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Improved Phytate Phosphorus Utilization by Japanese Medaka Transgenic for the *Aspergillus niger* Phytase Gene

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ABSTRACT

The inefficient digestion of phytate phosphorus by fish has created environmental concerns associated with phosphorus pollution from aquaculture production facilities. To further complicate this situation, phytate is known to chelate minerals and proteins, making them nutritionally unavailable. The enzyme phytase degrades phytate into inorganic phosphorus, which can be directly utilized by fish. As a model to examine the feasibility and efficacy of producing fish capable of degrading phytate, Japanese medaka (*Oryzias latipes*) transgenic for an *Aspergillus niger* phytase gene were produced and their ability to utilize phytate phosphorus tested. Cell culture techniques, including transfection, RT-PCR, Northern blot, Western blot, and enzyme activity analysis demonstrated that the protein was expressed, active, and secreted. Survival of transgenic fish was significantly greater on all examined diets than their nontransgenic siblings and up to six-fold higher on a diet with phytate as the main phosphorus source. Similar results were obtained with nontransgenic fish when fed the same diet supplemented with phytase, suggesting that phytase, whether ingested or produced by the fish, is effective in degrading phytate and overcoming many of the known antinutritional factors.

INTRODUCTION

MOST AQUACULTURED SPECIES can be raised on grain-based diets, such as corn, soybean, and wheat. Unfortunately, the major storage form of phosphorus in grain exists as phytate (*myo*-inositol hexakisphosphate), which is not utilizable by monogastric animals and even has antinutritional properties. In order to overcome phosphorus deficiencies, plant-based diets for agricultural animals are supplemented with inorganic phosphorus. Although several studies have been conducted to determine the minimal phosphorus concentration required, it has been shown that maximum mineralization in fish cannot be achieved without the generation of significant levels of dissolved phosphorus waste.[1] The excess phosphorus in the water causes an increase in microorganisms and aquatic plant growth. The microorganisms degrade the new plant material, which floats to the water's surface and causes a decrease in the dissolved oxygen level, leading to eutrophication.[2] The indigestible phytate is also discharged into the environment where microorganisms that are capable of degrading

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phytate are located, thereby increasing the phosphorus load in the environment further.

The distinctive structure of phytate, a cyclic alcohol inositol with six phosphate groups, allows chelation with divalent and trivalent minerals, thereby reducing their availability.[3] Phytate forms complexes with several dietary minerals such as calcium, magnesium, zinc, copper, and iron,[3,4] and such complexes have been shown to be the cause of mineral deficiencies in several species.[3,5] Phytate has also been shown to contribute to poor protein digestibility,[6] which may be due to phytate's ability to bind to, and thereby inhibit, several enzymes including α-amylase, pepsin, and trypsin.[7]

The enzyme phytase, *myo*-inositol-hexakisphosphohydrolase (E.C. 3.1.3.8), degrades phytate into inorganic phosphorus, which is utilizable by fish.[8] Several studies have involved the addition of phytase enzyme directly to plant-based diets in an attempt to degrade the phytate phosphorus, and these results have met with varying success.[2,9–11] However, the phytase enzyme is unstable at the high temperature required for feed processing, specifically the pelleting process, and as a consequence there is a loss in phytase activity. For this reason, post-pelleting application of phytase is often used, which may account for the variation in such supplementation studies.

The goal of this research was to generate transgenic fish capable of producing phytase. If fish could produce their own phytase enzyme, dietary supplementation with inorganic phosphorous could be reduced or eliminated while the bioavailability of other dietary minerals would increase. In an attempt to test this theory, we produced Japanese medaka transgenic for the *phy*A gene from *Aspergillus niger* driven by either the human cytomegalovirus (CMV) or the salmon histone III (OnH3) promoters. After extensive studies to test for protein production, secretion, and glycosylation, phytate phosphorus utilization was tested in these fish as compared to nontransgenic siblings. The primary emphasis of this research is the phenotypic effects of the transgene (i.e., does the transgene alter growth and survival characteristics, rather than an expression profile).

MATERIALS AND METHODS

Transgene Plasmids

Plasmids were constructed as previously described.[12] Each plasmid consists of the phytase (*phy*A) gene from *Aspergillus niger*[13] linked to the secretion signal from the sockeye salmon growth hormone type I gene in an enhanced green fluorescence protein (EGFP) plasmid. Each plasmid is driven by either the human cytomegalovirus (CMV-IE) promoter or sockeye salmon histone III (OnH3) promoter.

Cell Culture

Cells derived from Chinese Hamster Ovary (CHO-K1, American Type Culture Collection, Manassas, VA) were grown in FD medium (50% Ham's F-12 and 50% Dulbecco's modified Eagle's media, GIBCO, Carlsbad, CA) with supplementation as previously described[14] at 37°C in a humidified incubator with an atmosphere of 5% carbon dioxide. Both phytase constructs were transfected into CHO cells through the calcium-phosphate method.[14] Stable cell lines were selected for through G-418 resistance at 500 μg/mL and EGFP expression. Green fluorescence protein was observed through a Nikon Diaphot inverted phase-contrast fluorescence microscope fitted with a standard fluorescein isothiocyanate filter set (HQ FITC-GFP, Chroma Technology Corp., Rockingham, VT) and camera.

RT-PCR and Northern Blot Analysis

Total RNA was isolated from each of five stable clones of CMV-PHY-GH1t-CMV-EGFP or OnH3-PHY-GH1t-OnH3-EGFP CHO cells, as well as nontransfected CHO-K1 cells, with a total RNA isolation kit (Sigma, St. Louis, MO). Total RNA was DNase-treated in the presence of an RNase inhibitor, and cDNA was synthesized from DNase-treated RNA from each sample. A sample of the DNase-treated RNA was used as a control to test for nondegraded DNA. PCR was performed using both EGFP-specific primers (5′-CGTAAACGGCCACAAGTTCAGCGT-3′, 5′-GTTGTGGCTGTTGTAGTTGTACTCC-3′, 400 bp product) and phytase-specific primers (5′-TACGTCGAGATGATGCAGTG-3′, 5′-TCAGCTAAGCAAAACACTCCGCC-3′, 200bp product).

After analysis of RT-PCR results, the clone with the strongest expression for each construct (clone #2 for CMV-PHY-GH1t-CMV-EGFP and clone #5 for OnH3-PHY-GH1t-OnH3-EGFP) was utilized for Northern blot analysis. Total RNA (20 μg) from each sample was electrophoresed through a formaldehyde-agarose gel and transferred to nylon membrane through downward transfer. A 1.5 kb PCR product of the phytase gene was ^{32}P-labeled and used as a hybridization probe. Hybridization protocols and washes were performed as described by Strauss,[15] and autoradiography was performed with Kodak X-OMAT film and developed using standard techniques.

Transgenic Fish

Transgenic fish were produced by electroporation and tested by PCR as previously described.[12] Three germ-line transgenic founder fish for each construct were back-crossed to the wild-type population to produce the BC_1 generation. BC_2 fish used for protein analysis and testing of alternative diets were produced by back-crossing five transgenic BC_1 fish from each founder to the wild-type population. Eggs from each BC_1 mating (BC_2) were collected daily for approximately 1 week per replicate, and eggs were hatched as previously described.[12] Fry from the same founder line were pooled and grown in 9.5 L aquaria, at 25 fry per tank, and fed a combination of live artemia and larval AP100 (Zeigler Brothers, Gardners, PA). The use of these animals and the experimental protocols described herein were approved by the Purdue University Animal Care and Use Committee.

SDS-PAGE and Western Blot Analysis

Two days prior to protein collection, cell culture media was replaced with clear, serum-free media. Media was removed and concentrated four-fold using Centricon 30 MW cut-off centrifugal filters (Millipore, Billerica, MA). Cells were rinsed twice with 5 mL of ice-cold phosphate buffered saline (PBS, pH 7.4), harvested by scraping in 1 mL of PBS, and collected by centrifugation. Total protein was precipitated from cells[16] and resuspended in 10 mM Tris, pH 8.0. Protein concentration was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA), and 20 μg of total protein was mixed with sample loading buffer[17] and heated at 100°C for 5 min for Western analysis.

Fish were removed from food 36 hours prior to euthanization. The stomach, small intestines, and large intestines were removed, and crude protein extracted using a modified form of the EZ protein extraction method as described by Rider et al.[18] Protein was quantified by the RC DC assay (Bio-Rad, Hercules, CA). The samples were then heated for 5 min at 100°C, and 20 μg of each sample used for Western analysis.

Samples were electrophoresed through a 4–20% SDS-polyacrylamide gel and electrophoretically transferred to membrane (PVDF, Bio-Rad, Hercules, CA). Western blot procedures were conducted as described by Gallagher et al.[19] and stained using a chemiluminescent substrate (SuperSignal West Pico, Pierce, Rockford, IL). The primary antibody (chicken antiphytase) was a polyclonal antibody produced by injecting an emulsion of phytase enzyme (Natuphos, BASF, AG Ludwigshafen Germany) with Freund's complete adjuvant (Sigma, St. Louis, MO) intramuscularly into the breast of white leghorn hens (*Gallus gallus*) approximately 21 weeks of age, followed by three more injections administered 2 weeks apart with incomplete adjuvant and phytase. Polyclonal antibodies were purified from egg yolks following the methods of Svendsen et al.[20] and tested for activity by ELISA as described by Hornbeck et al.[21]

Phytate Hydrolyzation in Cell Culture

The amount of phytate hydrolysis by protein samples from CHO cells and media was measured by spectrometry as described by Heinonen and Lahti.[16] Samples were prepared as described above for "SDS-PAGE and Western blots." Phytate solution (180 μL of 5mM) was mixed with crude protein extracts (10 μg in 20 μL), incubated at 37°C, and stopped at 0 or 20 min by the addition of 100 μL 30% TCA. For color development, the resulting solutions were mixed with 1 mL of acetone-acid molybdate-citric acid solution (2.25 mM ammonium molybdate, 45% acetone, 22.5% sulfuric acid, 100 mM citric acid). Triplicate biological samples were examined for

each sample for each time point. Phosphorus levels were deduced using absorbance readings of samples at 355 nm and the equation of the best-fit line derived from a standard curve of potassium phosphate controls.[18]

Immunohistochemistry of Transgenic Fish

Fish were euthanized in 10% tricaine methane sulfonate. Whole fish were flash frozen in liquid nitrogen, embedded in O.C.T. compound (Tissue-Tek, Sakura, Tokyo, Japan), and sectioned on a cryotome to produce approximately 8 μm sections. Immunohistochemical staining was carried out as described by Watkins[22] using the primary antibody described under "SDS-PAGE and Western Blots" and an HRP-conjugated secondary antibody. Immunolocalization was detected with a diaminobenzidine (DAB) solution to produce a brown coloration, and sections were background stained with hematoxylin to produce a purple color.

Experimental Design and Diets

Due to limitations in facilities, efficacy of each construct was tested separately; however, two types of controls were utilized at each time making comparisons possible. The 36 tanks were divided into 12 sets of three adjacent tanks. The three founders were randomly assigned to a set of three adjacent tanks (four replicates per founder). The three diets were randomized onto the three adjacent tanks. At 3 weeks of age, 50 fish from the appropriate founder line were randomly added to each tank. Fish were fed their assigned diets twice a day *ad libitum* for 10 weeks. A total of 1800 fish were used per construct (3 founders [multi] 4 replicates [multi] 3 diets [multi] 50 fish).

Three dietary treatments (basal diet, BD; basal diet with inorganic phosphorus supplementation, BDP; and basal diet with phytase supplementation, BDPY) were designed to test for transgene expression in fish. All diets were designed and produced by Zeigler Brothers (Gardners, PA) to be isocaloric and isonitrogenous, differing only in the phosphorus amount and source. Corn grain, corn gluten meal, soybean meal, and wheat midlings served as the protein, carbohydrate, and phytic acid source for all three diets (Table 1). Although approximately 20%–30% of the phosphorus present in the basal diet was comprised of inorganic phosphorus, this amount was considered insufficient with regards to the medakas' phosphorus requirement (National Research Council).[23] In the BDP, the calcium carbonate from the basal diet was replaced (1:1) with calcium phosphate, to produce a plant-based diet with phosphorus

Table 1. Basal Diet Formulation

Ingredients	*Percent of diet (%)*	*% Crude protein*	*% Crude fat*	*% Total phosphorus*	*% Free phosphorus*
Soybean meal, solvent extract	37	17.9	0.33	0.24	0.06
Corn gluten meal, 60%	22	14.5	0.43	0.10	0.03
Corn grain	20	5.5	1.86	0.12	0.028
Wheat midlings	6	1	0.26	0.05	0.008
Canola oil	5	—	5	—	—
Carboxymethyl cellulose (CMC)	2	—	—	—	—
L-Lysine	1.8	—	—	—	—
Arginine	0.7	—	—	—	—
L-Methionine	0.2	—	—	—	—
Calcium carbonate	2.2	—	—	—	—
Sodium chloride	0.48	—	—	—	—
Mineral premix[a]	0.12	—	—	—	—
Vitamin premix[b]	2	—	—	—	—
Ascorbic acid	0.5	—	—	—	—
Total	100	39	8	0.52	0.126

[a]Mineral premix consists of trace amounts of copper, iron, manganese, and zinc.

[b]Vitamin premix consists of 0.65 mg/kg biotin, 274.5 mg/kg choline, 0.38 mg/kg folacin, 4.5 mg/kg niacin, 9.8 mg/kg pyridoxine, 2.5 mg/kg riboflavin, 6.9 mg/kg thiamin, 1.0 mg/kg vitamin B12, 33.2 mg/kg vitamin E, 10.0 mg/kg vitamin K, and 1000 IU/mg vitamin A.

supplementation at a level comparable to standard fish diets. For the BDPY diet, *Aspergillus niger* phytase enzyme was applied to the basal diet at 1,000 U/kg of feed. Due to the inactivation of phytase at the high temperatures required for feed processing and pelleting, the phytase was sprayed onto the feed after production and allowed to dry.

Testing of Fish

After 10 weeks on their assigned diets, fish were euthanized in 10% tricaine methane sulfonate. Fish were weighed and tested for the presence or absence of the transgene by slot blot analysis. Genomic DNA was isolated from transgenic and nontransgenic medaka according to the methods of Jowett.[24] Tissue was digested in lysis buffer (100 mM Tris, pH 8.0, 1% SDS, 100 mM EDTA, 0.2 mg/mL proteinase K) at 60°C for 1 hour, and DNA purified using phenol-chloroform extraction and ethanol precipitation. Approximately 2 μg of DNA from each fish was blotted onto membrane with a Bio-Dot SF microfiltration device (Bio-Rad Laboratories).[25] Approximately 50 ng of ^{32}P-labeled phytase PCR product was used as probe. Nucleic acid hybridization protocols and washes followed the methods of Strauss.[15] Autoradiography was performed with Kodak X-OMAT film and developed using standard procedures.

Statistical Analysis

The average of all fish in a tank for each genotype was used as the experimental unit. The design was a split-split plot with three error terms, replicates within founders, which tests founder differences; treatment by replicate within founder, which tests for treatment differences; and residual, which tests for genotype and treatment by genotype interactions. Statistical analysis was conducted on data from each construct, as well as the entire data set, using the proc. GLM option of SAS (SAS Institute, NC). Differences among means for genotypes, genotype by diet, and genotype by diet by founder were identified using Fisher's least significant differences (LSD). Except where noted, differences were declared significant at the 0.05 level of probability.

RESULTS

Expression of Phytase in Cell Culture

Expression of the *Aspergillus niger phy*A gene was driven by either the CMV or OnH3 promoter, and both were cloned into a pEGFP construct as previously described.[12] Green fluorescence protein expression was noted in CHO cells for both constructs, although variation in expression level and pattern was observed (Fig. 1A, B). RT-PCR results showed much variation between clones of the same and different constructs. Since RT-PCR was conducted for both EGFP (Fig. 1C) and phytase (Fig. 1D), the band patterns and intensity of both genes can be compared. The intensity of the bands appears to be slightly lower for the phytase gene than for EGFP, however, the banding pattern seems similar. Northern blot analysis detected a 1.8 Kb band, indicating strong expression from the OnH3-PHY-GH1t-OnH3-EGFP cells and weaker expression from the CMV-PHY-GH1t-CMV-EGFP cells (Fig. 1E).

Two different clones from each of the cell lines were used for protein analysis, and both cells and media from each clone, as well as a nontransfected cell line, were used to test for the presence and activity of the phytase protein. Western blot analysis resulted in two bands of approximately 40 kDa and 85 kDa in the media from both transfected cell lines; however, no phytase expression was noted from the cells (Fig. 1F). The absence of phytase within the cells suggests that the protein is quickly secreted into the media. The smaller band seen in the media coincides with the expected size of the native protein; whereas the larger band is similar to that reported for the glycosylated *A. niger* phytase enzyme. Since a high degree of glycosylation is required for proper protein folding and transportation through the membrane, it is most likely that the smaller band is due to protein degradation.

Enzyme activity analysis by phytate hydrolysis showed that media from nontransfected cells resulted in 6.0 ± 0.7 μM of phosphorus liberation; similar to that obtained with controls of media from transfected cells with no incubation time. This amount of phosphorus probably represents the free phosphorus in the media. Media from both the CMV-PHY-GH1t-CMV-EGFP and

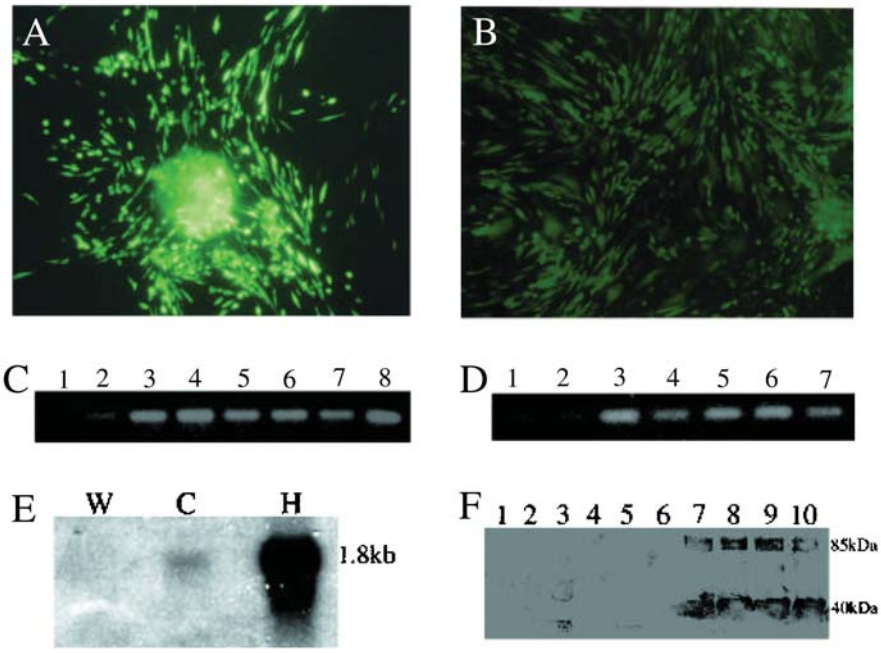

FIG. 1. Cell culture expression results. CMV-PHY-GH1t-CMV-EGFP (**A**) and OnH3-PHY-GH1t-OnH3-EGFP (**B**) transfected CHO cells expressing EGFP. Images were taken with an inverted phase-contrast fluorescence microscope fitted with the HQ FITC-GFP filter set and camera. RT-PCR products for the EGFP (**C**) and phytase (**D**) gene; *lane 1*, nontransfected CHO-K1 cells; *lanes 2–4*, CMV-PHY-GH1t-CMV-EGFP CHO cells; *lanes 5–7*, OnH3-PHY-GH1t-OnH3-EGFP CHO cells; *lane 8*, positive control for EGFP. Northern blot probed for phytase RNA expression (**E**); W, CHO-K1 nontransfected cells; C, CMV-PHY-GH1t-CMV-EGFP; H, OnH3-PHY-GH1t-OnH3-EGFP. Western blot analysis for phytase protein in cells and media (**F**); *lanes 1–5* are of cell extracts, *lanes 6–10* are of concentrated media. *Lanes 1* and *6*, CHO-K1 nontransfected cells; *lanes 2, 3, 7*, and *8*, CMV-PHY-GH1t-CMV-EGFP CHO cells, *lanes 4, 5, 9*, and *10*, OnH3–PHY-GH1t-OnH3–EGFP CHO cells.

OnH3-PHY-GH1t-OnH3-EGFP lines showed approximately three times more phosphorus liberation than controls; with CMV-PHY-GH1t-CMV-EGFP media at 16.6 $\pm$ 5.6 μM and OnH3-PHY-GH1t-OnH3-EGFP media at 19.5 $\pm$ 5.4 μM. Phytate hydrolysis from cell extracts resulted in 41.3–47.1 μM phosphorus liberation with no significant differences between controls and samples for any of the lines (data not shown).

Expression of Phytase in Japanese Medaka

Over 100 embryos were electroporated with both constructs, and of the 30 embryos tested from each construct, 100% somatic gene transfer was obtained. After two generations of backcrossing, resulting in the BC_2 population, a 1:1 ratio of transgenic to wild-type fish was expected at hatch. This BC_2 population was used to test for phytase expression and phytate phosphorus utilization. Western blot analysis of the gastrointestinal tissue from transgenic medaka fish resulted in a band of approximately 85 kDa for all transgenic samples (Fig. 2), suggesting the phytase protein was highly glycosylated. A smaller 40 kDa fragment is noted in several samples, suggesting that all of the phytase enzyme may not be glycosylated simultaneously or that some degradation of the phytase enzyme occurred, possibly due to protease activity during protein extraction. Immunohistochemistry of sections of whole mount fish show specific staining in the gastrointestinal region, specifically in the coils of the large intestines and stomach (Fig. 3A,B) as compared to a wild-type control (Fig. 3C).

Phytate Phosphorus Utilization by CMV-PHY-GH1t-CMV-EGFP Transgenic Fish

For fish transgenic for the CMV-PHY-GH1t-CMV-EGFP construct, no significant differences in average weight were noted on any of the three diets (Fig. 4A). In contrast, survival was significantly higher for transgenic fish than nontransgenic siblings on all three diets ($P <$

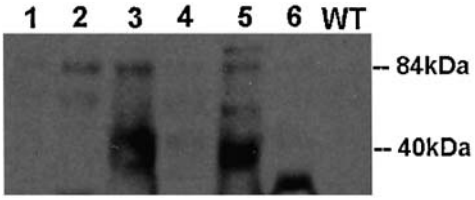

FIG. 2. Western blot of phytase protein isolated from the gastrointestinal tissue of one BC_2 fish from each founder line and one wild-type control. Blot was probed with a polyclonal chicken anti-phytase primary antibody and a goat anti-chicken IgY HRP-conjugated secondary antibody and developed with West Pico chemiluminescent substrate. *Lanes 1–3*, CMV-PHY-GH1t-CMV-EGFP fish; *lanes 4–6*, OnH3-PHY-GH1t-OnH3-EGFP fish; WT, wild-type fish.

0.001). The greatest difference occurred on the basal diet, in which transgenic fish had a 2.5-fold higher survival than nontransgenic siblings, and the smallest difference (1.5-fold) was noted for the phytase-supplemented diet (Fig. 4B). Data was also analyzed by founder line for weight and survival, and no significant differences in weight were noted, although the greatest variation occurred on the basal diet (Fig. 5A). A significantly higher survival was noted for transgenic fish from each founder line on the basal diet, with the largest difference, a six-fold higher survival on the basal diet, noted for transgenic fish from founder #1. Transgenic fish from founder line #1 also exhibited a four-fold higher survival on the phosphorus supplemented diet than their nontransgenic siblings, whereas transgenic fish from founder lines #2 and #3 both displayed only a two-fold higher survival on the basal diet than their nontransgenic siblings (Fig. 5B).

Phytate Phosphorus Utilization by OnH3-PHY-GH1t-OnH3-EGFP Transgenic Fish

No significant differences in average weight values were noted for fish transgenic for the OnH3-PHY-GH1t-OnH3-EGFP construct (Fig. 6A). Transgenic fish had a significantly higher survival rate than nontransgenic fish on all three diets ($P < 0.001$), with a 1.5-fold higher survival of transgenic fish on the basal diet than nontransgenic siblings (Fig. 6B). Again, the smallest difference between transgenic and nontransgenic siblings occurred on the phytase-supplemented diet. No significant differ-

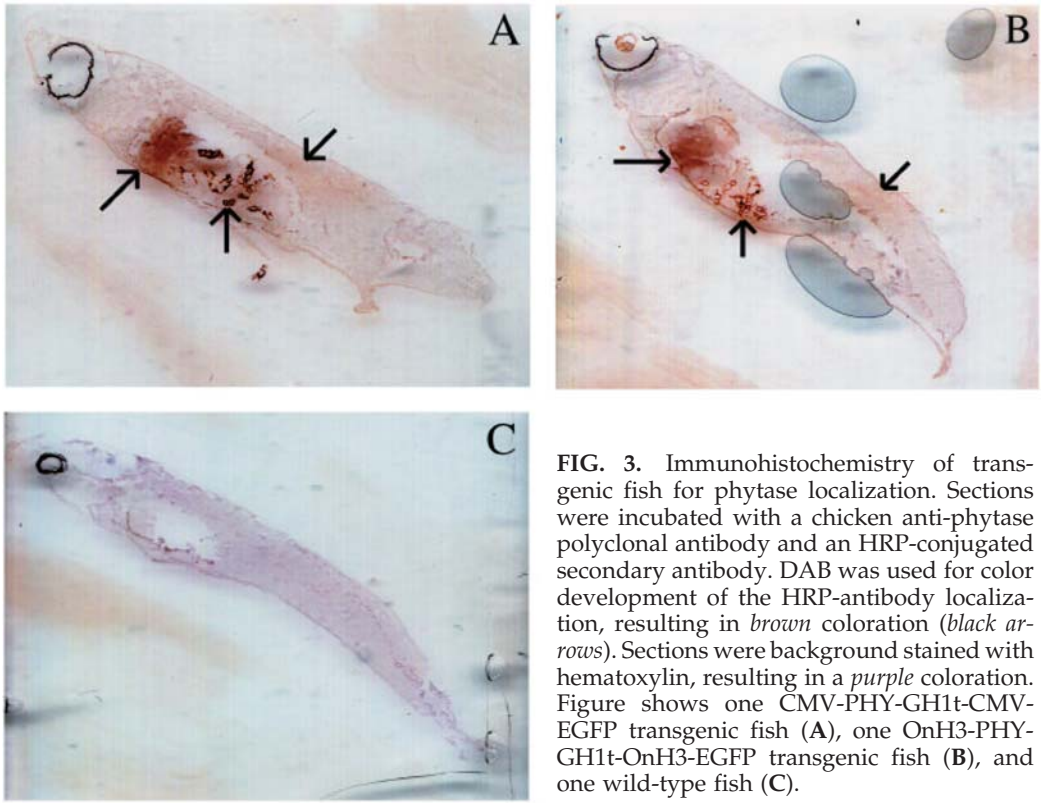

FIG. 3. Immunohistochemistry of transgenic fish for phytase localization. Sections were incubated with a chicken anti-phytase polyclonal antibody and an HRP-conjugated secondary antibody. DAB was used for color development of the HRP-antibody localization, resulting in *brown* coloration (*black arrows*). Sections were background stained with hematoxylin, resulting in a *purple* coloration. Figure shows one CMV-PHY-GH1t-CMV-EGFP transgenic fish (**A**), one OnH3-PHY-GH1t-OnH3-EGFP transgenic fish (**B**), and one wild-type fish (**C**).

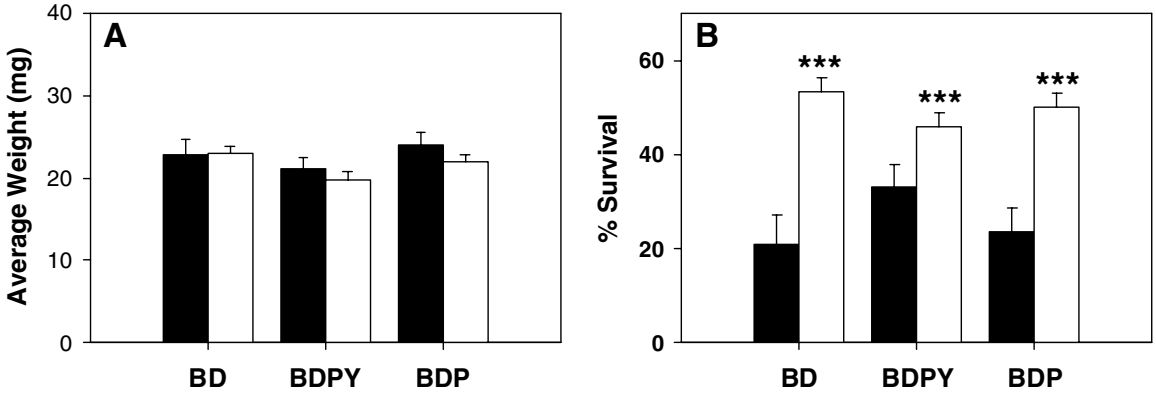

FIG. 4. Phytate utilization results for CMV-PHY-GH1t-CMV-EGFP transgenic (*white bars*) and nontransgenic (*black bars*) fish. Bar graphs show a comparison of transgenic and nontransgenic fish for overall average weight (**A**) and percent survival (**B**) with standard error bars on each of three diets; BD, basal diet; BDPY, basal diet with phytase supplementation; BDP, basal diet with phosphorus supplementation. *Asterisk symbols* (***) indicate significance differences between transgenic and nontransgenic fish on the same diet ($P < 0.001$).

ences in average weight were observed for any of the three founder lines on any of the three diets (Fig. 7A). Transgenic fish from founder line #4 showed the greatest difference in survival ($P < 0.002$) on the basal diet and the diet supplemented with phytase, with a two-fold higher survival than their nontransgenic siblings on the basal diet. Transgenic fish from founder #5 had significantly greater survival than nontransgenic fish on both the basal diet and the diet supplemented with phosphorus, whereas transgenic fish from founder #6 exhibited higher survival only on the diet supplemented with phosphorus (Fig. 7B).

DISCUSSION

Both growth and survival are critical variables indicating phosphorus utilization, as this compound is extremely important for many life processes. At the young age these fish were tested, large amounts of phosphorus would have been required for proper bone growth and

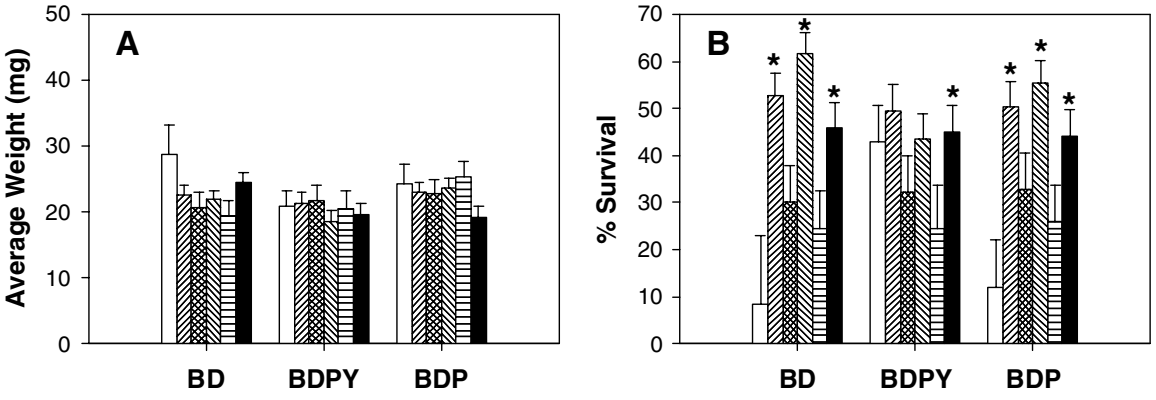

FIG. 5. Phytate utilization results by founder line for CMV-PHY-GH1t-CMV-EGFP fish as compared to nontransgenic siblings. Bar graphs show a comparison of transgenic and nontransgenic fish for overall average weight (**A**) and percent survival (**B**) with standard error bars on each of three diets; BD, basal diet; BDPY, basal diet with phytase supplementation; BDP, basal diet with phosphorus supplementation. *White bars*, founder #1 nontransgenic fish; *diagonal up bars*, founder #1 transgenic fish; *hatched bars*, founder #2 nontransgenic fish; *diagonal down bars*, founder #2 transgenic fish; *horizontal bars*, founder #3 nontransgenic fish; *black bars*, founder #3 transgenic fish. *Asterisk symbols* (*) indicate significant differences between transgenic and nontransgenic siblings on the same diet for a given founder line ($P < 0.05$).

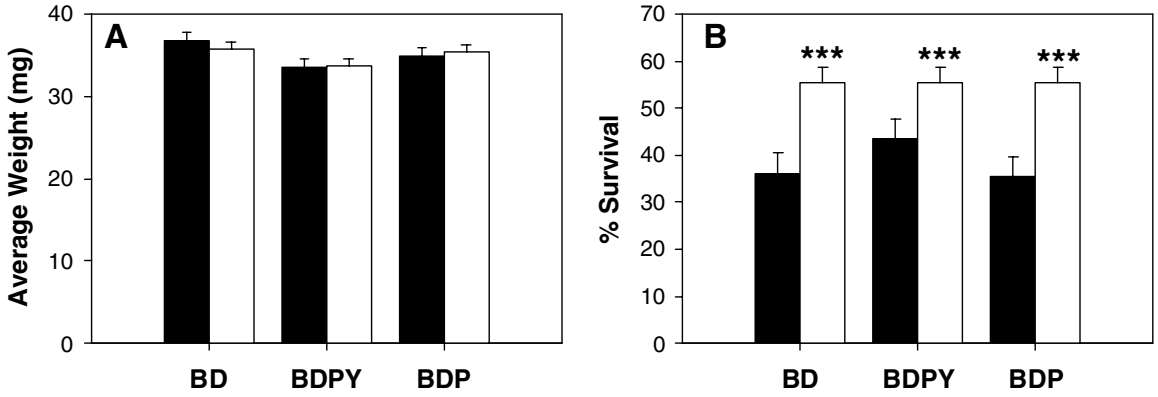

FIG. 6. Phytate utilization results for OnH3-PHY-GH1t-OnH3-EGFP transgenic (*white bars*) and nontransgenic (*black bars*) fish. Bar graphs show a comparison of transgenic and nontransgenic fish for overall average weight (**A**) and percent survival (**B**) with standard error bars on each of three diets; BD, basal diet; BDPY, basal diet with phytase supplementation; BDP, basal diet with phosphorus supplementation. *Asterisk symbols* (***) indicate significance differences between transgenic and nontransgenic fish on the same diet ($P < 0.001$).

development. Survival of transgenic fish was significantly greater than survival of nontransgenic fish, suggesting that fish transgenic for these constructs were more efficient at obtaining phosphorus from their environment, most likely by the utilization of phytate, than their nontransgenic siblings. However, no difference in growth was observed between phytase transgenic and nontransgenic siblings. These observations indicate fish in the current experiments were able to obtain enough phosphorus from the environment to sustain growth, but differed in their ability to acquire this limited nutrient at levels necessary to sustain viability.

Although results from phytase supplementation studies in aquatic species have varied greatly, several have resulted in changes without a significant increase in weight gain. Studies involving the addition of phytase to rainbow trout diets have shown improved phosphorus utilization; yet in some instances, weight gain, protein utilization, and mineral utilization were unchanged[11,26] and in other cases these factors were improved.[27] Similar studies in catfish re-

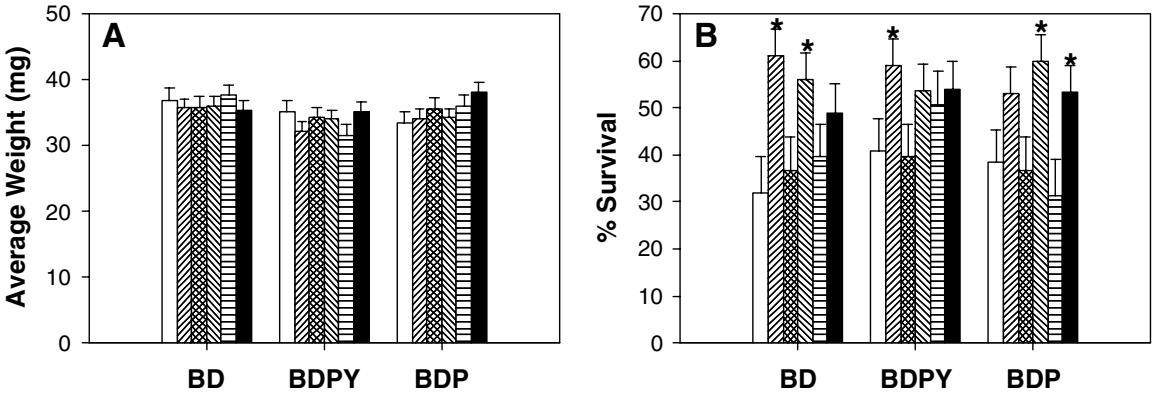

FIG. 7. Phytate utilization results by founder line for OnH3-PHY-GH1t-OnH3-EGFP fish as compared to nontransgenic siblings. Bar graphs show a comparison of transgenic and nontransgenic fish for overall average weight (**A**) and percent survival (**B**) with standard error bars on each of three diets; BD, basal diet; BDPY, basal diet with phytase supplementation; BDP, basal diet with phosphorus supplementation. *White bars*, founder #4 nontransgenic fish; *diagonal up bars*, founder #4 transgenic fish; *hatched bars*, founder #5 nontransgenic fish; *diagonal down bars*, founder #5 transgenic fish; *horizontal bars*, founder #6 nontransgenic fish; *black bars*, founder #6 transgenic fish. *Asterisk symbols* (*) indicate significant differences between transgenic and nontransgenic siblings on the same diet for a given founder line ($P < 0.05$).

sulted in an increase in mineral utilization without a change in weight gain or protein utilization.[28]

These data suggest that the nontransgenic fish that did survive were able to obtain enough phosphorus from their environment to grow comparable to their transgenic siblings. One possibility is that the high mortality of the nontransgenic fish was due to improper bone formation or development. Previous experiments in rainbow trout suggest that fish require higher levels of phosphorus for bone growth than for weight gain.[1,29] If the nontransgenic fish were only able to obtain minimal amounts of phosphorus from their environment, whether from the low amount of inorganic phosphorus present in the diet or from ingesting feces from their transgenic siblings, it may have been adequate for weight gain while insufficient for proper development. Another possible explanation for the difference in survival with no change in weight is that fish growth is limited by the amount of space they have to grow in. This competition may have slowed the growth of the transgenic fish to a rate comparable to that of the nontransgenic siblings.

Although the exact age of the fish at mortality in this study is unknown, most of the mortality must have occurred early in the experiment. Tanks were checked twice daily for deceased fish, and none were ever noticed. This suggests that the dead fish must have been small enough to degrade rapidly or be consumed by surviving siblings. This supports the idea that improper bone growth and development may have caused the high mortality in the nontransgenic fish. If fish were able to obtain adequate phosphorus levels early in the experiment, then bone formation would be normal and lower levels of phosphorus would be required later for growth.

Since the total available phosphorus was theoretically the same for the control diets and the basal diet for transgenic fish capable of liberating phosphorus from phytate, it was expected that transgenic fish expressing the phytase protein would show equal survival on all three diets. It was also expected that the nontransgenic fish would show high mortality on the basal diet with normal survival on both supplemented diets. The transgenic fish followed these expectations, doing well on all three diets, but the wild-type did not. Since all three diets were high in phytate, which has the antinutritional property of chelating to dietary minerals and proteins, these elements may not have been available to the nontransgenic fish for survival. The addition of phytase enzyme to the basal diet reduced the negative effects of the phytate in nontransgenic fish; resulting in increased survival. Fish transgenic for the phytase gene seem able to obtain micronutrients and other nutrients required for survival better from their environment than nontransgenic siblings, even on the phytase supplemented diet. Since the phytase was added post-processing to the diet, it is possible that the phytase enzyme activity decreased with time, thus fish producing fresh enzyme all the time would have a greater advantage.

Overall survival varied between founder lines for both constructs, indicating that the amount of phytate phosphorus utilization was affected by more than just promoter choice. This variation may be due to several factors and suggests that protein quantity and/or activity varied among founder lines. Although all founder lines were backcrossed to the wild-type population, some genetic variability may exist between the founder lines. For example, founder line #1 for the CMV-PHY-GH1t-CMV-EGFP construct shows the most survival variation in nontransgenic fish on the three diets, whereas nontransgenic fish from founder line #3 showed the same amount of survival on all three diets. It also shows that although actual results of a transgene may vary with each founder, the potential for altering phytate phosphorus utilization in these fish may be greater than these results indicate, as seen by a six-fold increase in survival for one of the founder lines as compared to nontransgenic siblings.

Previous experiments have shown that an *E. coli* phytase transgene can increase phosphorus utilization and decrease phosphorus waste in swine fed a high phytate diet.[30] However, that transgenic protein was secreted by the salivary glands and found to be partially inactivated by pepsin and sensitive to pancreatic proteases in the small intestine.[31] Since fish have no salivary glands, a phytase protein was needed that

would have high activity in the stomach or intestines. The *A. niger phy*A gene has two optima pH values, 2.5 and 5.0,[32] and was chosen for this study because these values correspond to the pH values within the stomach and intestine of several fish species.[33] Although it has been suggested that a phytase enzyme would be degraded within the digestive tract before it could function to degrade phytate phosphorus, Phillippy[34] has shown that *A. niger* phytase is relatively resistant to inactivation by pepsin and pancreatin in a laboratory setting, and our results suggest that this is also the case *in vivo*. It should be noted that one of the difficulties in creating a transgenic organism with an active *A. niger* phytase gene has been the inability for this transgene to be properly glycosylated, which is needed for phytase activity.[35] The ability of these fish to glycosylate the transgene product represents a fundamental advantage of placing this gene in fish to produce an active phytase enzyme.

Since it was unknown where phytase expression would cause the greatest effect, strong ubiquitous promoters were chosen for this experiment. Although neither construct resulted in completely ubiquitous expression, the greatest amount of phytase expression was located within the coils of the intestines with moderate expression in the stomach and dorsal tissue of fish transgenic for both constructs. Moderate expression was also noted in the eye tissue of fish transgenic for the OnH3-PHY-GH1t-OnH3-EGFP construct. Although the reason for the strongest expression being noted in the intestines is unknown, this is not an uncommon feature of ubiquitous promoters. Previous work[36] has shown similar effects with the sockeye salmon metallothionein (OnMT) promoter driving expression of the sockeye salmon type I growth hormone construct in salmon. In this case, RT-PCR results showed an almost 100-fold increase in expression in the pyloric caeca than muscle tissue, with high levels of expression also in the kidney, spleen, and intestine.

Overall, the results of this study are very promising. The production of fish with improved phytate phosphorus utilization is possible and has been accomplished. Although beyond the scope of this study, the production of a commercial species transgenic for the phytase gene may prove very important. Medaka are models and as such have many benefits for preliminary testing; however, medaka have very short intestines, which may not allow adequate time for full phytate degradation. Fish that regularly feed on plant material, such as tilapia, have larger intestines, and more phytate degradation may be possible in such species. Preliminary data from our laboratory using the same constructs showed that phytase transgenic tilapia fish compared to nontransgenic tilapia fed an all-plant diet was able to significantly reduce fecal phytate by 43.5%.[37] In a production setting, the amount of waste is higher and the effect of phosphorus waste is more of a concern. The ability to reduce phosphorus waste in these settings through improved phytate phosphorus utilization would not only be advantageous from an ecological perspective, but should allow for noticeable decreases in feed costs.

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