response genes in cfIL-22 treated CCO cells were identified and assessed as a measure of cfIL-22 function: Fibronectin, for assessing tissue repair; interferon, a general immune response protein; and natural killer Lysin-1 an antimicrobial peptide involved in directly killing invading bacteria. These transcript levels of genes were assessed 24 hours after treatment with cfIL-22 or pBK plant background control at two concentrations (Figure 4). All response genes were assessed relative to a set of reference genes (alpha smooth muscle actin and alpha tubulin) established as suitable for this experiment. Interferon and NK-Lysin 1 showed significant increases in gene expression in both the 100 and 500ng/ml cfIL-22 treatments over pBK plant background control. Fibronectin was significantly increased in the 500ng/ml treatment.

DISCUSSION

This study serves as the first report supporting the use of plants to express bioactive therapeutic proteins with application for the aquaculture industry. Considering the inherent instability and rapid turnover of cytokines relative to most proteins, plants may offer a useful platform for expressing this class of protein. In addition, previous studies have reported expression of other fish IL-22 homologs with relatively low yields (Monte et al, 2011; Costa et al, 2013, Siupka et al, 2014, Qi et al, 2015). Using this well-described and tested transient expression system, plants are capable of expressing this protein at levels approaching 25.5 µg/gFW. Although the aim of this study did not focus on optimizing cfIL-22 expression levels, the transient plant expression platform is scalable and has been shown to support therapeutic protein production appropriate for commercialization (Sukenik et al, 2018; Holtz et al, 2015; D'Aoust et al, 2010). An alternative plant production platform in consideration of meeting a price acceptable to this commodity market are generating cfIL-22 stable transgenic plants wherein the recombinant therapeutic protein expressed in leaves or seeds could be directly incorporated into the fish's diet without the need for extensive processing (Sissener et al, 2011).

While sequence analysis predicts ˜20% identity between human and catfish IL-22, it is somewhat surprising how divergent the fish homologs are. Human shares 95.5% identity with resus macaque, while channel catfish shares only 83.1% identity with yellow catfish. The divergence is even more pronounced when comparing the second most related species. For human this is mouse with an 81% identity compared to channel catfish and zebrafish which share only 39.7% identity. Despite this sequence divergence among IL-22, all homologs do contain the IL-10 family signature, with ˜80% identity in this region of the protein. Structural predictive software indicates the catfish protein also shares zebrafish and human IL-22's globular structure (Siupka et al, 2014, Nagem et al, 2002; Supplement Figure 8).

The predicted monomer size of cfIL-22 based on amino acid sequence is 20kDa and is similar to that of other IL-22 homologs (Dudakov et al, 2015). Recombinant expression of this sequence in plants reveals two bands, ˜20kDa and 24kDa (Fig. 2.2), which were confirmed by MS/MS sequencing to both be cfIL-22. Multiple bands have been previously reported for human IL-22 expressed in plants and associated with alternate glycoforms (Wilbers et al, 2016). While glycosylation was shown in this case as well as other recombinant fish IL-22 to not be necessary for its activity (Monte et al, 2011; Costa et al, 2013; Siupka et al, 2014; Qi et al, 2015), a Asn-Asn-Thr glycosylation site at aa 148 of cfIL-22 (Supporting material Figure S5) was confirmed to account for the two monomers observed (Supporting material Figure S6). A third band of ˜44kDa that was consistently detected by western immunoblotting and confirmed by MS/MS analysis to be cfIL-22, is likely a dimer. This is consistent with human IL-22, which is known to produce dimers (Nagem et al, 2002; Neto et al, 2008).

After successfully producing catfish IL-22 in plants, it was important to establish that this recombinant protein had the associated predicted functional activity. To assess activity, cfIL-22 was purified to >90%. Although this three step purification scheme produced a low recovery (21%) of cfIL-22, achieving highly pure product is necessary for establishing accurate activity measurements and important for proof of concept of a plant host producing an active fish protein. As significant lipopolysaccharide levels can be associated with*Agrobacteria* -mediated production (gram negative soil bacteria) it was important to establish endotoxin levels in the purified protein products. Levels <0.1 EU/µg protein were typical and below levels accepted for commercialized recombinant proteins. Notably this endotoxin level aligns with commercial human IL-22 and lower than EU of mouse IL-22 product (Genscript, R&D Systems). Ensuring low or unmeasurable endotoxin was paramount in validating the bioactivity of our plant-made recombinant protein was indeed exclusive to catfish IL-22 (Gao and Tsan, 2003).

At the onset of this study, no *in vitro* bioassays to establish catfish IL-22 function had been reported. In general many cytokines are known to induce cell growth and division which is often used as an indicator of cytokine activity including IL-22 (Cai et al, 2010). The recombinant cfIL-22 notably increased the proliferation of catfish cells, highlighting the tissue preservation capabilities of this protein. Recombinant cfIL-22 dose dependent response induced proliferation over a broad concentration range with high levels of the cytokine resulting in expected cytotoxicity (Park et al, 2015; Park et al, 2011; Liang et al, 2010).

Activity for the majority of animal IL-22s including fish (Monte et al, 2011; Costa et al, 2013; Qi et al, 2015) has been validated using gene expression analyses. This along with the recent release of a channel catfish reference genome (Liu et al, 2016; Chen et al, 2016), made RTqPCR a credible approach for establishing the activity of our plant-made cfIL-22 protein. Using catfish fibroblast cells as our bioassessment model system, cfIL-22 upregulated expression of genes from three major categories important to combating disease. Consistent with the mammalian literature, cfIL-22 resulted in upregulation of genes encoding a tissue repair protein, an antimicrobial peptide, and a common innate immune protein. Fibronectin, an extracellular matrix protein involved in wound healing and tissue repair, expression was increased ˜1-fold and corresponded with the well-known tissue preservation and repair activity of IL-22 (Sabat et al, 2014). Natural killer Lysin-1, involved in directly killing invading bacteria, is one of a number of antimicrobial peptides to be triggered by IL-22 and reported here to be increased ˜2.5-fold. Finally, interferon, an important protein involved in modulating host immunity to different pathogens, was markedly upregulated (˜2-fold) by cfIL-22. These findings are consistent with other studies of fish IL-22 in rainbow trout (Monte et al, 2011), turbot (Costa et al, 2013), zebrafish (Siupka et al, 2014) and mullet (Qi et al, 2015).

Channel catfish (*Ictalurus punctatus*) is a warm water species and is currently the number one farmed fin fish in the United States with Alabama, Arkansas, and Mississippi accounting for ˜60% of total US produced aquaculture products (Saroglia and Liu, 2012). Stress and disease-induced losses are estimated to account for more than $70 million annually (Welker, Lim, Yildirim-Aksoy, Shelby, & Klesius, 2007; USDA, 2011). There is opportunity for developing novel tools to aid catfish farmers in managing fish health. A notable advantage of producing a recombinant therapeutic protein for fish in plants is the ability to incorporate the active therapeutant directly into a component of their diet (e.g. soybean, corn) that is readily consistent with medicated feed applications currently used in the aquaculture industry.

Another significant advantage to exploring protein-based therapeutics in general is they offer greater selectivity and specificity over currently used chemicals and antibiotics. A cfIL-22 therapeutant should not be active on other animals or organisms comprising the aquaculture environment providing better environmental safety profiles. In fact cfIL-22 was tested in a cell line of a related species to channel catfish (walking catfish, G1B) and cfIL-22 was not active. Furthermore, with economic incentives to increase fish density that further complicates disease management and increases antibiotic usage in the aquaculture industry, recombinant immune proteins could offer a safer alternative. With increased consumer demand for antibiotic-free food, and interest in avoiding antibiotic resistant bacterial strains associated with food production, alternatives to antibiotics for the aquaculture industry are much needed. Interestingly, one of the most valuable traits of IL-22 as a therapeutic target is potentially its associated antimicrobial activity.

While more fully defining the antimicrobial effects of cfIL-22 is required, this study is the first to suggest that at least one antimicrobial gene (NK1 lysin) is induced by the catfish homolog of IL-22. Unfortunately with limited *in vitro* based tools to study catfish immunity, the commercially available channel catfish fibroblast cell line used in this study is not ideal. There are IL-22 receptors located on fibroblasts in mouse cells however it is known that the majority of IL-22 receptors are located on epithelial cells (Sabat et al, 2014; McGee et al, 2013). Therefore, to adequately evaluate the function of cfIL-22 in channel catfish our group is in the process of developing epithelial cell lines.

In this study, we successfully produced cfIL-22 in plants at adequate amounts and purity to enable biochemical characterization and to conduct*in vitro* bioactivity of this proposed therapeutant. Biochemical analysis confirmed signal peptide processing and dimer formation. Bioassays designed to test cfIL-22 function confirmed both the tissue preservation and repair properties of IL-22 as well as immune stimulation and antimicrobial production. These findings support plant made recombinant catfish interleukin-22 as a possible therapeutic for the aquaculture industry and support further analysis of this protein for therapeutic use.

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TABLES

FIGURE LEGENDS

Figure 1. Phylogenetic tree of many sequenced IL-22 genes.

Tree made with Geneious Tree Builder. Cost matrix: Blosum62, Alignment type: Global alignment with free end gaps, Genetic Distance model: Jukes-Cantor, Tree Build Method: Neighbor-joining, Gap open penalty: 12, Gap extension penalty: 3

Figure 2. Cloning strategy for cfIL-22 expression constructs. Coding region of cfIL-22 was synthesized using both the native and*N. benthamiana* codon optimized sequence with the native signal peptide (SP) and a 3' histidine tag. The gene was cloned into the pBIBKan plant expression vector containing the 35S duel enhanced CaMV promoter and the tobacco etch virus translational enhancer (TEV).

Figure 3. Three-step purification process of plant-expressed cfIL-22. Western blot detected using an anti-His antibody (A) and Coomassie blue total protein stain (B) were used in monitoring purification of cfIL-22. Crude leaf extracts (1) from transient tobacco plants expressing cfIL-22 were subjected to a two-step ammonium sulfate (AS) precipitation. IL-22 recovered in the 35% saturated supernatant (2) was precipitated with 63% AS saturation (3). IL-22 containing fraction was subjected to nickel affinity chromatography; Flow through (4), wash (5), and elution (6-9) fractions. The 300mM imidazole elution fraction (8) enriched with IL-22 was concentrated (10) and further purified by size exclusion chromatography (11). The purified cfIL-22 protein (C) was concentrated, sterilized and analyzed by coomassie (a), silverstain (b), and western (c) for downstream bioassays. All samples were resolved on a 12% SDS-PAGE.

Figure 4. Cell proliferation as a measure of cfIL-22 bioactivity. Catfish fibroblast cells **(** CCO) seeded on a 96-well plate at $8x10^4$ cells/well were incubated with varying amounts of cfIL-22 and equivalent volumes of plant control protein, pBK, (as a negative control) for 24 hours. Resazurin assay and monitoring fluorescence intensity is used as a measure of cell growth and metabolism. Data represent eight biological replicates, error bars represent standard error mean. Difference between pBK and cfIL-22 significant from 100-1500 ng treatments $P< 0.05$).

Figure 5. IL-22 induced gene expression in catfish cell line. Recombinant cfIL-22 induced the expression of three key response genes-fibronectin, interferon, and NK Lysin-1 in CCO fibroblast cells. IL-22 induction of these genes was dose-dependent (100ng vs 500ng) and compared relative to media only treated cells (NT) and cells treated with a plant protein negative control (pBK). *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$

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