國立臺灣大學生物資源暨農學院動物科學技術學系

博士論文

Department of Animal Science and Technology
College of Bioresources and Agriculture
National Taiwan University

Doctoral Thesis

# 藉由豬胰澱粉酶啟動子基因轉殖策略賦予轉基因豬同時表現外源植酸分解酶與纖維素分解酶之可行性

# The Feasibility of a Transgenic Strategy for Co-expression of Phytase and Cellulase Transgenes Driven by the Porcine Pancreatic Amylase Promoter in Pigs

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中華民國 103 年 10 月
October 2014

# 國立臺灣大學博士學位論文
# 口試委員會審定書

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本論文係林育聖君（D95626001）在國立臺灣大學動物科學技術學系完成之博士學位論文，於民國 103 年 10 月 22 日承下列考試委員審查通過及口試及格，特此證明

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（是否須簽章依各院系所規定）

# 誌 謝

本論文能順利完成並付梓，乃承蒙恩師 鄭登貴教授在研究、課業及生活上給予極大之指導、關懷與鼓勵， 徐濟泰教授不辭辛勞悉心提供諸多寶貴建議， 黃木秋教授、 陳全木教授、與 吳信志教授在學位考試及論文上之詳細審閱斧正；老師們勉勵提攜之情，讓學生在博士班訓練過程中獲益良多並感念於心，謹此致最深之謝忱。

在研究過程中，同時非常感謝 徐啟真博士、與 楊卓真博士於實驗細節上之提點以及論文的校審；朱有田教授在實驗儀器上的支援；劉嘉睿教授、余碧教授提供酵素活性測試方法指導；農場鄭位明學長、重威、及阿芬阿姨在樣本採樣以及試驗動物照顧之協助；劉秀洲博士、廖奕雯學姐、游位育學長於平日生活上的激勵和學務上的協助；以及動物生殖科技研究室志任、淳淳、華雲、劭于、妍樺、廉本、碩甫、家駿、冠宇、偉誠，營養研究室瑋蕾、柏元、煜軒，分子生物研究室虹妏、晁偉、原佑等昔日與今日的實驗室成員在精神與試驗上的鼓勵與協助，能與各位共同學習以及切磋分享，實是人生中最珍貴的回憶。

最後要感謝我的家人，謝謝你們在這些年來給予的支持，遂使求學生涯能無後顧之憂，得以順利完成學業。

謹以本論文獻給我敬愛的家人，及所有關心我的朋友，謝謝你們！

謹識於
國立台灣大學 動物科學技術學系
中華民國 103 年 10 月 22 日

# 中文摘要

## 藉由豬胰澱粉酶啟動子基因轉殖策略賦予轉基因豬同時表現外源植酸分解酶與纖維素分解酶之可行性

林育聖

近年全球面臨氣候變遷與能源轉型、短缺，造成國際穀物供需失衡，國內進口大豆、玉米等主要飼料原料價格高漲，養豬產業飼料成本節節上揚，且畜體飼養過程中所產生之排泄物、廢棄物與其所衍生之環境污染更是目前養豬業者務必嚴正面對之環保課題；職是之故，部分研究先進乃嘗試從營養設計之角度，試圖改良動物對營養分之消化吸收，從而降低大量營養物質隨糞便排出之比例，進而得能有效減少飼料浪費與環境污染之目標。

本研究旨在釐清吾等先前已成功產製以豬胰澱粉酶基因啟動子在胰臟中表現外源之植酸分解酶與纖維素分解酶之轉基因豬，是否具備有效提高消化率與降低糞磷之排放能力；試驗之進行包括：1. 完成分析業經成功選殖2,488-bp之豬胰澱粉酶基因啟動子序列特性以及其活性；2. 依據業經建立之轉基因豬，完成分析並確認彼等外源植酸分解酶與纖維素分解酶等之轉殖基因，分別在其體內之組織表現性及其在性腺被傳承之能力；3. 進行表面消化率試驗，裨謀比較彼等非轉基因豬與轉基因豬，分別被飼予對照組日糧（control diet）與/或低磷日糧（low-P diet）時，各受試豬隻對於飼糧中包括乾物質、粗蛋白質、粗纖維、鈣、磷等之利用的差異性。

試驗結果證明：1. 前述業經選殖出之2,488-bp序列片段被與綠色螢光蛋白基因共同構築成報導載體後，確實能在大鼠胰臟腫瘤細胞株（AR-42J cell line）中成功表現出螢光蛋白質，證明該選殖片段確定具備啟動子之活性，且能有效控制下游基因之表現;2. 以聚合酶反應(polymerase chain reaction, PCR)及南方吸漬法(Southern blot)檢測F1世代基因組，證明先前試驗所產製之轉基因豬具備性腺傳承之能力。此外藉由反轉錄聚合酶鏈鎖反應(reverse transcription-PCR)策略分析F1世代之器官及/或組織，結果證實包括植酸分解酶與纖維素分解酶兩者之mRNA表現，分別皆具備有組織之專一性，且僅在胰臟組織中被轉錄。進一步藉由西方吸漬（Western blot）分析包括F1與F2世代轉基因豬之胰臟組織及其腸內容物;結果證明彼等源自被轉殖之植酸分解酶與纖維素分解酶等外源基因，其所表現之蛋白質分別確能在胰臟被合成，且經由胰管分泌進入十二指腸中。此外，經活性試驗結果證明，前述兩種蛋白質在胰臟及十二指腸內容物，且分別具備分解植酸與纖維素之活性，並以十二指腸內容物活性較高；植酸分解酶活性經過鉬藍法（molybdate-blue method）定量計算結果，證明其在十二指腸中存在，且高達到8.2 U/mL之譜；3. 在以玉米、大豆及大麥為主之對照組日糧中，相較於彼等非轉基因豬而言，轉基因豬在乾物質、中性洗滌纖維、粗蛋白質、鈣、及磷等之表面消化率，分別均有較佳之表現，特別是乾物質及中性洗滌纖維等，更呈現有顯著性之差異（$P < 0.05$）；就飼予玉米、大豆及大麥等為主之低磷組日糧者而言，轉基因豬在乾物質、中性洗滌纖維、粗蛋白質、鈣、及磷等之表面消化率，分別均較彼等非基因轉殖豬者，有較佳的表現（$P < 0.05$）。其屬有趣者，乃非基因轉殖豬比較之被飼予對照組及/或低磷組日糧者，並進一步分析其糞便中之氮及磷含量者，試驗

結果顯示轉基因豬之被餵飼對照組與低磷組日糧者，其糞便中磷之排出量分別被減少29%和48%之譜，此外其糞便中氮排出量亦分別下降多達31%和49%之譜。

綜合上述試驗結果，本研究證明，試驗所構築之2,488-bp豬胰澱粉酶基因啟動子序列不僅具有啟動轉錄之能力，且在先前產製之轉基因豬所攜帶之外源基因，確定可經由性腺被傳承至其子代。此外，彼等在胰臟組織專一表現植酸分解酶及纖維素分解酶之轉殖基因，其表現之外源轉基因之蛋白質，且分別具備有分解酶之活性，遂能有效增進其對飼料之消化效率從而顯著減少糞中氮及磷之排出量。未來之試驗研究冀望得能進一步完成豬隻在各不同生長階段之相關營養試驗，並針對此等轉基因豬隻各階段之生長速率，對於各不同飼料原料之消化能力，及豬隻在腸道中被免疫相關基因之調控等，可能分別扮演有重要之角色；深盼透過本轉基因殖豬模式之完成建立，不僅得能賦予豬隻具備有效利用高纖維原料替代物（fiber-rich by products）之固有營養分，且能有效維持豬隻生長與繁殖性能，從而更能兼具臻於環保目標之要求。

**關鍵字**: 轉基因豬, 豬胰澱粉酶基因啟動子, 植酸分解酶, 纖維素分解酶

# Abstract

The Feasibility of a Transgenic Strategy for Co-expression of Phytase and Cellulase Transgenes Driven by the Porcine Pancreatic Amylase Promoter in Pigs

Lin, Yu-Sheng

Competition between humans and livestock for cereal and legume grains makes it challenging to provide economical feeds to livestock animals. Recent increases in both corn and soybean prices have had a significant impact on the cost of feed for pig producers. The utilization of by-products and alternative ingredients in pig diets has the potential to reduce feed costs. However, unlike ruminants, pigs have limited ability to utilize diets with high fiber content because they lack endogenous enzymes capable of breaking down non-starch polysaccharides into simple sugars.

In addition, pigs do not produce sufficient endogenous phytase to hydrolyze P from phytate in cereals, making it necessary to supplement with inorganic phosphorus (P) to meet the requirements for optimum pig production, resulting in higher fecal concentrations of P and increasing potential environmental pollution. Thus, increased regulatory pressures are being imposed on animal agriculture to minimize P and nitrogen emissions. Improvement of the digestive efficiency of pigs is the most logical approach for reducing nutrient excretion and achieving pollution control. This study investigated a biological method for reducing fecal P excretion and improving nutrient digestibility, and utilized a transgenic strategy in which

expression of bacterial phytase and fungal cellulase in the gastrointestinal tract to establish an eco-friendly pig model.

A 2,488-bp 5′-flanking region of the porcine pancreatic amylase gene was cloned and its structural features were characterized. Using green fluorescent protein as a reporter, we found that this region contained promoter activity and had the potential to control heterologous gene expression. Germ-line transmission and tissue-specific expression of phytase and cellulase transgenes in the pancreas of F1 and F2 transgenic pigs were identified. Both enzyme activities were also detectable in pancreas and duodenal contents. Up to 8.233 U/mL phytase activity was detected in the duodenal contents of transgenic pigs, which represents 5−8 times the activity in non-transgenic pigs. On a corn, barley, and soybean-meal-based control diet, the apparent fecal digestibility of dry matter (DM), neutral detergent fiber (NDF), crude protein, calcium, and P was higher in transgenic pigs than in non-transgenic pigs ($P < 0.05$ for both DM and NDF digestibility). On a low-P basal diet, the transgenic pigs exhibited significantly increased digestibility of these nutrients ($P < 0.05$).

Conclusions come to the above studies indicated that those transgenic pigs fed with the control and/or the low-P diet(s) would result in significantly decrease of their fecal P outputs by 29% and 48%, respectively, and also their fecal N outputs was reduced by 31% and 49%, respectively, when comparisons were made to the fecal P and/or N outputs from those non-transgenic pigs. Based on evidences demonstrated above, the establishment of a tissue-specific promoter of the porcine pancreatic amylase gene and suggests our transgenic pigs may represent a way to increase nutrient utilization and reduce fecal P and N contents.

**Keywords**: transgenic pig, pancreatic amylase promoter, phytase, cellulase

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# Introduction

The environmental issues surrounding pig production present a growing public-health concern, particularly with regard to the disposal of feces. Feeds are supplemented with minerals and inorganic phosphorous (P) to meet the nutritional requirements of the porcine diet; excess intake is readily excreted in the feces and results in water pollution and soil erosion (Carpenter, 2008). Although phase feeding is effective for reducing P and nitrogen (N) excretion in porcine feces, the low digestibility of P-bound phytate (phytate-P) is the primary concern (Dourmad and Jondreville, 2007). The use of phytase, which releases phytate-bound minerals in feeds, has gained popularity in the industry (Harper et al., 1997; Chu et al., 2009).

The corn and soybean meal in Taiwan are imported products. The fluctuation of their prices becomes one of the most influencing factors for pig production (Saengwong et al., 2011). In 2008, the prices of grain and soybean has increased, substantially causing pig production cost per 100 kg live hog weight raised from NT$ 5,146 (in 2007) to NT$ 6,566 (National Animal Industry Foundation, 2012). To overcome the global increase in the cost of traditional feeds and limited cereal production, local and

inexpensive ingredients are to be used as substitutes for more expensive conventional ingredients in porcine diets (Esteban et al., 2007; Weber et al., 2008; Taiwan Grain and Feed Annual, 2014); however, most of these alternatives comprise fiber-rich by-products and have distinct nutritional profiles in comparison to common traditional ingredients (Varel and Yen 1997). Therefore, improved fiber conversion for energy utilization will provide critical reductions in grain consumption and digestive waste in the pig industry.

Feedstuffs of plant origin contain phytic acid within the cell, and non-starch polysaccharides (NSPs) are located in the cell walls. Phytase and carbohydrates may act in synergy to improve nutrient utilization, as the carbohydrases hydrolyze the NSP in cell walls to increase phytase access to phytic acid (Woyengo and Nyachoti, 2011). In the previous study (Lin, 2004), transgenic pigs harboring transgenes for bacterial phytase and fungal cellulase were generated by using a direct co-injection strategy. These animals have since been anticipated being capable of showing much better to digest those fibers, use P more efficiently, and produce much less solid waste; ultimately bringing much more profit to farmers. To allow spatial and temporal control of transgene being expressed in this type of pig, a promoter DNA fragment was cloned

from the porcine pancreatic amylase gene subsequently being designed for driving both of the exogenous phytase and cellulase genes. However, it remains to be determined that if the cloned amylase promoter is sufficient to drive both of the phytase and cellulase transgenes, and whether these secreted proteins can maintain their enzymatic properties in the duodenal lumen. The objective of Experiment I was to characterize the properties of this putative promoter as well as being able of providing fundamental information regarding transgene expression in those transgenic pigs generated. Experiment II was designed to verify the expression and activity of phytase and cellulase in the pancreas and gastrointestinal tract. To verify the feasibility of producing adequate digestive enzymes for efficient digestion in transgenic pigs carrying transgenes driving by the porcine pancreatic amylase promoter, further studies related to the digestive efficiency was measured in Experiment III. The valuable information obtained from these studies should be conducted for providing those important clues regarding to the utility of these transgenic lines of pigs as a promising alternative strategy for ensuring though events related to hydrolysis of partial anti-nutritional compounds in diets.

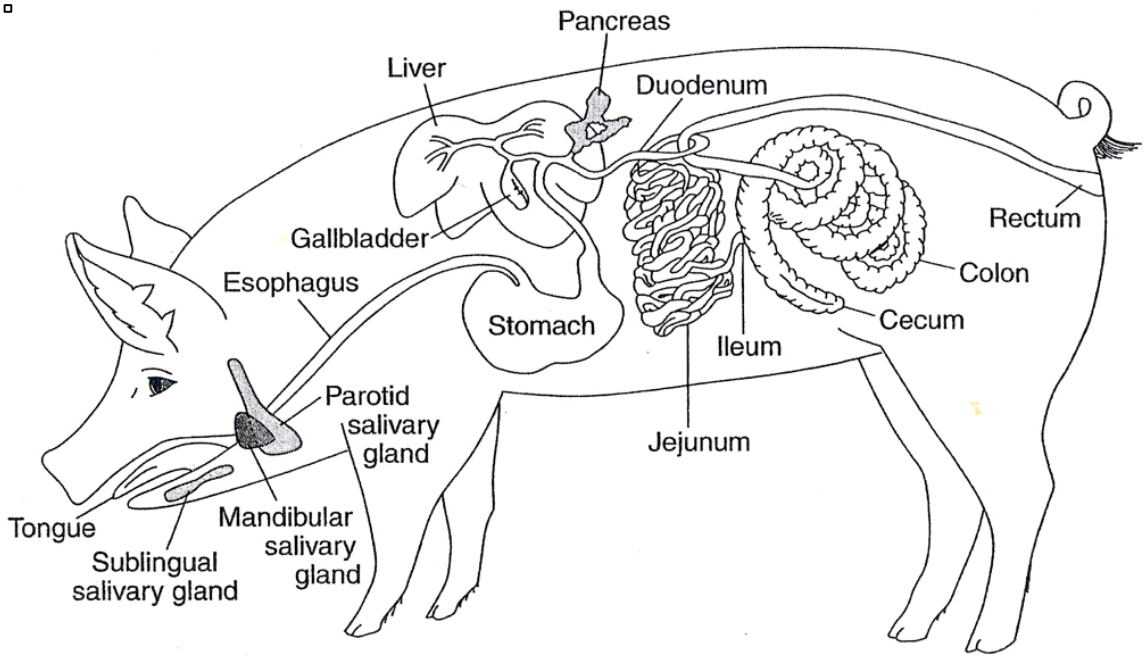
# Chapter 1 Review of the Literature

## 1.1 The Digestive Tract of Pigs

Pigs have a monogastric or called as the non-ruminant digestion system. Similar to all other monogastric animals, their digestive tract is highly developed and has five main parts, including the mouth, esophagus, stomach, and small and large intestines (Yen, 2001a; Figure 1).

### 1.1.1 Mouth and Salivary Glands

The digestive process starts with feed entering the mouth of the pig. The physical action of chewing promotes salivary glands secretion by reflex nervous stimulation (Strube, 2013). More than 90% of the saliva is derived from the secretion of three pairs of major glands, the parotid, mandibular, and sublingual glands, which secrete proteins belonging to the classes of proline-rich proteins, mucins, lysozyme, immunoglobulin (Ig) A, and α-amylase (Nagler, 2008). The activity of porcine salivary α-amylase, which hydrolyzes the α-1,4-glycosidic linkages of starch and oligosaccharide and is secreted




(Yen, 2001a)

**Figure 1. Diagrammatic representation of the porcine digestive system.**

by the sublingual glands, is low and largely inactivated by the acidic gastric contents (Araki et al., 1990). From a quantitative viewpoint, salivary α-amylase is not considered important because the ratio of total salivary amylase to total pancreatic amylase in the postprandial phase (0–5 h after feeding) is only about 1:250,000 (Lærke and Hedemann, 2012). Thus, although α-amylase is secreted in the oral cavity, starch digestion is not of quantitative importance here because the time spent by the feed in the mouth is too short (Yen, 2001b).

### 1.1.2 Stomach

The pig stomach is a reservoir hold about 30% of the total volume of the digestive tract (Wenk, 2001). Compared to ruminants, pigs have a “simple” stomach in which only slight microbial modification of available nutrients takes place before absorption occurs. Digestion of ingested proteins begins with pepsin activity within an optimum pH range from 2 to 3 (Rajagopalan et al., 1966). When feed enters the stomach, it encounters hydrochloric acid and pepsin, both of which may compromise exogenous enzyme stability and activity *via* acid denaturation and proteolytic digestion (Strube et al., 2013). However, pepsin is not essential for normal protein digestion

because it only acts on approximately 10–15% of dietary proteins before it is inactivated in the duodenum, where pH increases due to pancreatic bicarbonate ion secretion (Lærke and Hedemann, 2012). Therefore, in the stomach, only a limited mixing of feed occurs and is subject to the action of pepsin, which may partially degrades the dietary proteins.

### 1.1.3 Small Intestine

The pig small intestine is a long digestion tube and shaped like a spiral. Its wall has millions of tiny finger-like protrusions known as villi, which increase the absorptive surface area. Epithelial cells lining the wall of the small intestine also secrete enzymes that break down feed particles and facilitate nutrient absorption (Lærke and Hedemann, 2012). The pig small intestine is longer in comparison to those of other farm animals and permits intensive endogenous digestion under near-neutral conditions (Wenk, 2001). The first section of the small intestine is known as the duodenum. It is here that liver and pancreas secretions enter the digestive system, where they are essential for digestion and absorption of various nutrients utilized in intermediary metabolism. Liver secretions digest fats, while the pancreatic secretions digest lipids, proteins, and

carbohydrates. The products of digestion, such as fatty acids, amino acids, and glucose, are absorbed in the jejunum and ileum, which are the second and third sections of the small intestine. The activity of the enzymes contained in feed may disrupt the natural process of digestion (Strube et al., 2013).

### 1.1.4 Large Intestine

The large intestine begins with the cecum. In this part of the digestive system, undigested feed components such as dietary fibers, lipids with high melting point, insoluble proteins, and endogenous secretions are fermented by the intestinal microflora and most of the remaining water from undigested feed is reclaimed back into the circulation (Wenk, 2001).

While the fermentative capacity of the large intestine in pigs is not comparable to the rumen-reticulum of ruminants, volatile fatty acids (VFA) such as acetate, propionate, and butyrate produced during fermentation in the large intestine can supply up to 30% of the energy requirements of growing/finishing pigs (Rérat, 1985; Yen et al., 1991). However, fermentative energy production is frequently underestimated because the formation of methane, hydrogen, and fermentative heat decreases the amount of energy

available to the pig (Grieshop et al., 2001), hence decreasing the efficiency of energy utilization (Giusi-Perier et al., 1989; Noblet et al., 1994).

The intestinal microflora also depends on the host animal's diet as the main source of metabolic substrates. Increasing levels of dietary fiber subsequently increase the amount of substrate that moves into the large intestine for microbial fermentation, further promoting the activity of carbohydrate-fermenting bacteria (Haberer et al., 1999; Diebold et al., 2004). Increasing lactic acid and VFA products cause a decrease in the digesta pH, which creates unfavorable conditions for the growth of protein-fermenting bacteria (Smith and MacFarlane, 1997). By limiting microbial proteolysis and increasing carbohydrate fermentation in the large intestine, some of the most offensive-smelling compounds emanating from pig production are reduced (Mackie et al., 1998; O'Connell et al., 2005).

## 1.2 Pancreatic Secretions in the Pig

### 1.2.1 Structure of the Pancreas

The pancreas is a major part of the digestive system and has an integral role in

absorption and metabolism (Rinderknecht, 1993). It contains 90–95% exocrine tissue and about 2–3% endocrine tissue (Brannon, 1990), and exhibits the highest rate of protein synthesis and secretion per gram of tissue than any other organ (Logsdon and Ji, 2013). Acinar (>80%) and ductal cells of the exocrine pancreas form a close functional unit (Fredirick and Jamieson, 1994). The pancreatic acinar cells synthesize and secrete a variety of digestive enzymes, water, and electrolytes into the duodenum, while cells lining the pancreatic duct produce bicarbonate-rich secretions that neutralize the acid chyme entering the duodenum from the stomach and generate a slightly alkaline environment in the duodenum for optimum pancreatic enzyme activity (Hegyi et al., 2011). Acinar cell and duct cell secretions constitute the pancreatic juice.

### 1.2.2 Enzyme Secretion of the Exocrine Pancreas

The main regulatory pathways controlling exocrine pancreatic secretion are linked to hormone–hormonal and neural–hormonal interactions of regulatory peptides such as secretin and cholecystokinin (CCK), as well as neurotransmitters from the gut, pancreas, and vagus nerve (Cook and Young, 1996; Evilevitch et al., 2003; Chandra and Liddle, 2014). Enzyme secretion also depends on dietary composition, age, feeding

regimen, and postprandial sampling time (Corring et al., 1989; Makkink and Versiegen 1990). Changes in pancreatic synthesis of individual exocrine proteins in response to polypeptide hormones or nutritional substrates are summarized in Table 1 (Scheele, 1993). Positive feedback mechanisms appear to exist between nutritional substrates in the diet and the synthesis of pancreatic enzymes required to digest those substrates. Basal secretion occurs between meals; ingestion of a meal stimulates secretion for 3–7 h, depending on the frequency of feeding (Lærke and Hedemann, 2012). Average basal pancreatic secretion is only 0.5 mL/kg/h in suckling pigs, and there is no increase in response to milk intake (Pierzynowski et al., 1990). Basal pancreatic secretions are much higher after weaning and increase by 3-fold after feed intake (Pierzynowski et al., 1990; Rantzer et al., 1997); the most important of these are proteolytic, lipolytic, and amylolytic enzymes (Ohlsson et al., 1982).

All proteolytic enzymes are secreted as inactive proenzymes from pancreatic acinar cells to protect the glands from autodigestion (Lærke and Hedemann, 2012). Activation of these proenzymes is initiated by a regulatory cascade of enteropeptidases secreted by the duodenal glands within the walls of the small intestine (Lowe, 1994; Braud et al., 2012; Figure 2). Proteolysis of trypsinogen to trypsin activates other

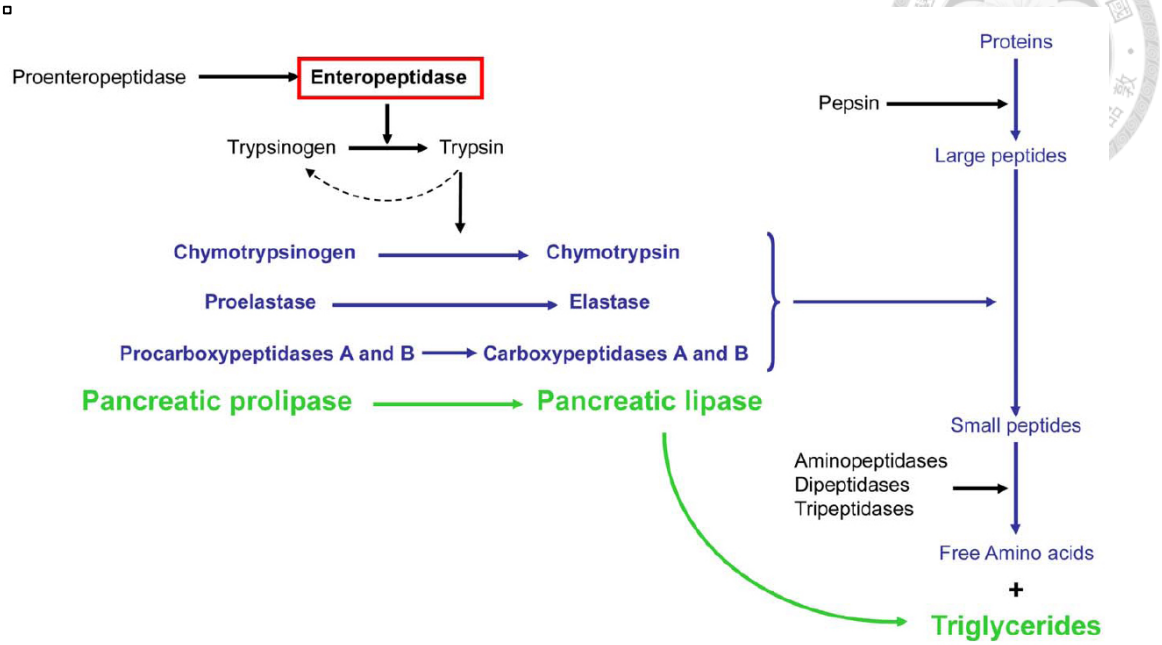
## Table 1

## Regulation of protein synthesis in rat exocrine pancreas

(Scheele, 1993)

| Nutritional substrates | Digestive end products | Hormones | (Pro)enzyme synthesis | | |
|---|---|---|---|---|---|
| | | | Proteases | Lipases | Amylases |
| Protein | Amino acids | CCK | Trypsinogen 1, 2, 3 ↑<br>Chymotrypsinogen 1, 2 ↑<br>Proelastase 1 ↑<br>Procarboxypeptidase A, B ↑ | | Amylase 1, 2 ↓ |
| Triglyceride | Fatty acids | Secretin<br>GIP | Proelastase 2 ↑ | Lipase ↑<br>Colipase ↑ | |
| Starch<br>Glycogen | Glucose | Insulin[a] | | | Amylase 1, 2 ↑ |

↑, increase; ↓, decrease; CCK, cholecystokinin; GIP, gastric inhibitory peptide.
[a]Insulin effect observed only in diabetic animals.




(Braud et al., 2012)

**Figure 2. Cascade of biochemical events starting with proenteropeptidase action.** Enteropeptidase converts inactive trypsinogen into active trypsin, which in turn converts the other pancreatic zymogens—chymotrypsinogen, proelastase, carboxypeptidases A and B, and prolipase—into active enzymes.

digestive enzymes in the alkaline environment (Light and Janska, 1989).

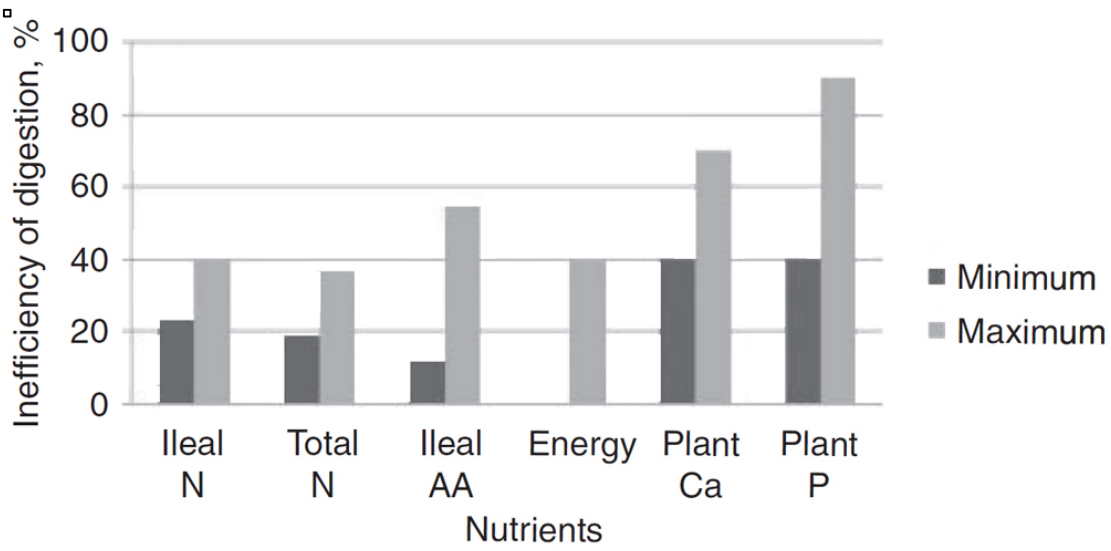
The pancreatic juice contains three lipolytic enzymes, including lipase, carboxylester hydrolase, and phospholipase A2 (Lærke and Hedemann, 2012). Similar to proteolytic enzymes, the lipolytic enzymes are excreted in an inactive form and activated by trypsin. All lipolytic enzymes hydrolyze triacylglycerides to fatty acids, glycerol, and mono- or di-acylglycerides and, therefore, are the most important enzymes in the digestion of dietary fats (Jensen et al., 1997).

The luminal phase of carbohydrate digestion by pancreatic α-amylase in the small intestine applies only to starches (Lærke and Hedemann, 2012), which are the principal dietary carbohydrate and the single most abundant feed energy in diets for piglets, growing pigs, and sows, comprising 60–70% of the total energy intake (Bach Knudsen et al., 2013). Pancreatic α-amylase, like salivary amylase, cleaves the α-1,4 glycosidic linkages of starch substrates, reducing them to glucose, maltose, maltotriose, and dextrin (Hizukuri et al., 1996). Alpha-amylase activity in pancreatic tissue is extremely low at birth and rapidly increases with age. Pigs exhibit adaptive secretion, sensitive to the type and level of starch and dietary fiber in the diet. Weaned piglets show a sharp increase in basal and postprandial amylase output after the diet is changed

from milk to a diet high in starch (Aumaitre, 1972; Corring, and Saucier, 1972). The increase in α-amylase activity is also observed when the daily intake of dietary starch is up to 400%; total α-amylase activity increases by 2.3-fold in 1–2 post-prandial hours (Corring and Chayvialle, 1987; Corring et al., 1989). However, replacing dietary starches with monomeric carbohydrates such as glucose or dextrose had no influence on α-amylase activity (Corring, 1977). The replacement of starch by cellulose, straw meal, or pectin significant reduces total α-amylase activity (Mosenthin and Sauer, 1991; 1993).

## 1.3 Exogenous Enzymes in Porcine Feeding Programs

The efficiency of nutrient digestibility has been assessed in the absence of exogenous enzymes (Olukosi and Adeola, 2012; Figure 3). Exogenous enzymes such as carbohydrases and phytase are used worldwide as additives in non-ruminant diets. Supplementation of porcine diets with these exogenous enzymes is a proven alternative strategy for improving animal performance by reducing or eliminating anti-nutritional compounds presents in feedstuffs, thereby increasing nutrient digestibility and availability (Suga et al., 1978; Xia, 2000; Omogbenigun et al., 2004; De Lange et al.,




(Olukosi and Adeola, 2012)

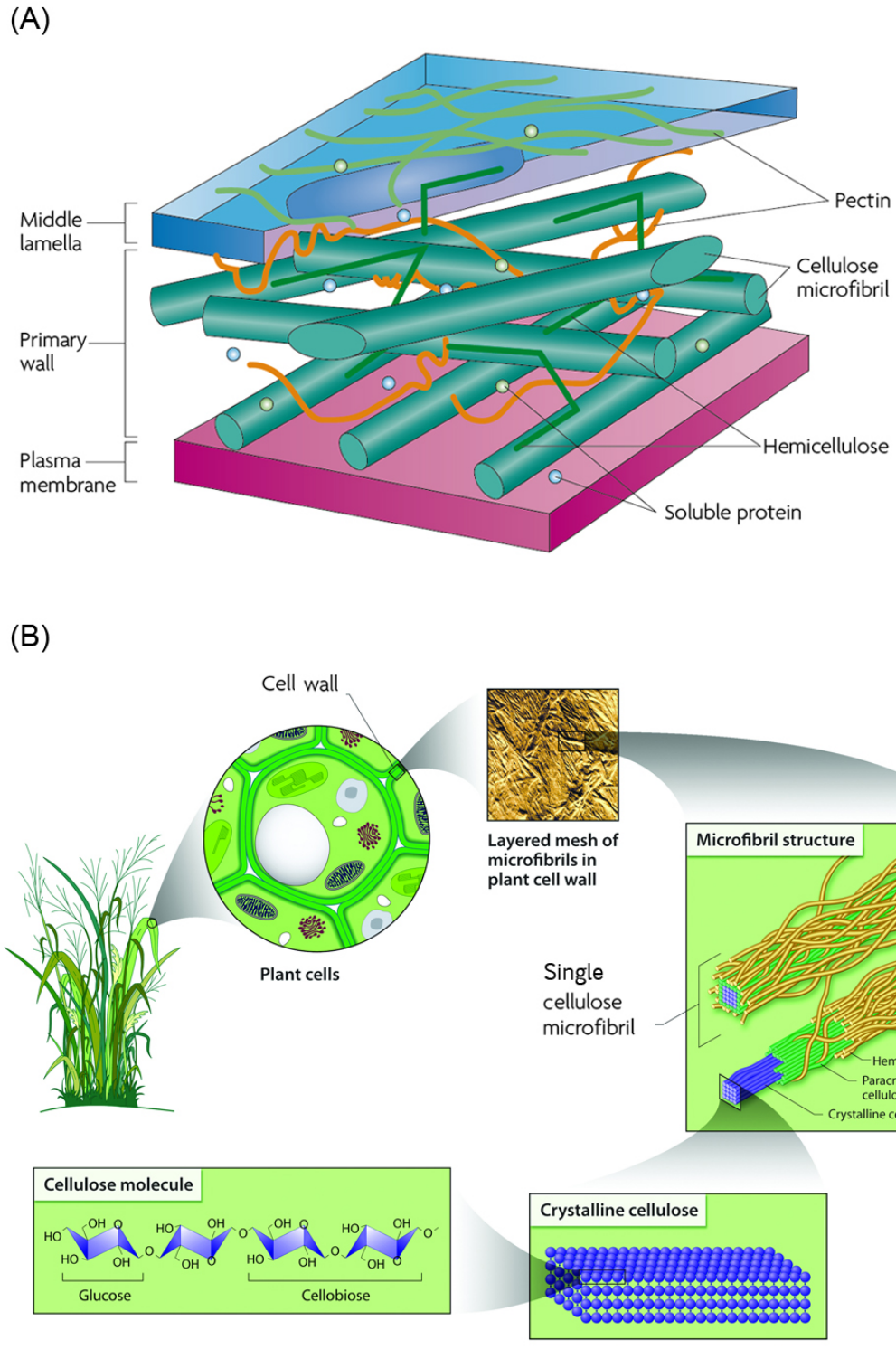
**Figure 3. Inefficiency associated with digestion in pigs.** The inefficiency (%) is calculated using the percentage digestibility of the nutrients. The inefficiency associated with nutrient digestion without exogenous enzyme can be up to 90% for plant calcium (Ca) and phosphorus (P), whereas nitrogen (N) and energy are used with greater efficiency. AA, amino acid

2010). Enzyme effectiveness in animal nutrition depends on type, source, and level of supplementation, as well as on the type of diet, animal health, and animal productivity. Since enzymes are protein catalysis, their activity is also susceptible to variations in pH and may be hydrolyzed by proteases acting inside the gastrointestinal tract. Successful enzyme supplementation therefore requires an assessment of the factors affecting enzyme activity and stability during passage (Strube et al., 2013).

### 1.3.1 Carbohydrase Supplementation

Cell wall NSPs are the major components of dietary fibers and are composed of cellulose and non-cellulosic polysaccharides (Figure 4). Table 2 shows NSP and cellulose levels in various cereals. Pigs obtain energy from fiber but only after microbial fermentation in the gastrointestinal tract and subsequent absorption of VFA (Urriola et al., 2012), because they have a limited ability to produce endogenous enzyme to digest NSP. Although soluble fiber is easily fermented, insoluble fiber is not well utilized by pigs. The greater the concentration of fiber contained in the diet, the lower overall energy digestibility becomes.

Carbohydrases such as β-glucanase, xylanase, and cellulase, make a significant




(Paloheimo et al., 2001; Sticklen, 2008)

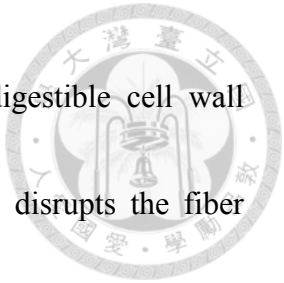
**Figure 4. The plant cell wall.** (A) Cell wall composition. A plant cell wall is arranged in layers and contains cellulose microfibrils, hemicellulose, pectin, lignin, and soluble protein. (B) Schematic representation of the cellulose structure.

**Table 2**

**Mean concentration of non-starch polysaccharides (NSP) in cereals and some legume grains (g/kg of dry matter)**

(Broz and Ward, 2007)

| Ingredient | Soluble NSP | Insoluble NSP | Cellulose | Total NSP |
|---|---|---|---|---|
| Wheat | 25 | 74 | 20 | 119 |
| Barley (hulled) | 56 | 88 | 43 | 186 |
| Rye | 42 | 94 | 16 | 152 |
| Oats (hulled) | 40 | 110 | 82 | 232 |
| Corn | 9 | 66 | 22 | 97 |
| Soybean meal | 63 | 92 | 62 | 217 |
| Peas | 52 | 76 | 53 | 181 |
| White lupins | 134 | 139 | 131 | 404 |



contribution to porcine diets by catalyzing the breakdown of indigestible cell wall components into simple sugars. Addition of dietary carbohydrases disrupts the fiber matrix embedding digestible carbohydrates, thus increasing accessibility of the digestive enzymes and improving outcomes in terms of diet conversion indexes (Bindelle et al., 2011). Many of these biologically active enzymes have been purified from plants, animals, and microorganisms. A large difference in affinity and turnover is observed for different enzyme sources with the same substrate; for example, xylanases share a common arabinoxylan substrate but their affinities and activities differ widely (Biely et al., 1997; Cuyvers et al., 2011). Exogenous enzyme activity is also influenced by the pH of the digesta. The optimum pH of most exogenous enzymes is between 4 and 5, but great variation may exist between enzymes from different sources, resulting in catalytic activity high at both lower and higher pH (Svihus, 2010). For example, *Aspergillus*-derived xylanase has an optimum pH between 4 and 6 (De Vries and Visser, 2001), but *Streptomyces*-derived xylanase functions over a pH range of 3–7 (Ding et al., 2008). This complicated matrix of conditions will determine the scale and variation of activity for an enzyme added to the diet and as it passes through the digestive tract. The efficacy of carbohydrase in pigs may be not the result of improvements in nutrient

digestibility alone (Omogbenigun et al., 2004; Ji et al., 2008), but to changes in digestive content characteristics, which indirectly affect the physiological status of the gastrointestinal tract. Hydrolysis of polysaccharides yields oligosaccharides such as xylose or arabinose that serve as prebiotic substrates which are capable of modulating microbial activity in the gastrointestinal tract, improving the health of the pig (Pluske et al., 2002; Kiarie et al., 2007). Such prebiotic substances favor the proliferation of lactic acid bacteria (Högberg and Lindberg, 2004; Kiarie et al., 2007), therefore inhibiting pathogen growth by competitive exclusion (Hillman et al., 1995).

### 1.3.2 Phytase Supplementation

Phytate is the main P-containing constituent of many seeds and tubers, which contain a P content of 282 g/kg (Maga, 1982). In general, phytates constitute about 1–2% by weight of many cereals and oilseeds. Approximately 36% (rapeseed meal) to 84% (wheat bran) of total P in these seeds is present in the phytate-bound form (Broz and Ward, 2007; Table 3). A possible structure of the phytate molecule (*myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate; IP6) when complexed with minerals, protein, and starch in acidic medium is shown in Figure 5A.

## Table 3

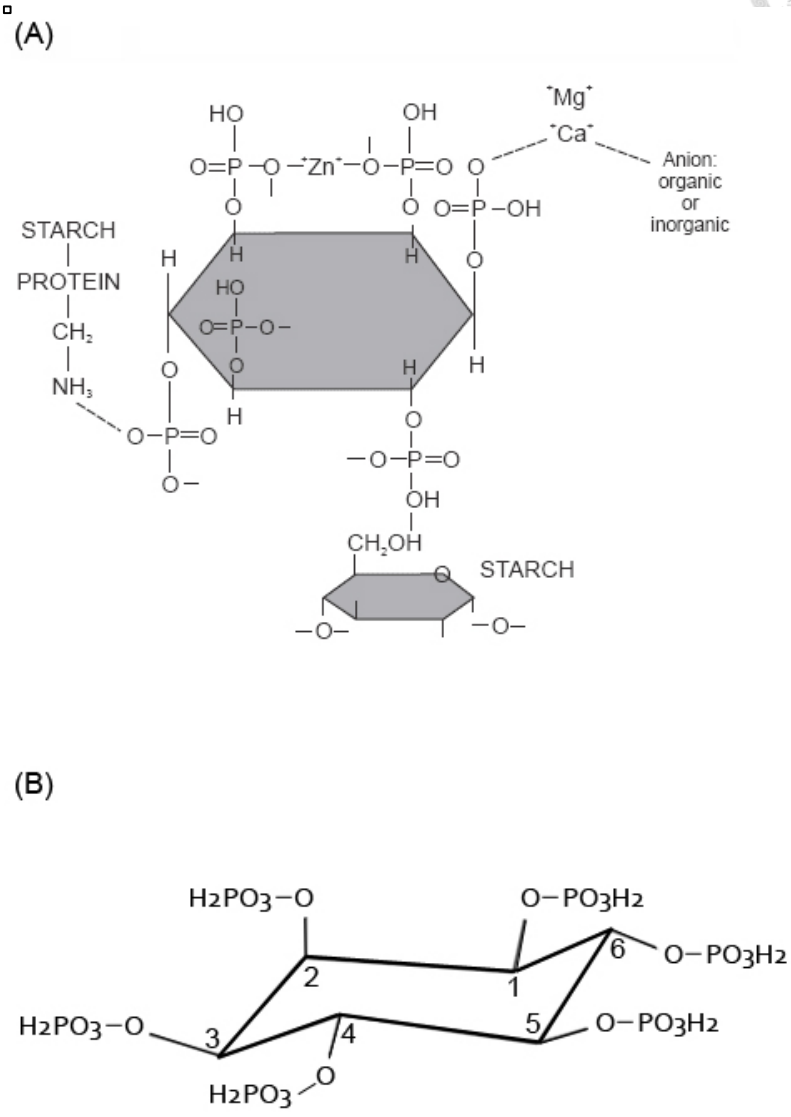
**Total and phytate phosphorus (P) content in feed**

(Broz and Ward, 2007)

| Feed ingredient | Total P (%) | Phytate P (%) | Phytate P (% of total P) |
|---|---|---|---|
| Wheat | 0.33 | 0.22 | 67 |
| Barley | 0.37 | 0.22 | 60 |
| Rye | 0.36 | 0.22 | 61 |
| Maize (corn) | 0.28 | 0.19 | 68 |
| Peas | 0.38 | 0.17 | 46 |
| Rapeseed meal | 1.12 | 0.40 | 36 |
| Soybean meal (44% CP) | 0.66 | 0.35 | 53 |
| Soybean meal (48% CP) | 0.61 | 0.32 | 52 |
| Wheat bran | 1.16 | 0.97 | 84 |
| Rice bran | 1.71 | 1.10 | 64 |

The P component of phytate is only partially available to pigs because the endogenous phytase activity in the small intestinal mucosa is insufficient to dephosphorylate phytate, leading to poor bioavailability of total P in grains (Hu et al., 1996). It is possible that the standard dietary calcium (Ca) levels have a substantial negative influence on phytate degradation by endogenous enzymes (Selle et al., 2009). To ensure an adequate supply of P, it is necessary to include inorganic P supplements in the porcine diet, although feces produced by these pigs contain high levels of P. Continuous application of fermented fertilizer made from pig feces causes a build-up of soil P, thus increasing the potential for P loss and possible water quality degradation (Adeola, 1999; Abioye et al., 2010).

The mode of action of phytase is illustrated in Figure 5B. Approximately 2–5 U/mL phytase activity is sufficient to satisfy the dietary P requirement (Golovan et al., 2001). One way to reduce fecal P content is through supplementation of exogenous phytase. Inclusion of phytase in porcine diets increases the digestibility of P and phytate-bound minerals, and enhances protein digestibility (Beers and Jongbloed, 1992), thus increasing the concentration of IP3–IP5 in the small intestine relative to IP6 (Hu et al., 1996) and reducing P excretion (Li et al., 1998). Another approach to reducing




(Kies et al., 2001; Yu et al., 2012)

**Figure 5. The structure of phytate.** (A) A putative phytate structure and its interaction with protein, starch, and cation. (B) Schematic of microbial phytase activity on dietary phytates. Phytate is numbered according to Agranoff's nomenclature, with the 2-phosphate axial end pointing upwards, with the carbon atoms numbered anticlockwise around the ring. Arrows indicate the phosphomonoester bonds under attack by the phytase.

phytate is exogenous expression of microbial phytase genes in pigs that seems to require almost no inorganic P supplementation for normal growth and excrete up to 75% less fecal P than non-transgenic pigs (Golovan et al., 2001); however, the activities of phytase decrease with age.

Bacterial phytase is typically more effective than fungal phytases in terms of the amount of P released per unit of phytase (Kornegay and Qian, 1996; Adeola et al., 2004; Augspurger et al., 2007). The characteristics of phytases from different sources are listed in Table 4. In general, manufacturer-recommended levels of commercially available phytases replace inorganic P levels by 0.12% in porcine diets (Jendza et al., 2006). Increasing amounts of phytase in the diet is associated with a curvilinear increase in the release of P from phytate (Kornegay, 2001).

Most phytases from microbial sources have optimum working pH values in the acidic range (Igbasan et al., 2000); the low pH environment in the gastric phase is ideal for making phytate susceptible to hydrolysis (Greiner and Konietzny, 2006). Therefore, phytase activity in digesta from the stomach is usually higher than in digesta from the upper small intestine (Jongbloed et al., 1992) and no phytase activity is detectable in lower small intestine digesta (Yi and Kornegay, 1996). Low phytase activity is also

## Table 4

### Common phytase enzymes used in swine nutrition[1]

(Olukosi et al., 2012)

| Sources | Characteristics | Weaknesses |
|---|---|---|
| Fungal: *Aspergillus* spp. | 1. Initiate dephosphorylation from position 3 on the inositols ring<br>2. Resistant to trypsin<br>3. May or may not dephosphorylate IP3 and lower<br>4. Leaves inositols monophosphate with phosphate group on C 2 as end product<br>5. Narrower pH range | 1. Susceptibility to pepsin<br>2. Weaker hydrolytic activity toward lower inositol |
| [2]Bacterial: *Escherichia coli* *Baccilus* spp. | 1. Initiates dephosphorylation from position 6 of the inositol ring (*E. coli*)<br>2. Resistance to pepsin<br>3. Wider pH range allowing for longer retention of activity in the digestive tract<br>4. Greater resistance to heat<br>5. May liberate all the phosphate groups | 1. Susceptibility to trypsin |
| Plant phytase | 1. Initiates dephosphorylation from position 6 of the inositols ring<br>2. Pollen phytase initiates dephosphorylation from position 5 of the inositols ring | 1. Lower resistance to proteases compared with fungal and bacterial phytases<br>2. Lower tolerance to heat; more likely to lose activity during pelleting<br>3. Lower pH range for hydrolytic activity; more likely to lose activity during transit in digestive tract<br>4. Lower activity toward IP3 and lower inositols |

[1]Other enzymes not listed include proteases and lipases of microbial origin, especially those of *Bacillus* spp. [2]Most of these characteristics are applicable only to *Escherichia coli* phytase.

detected in the intestinal contents of transgenic pigs carrying phytase gene driven by a mouse parotid secretory protein promoter (Golovan et al., 2001).

### 1.3.3 Enzyme Combinations

Feed enzymes are available as single-component enzymes or as multiple-component enzymes for which activity is generated in a single fermentation (Masey O'Neill et al., 2012). Addition of supplemental enzymes in combination is expected to produce synergistic effects; however, additional studies of multiple enzyme combinations are needed to elucidate their potential effect on the typical ingredients utilized in porcine diets.

# Chapter 2 Characterization of a Putative Pancreatic Amylase Gene Promoter from a Pig

## 2.1 Objective

Successful genetic engineering strategies require the use of promoters with appropriate characteristics for the desired objective (Potenza et al., 2004). In the laboratory, transgenic constructs are often designed with viral promoters and enhancer sequences to increase the efficiency of expression in most tissues and species (Betrabet et al., 2004). However, it remains unclear if there are risks associated with human consumption of integrated viral promoter or enhancer sequences within the transgenic pig genome. Combining spatial and temporal control of gene expression allows transgenic livestock production to be more useful in agricultural applications. Accordingly, the use of tissue-specific and its own promoters for heterologous gene expression may minimize the negative effects of transgenes on the normal growth and development of transgenic individuals (Zheng and Baum, 2008). In a previous study

(Lin, 2004), a 2,488-bp 5'-flanking region from a pig whose own pancreatic amylase gene was cloned by the genomic walking technique; however, its structural features were not characterized. To characterize this putative promoter, we used the cloning technique, a green fluorescent protein (GFP) reporter gene, bioinformatics, and functional analysis in a rat pancreatic tumor cell line.

## 2.2 Materials and Methods

### 2.2.1 Computer-based Analysis: the 5'-flanking Region of the Porcine Pancreatic Amylase Gene

A core promoter region was predicted in the 2,488-bp 5'-flanking region of the porcine pancreatic amylase gene by using the Neural Network Promoter Prediction program (NNPP) program, version 2.2 (Reese, 2001). Putative transcription factor binding sites were identified by using the Transcription Element Search System (TESS) database (http://www.cbil.upenn.edu/cgi-bin/tess/tess), a string-based search tool similar to local alignment software.

### 2.2.2 Cloning of the Fluorescent Protein Reporter Construct

The putative promoter region and N-terminal signal peptide-encoding region of the porcine pancreatic amylase gene (Darnis et al., 1999) were amplified with primers containing restriction sequences of *Not*I and *Eco*RV (forward-1, 5'-GCGGCCGCCTGACATAAGCTGAA; reverse-1, 5'-GATATCGGCCCAGCAGAA CCCAA) and with the following cycling conditions: 94°C for 5 min; 35 cycles at 94°C for 30 sec, 65°C for 30 sec, and 72°C for 2 min; and a final extension at 72°C for 7 min. Subsequently, the PCR products were digested with designate endonucleases, and purified with a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). The digested and purified PCR product was subcloned into the humanized *Renilla reniformis*-derived GFP (phrGFP) mammalian expression vector (Agilent Technologies, Cedar Creek, TX, USA) to generate the pAMY-hrGFP construct.

### 2.2.3 Culture of Rat Pancreatic AR-42J Cells

The rat pancreatic tumor cell line AR-42J was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were maintained at 37°C under an atmosphere of 5% $CO_2$/95% air in Ham's F12K medium (Invitrogen, Grand

Island, NY, USA) containing 1.5 g/L sodium bicarbonate and supplemented with 2 mM L-glutamine, 20% fetal bovine serum (FBS; Invitrogen), and 1% antibiotic-antimycotic mixture (Invitrogen). The medium was changed daily and cells were harvested at the indicated time points.

### 2.2.4 Transfection and Transient Expression of the AMY-GFP Reporter Construct

For transfection, cells were seeded and cultured in 35-mm dishes to 70–80% confluence. Attached cells were rinsed with PBS twice, then with serum-free Ham's F12K medium supplemented with 50 mM dexamethasone (Raffaniello et al., 2009). Two micrograms of pAMY-hrGFP were transfected into the cells by using a jetPEI cationic polymer transfection reagent (Polyplus-transfection, New York, NY, USA). Non-transfected cells were used as a negative control. At 48 h post-transfection, GFP expression was observed under an inverted fluorescence microscope (Axiovert 200; Carl Zeiss, Jena, Germany).

## 2.3 Results

### 2.3.1 Characterization of the Putative Porcine Pancreatic Amylase Gene Promoter Region

A DNA fragment containing 2,488-bp of the 5'-flanking region in conjunction with 45 bp signal peptide-coding sequence from the porcine pancreatic amylase gene was isolated and sequenced. Bioinformatics analysis indicated that the potential transcriptional start sites were located 11, 438, and 1,380 bp upstream of the ATG translational start site, and a TATAAA (TATA-box) sequence was located 43 bp away (Figure 6). Potential transcriptional factor binding sites in this region were specifically for insulin promoter factor 1 (IPF-1), glucocorticoid response element (GRE), hepatocyte nuclear factor 3-beta (HNF3-β), Opaque-2, and prolamin box binding factor (PBF).

### 2.3.2 *In Vitro* Activity of the Porcine Pancreatic Amylase Gene Promoter

Amylase promoter activity was examined in AR-42J cells transfected with the GFP reporter plasmid (Figure 7A). The results showed that this 2,488-bp 5'-flanking region was sufficient to activate reporter gene expression (Figure 7B).

## 2.4 Discussion

Digestive gene expression and enzyme activity are modulated in response to dietary chemical signals (Karasov et al., 2011). Pancreatic enzymes accommodate each individual's dietary composition via biosynthesis and secretion of specific enzymes (Owsley et al., 1986; Simoes Nunes, 1986; Flores et al., 1988); for example, pancreatic amylase secretion is sensitive to the amount of starch in the diet (Corring and Chayvialle, 1987; Mosenthin and Sauer, 1993). Low intake of carbohydrate usually results in a decrease in amylase expression at the transcriptional level (Giorgi et al., 1984). Fluctuations in amylase activity may be modulated at the level of gene expression. Therefore, the amylase gene promoter may be useful for gene expression through dietary stimulate.

Linking molecular mechanisms with physiological functions improves the understanding of hormonal control of the pancreatic amylase promoter in regulating its downstream gene expression (Ma et al., 2004; Scheele, 1993). GFP fluorescence was observed in AR-42J cells transfected with pAMY-GFP, suggesting this construct contained a functional promoter that was able to drive the downstream gene expression. Several *cis*-acting regulatory elements were predicted in the 5'-flanking region of the

porcine pancreatic amylase gene, including IPF-1, GRE, and HNF3-β. These observations are consistent with the results of previous studies in humans, mice, and sea bass (Liberzon et al., 2004; Ma et al., 2004). The close physical association of the three functional elements is consistent with the role of insulin in mediating dietary response (Hani et al., 1999; Lechner et al., 2001; Lee et al., 2002; Scheele, 1993). However, the insulin-response element is necessary but not sufficient for the regulation of amylase by dietary carbohydrate in mice (Schmid and Meisler, 1992). Glucocorticoid signaling also plays a key role in modulating the expression of many digestive enzymes in the exocrine pancreas (Kaiser et al., 1996; Logsdon et al., 1985). The hexanucleotide 5'-TGTCCT-3' is important for GRE activity (Slater et al., 1985), and pancreatic transcription factor (PTF-1) is required for glucocorticoid induction of mouse amylase expression (Slater et al., 1993). Glucocorticoids such as the synthetic dexamethasone typically bind the ligand-binding domain of GRE and increase GRE binding activity in a dose-dependent manner, thus promoting nuclear translocation and up-regulating amylase gene expression (Logsdon et al., 1985; Pratt etv al., 2004). Two hexanucleotides were present in the 5'-flanking region of the porcine pancreatic amylase gene, but no functional PTF-1 binding site had been identified. Instead, a functional

HNF3-β (also known as FoxA2) binding site was found in this region, where it recognizes an HNF3-binding site required for transcriptional activation of genes in exocrine acinar cells (Rausa et al., 1997). These observations are consistent with a previous study in sea bass, which showed that glucocorticoid stimulation of amylase promoter-driven gene expression is direct *via* GRE (Ma et al., 2004). Our results also revealed the presence of binding sites for the corn-transcriptional activators PBF and Opaque-2 scattered throughout the 5'-flanking region of the porcine pancreatic amylase gene. These proteins are expressed in parallel in corn endosperm and their putative orthologs have been identified in a large number of other cereals, including rice, barley, and wheat (Singh, 1998; Hwang et al., 2004). PBF and Opaque-2 act singly or in combination to promote transcription of numerous seed storage protein genes during seed development (Hwang et al., 2004). It remains unknown whether binding of these putative PBF and Opaque-2 transcription factors regulates the porcine pancreatic promoter following food intake.

## 2.5 Summary



We ligated the 2,488-bp 5'-flanking sequence of the porcine pancreatic amylase gene to GFP and found that this region contained promoter activity and had the potential to control heterologous gene expression. The structural features of the promoter include several potential transcriptional factor-binding sites for IPF-1, GRE, HNF3-β, Opaque-2, and PBF.


**Figure 6. The 5'-flanking region of the porcine pancreatic amylase gene.** Lowercase and uppercase letters represent the 5'-flanking region and coding sequence, respectively. The deduced amino acid sequence is presented below the coding region of exon 1, and the sequence encoding the signal peptide is boxed. Numbers alongside the sequence refer to the nucleotide position relative to the translation initiation site, designated as nucleotide +1. Three putative transcription start sites are indicated in bold with asterisks. The TATA box is highlighted in gray, and putative binding sites for insulin promoter factor 1 (IPF-1, dotted), glucocorticoid response element (GRE, broken), hepatocyte nuclear factor 3-beta (HNF3-β, double), Opaque-2 (thick), and prolamin box binding factor (PBF, thin) are underlined.

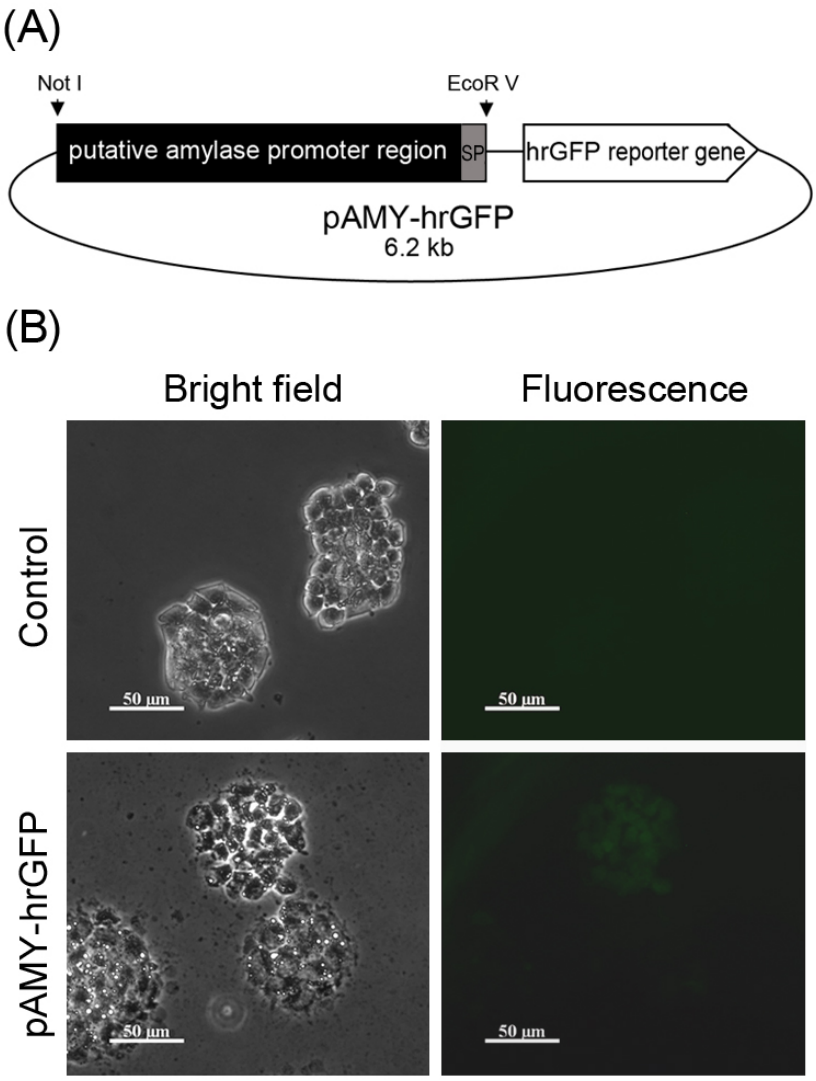



**Figure 7. Verification of promoter activity.** (A) Schematic diagram of the pAMY-GFP plasmid vector. The construct, consisting of the putative amylase promoter region (black box), porcine pancreatic amylase signal peptide (SP, gray box), and a humanized *Renilla reniformis*-derived GFP (hrGFP) gene (white box), was designed for transient transfection of AR-42J cells. (B) GFP expression in transfected cells. Cells were maintained in the presence of 50 mM dexamethasone. Negative controls (upper panel) were treated as above, but without transfection reagent or plasmid. After 48 h treatment, bright field (left panels) and fluorescence (right panels) images of the cultured cells were collected at 200× magnification. Scale bars represent 50 μm.

# Chapter 3 Evaluation of Transgenic Pigs Expressing AMY-PHY and AMY-CEL in the Gastrointestinal Tract

## 3.1 Objective

There are many potential applications of the transgenic methodology in the development of new and improved strains of livestock (Prather et al., 2008). Practical applications of transgenics in livestock production include enhanced prolificacy and reproductive performance (Rejduch et al., 2002), increased feed utilization and growth rate (Vize et al., 1988), improved carcass composition (Pan et al., 2010), improved milk composition (Yang et al., 2011), and increased disease resistance (Donovan et al., 2005). Accordingly, genomic integration of exogenous phytase and fibrolytic genes for direct expression in the gastrointestinal tract may be a rational and economical choice approach to reach the reduction of the fecal P and N contents. In a previous study (Lin, 2004), a transgenic founder was generated by co-injection of the pronuclei of fertilized pig eggs with two constructs in which the bacterial phytase transgene (AMY-PHY) and fungal cellulase transgene (AMY-CEL) were under the control of the 2,488-bp

5'-flanking region of the pancreatic amylase gene. However, it remained unclear whether the pancreatic amylase promoter could be used to induce transgene expression in the porcine gastrointestinal tract. The activity of secreted enzymes in the gastrointestinal tract has also not been established. The objective of this study was to evaluate AMY-PHY and AMY-CEL expression by RT-PCR, western blotting, and specific enzyme activity assays in pancreatic tissue and gastrointestinal tract contents.

## 3.2 Materials and Methods

All surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee, National Taiwan University, Taiwan.

### 3.2.1 PCR and Southern Blot Analyses

Genomic DNAs were isolated from the ear tissue of each newborns pig by using standard proteinase K-sodium dodecyl sulfate digestion followed by the phenol-chloroform extraction (Herrmann and Frischauf, 1987). One microgram of genomic DNA was employed as the template for a PCR reaction. Two sets of primers

were used to amplify the full-length DNA of the transgenes to confirm the presence of the AMY-PHY (forward-2, 5'-GGATCCCAGAGTGAGCCGGAGCT; reverse-2, 5'-CTCGAGTTACAAACTGCACGCCGGTA) and AMY-CEL (forward-3, 5'-GGATCCATTATGAAACCCGAACCA; reverse-3, 5'-CTCGAGTTATTCCTTTGG TTTTTC) cassettes in the transgenic pigs versus their nontransgenic littermates. Southern blotting was performed as previously described (Yang et al., 2004). In brief, radio-active probes were generated with PCR-amplified fragments and labeled with [α-$^{32}$P]dCTP by using the Amersham Rediprime II DNA labeling system (GE Healthcare, Fairfield, CT, USA). Two probes were designed to target the structural genes of the AMY-PHY (forward-2; reverse-4, 5'-TCAGTCACGTTCGCGTTATCT) and AMY-CEL (forward-3; reverse-5, 5'- TCCGTTCCATTCAACTGGTG) cassettes and were expected to amplify 431- and 599-bp products, respectively. After hybridization and variable stringency washing, membranes were subjected to phosphor-image analysis with a Typhoon 9200 scanner (GE Healthcare).

### 3.2.2 RNA Extraction and Analyses of RT-PCR

Total RNA was extracted from snap frozen pancreas, heart, liver, lung, kidney,

muscle, stomach, and duodenum tissues of transgenic pigs using an RNeasy Lipid Tissue Mini kit (Qiagen) with on-column DNase digestion (RNase-Free DNase Set; Qiagen) according to manufacturer instructions. RT-PCR was performed using a SuperScript III One-Step RT-PCR System with a Platinum Taq DNA polymerase kit (Invitrogen). In brief, 50 ng of each purified RNA sample was used as the starting material. Two primer sets were designed to target the structural genes of the AMY-PHY (forward-2; reverse-4) and AMY-CEL (forward-3; reverse-5) cassettes, and were expected to amplify 431- and 599-bp products, respectively. Beta-2 microglobulin (B2M; forward-4, 5'-AACGGAAAGCCAAATTACCTG; reverse-6, 5'-GTGATGCCG GTTAGTGGTCTG) served as a positive internal control, yielding a 259-bp fragment. Reactions were performed as follows: 55°C for 40 min; 94°C for 2 min; 35 cycles at 94°C for 30 sec, 64°C for 30 sec, and 68°C for 2 min; and a final extension at 68°C for 7 min. Amplified products were separated by 0.8% agarose gel electrophoresis and visualized by EtBr staining.

### 3.2.3 Protein Extraction

All pigs had body weights in excess of 120 kg prior to sampling. Porcine tissue samples as well as digesta samples from the duodenum and ileum were collected at least 6 h after feeding and then stored in liquid nitrogen. A piece of frozen porcine pancreatic tissue was pulverized within liquid nitrogen and its total protein was extracted through homogenization in ice-cold RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor (Millipore, Billerica, MA, USA) under gently rotation for 2 h at 4°C. The supernatants from either the RIPA-soluble pancreatic protein or duodenal content preparations were collected by centrifugation at 13,500 ×*g* for 20 min at 4°C. Total protein concentrations were determined with a BCA Protein Assay kit (Pierce, Rockford, IL, USA). All samples were stored at −20°C until SDS-PAGE analysis.

### 3.2.4 Cloning and Purification of Recombinant Proteins

Recombinant phytase and cellulase were cloned using a pET-52 3C/LIC cloning kit (Merck, Darmstadt, Germany) according to the manufacturer instructions. Primer sets were used to generate the full-length phytase coding region (forward-5,

5'-CAGGGACCCGGTCAGAGTGAGCCGGAGCT; reverse-7, 5'-GGCACCAGAGC GTTTTACAAACTGCACGCCGGTA) and cellulase coding region (forward-6, 5'-GACGACGACAAGATATTATGAAACCCGAACCA; reverse-8, 5'-CTCGAGTTA TTCCTTTGGTTTTTC) with N-terminal Strep-tag II and C-terminal 10×-His tag-specific sequences (underline). The resulting fragments were ligated into the pET-52b(+) 3C/LIC vector (Merck) and transformed into BL21(DE3) competent cells (Stratagene, La Jolla, CA, USA), respectively. Transformants were selected for kanamycin resistance and resulting clones were sequenced to confirm the insert orientation. Each selected clone containing the recombinant plasmid pET52-CEL was grown in 4 mL LB medium before being transferred to 200 mL Overnight Express Instant TB Medium (Merck) supplemented with 1% glycerol and 1 μg/mL kanamycin. The expressed His-Strep-tagged protein from bacterial lysates was purified by using a Ni-NTA Spin kit (Qiagen) followed by Strep-Tactin Magnetic Beads (Qiagen) according to manufacturer instructions. Fractions from each step of purification were fractionated on 10% SDS-PAGE following Coomassie blue staining. Purified recombinant protein was quantified using a BCA protein assay reagent (Bio-Rad, Hercules, CA, USA).

### 3.2.5 Western Blotting Analyses

Protein samples (50 μg) were boiled for 10 min in 5× sample buffer (60 mM Tris-HCl [pH 6.8], 25% glycerol, 2% SDS, 5% beta-mercaptoethanol, 0.1% bromophenol blue) and loaded onto an 8% polyacrylamide gels (TGX Acrylamide Starter Kit, Bio-Rad). After electrophoresis, the proteins were electrotransferred to Hybond-P polyvinylidene difluoride (PVDF) membranes (GE Healthcare) using the Trans-Blot Turbo Transfer System (Bio-Rad). Blocking was performed in Tris-buffered saline with Tween 20 (TBS-T; 20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 0.1% Tween-20) containing 10% skim milk overnight at 4°C before incubating 2 h in our custom-made rabbit polyclonal antiserum generated against recombinant cellulase (1:2,000; GeneTex, Hsinchu, Taiwan) or phytase (1:2,500; GeneTex) at room temperature with gentle agitation. Membranes were then washed with TBS-T and incubated with horseradish peroxidase (HRP)-conjugated chicken anti-rabbit IgG (1:20,000; Abcam, Cambridge, UK) for 1 h at room temperature. All primary and secondary antibodies were diluted in 5% skim milk in TBS-T. Bound horseradish peroxidase (HRP)-conjugate was detected using a Western Lightning Plus ECL kit

(PerkinElmer, Waltham, MA, USA) and chemiluminescent signals were detected with a ChemiDoc XRS gel imaging system (Bio-Rad). Equal protein loading was confirmed by stripping and re-probing the blots with a mouse anti-GAPDH antibody (1:20,000; Novus Biologicals, Littleton, CO, USA) and HRP-conjugated goat anti-mouse IgG (1:20,000; Abcam).

### 3.2.6 Phytase Enzyme Extraction

The contents of duodenum and ileum were collected and centrifuged at 13,000 ×*g* for 10 min at 4°C. The supernatants were immediately used to determine enzyme activity. For the solid sample, a piece of frozen porcine pancreatic tissue was pulverized under liquid nitrogen, weighted, and homogenized in phytase extraction buffer (0.25 M sodium acetate buffer containing 0.05% Triton X-100 and 0.05% bovine serum albumin) under shaking for 30 min at 4°C. After extraction, the sample was centrifuged at 13,000 ×*g* for 10 min at 4°C and the supernatant was immediately used to determine enzyme activity.

### 3.2.7 Assessment of Phytase Enzyme Activity

Phytase activity in the pancreas and the duodenal and ileal contents was assayed at 37°C and pH 5.5 by the ammonium molybdate method for absolute analysis (Engelen et al., 1994). Briefly, three 20-μL aliquots of each sample were mixed with 180 μL of 0.25 M sodium acetate buffers (pH 5.5) supplemented with 400 μL sodium phytate substrate [7.5 mM sodium phytate (catalogue number P8810; Sigma-Aldrich, St. Louis, MO, USA) in 0.25 M sodium acetate buffer] and incubated at 37°C for 60 min. The reaction was terminated via adding 400 μL color-developing reagent [200 μL 21.67% nitric acid, 100 μL ammonium hepta-molybdate solution (10% ammonium molybdate in 0.25% ammonia solution), 100 μL ammonium metavanadate solution (0.235% ammonium vanadate in 0.43% nitric acid solution)] for 10 min at room temperature. All reactions were centrifuged at 13,000 ×*g* for 10 min at 4°C and 200 μL of the supernatant was transferred to a 96-well microplate, then absorbance was measured at 415 nm with a SpectraMax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Quantitation was performed with phytase extraction buffer as a blank and series-diluted potassium dihydrogen phosphate standards. One unit of phytase activity (U) was defined as 1 μmol of inorganic phosphate produced per min per mL (or gram) from the 5.0 μmol/mL sodium phytate solution under the assay condition. The calculation was

performed based on the following formula:

$$U = \frac{C}{m \times 60} \times F$$

U—Activity of phytase in sample (U/g or U/mL );

C—Enzymatic activity calculated by linear regression equation according to actual absorbency of sample solution;

F—Total dilution multiples of sample solution before reaction;

m—Mass of sample (in g or mL);

60—Reaction period (in min)

### 3.2.8 Assessment of Cellulase Enzyme Activity

Cellulase activity in the pancreas, salivary gland, stomach, liver, and heart was analyzed with a MarkerGene fluorescence cellulase assay kit (Marker Gene Technologies, Eugene, OR, USA). Three samples from each tissue were collected, snap-frozen in liquid nitrogen, and pulverized into a fine powder using a chilled mortar and pestle. One milligram of powdered tissue was suspended in 200 μL reaction buffer and centrifuged at 13,000 × *g* for 10 min at 4°C. Then 50 μL of the supernatant was added to a clear, flat-bottomed 96-well plate. A blank sample containing only 50 μL reaction buffer was also prepared. A 0.5 mM substrate reagent solution (from the kit)

was prepared and added to each well to a final concentration of 0.25 mM. The plate was immediately placed in a SpectraMax microplate spectrophotometer (Molecular Devices); fluorescence was recorded at EX/EM = 570/590 at 3-min intervals for 90 min and recorded.

### 3.2.9 Statistical Analyses

All statistical analyses were conducted using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Values are presented as the mean ± SD.

## 3.3 Results

### 3.3.1 Germ-line Transmission in Transgenic pigs

The constructs encoding bacterial phytase or fungal cellulase used in generating transgenic pigs are shown in Figure 8A. The male founder line carrying AMY-PHY and AMY-CEL transgenes was successively backcrossed with wild-type females to produce F1 offsprings. Mendelian inheritance was confirmed, as 50% (32/64) of the F1 generation pigs inherited the exogenous transgenes of AMY-PHY and AMY-CEL

(Table 5). Figure 8B shows the representative results of the PCR screen. The PCR-positive transgenic pigs also showed positive results in the Southern blot analysis (Figure 8C).

### 3.3.2 Transgene Expression in the Pancreas

Reverse transcription-PCR showed PHY and CEL transcripts were exclusively expressed in the pancreatic tissue of a representative transgenic pig (Figure 9). The RT-PCR-positive F1 individual and its F2 generation were assessed for transgenic protein expression in the pancreatic tissue by western blotting. The AMY-PHY transgene appeared as a single band at around 43−55 kDa in the pancreatic tissue but was not detected in samples from a nontransgenic individual (Figure 10A). The AMY-CEL transgene also produced a single band at approximately 72−95 kDa in a transgenic individual of the same line (Figure 10B). All detected bands were larger than the predicted size of recombinant PHY or CEL proteins in *Escherichia coli*. These results indicated that the cloned porcine pancreatic amylase promoter can effectively drive transgene expression *in vivo*.

### 3.3.3 Phytase Activity Analyses for Transgenic Pigs

Phytase activity values are presented in Table 6. The activity of the expressed phytase in the pancreas tissue from the transgenic pig was 0.437 U/g, whereas no activity was detectable in nontransgenic individuals. In the duodenal content of transgenic pig, activity was 5−8 times higher than that of nontransgenic pigs. The ileal content of non-transgenic and transgenic pigs exhibited phytase activity, although that activity was higher in the transgenic pigs. Duodenal contents were also assessed by western blotting. Phytase expressed in the duodenal contents of transgenic pigs appeared as two bands at 34−55 kDa and a faint band near 55 kDa that was also seen in nontransgenic pigs (Figure 11).

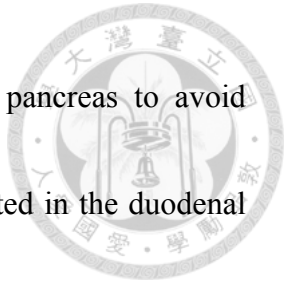
### 3.3.4 Cellulase Activity Analyses for Transgenic Pigs

The time course of expression of cellulase in the transgenic pig is presented in Figure 12. Figure 13 shows cellulase in the duodenal contents of a transgenic individual as two distinct bands in the range of 43−55 and 72−95 kDa.

## 3.4 Discussion

The porcine amylase signal peptide sequence was included in transgenes, AMY-PHY and AMY-CEL, which was used to guide the exportation of phytase and cellulase export. This strategy was similar to one used in transgenic mice expressing exogenous glucanase from the mouse amylase gene promoter and its homologous signal sequence (Zhang et al., 1999). Fusion of a recombinant protein with an N-terminal signal peptide is generally used to achieve translocation of the nascent preprotein to the endoplasmic reticulum (Tsuchiya et al., 2003). Increasing evidence indicates that presence of a eukaryotic signal peptide efficiently directs secretion of prokaryotic digestive enzymes in mammalian cells (Soole et al., 1993; Yin et al., 2006). Expression of phytase and cellulase enzymes in the pancreas of our transgenic pigs also supports this hypothesis. Moreover, secreted pancreatic phytase and cellulase were observed in the duodenal contents, indicating these exogenous enzymes were expressed, secreted, and then transported from the pancreas into the duodenum.

Transgenic pig lines were established through germ-line transmission of tissue-specific phytase and cellulase transgenes. Under natural physiological conditions,



it is essential that only inactive enzymes are synthesized in the pancreas to avoid auto-degradation (Lüthen et al., 1995); these proenzymes are activated in the duodenal lumen by proteolytic cleavage (Rothman et al., 2002). In this study, the proteins expressed in the pancreas were larger than the same full-length cellulase and phytase proteins expressed in *E. coli*, suggesting the proteins may have undergone post-translational modifications required for efficient secretion and stability of exogenously expressed enzymes in mammalian cells (Golovan et al., 2001; Assenberg et al., 2013). In addition, both enzymes exhibited much greater activity in the duodenal contents; their molecular weights were similar to the expected size of the recombinant protein in *E. coli*. Although we did not verify that the exogenous enzymes underwent enzymatic cleavage during their transit into the duodenum, our observations are consistent with the production of most pancreatic enzymes, which are synthesized as inactive precursors and become activated upon cleavage of one or a few specific peptide bonds (Niederau et al., 1986; Whitcomb and Lowe, 2007).

The catalytic properties of an exogenous enzyme are affected by its environmental conditions, such as temperature and pH. The optimum pH for plant phytase ranges from 5.0 to 6.0, whereas that for *Aspergillus niger*, *Peniophora lycii* and

*E. coli* derived phytase ranges from 2.0 to 5.5 (Woyengo and Nyachoti, 2011). In the present study, bacterial phytase (from *E. coli* K12 ATCC33965; Igbasan et al., 2000) and fungal cellulolytic enzymes (US Patent 6,428,996 B1) function best in an acidic environment, and phytase activity is predominantly higher and more stable in the stomach (Lim et al., 2000; Liu et al., 2001; Forsberg et al., 2003). Unfortunately, pepsin acts as a potent proteolytic enzyme that induces greater exogenous enzyme degradation in the lower stomach pH environment (Pagano et al., 2007). The intraduodenal pH in fed pigs is maintained between pH 4 and 6 with normal secretion of pancreatic juice and bile (Abello et al., 1987). Thus, the duodenal cavity would be an ideal location to promote phytase and cellulase activity following heterologous exogenous enzyme expression.

## 3.5 Summary

The purpose of this study was made to explore the feasibility of expressing two transgenes, AMY-PHY and AMY-CEL, in the pancreas of pigs. Tissue-specific mRNA expression of both phytase and cellulase were observed in those transgenic pigs. Enzyme activities were also observed in the duodenal contents of transgenic pigs.

Evidences of Western blotting showed the molecular weight of the secreted enzymes in the duodenal lumen was similar to the expected size of the recombinant protein in *E. coli*. Therefore, it is confirmed that the putative amylase promoter appeared to be sufficient to drive pancreas-specific expression of the exogenous bacterial phytase and fungal cellulase genes in those of transgenic pigs.

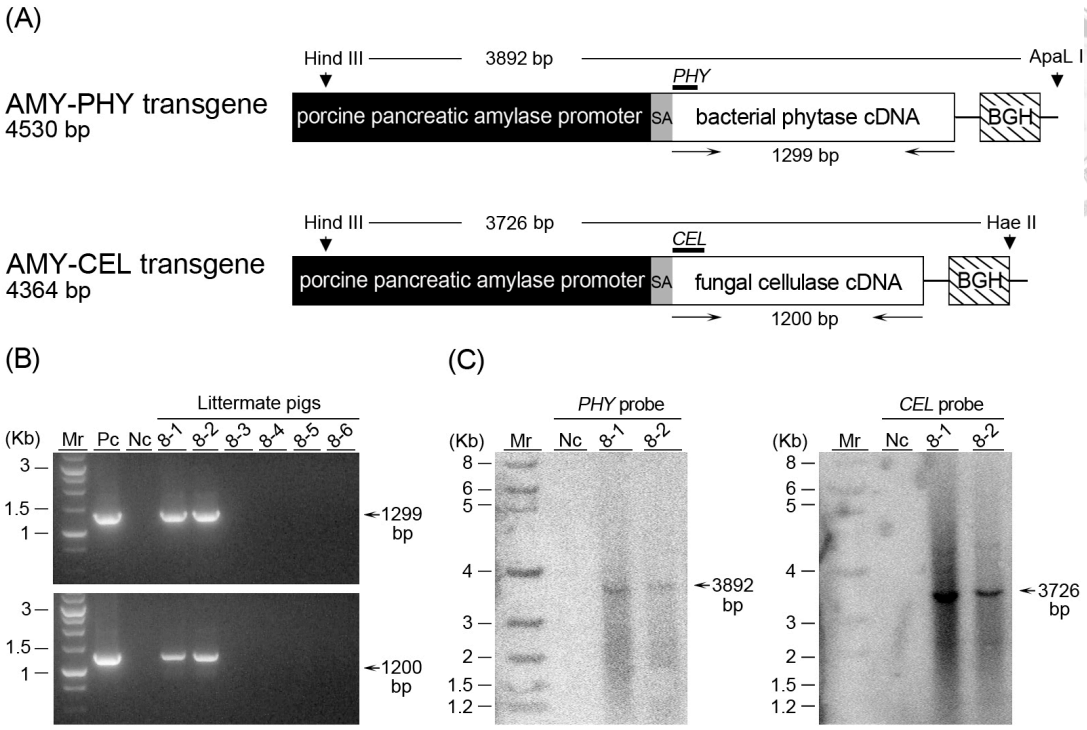



**Figure 8. Identification of transgenic pigs harboring phytase and cellulase transgenes.** (A) Schematic diagrams of the AMY-PHY and AMY-CEL transgenic cassettes (not to scale). Each construct consists of the 2,488-bp porcine pancreatic amylase promoter (black box), porcine pancreatic amylase signal peptide (SP, gray box), structural gene (white box), and bovine growth hormone polyadenylation site (BGH polyA; hatched box). Horizontal arrows represent primers used to distinguish the transgenic pigs from their wild-type littermates. Vertical arrowheads show the position of the restriction enzyme sites. The short horizontal bar above each drawing indicates the probe used in the Southern blot analysis; (B) A representative result of PCR screening of the transgenic pigs. The number of littermate F1 generation pigs is indicated at the top of the figure, from Lane 4 to Lane 9. The results from two positive pigs (Tg. 8-1 and Tg. 8-2) carrying dual transgenes of phytase (upper panel) and cellulase (lower panel) are shown. Mr, DNA marker; Pc, founder transgenic pig; Nc, wild-type pig; (C) Verification of the transgenic pigs by Southern blot analysis. Genomic DNAs confirmed by PCR were extracted from the F1 transgenic pigs. Twenty micrograms of each genomic DNA were digested with two restriction enzymes (*Hind*III-*Hae*I or *Hind*III-*Apa*LI, respectively) to detect the integration of the transgenic cassettes of AMY-PHY (left panel) and AMY-CEL (right panel) by using the probe specific to the structural gene. Mr, DNA marker; Nc, wild-type pig.

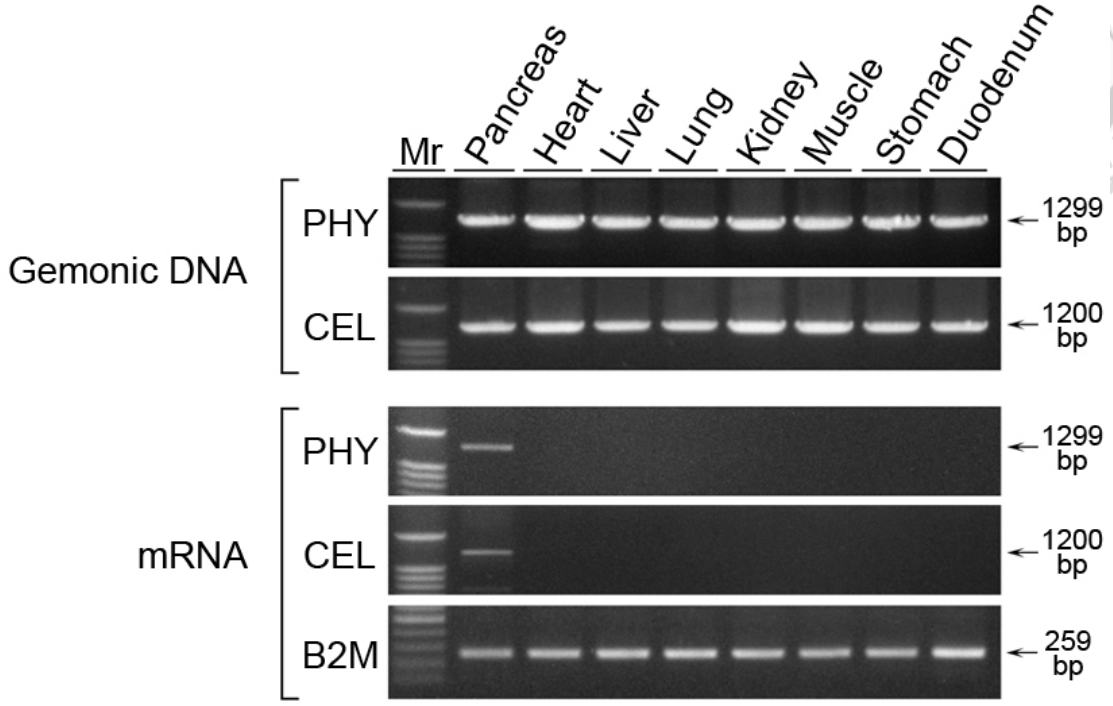



**Figure 9. RT-PCR analysis of phytase and cellulase expression in the F1 generation transgenic pig.** Fifty nanograms of genomic DNA and total RNA were isolated from tissue samples (pancreas, heart, liver, lung, kidney, muscle, stomach, and duodenum tissues). In the upper panel, genomic DNA derived from eight tissue types served as controls for RT-PCR-mediated detection of the phytase (PHY) and cellulase (CEL) transgenes. The 1,299- and 1,200-bp bands represent expression of the full-length phytase and cellulase transgenes, respectively (positive control). In the lower panel, tissue expression of transgene mRNA were specific to the pancreatic tissue of the transgenic pig, and was absent from the other tissues. Beta-2 microglobulin (B2M) was used as an internal reference. Mr, DNA marker.

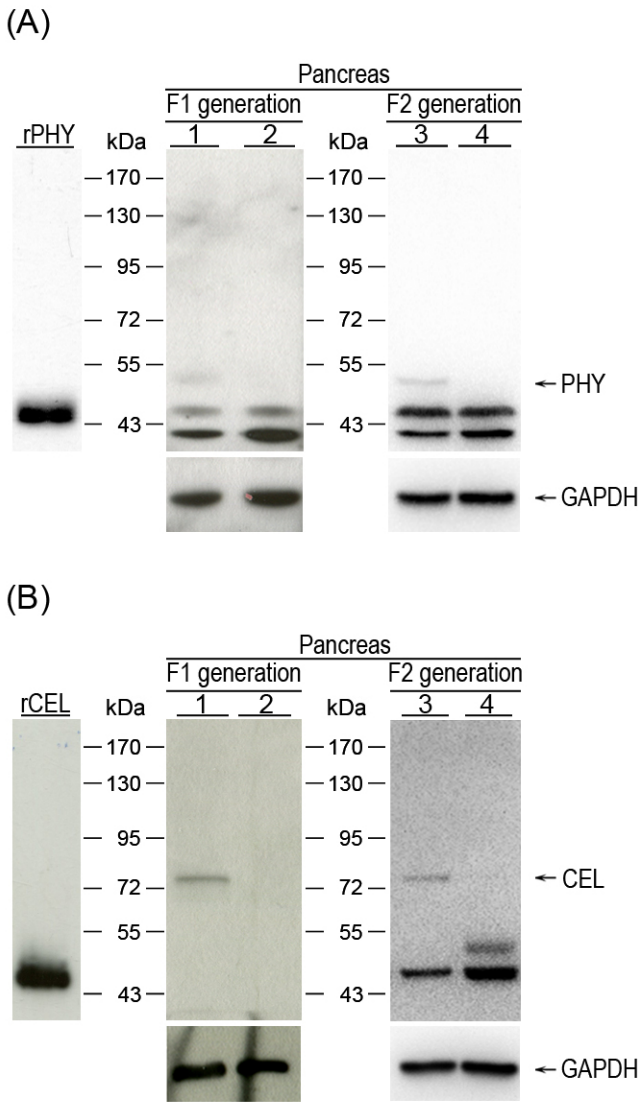



**Figure 10. Western blot analysis of phytase and cellulase expression in the pancreas of F1 and F2 generation transgenic pigs.** (A) Anti-PHY antibody was used to probe for phytase protein extracted from the pancreatic tissues of transgenic (middle panel, lane 1 and right panel, lane 3) and nontransgenic pigs (middle panel, lane 2 and right panel, lane 4) as well as recombinant phytase (rPHY) purified form *E. coli* (left panel). The arrow indicates the phytase-specific band. (B) Anti-CEL antibody was used to probe expression of cellulase protein extracted from the pancreatic tissues of transgenic (middle panel, lane 1 and right panel, lane 3) and nontransgenic pigs (middle panel, lane 2 and right panel, lane 4) as well as recombinant cellulase (rCEL) purified from *E. coli* (left panel). The arrow indicates the cellulose-specific band. GAPDH was used as a loading control.

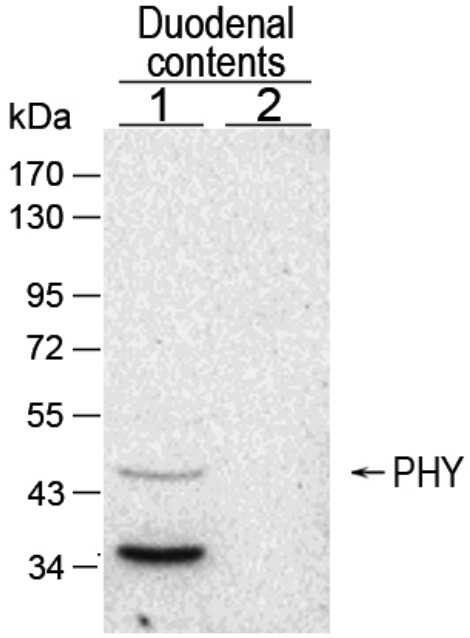



**Figure 11. Western blot analysis of phytase expression in the duodenal contents of an F2 generation transgenic pig.** Anti-PHY antibody was used to probe expression in the supernatant from the duodenal lumen of transgenic (lane 1) and nontransgenic pigs (lane 2). The arrow indicates the phytase-specific band.

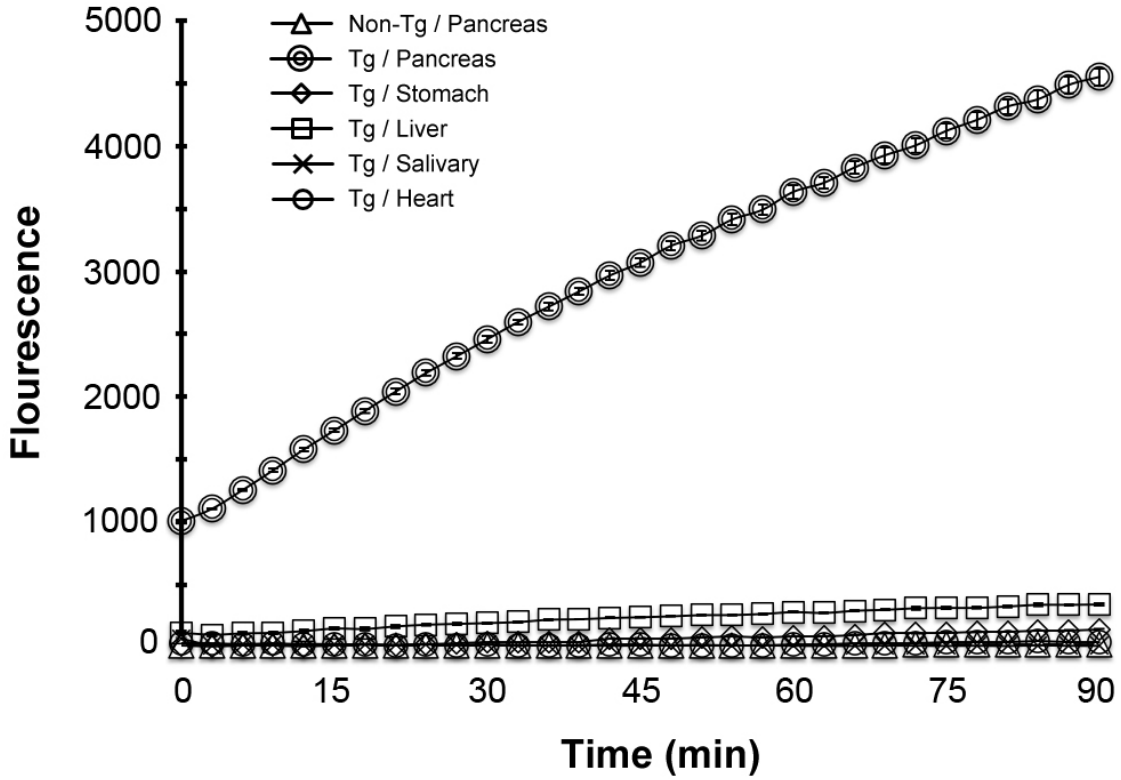



**Figure 12. Time course of expression of cellualse enzyme in porcine tissues.** Continuous fluorescent analyses of soluble lysates from transgenic porcine pancreas (◎), stomach (♢), liver (□), salivary gland (×), and heart (○), as well as the nontransgenic (Non-Tg) pancreas (△) were used for detection of the substrate resorufin-β-D-cellobioside. The fluorescence of a blank sample (50μL of substrate reagent added to 50 μL of reaction buffer) was set as the background and analyzed over the same time period. Fluorescence emission was measured at 590 nm with excitation at 570 nm for 90 min at 3-min intervals. Each tissue was sampled in triplicate. Data points represent the mean ± SD. Background fluorescence has been subtracted.

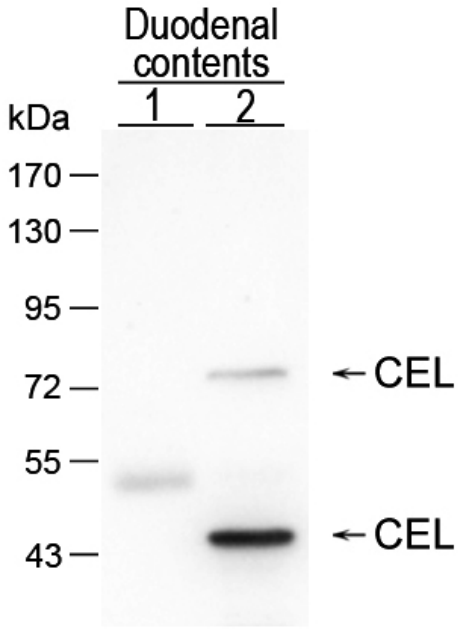



**Figure 13. Western blot analysis of cellulase expression in the duodenal contents of an F2 generation transgenic pig.** Anti-CEL antibody was used to probe expression in the supernatant of samples from the duodenal lumen of nontransgenic (lane 1) and transgenic (lane 2) pigs. Arrows indicate the cellulose-specific bands.

## Table 5

**Rate of germline transmission of F0 generation transgenic pigs expressing AMY-PHY and AMY-CEL**

| F0 Founder | | No. of F1 offspring | No. of positive F1 offspring | Germ-line transmission rate (%) |
|---|---|---|---|---|
| ID | Sex | | | |
| 7 | Female | 11 | 3 | 3/11 (27.3) |
| 8 | Male | 53 | 29 | 29/53 (54.7) |
| Total | | 64 | 32 | 32/64 (50.0) |

## Table 6

**Phytase activity in the pancreas and gastrointestinal tract lumen of F2 generation transgenic pigs**

| Sample source | Phytase Acticity [1,2] | | |
|---|---|---|---|
| | Transgenic pig | Nontransgenic pig | |
| | | #1 | #2 |
| Pancreas (U/g) | 0.437±0.004 | ND | ND |
| Duodenal content (U/mL) | 8.233±0.263 | 1.432±0.109 | ND |
| Ileal content (U/mL) | 3.581±0.055 | 1.642±0.036 | 1.424±0.094 |

[1]Activity is expressed in units (U). One phytase activity unit is defined as the amount of enzyme per milliliter of gastrointestinal tract content or gram of pancreas tissue that liberates 1 μmol inorganic phosphorus from 5.0 mmol/L sodium phytase solution per minute at 37°C and pH 5.5.
[2]Data represent mean ± SD. ND, not detected.

# Chapter 4 Potential of the Transgenic Pig for Improvement of their Digestive Efficiency

## 4.1 Objective

Animal manure has been identified as a large contributor to water pollution due to its overabundance (Jongbloed and Lenis, 1998). Improving digestive efficiency may be a logical approach to reduce nutrient excretion and achieve pollution control. With a global increase in the cost of traditional feeds, alternative ingredients are required for partial substitution of the conventional ingredients in porcine diets (Esteban et al., 2007; Weber et al., 2008). Moreover, most of these alternatives comprise fiber-rich carbohydrates and have distinct nutritional profiles compared to traditional ingredients. Application of commercially available phytase, fibrolytic enzymes, or a multi-enzyme complex in porcine diets is a popular method for improving the environmental impact and digestibility of cereal-based ingredients (Yi et al., 1996; Diebold et al., 2005; Nortey et al., 2007); however, the use of such supplements is limited by the feed-processing temperature and storage stability. Preliminary studies in pigs carrying

AMY-CEL and AMY-PHY transgenes showed enzyme activity in the duodenal contents. In this study, further attempt was made to evaluate the digestive efficiency in those of transgenic pigs.

## 4.2 Materials and Methods

All surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee, National Taiwan University, Taiwan.

### 4.2.1 Phosphorus-digestibility Trials

Eighteen-month-old F1 transgenic female pigs were randomly distributed into two groups ($n$ = 3 each). Female individuals without transgenic inserts were used as the controls and randomly distributed in matched pairs. All pigs were fed a typical commercial grower diet prior to the feeding trials. Two corn, barley, and soybean-meal-based diets with the same metabolizable energy content were formulated to contain relatively high and/or low concentrations of total P (Table 7). All diets contained 0.5% chromic oxide as an inert marker for nutrient digestibility determination.

Each group of pigs received one of the following diets: either the control diet comprised the basal diet supplemented with monocalcium phosphate to meet the National Research Council nutrient requirements (NRC, 1998), or the low available P (low-P) diet comprising the basal diet formulated without additional P supplementation. Fresh feed (1.5 kg) was provided twice daily at regular intervals between 0800 h and 1700 h, and water was provided *ad libitum* throughout the 4 weeks of experimental period. At the beginning of the 4th week, total feces were collected individually from each pig for 7 consecutive days. The fecal materials were immediately weighed, frozen at −20°C, and stored. On the last day of the experiment, 3 mL blood was drawn from each pig at 1900 h and collected in tripotassium EDTA-containing tubes (BD Bioscience, Franklin Lakes, NJ, USA). Each blood sample was centrifuged at 1,100 ×*g* for 10 min, and serum was collected and stored at −80°C.

### 4.2.2 Chemical Analyses of Diets, Feces, and Blood Serum

Diets and feces were analyzed for proximate constituents according to the methods of AOAC International (AOAC, 1984). The samples were dried in a forced-air drying oven at 60°C for 72 h and ground to a fine powder before compositing. The total

dry matter (DM) content was determined by drying 2 g of a representative sample at 105°C until constant weight was achieved. The ash content was produced by incinerating the 2 g sample at 600°C until light-grey ash was obtained. An N-to-protein conversion factor of 6.25 was used to calculate the crude protein (CP) content from the total N content estimated by employing the Kjeldahl method (Kjeldahl, 1883). Neutral detergent fiber (NDF) was assayed according to Van Soest (Van Soest et al., 1991) with sodium sulfite and α-amylase, and expressed with residual ash. Oxalic acid was used to precipitate Ca, which was subsequently quantified by titration with a 0.1 N potassium permanganate solution. The P content was detected colorimetrically as a yellow phospho-vanado-molybdate complex by spectrophotometry at 400 nm (Metertek, Taipei, Taiwan). The concentration of P in the blood serum was measured with an automated Vitros 350 clinical chemical analyzer (Johnson & Johnson, Rochester, NY, USA).

### 4.2.3 Statistical Analyses

All statistical analyses were conducted with SAS software, version 9.1.3 (SAS Institute). Comparisons between genotypes were conducted with unpaired *t*-tests. Values are presented as the mean ± SEM, and $P < 0.05$ denotes a statistically significant

difference.

## 4.3 Results

### 4.3.1 Chemical Analyses of Feces in Transgenic Pigs Expressing the AMY-CEL Transgene

Apparent nutrient digestibility was calculated for control diet-fed nontransgenic and transgenic pigs (Figure 14). Digestibility of DM, NDF, CP, Ca, and P was higher in the transgenic pigs than in the nontransgenic pigs; a statistically significant difference ($P < 0.05$) was found in the case of DM and NDF digestibility. On the DM basis, the fecal P and N outputs (g/day) are shown in Figure 15. The percentage of fecal P output from the transgenic pigs decreased by 29% relative to nontransgenic pigs; fecal N output was reduced by 31% in the transgenic pigs.

### 4.3.2 Chemical Analyses of Feces in Transgenic Pigs Expressing the AMY-PHY Transgene

Apparent nutrient digestibility was calculated for nontransgenic and transgenic pigs fed the low-P diet (Figure 16). The transgenic pigs on the low-P diet showed

significantly increased digestibility ($P < 0.05$) of DM, NDF, CP, Ca, and P. Mean serum P concentrations were higher in transgenic pigs than in the nontransgenic pigs (6.6 ± 0.3 mg/dL and 5.9 ± 0.3 mg/dL, respectively). Fecal P and N outputs (g/day) from the nontransgenic and transgenic pigs are shown in Figure 17. The percentage of fecal P output from transgenic pigs appeared to be significantly decreased by 48% relative to those of the nontransgenic pigs; fecal N output was also found to be reduced by 49% in those of the transgenic pigs.

## 4.4 Discussion

Genetically modified pigs are already being used to develop new or altered physiological traits to reduce agricultural pollution (Golovan et al., 2001). Efforts also include enhancing productivity by increasing piglet survival (Wheeler et al., 2001; Tong et al., 2011); altering the composition of the carcass to make it healthier for human consumption (Pursel et al., 2004; Lai et al., 2006); studying molecular mechanisms related to growth, disease, and development (Pursel et al., 2004; Miles et al., 2013); and applications in biomedical fields such as bioreactors (Paleyanda et al., 1997) and organ transplantation (Zeyland et al., 2013). In this study, attempts were made to establish the

double transgenic pig model equipped with potentiality co-expression of bacterial phytase and fungal cellulase transgenes to reduce P pollution from feces and to improve their nutrient digestibility as well.

### 4.4.1 Phytase Enzyme Activity in Transgenic Pigs

Approximately two-thirds of the P content in cereals and oilseeds is in the form of phytate-P, which has been confirmed to be poorly digestible due to a lack of sufficient phytase-producing microorganisms in the porcine gastrointestinal tract that can release P from the inositol ring of the phytate molecule (Viveros et al., 2000). Phytate is a potent chelating agent. The negatively charged phytate binds to various proteins, cationic minerals, and digestive enzymes to form stable complexes, resulting in nutrient unavailability for gastrointestinal absorption and utilization (Kies et al., 2005). Excess fecal excretion of P and N can aggravate freshwater eutrophication and soil erosion (Carpenter, 2008). Supplementation of microbial phytase in porcine diets is a useful practice to increase phytate-P bioavailability and to decrease P excretion in feces without incurring negative effects on growth performance (Harper et al., 1997; Sands and Kay, 2007). Utilization of phytase-transgenic grains may also be able to

improve nutritional efficiency and reduce feed costs, though dietary phytase activity is reduced when feeds are manufactured *via* pelleting or extrusion processes (Spencer et al., 2000). They are, however, not as effective as microbial phytase. It is reported that phytic acid hydrolysis in the stomach of minipigs fed a diet supplemented with the phytate derived from *A. niger* is higher than in those fed a diet with supplemented wheat phytase; moreover, a higher recovery of microbial phytase than plant phytase (70% vs. 45%) is observed in the duodenum of the same animals (Rapp et al., 2001). For economic reasons, however, inorganic P supplementation is still chosen by most pig producers.

Complete removal of inorganic P supplementation from the basal diet in the present study led to a significant increase in P digestibility associated with a decrease in fecal P output in the transgenic pigs because phytase has been retained much more efficiently in those diets with low and/or no inorganic P, which have been confirmed to inhibit the phytase synthesis (Konietzny and Greiner, 2004; Selle and Ravindran, 2008). Consequently, the amount of fecal P can be substantially reduced by avoiding excess P supplementation. Similar results have also been achieved by using the strategy of exogenous phytase instead of the reducement of inorganic P in those of porcine diets

(Kornegay and Qian, 1996; Jendza et al., 2005; Augspurger et al., 2009). However, when comparisons were made to those of exogenous phytase supplementation, the use of transgenic pigs equipped with potentiality of expression the bacterial phytase could be a cost-effective solution for improvement of P digestibility and subsequently result in limitation the P pollution (Golovan et al., 2001).

### 4.4.2 Cellulase Enzyme Activity in Transgenic Pigs

Plant cell walls constitute most of the dietary fiber in fruits, forages, and whole-grain cereals (Harris and Smith, 2006). Diets containing more than 7–10% fiber generally result in an inhibitory effect on growth in those of growing pigs (Kass et al., 1980), presumably due to fiber interactions with the digestion of energy-containing nutrients (Dégen et al., 2009).

Cellulose and hemicellulose are the principal constituents of the plant cell wall, where the complex structure acts as a barrier against degradation by pure cellulases or hemicellulases (Murashima et al., 2003). The recombinant enzymes secreted in those transgenic pigs generated in the present study had plant cell wall-degrading capabilities

and may hence enhance the hydrolysis of indigestible cell wall fiber, improving digestibility. Several groups of investigators have reported nutrient digestibility in older pigs fed a diet supplemented with exogenous NSP enzymes (Thacker et al., 1988; Baas and Thacker, 1996; Lindberg et al., 2007; Kim et al., 2008). Supplementation with carbohydrases can improve nutrient availability and reduce digesta viscosity, suggesting they may also increase phytase efficacy by increasing the accessibility of phytase to phytic acid and absorption of nutrients released by phytase in poultry (Woyengo and Nyachoti, 2011). However, the preliminary evidence in the present study suggested that the transgenic pigs carrying AMY-PHY and AMY-CEL transgenes on a normal or low-P diet have improved DM and NDF digestibility, although there was no effect of dietary P. Possible reasons for these discrepancies could be differences in dietary composition, enzyme source, enzyme activity, and method of application (Li et al., 1996; Omogbenigun et al., 2004). The response to supplementation with phytase and carbohydrases is variable and depending on the type of carbohydrases supplement, NSP composition, Ca and non phytate-P contents, and endogenous phytase activity (Woyengo and Nyachoti, 2011). On the other hand, transgenic pigs generated from this present study appeared to be continuously to secrete the recombinant fungal cellulase,

which further to alter the population and the activity of microbes that may decompose the fiber portion of those feed within the gastrointestinal tract. The recombinant fungal cellulase may able to decompose the cellulose found in the transgenic pigs and perform their functions well within the porcine gastrointestinal tract.

### 4.4.3 Digestibility of Nutrients in Transgenic Pigs

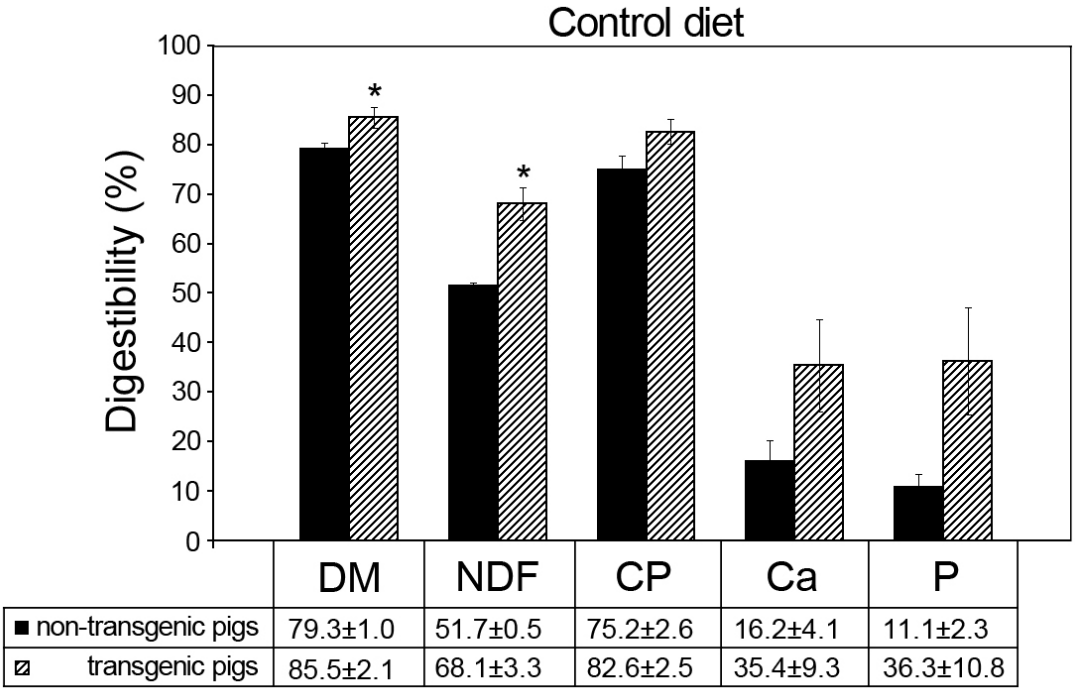
In the present studies, increased Ca digestibility was found in those of transgenic pigs fed with the low-P diet, consistent with those of prior studies (Traylor et al., 2001; Guggenbuhl et al., 2007). Previous studies conducted to evaluate the Ca digestibility have confirmed to be significantly improved by using the phytase-driven strategies to increase the phytate hydrolysis and resulting the decomposition of the phytate-Ca complex (Spencer et al., 2000). In addition, cellulase supplementation has also been confirmed to show significantly improves the digestibility of those cell wall components, suggesting Ca associated with the plant cell wall can be solubilized (Nahm and Carlson, 1985). Therefore, the significant increase in Ca digestibility found in this present study may also have been due to the action of cellulase generated in those of

transgenic pigs generated.

On the other hand, it is possible that phytate and fiber may reduce protein digestibility through the formation of insoluble phytate-protein and fiber-protein complexes (Adeola and Sands, 2003). The efficacy of phytase in improving the N or CP digestibility in pigs, however, remains a matter of debate (Bruce and Sundstol, 1995; Traylor et al., 2001). A model of combined phytase and xylanase produces a potential additive effect in pigs fed by the wheat-based diet (Kim et al., 2008; Lyberg et al., 2008). In this case, the transgenic pigs fed low-P diets tended to have much better in CP digestibility, though the mechanism remains unclear. A possible cause for the additive effect could be that the fibrolytic enzyme may play important role in disrupt the cell wall matrix, permitting endogenous enzymes to hydrolyze substrates, while simultaneous phytase action enables cleavage of the phytase-bound complexes, thereby resulting in greater nutrient digestibility (Oryschak et al., 2002; Nortey et al., 2007). Although these findings and our results suggest the positive combinatorial effect of the NSP enzyme and phytase, other reports have been inconsistent (Kim et al., 2005a; Woyengo et al., 2008), perhaps due to differences in dietary substrate, gastrointestinal viscosity, and enzyme activity in the luminal phase (Kim et al., 2005b).

## 4.5 Summary

In order to evaluate the performance of digestive efficiency in transgenic pigs harboring with both of phytase and cellulase transgenes, four treatment groups were designed with two lines of pigs fed with and without monocalcium phosphate supplementation. On a corn, barley, and soybean-meal-based control diet, the apparent fecal digestibility of DM, NDF, CP, Ca, and P was higher in those of transgenic pigs than those found in nontransgenic pigs ($P < 0.05$ for both DM and NDF digestibility). On a low-P basal diet, those transgenic pigs exhibited significantly increased their digestibility of these nutrients ($P < 0.05$). However, in those of transgenic pigs fed control and low-P diets, the fecal P output appeared to be much decreased by 29% and 48%, respectively, and the fecal N output was also reduced by 31% and 49%, respectively, when comparisons were made with the fecal N outputs from those of the nontransgenic individuals.




| | DM | NDF | CP | Ca | P |
|---|---|---|---|---|---|
| ■ non-transgenic pigs | 79.3±1.0 | 51.7±0.5 | 75.2±2.6 | 16.2±4.1 | 11.1±2.3 |
| ▨ transgenic pigs | 85.5±2.1 | 68.1±3.3 | 82.6±2.5 | 35.4±9.3 | 36.3±10.8 |

**Figure 14. Apparent nutrient digestibility in nontransgenic and transgenic pigs.** During the 4th week of the experiment, we calculated the apparent digestibility (%) of dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), calcium (Ca), and phosphorus (P) in the feces of pigs on a corn, barley, soybean-meal-based control diet. Values represent the mean ± SEM of three independent experiments, each with feces collected from a different pig, with the analyses performed in triplicate. *$P < 0.05$ and **$P < 0.01$ versus a nontransgenic control via unpaired t-test. Black bar, non-transgenic pigs; hatched bar, transgenic pigs.

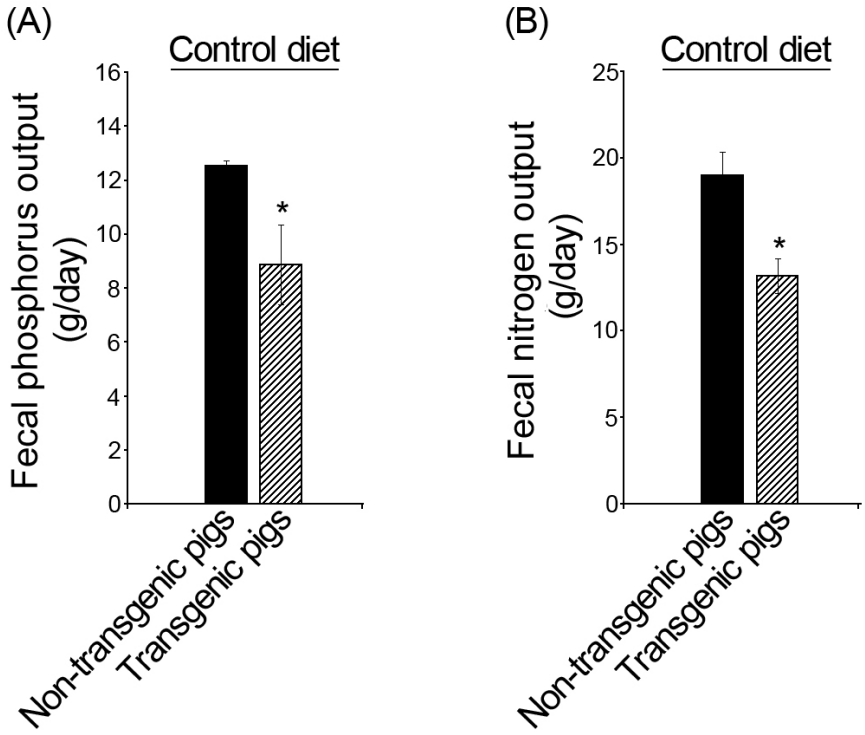
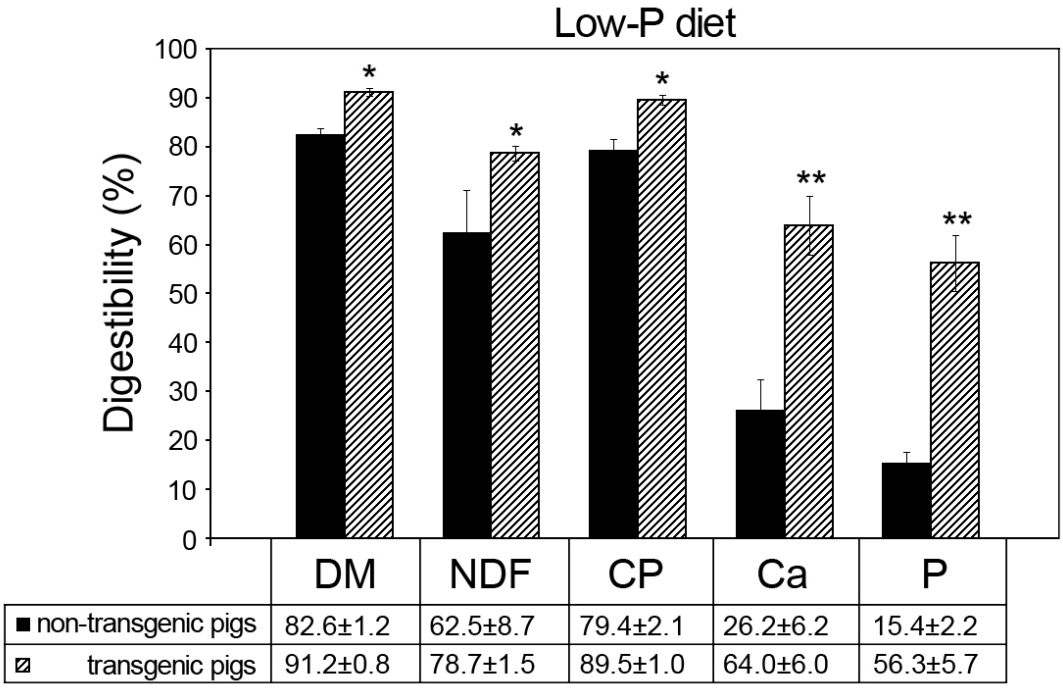



**Figure 15. Impact of genetic modification on porcine fecal phosphorus (P) and nitrogen (N) outputs on a control diet.** The fecal (A) P; and (B) N outputs (on dry matter basis) from transgenic pigs on a corn, barley, soybean-meal-based control diet were compared to nontransgenic individuals. Values represent the mean ± SEM of three independent experiments, each with feces collected from a different pig. In transgenic pigs on the control diet, fecal P output decreased by 29% and fecal N output was reduced by 31% relative to the outputs from nontransgenic pigs. Analyses were performed in triplicate. *$P < 0.05$ versus a nontransgenic control via unpaired t-test. Black bar, non-transgenic pigs; hatched bar, transgenic pigs.




| | DM | NDF | CP | Ca | P |
|---|---|---|---|---|---|
| ■ non-transgenic pigs | 82.6±1.2 | 62.5±8.7 | 79.4±2.1 | 26.2±6.2 | 15.4±2.2 |
| ▨ transgenic pigs | 91.2±0.8 | 78.7±1.5 | 89.5±1.0 | 64.0±6.0 | 56.3±5.7 |

**Figure 16. Apparent nutrient digestibility in nontransgenic and transgenic pigs fed low-P diet.** During the 4th week of the experimental period, we calculated the apparent digestibility (%) of dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), calcium (Ca), and phosphorus (P) in the feces of pigs fed a low-P basal diet. Values represent the mean ± SEM of three independent experiments, each with feces collected from a different pig, with analyses performed in triplicate. *$P < 0.05$ and **$P < 0.01$ versus a nontransgenic control *via* unpaired t-test. Black bar, non-transgenic pigs; hatched bar, transgenic pigs.

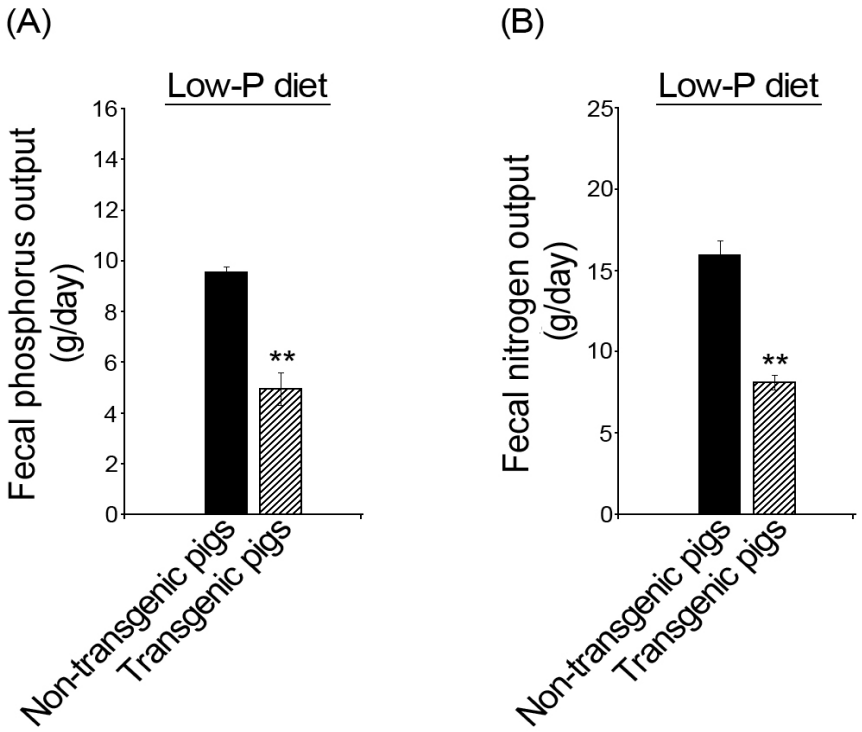



**Figure 17. Impact of genetic modification on porcine fecal phosphorus (P) and nitrogen (N) outputs on a low-P diet.** The fecal (A) P and (B) N outputs (on dry matter basis) from transgenic pigs fed a low-P basal diet were compared against nontransgenic individuals. Values represent the mean ± SEM of three independent experiments, each with feces collected from a different pig. In the transgenic pigs fed a low-P diet, the fecal P output decreased by 48%, and the fecal N output was reduced by 49%, relative to the outputs from nontransgenic pigs. Analyses were performed in triplicate. $^{**}P < 0.01$ versus a nontransgenic control *via* unpaired t-test. Black bar, non-transgenic pigs; hatched bar, transgenic pigs.

## Table 7

**Composition of the experimental diets**

| Item | Control diet | Low-P basal diet |
|---|---|---|
| Ingredient, % | | |
| Corn | 43.40 | 43.40 |
| Barley | 30.00 | 30.00 |
| Soybean meal | 15.00 | 15.00 |
| Cornstarch | 8.00 | 8.00 |
| Casin | 2.00 | 2.00 |
| Monocalcium phosphate | 0.45 | |
| Calcium carbonate | 0.55 | 0.55 |
| Sodium chloride | 0.20 | 0.20 |
| Zinc oxide | 0.004 | 0.004 |
| Vitamin premix[1] | 0.40 | 0.40 |
| Calculated analysis | | |
| Calcium, % | 0.46 | 0.38 |
| Total phosphorus, % | 0.44 | 0.35 |
| Available phosphorus, % | 0.14 | 0.09 |
| Crude protein, % | 15.03 | 15.03 |
| ME[2], kcal/kg | 3,230 | 3,230 |

[1]Supplied per kg of diet: vitamin A, 1,400 IU; vitamin D, 160 IU; vitamin E, 6 IU; vitamin K, 0.5 mg; vitamin $B_{12}$, 6 mg; riboflavin, 0.8 mg; and thiamin, 0.8 mg. [2]ME, metabolizable energy.

# General Conclusion and Future Works

Spatial and temporal control of gene expression allows transgenic livestock production to be more useful in agricultural applications. It is essential to use an adequate promoter for successful engineering of transgenic constructs. Here in the present study, attempts were made to investigate the feasibility of adopting a transgenic strategy in which co-expression of the bacterial phytase and/or fungal cellulase transgenes was designed to be drive by the porcine pancreatic amylase promoter in pigs. The putative promoter was sufficient to trigger pancreas-specific expression of these exogenous enzymes and produce activity in the duodenal lumen. Our preliminary evidence suggests the fecal P and N output decreased in comparison to the nontransgenic pigs and the apparent fecal digestibility of DM, NDF, CP, Ca, and P was higher in transgenic individuals. Our next goal is to design and manage porcine feeding programs to maximize nutrient digestion, absorption, and utilization, and to minimize P and N excretion. Feeding low-P and high fiber corn-soybean meal-based diets may improve the growth performance, bone strength, and carcass characteristics for this type of pig throughout the growing-finishing period. We must also explore the physiology of

the gastrointestinal tract, including the size and diversity of the resident microbial population and expression of immunity-related genes in the intestinal tissues of these transgenic pigs.

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# Appendices

A. Accepted Paper

Lin, Y. S., C. C. Yang, C. C. Hsu, J. T. Hsu, S. C. Wu, C. J. Lin, and W. T. K. Cheng. 2014. Establishment of a novel, eco-friendly transgenic pig model using porcine pancreatic amylase promoter-driven fungal cellulase transgenes. Transgenic Res. (in press)

B. Approved patents

1. Cheng, W. T. K., S. C. Wu, C. C. Hsu, Y. S. Lin, C. J. Lin. K. J. Cheng, and J. T. Hsu. 2011. Porcine pancreatic amylase gene promoter and transgenic pigs expressing heterologous digestive enzymes. US Patent: US 7,956,238 B2.

2. 鄭登貴、吳信志、徐啟真、林育聖、林之任、鄭國展、徐濟泰。2011。新穎之豬胰澱粉酶基因啟動子及表現異源性消化酵素之基因轉殖豬。中華民國專利。專利期間：2011 年 10 月 21 日～2026 年 5 月 16 日。專利證書編號：發明第 I 350851 號。

Transgenic Res
DOI 10.1007/s11248-014-9817-9

ORIGINAL PAPER

# Establishment of a novel, eco-friendly transgenic pig model using porcine pancreatic amylase promoter-driven fungal cellulase transgenes

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Received: 16 February 2014 / Accepted: 11 July 2014


**Abstract** Competition between humans and livestock for cereal and legume grains makes it challenging to provide economical feeds to livestock animals. Recent increases in corn and soybean prices have had a significant impact on the cost of feed for pig producers. The utilization of byproducts and alternative ingredients in pig diets has the potential to reduce feed costs. Moreover, unlike ruminants, pigs have limited ability to utilize diets with high fiber content because they lack endogenous enzymes capable of breaking down nonstarch polysaccharides into simple sugars. Here, we investigated the feasibility of a transgenic strategy in which expression of the fungal *cellulase* transgene was driven by the porcine pancreatic amylase promoter in pigs. A 2,488 bp 5′-flanking region of the porcine pancreatic amylase gene was cloned by the genomic walking technique, and its structural features were characterized. Using GFP as a reporter, we found that this region contained promoter activity and had the potential to control heterologous gene expression. Transgenic pigs were generated by pronuclear microinjection. Founders and offspring were identified by PCR and Southern blot analyses. Cellulase mRNA and protein showed tissue-specific expression in the pancreas of F1 generation pigs. Cellulolytic enzyme activity was also identified in the pancreas of transgenic pigs. These results demonstrated the establishment of a tissue-specific promoter of the porcine pancreatic amylase gene. Transgenic pigs expressing exogenous cellulase may represent a way to increase the intake of low-cost, fiber-rich feeds.

**Keywords** Transgenic pig · Pancreas · Amylase promoter · Cellulase

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## Introduction

Taiwan's demand for feed corn and soybeans meal is nearly dependent on imported supply. The fluctuations in the price from feedstuff including both raw materials have an importance to pig production (Saengwong et al. 2011). For example, the prices of grain and soybean are increased in 2008, and subsequently the pig production cost per 100 kg live weight of hog increased from NT$ 5,146 in 2007 to NT$ 6,566 (National Animal Industry Foundation 2012).

Published online: 26 July 2014

To overcome the global increase in the cost of traditional feeds and limited cereal production, local and inexpensive ingredients are required to substitute in part for expensive conventional ingredients in porcine diets (Esteban et al. 2007; Weber et al. 2008; Taiwan Grain and Feed Annual 2014). Most of these alternatives comprise a high proportion of fiber-rich by-products and have distinct nutritional profiles when compared to common traditional ingredients (Varel and Yen 1997). Consequently, improvement of fiber conversion for increased energy utilization may allow non-ruminants, such as pigs, to reduce grain consumption and digestive waste.

Exogenous microbial fibrolytic enzymes as feed additives have applications in the pig industry and have improved the environmental impact of the pig industry and digestion of cereal-based ingredients (Kim et al. 2003; Kiarie et al. 2007; Woyengo et al. 2008). However, the use of such supplements is limited by feed production costs, processing temperature, and storage stability (Walsh et al. 1993). Genetically engineered mice that express specific digestive enzyme genes have the potential to provide an alternative strategy for dietary enzyme supplementation (Hall et al. 1993; Guan et al. 2013; Huang et al. 2013). The use of a tissue-specific promoter in a heterologous expression cassette may minimize the negative effects of transgenes on the normal growth and development of transgenic individuals (Zheng and Baum 2008).

Additionally, promoter-controlled transgenes should be preferentially expressed in the intestines because some enzyme supplements are destroyed by stomach acid and resist degradation by proteases before they reach the small intestine. Pancreatic amylase is one of the most important digestive enzymes. This enzyme is present in pancreatic juice, which is secreted into the duodenum of the small intestine and catalyzes the hydrolysis of starch into simpler compounds (Alkazaz et al. 1996). The expression of the heterologous bacterial glucanase gene in transgenic mice under control of the amylase promoter has been demonstrated (Fontes et al. 1999). However, no studies have yet reported controlled expression of target genes by the pancreatic amylase promoter in pigs.

In the present study, we isolated and characterized a novel promoter of the porcine pancreatic amylase gene. We also generated pigs expressing the *cellulase* transgene driven by the porcine pancreatic amylase protein promoter specifically in digestive tract tissues. These pigs enabled us to evaluate a novel biological method for potentially improving nutrient digestibility in pigs, thereby reducing feed costs for pig producers.

## Materials and methods

All surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee, National Taiwan University, Taipei, Taiwan.

### Isolation of the 5′-flanking region of the porcine pancreatic amylase gene

Genomic DNA was extracted from porcine peripheral blood mononuclear cells using a genomic DNA isolation reagent (Genomarker, Taipei, Taiwan) in accordance with the manufacturer's protocol. Genome walking was performed using a GenomeWalker Universal kit (Clontech, Mountain View, CA, USA) following the manufacturer's instructions. Based on the published porcine pancreatic amylase cDNA sequence (Darnis et al. 1999), two non-overlapping antisense gene-specific primers (forward-1, 5′-TACCGCTCA CATTCAAGAGCAATGTCA; reverse-1, 5′-ATGAA CAATAGACGTTCGTCCAGACT) were designed to amplify the region upstream from the coding sequence with GenomeWalker adapter-specific primers (AP1 and AP2) in the primary and secondary PCRs, respectively. The annealing temperatures for primary and secondary PCRs were 60 and 70 °C, respectively. Gel-purified secondary PCR fragments were ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA) before sequencing. The resulting 5′-flanking region of the porcine pancreatic amylase gene was used to deduce the core promoter sequences with a Neural Network Promoter Prediction program (NNPP), version 2.2 (Reese 2001) and to predict transcriptional regulator-binding sites using the Transcription Element Search System (TESS) database (http://www.cbil.upenn.edu/cgi-bin/tess/tess).

### Cloning and transient expression of the GFP reporter gene construct

Nucleotide sequences of the putative promoter region and N-terminal signal peptide-encoding region of the porcine pancreatic amylase gene (Darnis et al. 1999) were amplified with specific primers (forward-2, 5′-

GCGGCCGCCTGACATAAGCTGAA; reverse-2, 5′-GATATCGGCCCAGCAGAACCCAA), and additional restriction enzyme sequences (underlined), *Not*I or *Eco*RV, were introduced for cloning purposes. Thermal cycling conditions were as follows: 94 °C for 5 min; 35 cycles at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 2 min; and a final extension at 72 °C for 7 min. The enzyme-digested and purified PCR product was subcloned into the *Not*I-*Eco*RV site of the phr*GFP* mammalian expression vector (Agilent Technologies, Cedar Creek, TX, USA) to generate the pAMY-hr*GFP* construct. DNA sequencing was performed to verify the correct promoter sequence. The cultured rat pancreatic acinar carcinoma cell line, AR-42J, was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC) and maintained in Ham's F12K medium (Invitrogen, Grand Island, NY, USA) containing 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, 20 % fetal bovine serum (FBS; Invitrogen), and 1 % antibiotic–antimycotic mixture (Invitrogen). For transfection, cells were seeded and cultured onto 35 mm dishes until the cell density reached 70–80 % confluence. Attached cells were subsequently rinsed with PBS twice, followed by the addition of serum-free Ham's F12K medium supplemented with 50 mM dexamethasone (Raffaniello et al. 2009). Two micrograms of the pAMY-hr*GFP* plasmid were successively transfected into the cells using a jetPEI cationic polymer transfection reagent (Polyplus-transfection, New York, NY, USA) following the manufacturer's instructions. Untransfected cells were used as a negative control. At 48 h post-transfection, the expression of green fluorescence in the cells was observed under an inverted fluorescence microscope (Axiovert 200; Carl Zeiss, Jena, Germany).

## Construction of the transgenic expression vectors

Forward-3 (5′-GCTAGCCTGACATAAGCTGAACCAA) and reverse-3 (5′-GGATCCGGCCCAGCAGAACCCAA) primers combined with *Nhe*I and *Bam*HI restriction enzyme sites (underlined), respectively, were designed according to the sequence of the pAMY-hr*GFP* plasmid for promoter amplification. The amplified product and mammalian expression vector pCR3.1 (Invitrogen) were double digested with *Nhe*I and *Bam*HI. Upon removal of the constitutive cytomegalovirus promoter of pCR3.1, the porcine pancreatic amylase promoter and its signal peptide-coding sequence were inserted into the vector backbone to construct the porcine amylase promoter-driven vector pAMYR3.1. A 1,200 bp cDNA clone encoding *Piromyces rhizinflata cellulase*, which *hydrolyzes* a polysaccharide containing a β-1,3′ or β-1,4′ glycosidic linkage (modified from US Patent 6,428,996 B1 pPR2301-16 clone; unpublished), provided as a gift obtained from Dr. Kuo-Joan Cheng, Academia Sinica, Taipei, Taiwan, was used to generate the structural gene of the transgenic cassette. The optimal enzymatic hydrolysis condition was temperature of 40 °C, pH 6.5. The primer set used to generate the *cellulase* gene (forward-4, 5′-GGATCCATTATGAAACCCGAACCA; reverse-4, 5′-CTCGAGTTATTCCTTTGGTTTTTC) had recognition sites (underlined) for the restriction enzymes *Bam*HI and *Xho*I. The thermal cycling conditions were as follows: 94 °C for 5 min; 35 cycles at 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 2 min; and a final extension at 72 °C for 7 min. Gel-purified PCR fragments were ligated into the pGEM-T Easy vector (Promega) before sequencing. After digestion of the restriction enzymes, the purified double-digested *cellulase* gene was subcloned into the unique *Bam*HI-*Xho*I-cleaved pAMYR3.1 vector to construct the expression plasmid pAMY-*CEL*. The resulting plasmid was further sequenced to confirm the accuracy of the inserted sequences.

## Generation of transgenic pigs

The linear DNA fragment of AMY-*CEL*, obtained from *Nhe*I and *Xho*I double digestion of pAMY-*CEL*, was purified separately with cesium chloride-ethidium bromide (EtBr) gradient ultracentrifugation, followed by removal of EtBr using 1-butanol (Sigma, St. Louis, MO, USA) before dialysis against TE buffer [10 mM Tris (pH 8.0), 1 mM EDTA] at 4 °C. After ethanol precipitation, the linear DNA fragments were recovered and diluted to a final concentration of 3 ng/μL in an injection buffer [10 mM Tris (pH 7.4), 0.1 mM EDTA]. Sexually mature crossbred gilts [Duroc × (Yorkshire × Landrace)] between 6 and 12 months of age and weighing at least 90 kg were used as embryo donors or recipients. Estrus synchronization and superovulation were performed with a slight modification to the aforementioned method (Park et al. 2006). In brief, estrus was synchronized by oral administration of 20 mg Regumate (Intervet, Millsboro, DE,

USA) daily for a period of 18 days. Superovulation of donors was induced with a single intramuscular injection of 1,750 IU of pregnant mare serum gonadotropin (PMSG; China Chemical and Pharmaceutical, Taipei, Taiwan) 24 h after the last Regumate feeding and 1,000 IU of human chorionic gonadotropin (hCG; China Chemical and Pharmaceutical) 80 h after PMSG administration. Dosages of 1,000 IU PMSG and 500 IU hCG were administered to an equal number of recipients in the same time period as for the donors. Pronuclear-stage embryos were recovered by flushing oviducts of superovulated donors approximately 55–62 h after hCG injection using Dulbecco's phosphate-buffered saline (DPBS; Invitrogen) supplemented with 1 % FBS. The AMY-*CEL* transgene cassettes were then injected into the fertilized egg pronuclei. Morphologically surviving DNA-injected fertilized eggs were surgically transferred into the recipient oviducts on the same day of estrous cycle as in donor pigs.

## PCR and Southern blot analyses

Three-day-old transgenic pigs were examined by PCR and Southern blot analyses. Each genomic DNA sample was isolated from the ear tissue using standard proteinase K-sodium dodecyl sulfate digestion followed by the phenol–chloroform extraction (Herrmann and Frischauf 1987). One microgram of each genomic DNA sample was used as the template for a single PCR. Two primer sets (forward-4 and reverse-4 & forward-5, 5′-AGGGACCGAAGGAGAGTGTT and reverse-5, 5′-ACTCAGACAATGCGATGCA) (Fig. 3a) were used to amplify the transgene and confirm the presence of AMY-*CEL* cassettes in transgenic pigs versus their nontransgenic littermates. Next, Southern blot analysis was performed as previously described (Yang et al. 2004). In brief, probes were generated using PCR-amplified fragments and were labeled with $\alpha$-$^{32}$P by using the Amersham Rediprime II DNA labeling system (GE Healthcare, Fairfield, CT, USA). The primer set was designed based on the basis of the structural genes of the AMY-*CEL* cassettes (forward-4; reverse-4-1, 5′-TCCGTTCCATTCAACTGGTG) and was expected to amplify a 599 bp product. After hybridization and variable stringency washing, membranes were subjected to phosphor-image analysis using a Typhoon 9200 scanner (GE Healthcare).

## Chemical analyses of the feces

Three F1 transgenic pigs and three nontransgenic pigs (not littermates) with similar age and body weight were assigned for this experiment. These animals were fed with a corn, barley, soybean-meal-based diet. Their feces were collected and dried in a forced-air drying oven at 60 °C for 72 h and ground to a fine powder before compositing. The total dry matter (DM) content was determined by drying 2 g of a representative sample at 105 °C until constant weight was achieved. The ash content was produced by incinerating the 2 g sample at 600 °C until light-grey ash was obtained. The content of neutral detergent fiber (NDF) was assayed according to the van Soest method (van Soest et al. 1991) with sodium sulfite and α-amylase, and expressed with residual ash.

## RT-PCR analyses

Total RNA was isolated from frozen pancreas, heart, liver, lung, kidney, muscle, stomach, and duodenum tissues of transgenic pigs using an RNeasy Lipid Tissue Mini kit (Qiagen, Hilden, Germany) with on-column DNase digestion (RNase-Free DNase Set; Qiagen) according to manufacturer's instructions. RT-PCR analyses were performed using a SuperScript III One-Step RT-PCR System with a Platinum Taq DNA polymerase kit (Invitrogen). In brief, 50 ng of each purified RNA sample was used as the staring material. The primer set for β-2 microglobulin (B2M; forward-5, 5′-AACGGAAAGCCAAATTACCTG; reverse-5, 5′-GTGATGCCGGTTAGTGGTCTG) amplified a 259 bp segment, which served as an internal control. All reactions were carried out using the following thermal cycling conditions: 55 °C for 40 min; 94 °C for 2 min; 35 cycles at 94 °C for 30 s, 64 °C for 30 s, and 68 °C for 2 min; and a final extension at 68 °C for 7 min. Amplified products were then electrophoresed and visualized by EtBr staining.

## Western blot analyses

Total protein from pancreatic tissue was extracted by homogenization in ice-cold RIPA buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS] supplemented with protease inhibitor (Millipore, Billerica, MA, USA) under rotation for 2 h at 4 °C. After centrifugation at

13,500×*g* for 20 min at 4 °C, the supernatant was quantified as described above. Twenty-five micrograms of protein in each pancreatic sample was boiled for 5 min in 5 × sample buffer [60 mM Tris–HCl (pH 6.8), 25 % glycerol, 2 % SDS, 5 % beta-mercaptoethanol, 0.1 % bromophenol blue], separated by electrophoresis on 12 % SDS-PAGE gels, and transferred to Hybond-P polyvinylidene difluoride membranes (GE Healthcare) at 250 mA for 2 h. For immunoblotting, the membrane was blocked for 1 h at room temperature with 10 % skim milk in Tris-buffered saline with Tween 20 [TBS-T; 20 mM Tris–HCl (pH 7.6), 137 mM NaCl, 0.1 % Tween-20] and then cut into three sections. The top (size > 70 kDa) and middle (size between 40 and 70 kDa) membrane sections were incubated with our custom-made rabbit polyclonal antiserum generated against recombinant cellulase (1:2,000; GeneTex, Hsinchu, Taiwan), and the other one-third of the membrane (size < 40 kDa) was incubated with mouse anti-GAPDH antibodies (1:20,000; Novus Biologicals, Littleton, CO, USA) at 4 °C overnight. Membranes were then washed with TBS-T and subsequently incubated with either horseradish peroxidase-conjugated chicken anti-rabbit IgG (1:20,000; Abcam, Cambridge, UK) or goat anti-mouse IgG (1:20,000; Abcam) as the secondary antibodies for 1 h at room temperature. We also characterized a custom-purified recombinant cellulase protein (GeneTex) as an immunogen to generate our antibody using western blotting. Bound peroxidase-conjugate was detected using a Western Lightning Plus ECL kit (PerkinElmer, Waltham, MA, USA) and exposed to X-ray film (GE Healthcare) following the manufacturer's instructions.

### Assay of cellulase enzyme activity

Cellulase activities of the pancreas, salivary gland, stomach, liver, and heart in the 12-month-old transgenic pig were analyzed with a MarkerGene fluorescence cellulase assay kit (Marker Gene Technologies, Eugene, OR, USA) following the manufacturer's instructions. Three samples from each tissue were collected and snap frozen in liquid nitrogen and pulverized into a fine powder using a chilled mortar and pestle. Each powdered sample was processed as following steps. One milligram of the resulting powder was suspended in 200 μL reaction buffer and centrifuged at 13,000×*g* for 10 min at 4 °C. Fifty microliters of the supernatant fraction prepared as above was added to a clear, flat-bottomed 96 well plate. A blank sample containing only 50 μL of reaction buffer was also prepared. A 0.5 mM substrate reagent solution (from the kit) was prepared and added to each well to a final concentration of 0.25 mM. The plate was immediately placed in a SpectraMax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA), then the fluorescence was read at EX/EM = 570/590 at 3 min intervals for 90 min and recorded.

### Statistical analyses

All the statistical analyses were conducted using SAS software, version 9.1.3 (SAS Institute, Cary, NC, USA). Comparisons between genotypes were carried out with unpaired *t* tests. Values are presented as the mean ± SEM, and $P < 0.05$ denotes a statistically significant difference.

## Results

### Identification of the porcine pancreatic amylase gene promoter region

A DNA fragment containing 2,488 bp of the 5′-flanking region of the porcine pancreatic amylase gene was isolated and sequenced in conjunction with 45 bp of the signal peptide coding sequence of the gene. Bioinformatic analysis of the genomic region upstream of the transcriptional start site was predicted at 11, 438, and 1,380 bp upstream of the ATG translational start site, and a TATAAA sequence of the TATA box was located 43 bp away (Fig. 1). Potential transcriptional factor binding sites including insulin promoter factor 1 (IPF-1), glucocorticoid response element (GRE), hepatocyte nuclear factor 3-beta (HNF3-β), Opaque-2, and prolamin box binding factor (PBF), were present within this sequence.

### Evaluation of the in vitro activity of the porcine pancreatic amylase gene promoter

The promoter activity of the 5′-flanking region of the amylase gene was examined in AR-42J cells transfected with the plasmid contained *GFP* reporter gene (Fig. 2a). The result in Fig. 2b shows that this newly cloned 5′-flanking region of the amylase gene was

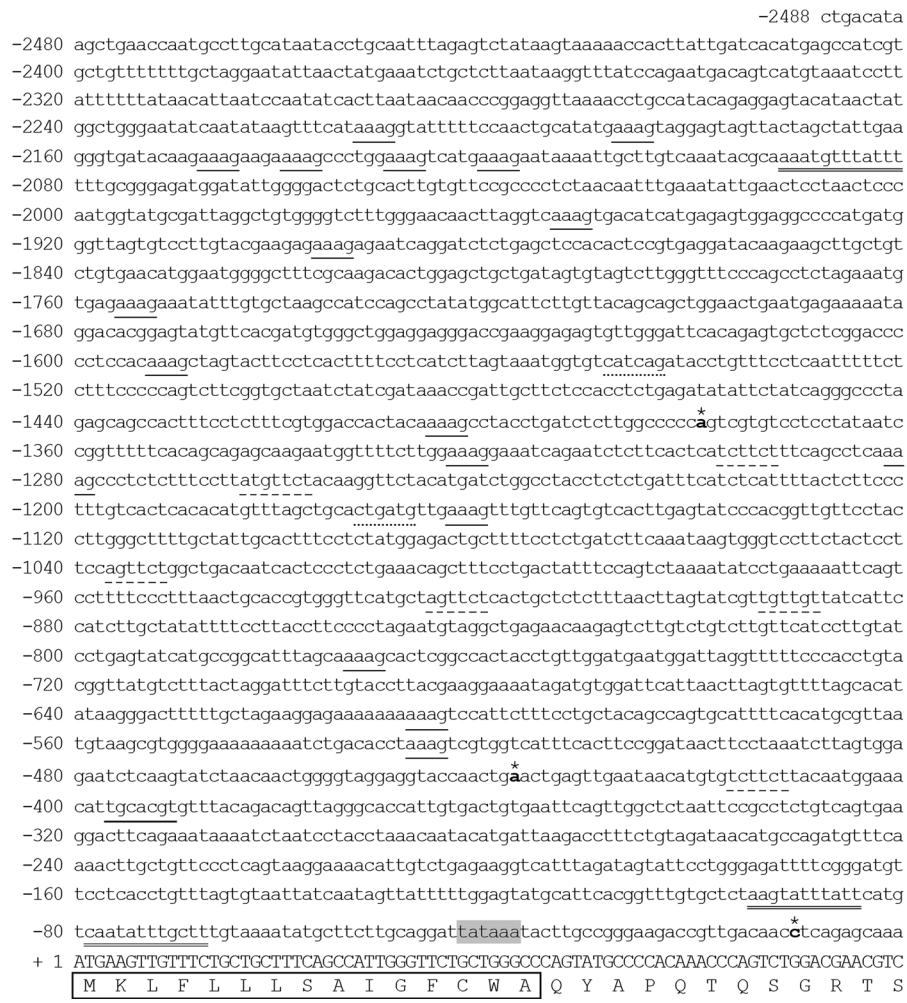


**Fig. 1** Nucleotide sequence analysis of the 5′-flanking region of the porcine pancreatic amylase gene. The *lowercase* and *uppercase letters* represent nucleotides of the 5′-flanking region and the coding sequence, respectively. The deduced amino acid sequence is presented *below* the coding region of exon 1, and the sequence encoding the signal peptide is *boxed*. The *numbers* alongside the sequence refer to the nucleotide position relative to the translation initiation site, designated as nucleotide +1. Three putative transcription start sites are indicated in *bold with asterisks*. The TATA box is highlighted in *gray*, and potential binding sites of transcription factors, insulin promoter factor 1 (IPF-1, *dotted*), glucocorticoid response element (GRE, *broken*), hepatocyte nuclear factor 3-beta (HNF3-β, *double*), Opaque 2 (O2, *thick*), and prolamin box binding factor (PBF, thin) are *underlined*

sufficient to direct reporter gene expression in cells of the rat pancreas.

## Establishment of transgenic pigs carrying AMY-*CEL*

Transgenic pigs were generated by microinjecting the 3.9 kb DNA fragment containing the 5′-flanking region linked to the amylase signal peptide coding sequence, fungi *cellulase* gene, and bovine growth hormone polyadenylation site (BGH polyA; Fig. 3a). In all, 145 zygotes were collected from 11 donors, of which 138 were microinjected and transferred to 6 recipients. Two recipients became pregnant and gave birth to a total of 13 piglets, which were subjected to PCR and Southern blot screening to confirm the presence of the transgene. The survival and birth rates of the fertilized eggs after microinjection were 95 % (138/145) and 9 % (13/138), respectively. DNA analyses identified that 5 piglets (2 male and 3 female) in the F0 generation were transgenic for the construct (5/13; 38 %). One of male founders was crossed with 6 wild-type females to generate the F1 hemizygous generation, and approximately 55 % (29/53) of the F1 generation pigs inherited the exogenous transgene of AMY-*CEL*. Figure 3b shows representative results of PCR screening of the transgenic pigs from the F1 hemizygous generation and their wild-type littermates. The PCR-positive F1 transgenic pigs were also confirmed via positive results in Southern blot analysis (Fig. 3c).

## Transgene expression of AMY-*CEL*

The statistically significant differences were found for the apparent nutrient digestibility of DM and NDF ($P < 0.05$) between transgenic and nontransgenic pigs

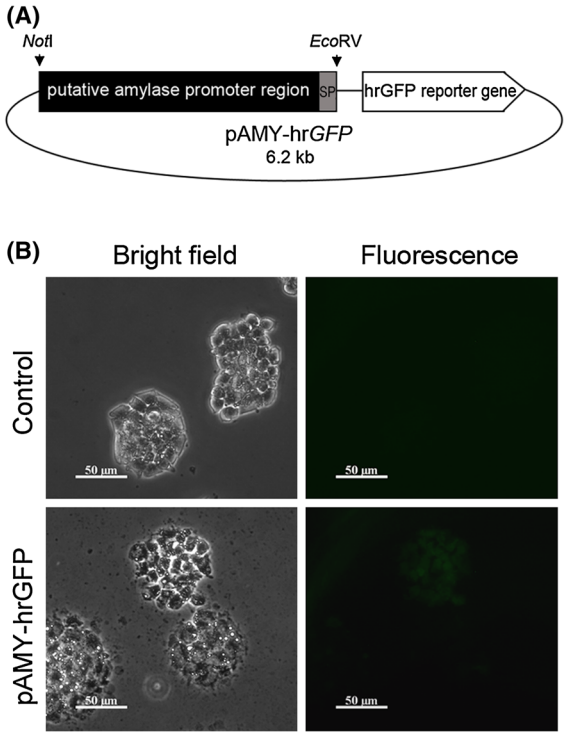


**Fig. 2** Verification of a 2,488 bp 5′-flanking region of the porcine pancreatic amylase gene possessing promoter activity. **a** Schematic diagram of the pAMY-*GFP* plasmid vector (not to scale). The construct, consisting of the putative amylase promoter region (*black box*), porcine pancreatic amylase signal peptide (SP, *gray box*), and a humanized Renilla reniformis-derived *GFP* (hr*GFP*) gene (*white box*), was designed for transient transfection of AR-42J cells. **b** Images of GFP in transfected cells. Cells were maintained in the presence of 50 mM dexamethasone (Dex). Negative control cells (*upper panel*) were treated as above, but without the addition of the transfection reagent or plasmid vector. After 48 h of treatment, bright-field (*left panels*) and corresponding fluorescence (*right panels*) images of the cultured cells were taken at a ×200 magnification. *Scale bars* represent at 50 μm in length

(Fig. 4). To analyze the transgene expression profiles of transgenic pigs, total RNA was collected from various tissues, including the pancreas, heart, liver, lung, kidney, muscle, stomach, and duodenum, from the F1 transgenic pig and was subjected to RT-PCR analyses. As shown in Fig. 5a, a 1,200 bp transgene transcript was detected exclusively in the pancreatic tissue of representative transgenic pigs. This RT-PCR-positive F1 individual was further examined for detection of transgenic protein expression in pancreatic tissue by western blotting (Fig. 5b). We observed a single band at around 70–100 kDa that was not detected in samples from a nontransgenic individual. This positive signal indicated that the 5′-flanking region of porcine pancreatic amylase gene sequences in this study had a potential for the regulation of transgene expression.

### Evaluation of cellulase enzyme activity in the transgenic pig

Cellulase activity was measured using the substrate resorufin cellobioside. As shown in Fig. 6, increased fluorescence over time was observed in assays of pancreatic tissue lysate of the transgenic pig, indicating that cellulase expressed in the pancreas was active.

## Discussion

Genetically modified pigs are already being used to develop new or altered physiological traits for reducing agricultural pollution (Golovan et al. 2001); enhancing productivity by increasing piglet survival (Wheeler et al. 2001; Tong et al. 2011); altering the composition of the carcass so that it may be healthier for humans to consume (Pursel et al. 2004; Lai et al. 2006); studying molecular mechanisms related growth, disease, and development (Pursel et al. 2004; Miles et al. 2013); and applications in biomedical fields such as bioreactors (Paleyanda et al. 1997) and organ transplantation (Zeyland et al. 2013). In some studies, the designed transgene vectors are based on viral promoter and enhancer sequences to boost the efficiency of expression in a variety of cell types (Betrabet et al. 2004). However, the potential risks associated with these viral-derived sequences are still of great concern, and it is possible that genetically altered products **are** not safe for human consumption. In this study, we developed an exogenous transgene (*cellulase*) expressed downstream under the control of a native porcine promoter in attempt to minimize possible risk factors.

Digestive gene expression and enzyme activity are often flexible in response to dietary chemical signals (Karasov et al. 2011). Pancreatic enzymes actually accommodate the composition of diets via the biosynthesis and secretion of specific enzymes (Owsley et al. 1986; Simoes Nunes 1986; Flores et al. 1988); for example, the secretion of pancreatic amylase is sensitive to the amount of starch in the diet (Corring and Chayvialle 1987; Mosenthin and Sauer 1993).

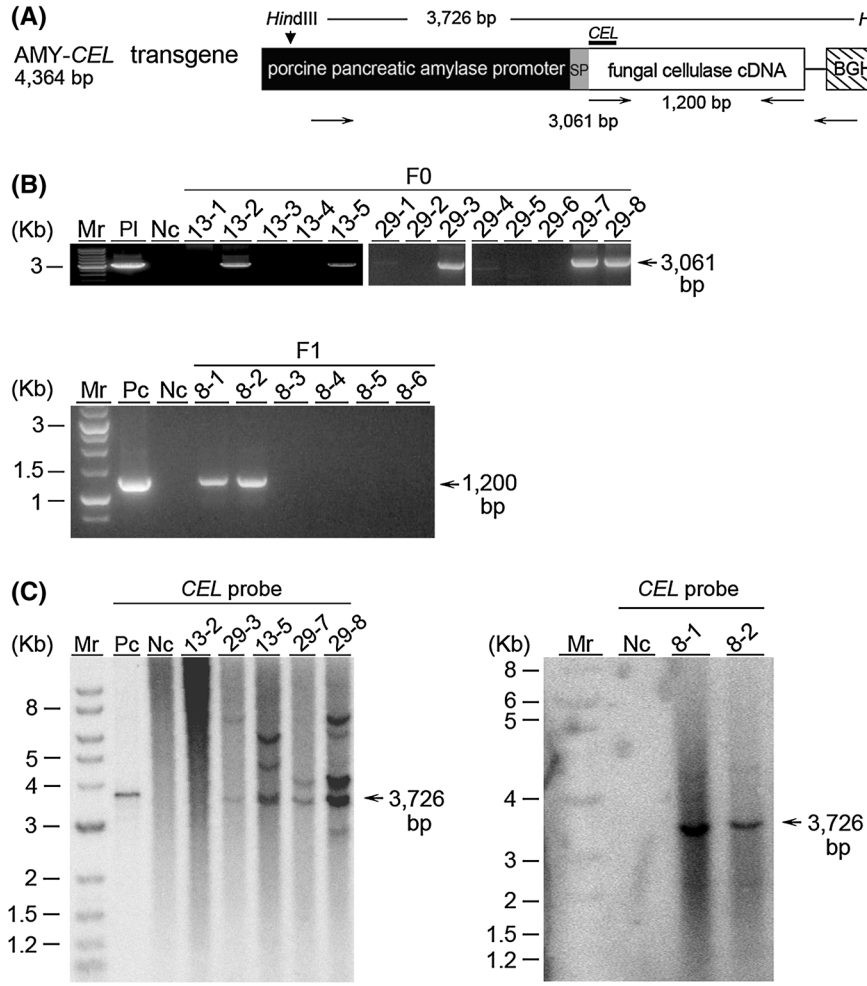


**Fig. 3** Identification of the transgene in pigs by genomic PCR and Southern blot analyses. **a** Schematic diagram of the AMY-*CEL* transgenic cassettes (not to scale). The construct consisted of a 2,488 bp porcine pancreatic amylase promoter (*black box*), porcine pancreatic amylase signal peptide (SP, *gray box*), *cellulase* gene (*white box*), and bovine growth hormone polyadenylation site (BGH polyA; *hatched box*). The *horizontal arrows* represent primer sets used in PCR for distinguishing transgenic pigs from their nontransgenic littermates. The *vertical arrowheads* show the position of the restriction enzyme sites. The *short horizontal bar* above each drawing indicates the probe used in Southern blot analysis. **b** A representative result of PCR screening of transgenic pigs. The *top panel* shows five out of thirteen pigs were positively transgenic in F0 and the *bottom panel* shows screening result of the offspring (F1) for one of the transgenic pigs (No. 29-8). The numbering of F0 is listed at the *top* of the figure from *lanes 3* to *15*. The numbering of F1 generation is indicated at the *top* of the figure from *lanes 3* to *8*, respectively. The results from two positive pigs (No. 8-1 and No. 8-2) carrying transgenes of *cellulase* are shown. Mr, DNA marker; Pl, plasmid contained AMY-*CEL*; Pc, founder transgenic pig (No. 29-8); Nc, nontransgenic pig. **c** Verification of transgenic pigs by Southern blot analysis. Twenty micrograms of each genomic DNA sample was digested with two restriction enzymes, *Hin*dIII and *Hae*II, for detecting the integration of the transgenic cassette of AMY-*CEL* using a probe specific to the structural gene. Mr, DNA marker; Nc, nontransgenic pig

This enzymatic activity fluctuation is possibly regulated via their gene expression. Therefore, the promoter of the amylase gene may be useful for inducible promoter-controlled gene expression through modifications of diet composition. In addition, the appropriate catalytic properties of an exogenous enzyme are affected by its environmental conditions, such as temperature and pH value. In a previous study, this fungal cellulolytic enzyme was found to function best in an acidic environment (US Patent 6,428,996 B1). The intraduodenal pH in fed pigs is maintained mostly between pH 4 and 6 with normal secretion of

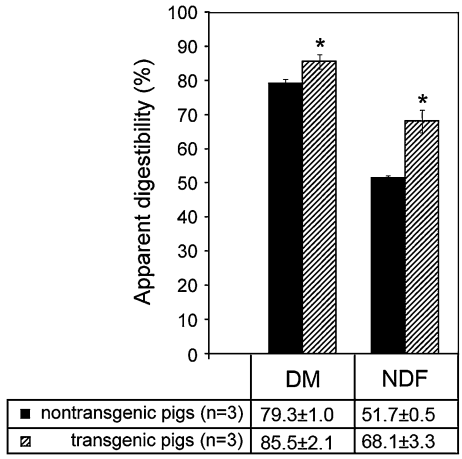


**Fig. 4** Comparison of the apparent nutrient digestibility of transgenic versus nontransgenic pigs. During the 4th week of the experimental period, this study measured and calculated the apparent digestibility (%) of dry matter (DM) and neutral detergent fiber (NDF) in feces of experimental animals fed with a corn, barley, soybean-meal-based control diet. Data points are means, and *error bars* represent standard errors. $*P < 0.05$ versus a nontransgenic control via unpaired *t* test. *Black bar* nontransgenic pigs, *hatched bar* transgenic pigs

pancreatic juice and bile (Abello et al. 1987). Thus, the duodenal cavity would be the most logical location to promote cellulase activity following heterologous exogenous enzyme gene expression.

Several *cis*-acting elements involved in regulating amylase gene expression, such as IPF1/PDX1, GRE, and HNF3-β, were predicted in the 5′-flanking region of the porcine pancreatic amylase gene in the current study. These observations are in agreement with those of previous studies in humans, mice, and sea bass (Liberzon et al. 2004; Ma et al. 2004). Our results also revealed the presence of binding sites of the corn-transcriptional activators PBF and Opaque-2 scattered throughout the 5′-flanking region of the porcine pancreatic amylase gene. Whether the potential specific transcription factor binding sites for PBF and Opaque-2 could affect porcine pancreatic promoter regulation following food intake remains unknown.

A signal peptide was used to guide the exported cellulase enzyme in our designed construct vector. This strategy was similar to that used in transgenic mice expressing exogenous glucanase under the

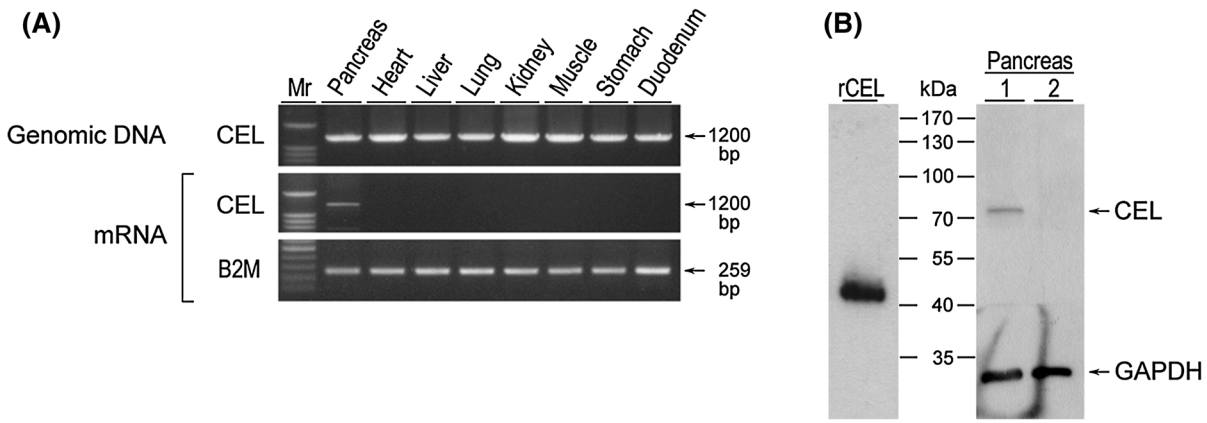


**Fig. 5** Detection of *cellulase* transgene expression by RT-PCR and western blot analyses. **a** Expression pattern of mRNA encoding the full-length transgene in a representative transgenic F1 generation pig. Fifty nanograms of genomic DNA and total RNA were isolated from tissue samples (pancreas, heart, liver, lung, kidney, muscle, stomach, and duodenum tissue). For the *upper panel*, genomic DNA derived from these eight tissue types served as controls for RT-PCR-mediated detection of the *cellulase* transgene (CEL). The 1,200 bp band represents expression of the *cellulase* transgene in pigs (positive control). For the *middle panel*, tissue expression of the transgene mRNA was specifically obtained in pancreatic tissue of the transgenic pig, but was absent in other tissues. The *lower panel* represents β-2 microglobulin (B2M) mRNA expression in RNA collected from various samples; this transcript was used as an internal reference. Mr, DNA marker. **b** Verification of *transgene* expression by measuring protein levels in the pancreas. Anti-CEL antibodies were used to probe expression of cellulase protein extracted from pancreatic tissues of a representative transgenic pig (*left panel*, *lane 1*) and nontransgenic littermate (*left panel*, *lane 2*) as well as purified recombinant cellulase (rCEL) expressed in *E. coli* (*right panel*). The *arrow* indicates the distinct band detected with antibodies against cellulase. No specific signal was observed in the nontransgenic pig. The GAPDH housekeeping protein was used as a loading control

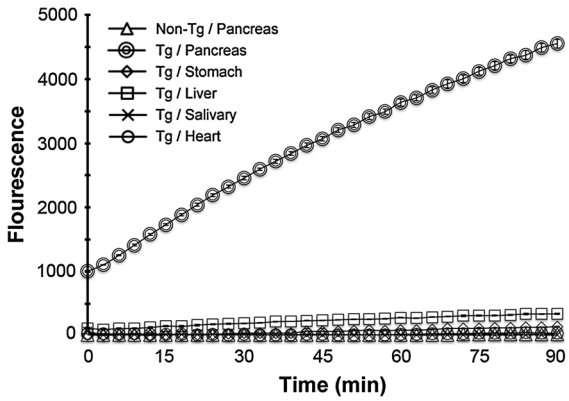


**Fig. 6** Assay of cellulase enzyme activity in porcine pancreatic tissues. Continuous fluorescent analyses of soluble lysates from transgenic porcine pancreas (*circle within circle*), stomach (*diamond*), liver (*square*), salivary gland (*cross symbol*), and heart (*circle*), as well as the nontransgenic (Non-Tg) pancreas (*triangle*) were used for detection of the substrate resorufin-β-D-cellobioside. The fluorescence of a blank sample (50 μL of substrate reagent added to 50 μL of reaction buffer) was set as the background and analyzed over the same time period. Fluorescence emission was measured at 590 nm with excitation at 570 nm for 90 min at 3 min intervals. Three samples from each tissue were processed for this experimental procedure. The fluorescent was measured for each sample. Data points are means, and *error bars* represent standard errors. Background fluorescence has been subtracted

promoter of the mouse amylase gene coupled with its homologous signal sequence (Zhang et al. 1999). Fusion of a recombinant protein with a signal peptide at the N-terminus is generally used to achieve translocation of the nascent pre-protein to the endoplasmic reticulum (Tsuchiya et al. 2003). Increasing evidence indicates that the presence of a eukaryotic signal peptide has the potential to efficiently direct secretion of prokaryotic digestive enzymes in mammalian cells (Soole et al. 1993; Yin et al. 2006). Expression of the cellulase enzyme in the pancreas of transgenic pigs in our study also supported this hypothesis. In addition, our results indicated that the size of the expressed transgene at the protein level appeared to be larger than that of the full-length cellulase protein expressed in *E. coli*, suggesting that the protein may have undergone post-translational modifications, which are often required for efficient secretion and stability of exogenously expressed enzymes in mammalian cells (Golovan et al. 2001; Assenberg et al. 2013).

In conclusion, we isolated and characterized a portion of the 5′-flanking region of the porcine pancreatic amylase gene. Using a transgenic approach, this novel cloned fragment was sufficient to drive pancreas-specific expression of the exogenous fungal *cellulase* gene in pigs. This line of transgenic pigs provided a new nonruminant animal model for future studies of dietary fiber utilization and heterologous expression of target proteins in vivo.

**Acknowledgments** This research was conducted using funds provided by grant NSC91-2317-B-002-017 awarded by the Ministry of Science and Technology of Taiwan, ROC. We thank Dr. Kuo-Joan Cheng (Academia Sinica, Taiwan) for the plasmid containing *Piromyces rhizinflata* cellulase cDNA and helpful advice during the preparation of this manuscript.

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US007956238B2

(12) **United States Patent**
**Cheng et al.**

(10) **Patent No.: US 7,956,238 B2**
(45) **Date of Patent: Jun. 7, 2011**

(54) **PORCINE PANCREATIC AMYLASE GENE PROMOTER AND TRANSGENIC PIGS EXPRESSING HETEROLOGOUS DIGESTIVE ENZYMES**

(75) Inventors: **Teng-Kuei Winston Cheng**, Taipei (TW); **Shinn-Chih Wu**, Taipei (TW); **Chi-Chen Hsu**, Taipei (TW); **Yu-Sheng Lin**, Taipei (TW); **Chih-Jen Lin**, Taipei (TW); **Kuo-Joan Cheng**, Hisn Chu (TW); **Jih-Tay Hsu**, Taipei (TW)

(73) Assignee: **National Taiwan University (An University of Taiwan, R.O.C.)**, Taipei (TW)

( * ) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 794 days.

(21) Appl. No.: **11/438,979**

(22) Filed: **May 23, 2006**

(65) **Prior Publication Data**

US 2007/0277253 A1 Nov. 29, 2007

(51) **Int. Cl.**

| | |
|---|---|
| *A01K 67/027* | (2006.01) |
| *C12N 15/52* | (2006.01) |
| *C12N 15/63* | (2006.01) |
| *C12N 15/85* | (2006.01) |
| *C07H 21/04* | (2006.01) |

(52) **U.S. Cl.** ..... **800/17**; 800/13; 800/21; 800/25; 536/24.1; 435/320.1

(58) **Field of Classification Search** ..... 800/13, 800/17, 21, 25; 536/24.1; 435/320.1
See application file for complete search history.

(56) **References Cited**

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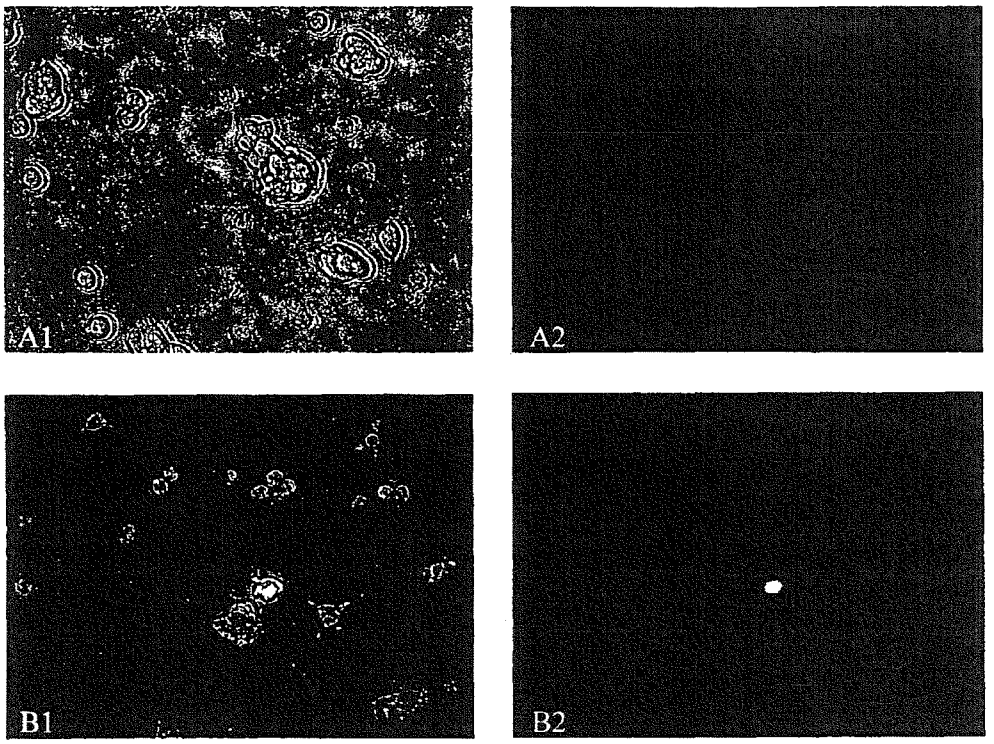
*Primary Examiner* — Anne Marie S Wehbe
(74) *Attorney, Agent, or Firm* — Occhiuti Rohlicek & Tsao LLP

(57) **ABSTRACT**

The present invention relates to a novel promoter and its use in driving expression of foreign genes in transgenic animals (especially pigs). Accordingly, the present invention provides a method for producing transgenic animals harboring heterologous genes regulated by the promoter of the present invention.

**11 Claims, 10 Drawing Sheets**

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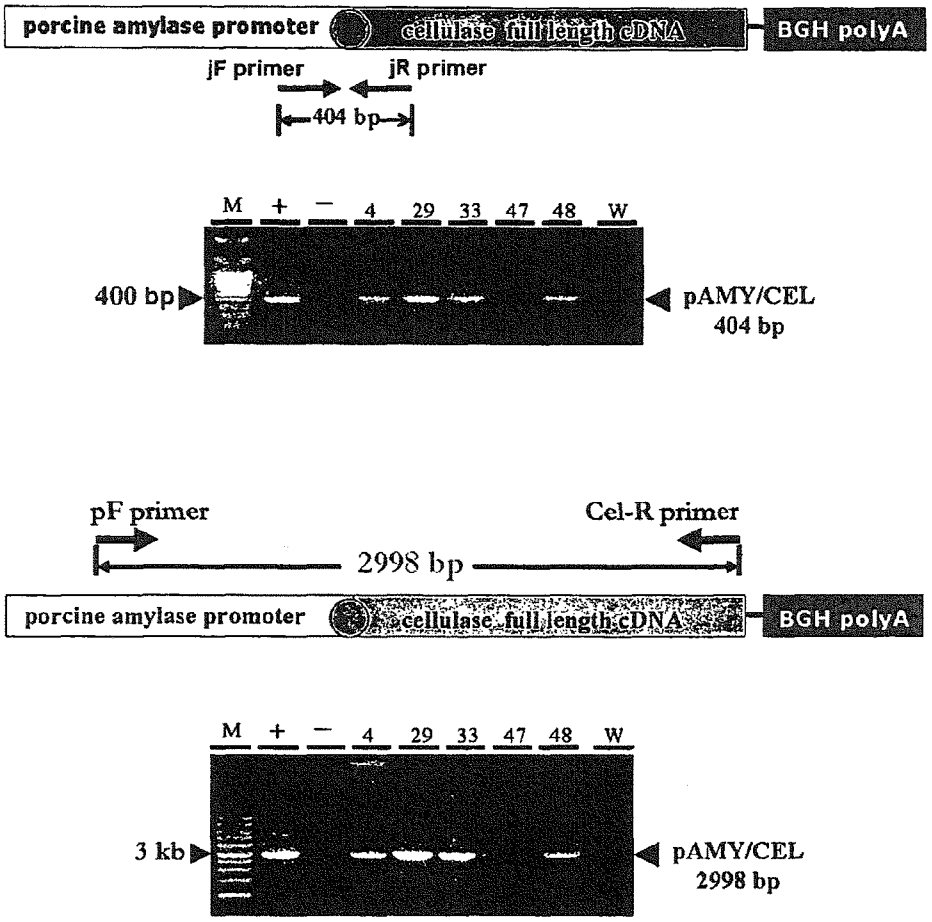
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pF primer
Cel-R primer
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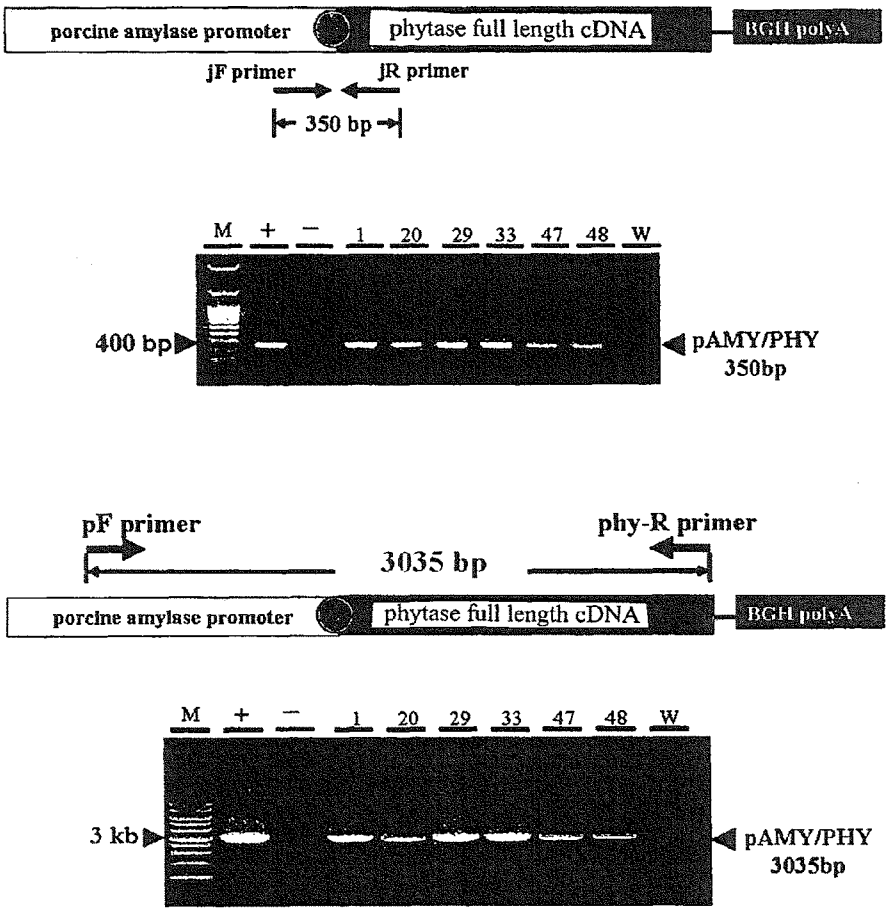
Figure 2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350bp
pF primer
phy-R primer
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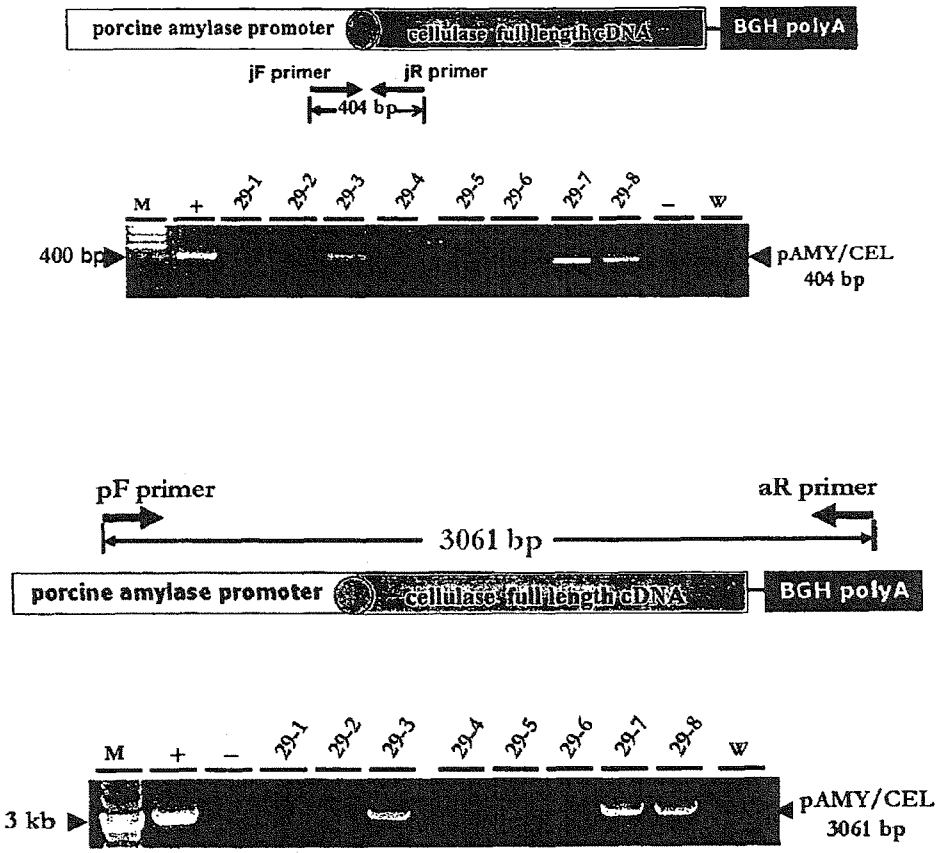
Figure 3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29-6
29-7
29-8
–
W
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aR primer
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–
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Figure 4A

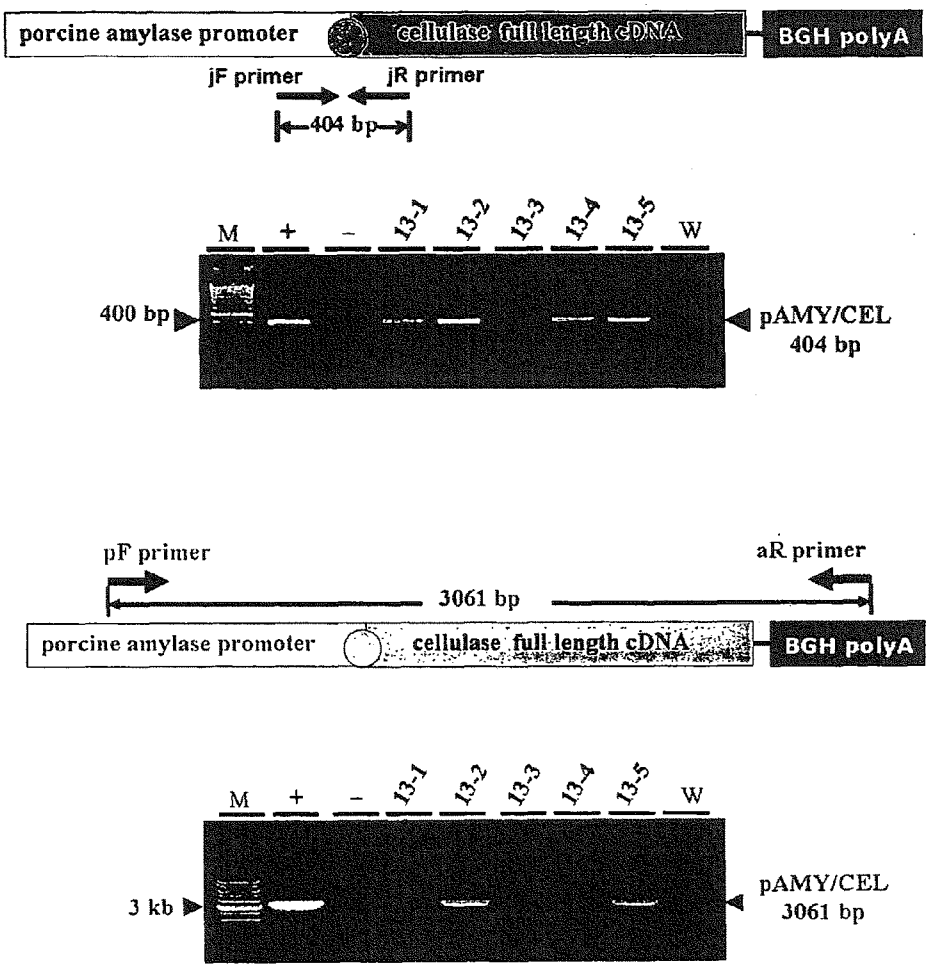
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Figure 4B

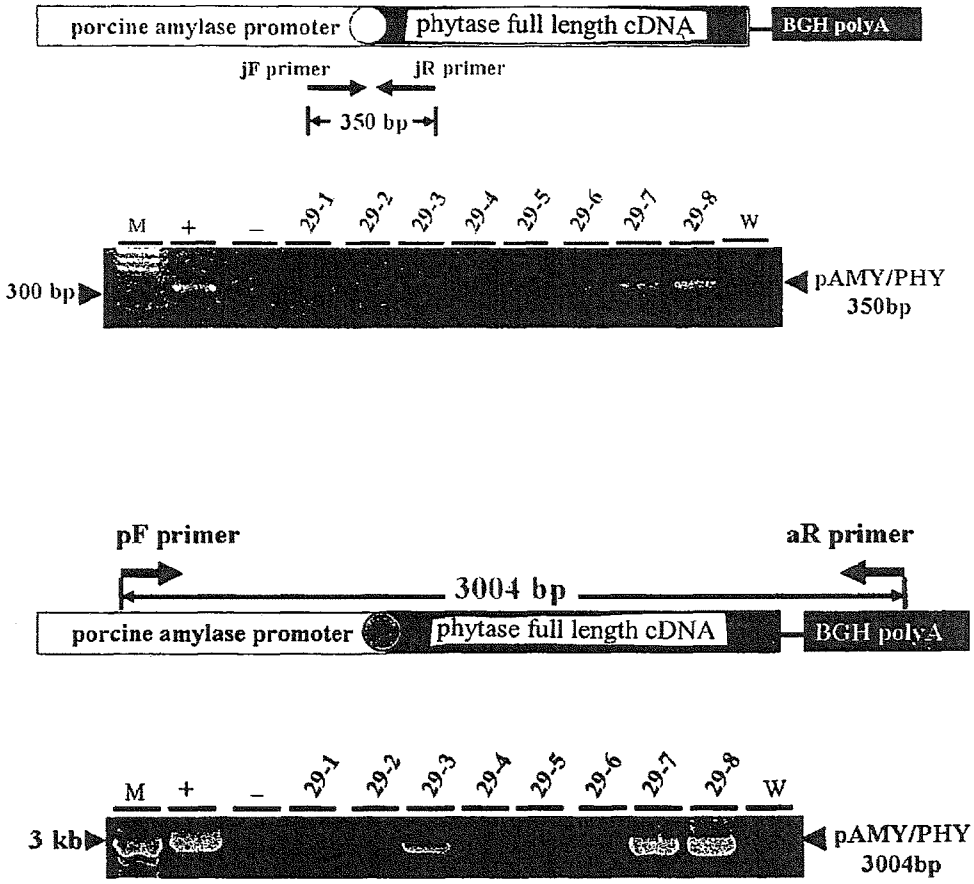
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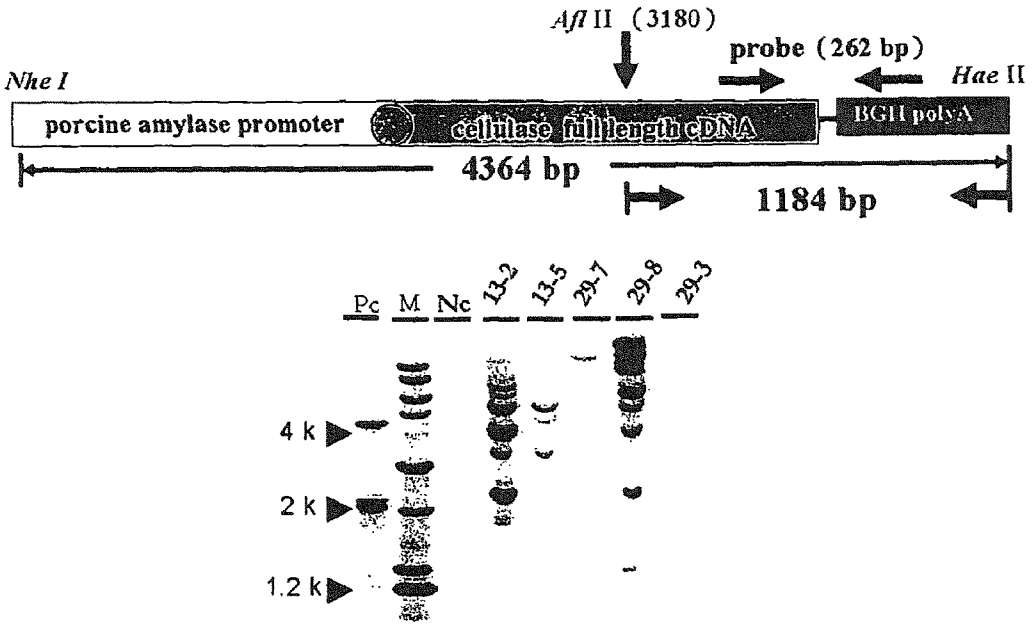
Figure 5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29-3
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2 k
1.2 k

Figure 6

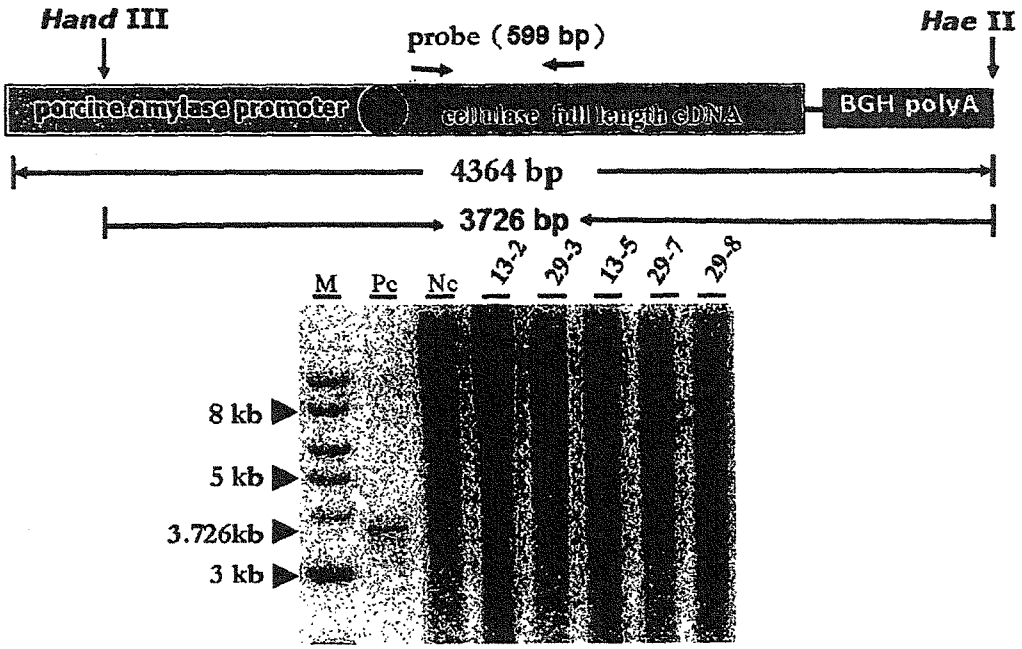
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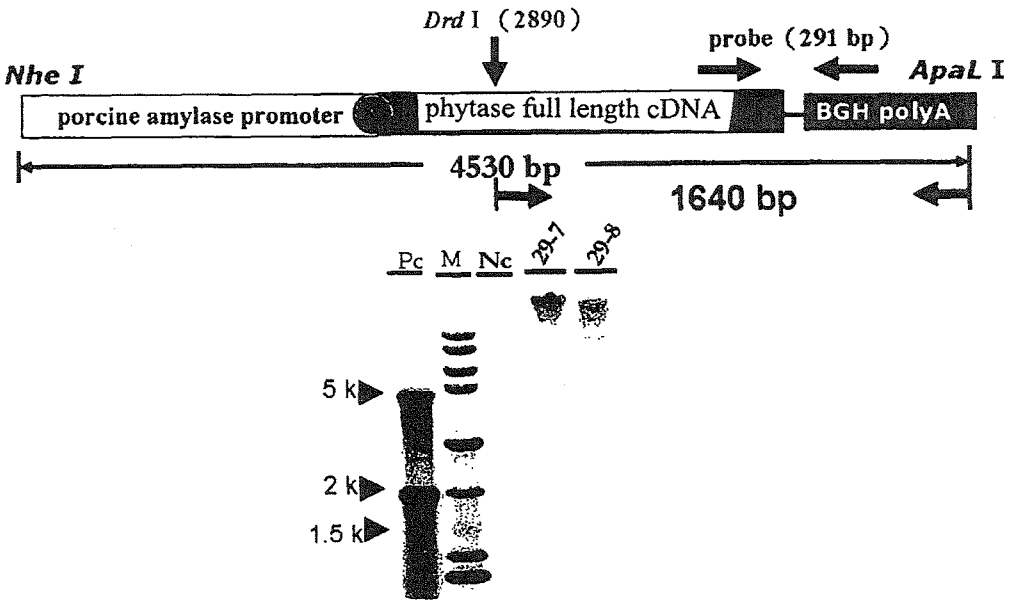
Figure 7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29-8
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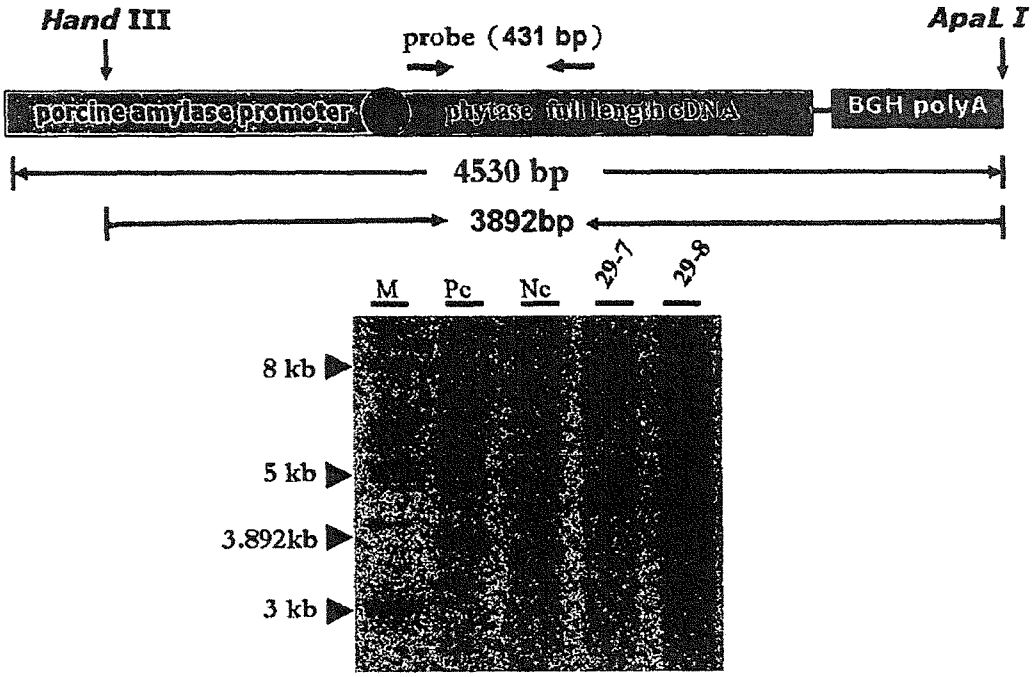
2 k
1.5 k

Figure 8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Pc
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29-7
29-8
8 kb
5 kb
3.892kb
3 kb

Figure 9

# PORCINE PANCREATIC AMYLASE GENE PROMOTER AND TRANSGENIC PIGS EXPRESSING HETEROLOGOUS DIGESTIVE ENZYMES

## FIELD OF THE INVENTION

The present invention relates generally to the expression of heterologous proteins in animals and to the production of transgenic animals.

## BACKGROUND OF THE INVENTION

Animal husbandry constitutes the biggest portion of agricultural production in Taiwan, and brings handsome and stable income to Taiwanese farmers. However, fecal waste produced by livestock in large amount is not properly disposed and thus results in environmental pollution. According to the statistical information from the Council of Agriculture, Executive Yuan, Taiwan, the headcount of farmed hogs was 6,794,000 by the end of the year 2002. Assuming that each hog produces 6 Kg of fecal waste per day, there will be about 15,000,000 metric tons of hog fecal waste per year, plus the fecal waste from poultry. The serious environmental pollution resulting from such a large amount of fecal waste has become the major problem in Taiwanese animal husbandry. How to reduce the production of fecal waste without jeopardizing farmers' income is an important issue.

In addition to the aforementioned environmental issue, Taiwanese animal husbandry has to compete with imported livestock products since Taiwan joined the World Trade Organization (WTO) in 2002. How to reduce the production cost while enhancing the production rate has become another important issue. Generally speaking, the cost of feed constitutes 60 to 70% of the total production cost. Therefore, the key to reduce the production cost is to improve feed utilization. Effective ways include utilizing biotechnology to develop feed additives for enhancing the digestion of feed, and breeding livestock animals able to digest cellulose to reduce fecal production.

The quantity and quality of animal feces depend on the digestion and utilization of the components of feed by the animal. Generally speaking, livestock animals do not have the ability to synthesize digestive enzymes such as cellulases and phytases, and thus cannot effectively digest the cellulose and plant phosphorus in the feed. Such inability affects feed utilization and results in a waste of feed. In addition, the undigested nutrients are excreted from the animals and become a cause of environmental pollution. A common method for enhancing utilization of the nutrients in feed by animals is to supplement the feed with certain digestive enzymes produced on large scale by microorganisms. Recently, the cDNAs of several digestive enzymes have been screened out from fungal and bacterial cDNA libraries, and the enzymes have been produced on large scale by *E. coli* (Ye et al., 2001, Inter. J. Biochem. Biol. 33:87-94; and Zhang et al., 1998, Biotechnol. Lett. 20: 1001-1005; both hereby incorporated herein by reference). However, the production cost of this method is high. In addition, the supplementary digestive enzymes are usually destroyed in the animal's digestive tract by the native enzymes or gastric acid and thus cannot carry out their function.

Since the 1980s, the generation of transgenic animals not only allows us to study gene functions in vivo, but also provides a new way to improve the genetics of livestock animals. In the conventional breeding method, it takes tens of or even over a hundred years to improve the genetics of livestock animals. However, utilizing the techniques of genetic transformation, the same improvement can be achieved within one generation. In 1985, Hammer et al. first published their results in transgenic pigs, sheep and rabbits (Hammer et al., 1985, Nature 315:680-683; hereby incorporated herein by reference). Afterwards, researchers have utilized genes encoding growth factors from various animals (including cattle, mice and humans) to generate transgenic pigs carrying heterologous growth factor genes, hoping to increase lean meat in pigs and to shorten rearing time (Pursel and Rexroad, 1993, J. Anim. Sci. 71:10-19; hereby incorporated herein by reference). For example, Pursel et al. have constructed several heterologous growth factor genes under the control of the mouse metallothionein-I promoter (Pursel et al., 1989, Science 244:1281-1288; hereby incorporated herein by reference), and thereby the transcription of the heterologous genes can be induced and regulated effectively by the addition of trace amounts of zinc in the feed.

As known in the art, manipulation of animals (such as pigs) to alter and/or improve phenotypic characteristics (such as productivity or quality) requires the expression of heterologous genes in animal tissues. Such genetic manipulation therefore relies on the availability of means to drive and to control gene expression as required; for example, on the availability and use of suitable promoters which are effective in animals and which regulate gene expression so as to give the desired effect(s) in the transgenic animal. It is advantageous to have the choice of a variety of different promoters so that the most suitable promoter may be selected for a particular gene, construct, cell, tissue, animal or environment.

There is a continuing need in the art for high-level expression promoters, as well as promoters which are spatially defined in their expression patterns.

## BRIEF SUMMARY OF THE INVENTION

In the present invention, a novel porcine pancreatic amylase gene promoter is operatively linked to either a fungal cellulase gene or a bacterial phytase gene to form an expression cassette, which is utilized in the generation of transgenic mice and pigs.

Accordingly, in one aspect, the present invention provides an isolated porcine pancreatic amylase gene promoter. In a preferred embodiment, the promoter has a nucleotide sequence as set forth in SEQ ID NO: 1.

In another aspect, the present invention provides a recombinant DNA construct comprising the porcine pancreatic amylase gene promoter of the present invention operatively linked to a nucleotide sequence of interest. In a preferred embodiment, the nucleotide sequence of interest codes for a digestive enzyme, such as a phytase, a cellulase, a glucanase and a xylanase.

In a further aspect, the present invention provides a method for producing a transgenic animal comprising the steps of providing an animal embryo and introducing into the animal embryo a transgene comprising the porcine pancreatic amylase gene promoter of the present invention operatively linked to a nucleotide sequence of interest, thereby transforming the embryo with the transgene.

In yet another aspect, the present invention provides a transgenic animal whose genome contains a transgene comprising a heterologous nucleotide sequence operatively linked to the promoter of the present invention. In a preferred embodiment, the transgenic animal is a pig.

## BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

The foregoing summary, as well as the following detailed description of the invention, will be better understood when

read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings embodiments which are presently preferred. It should be understood, however, that the invention is not limited to the precise arrangements and instrumentalities shown.

In the drawings:

FIG. **1** shows fluorescence microscopic images of AR-42J rat pancreatic tumor cells transfected with 412 pAMY-phrGFP or 1216 pAMY-phrGFP vectors. Panel A1 is the image of 1216 pAMY-phrGFP-transfected AR-42J cells under bright field. Panel A2 is the image of 1216 pAMY-phrGFP-transfected AR-42J cells under dark field. Panel B1 is the image of 412 pAMY-phrGFP-transfected AR-42J cells under bright field. Panel B2 is the image of 412 pAMY-phrGFP-transfected AR-42J cells under dark field.

FIG. **2** shows the results of PCR verification of transgenic mice harboring the cellulase transgene. "M" denotes markers; "+" denotes positive control; "−" denotes negative control; and "W" denotes wildtype. The numbers correspond to the identification numbers of the transgenic mice.

FIG. **3** shows the results of PCR verification of transgenic mice harboring the phytase transgene. "M" denotes markers; "+" denotes positive control; "−" denotes negative control; and "W" denotes wildtype. The numbers correspond to the identification numbers of the transgenic mice.

FIGS. **4**A and **4**B show the results of PCR verification of transgenic pigs harboring the cellulase transgene. "M" denotes markers; "+" denotes positive control; "−" denotes negative control; and "W" denotes wildtype. The numbers correspond to the identification numbers of the transgenic pigs.

FIG. **5** shows the results of PCR verification of transgenic pigs harboring the phytase transgene. "M" denotes markers; "+" denotes positive control; "−" denotes negative control; and "W" denotes wildtype. The numbers correspond to the identification numbers of the transgenic pigs.

FIG. **6** shows the results of Southern Blot analysis of transgenic pigs harboring the cellulase transgene, wherein the porcine genomic DNA was digested with Afl II. "M" denotes markers; "Pc" denotes positive control; and "Nc" denotes negative control. The numbers correspond to the identification numbers of the transgenic pigs.

FIG. **7** shows the results of Southern Blot analysis of transgenic pigs harboring the cellulase transgene, wherein the porcine genomic DNA was double digested with Hind III and HaeII. "M" denotes markers; "Pc" denotes positive control; and "Nc" denotes negative control. The numbers correspond to the identification numbers of the transgenic pigs.

FIG. **8** shows the results of Southern Blot analysis of transgenic pigs harboring the phytase transgene, wherein the porcine genomic DNA was digested with Drd I. "M" denotes markers; "Pc" denotes positive control; and "Nc" denotes negative control. The numbers correspond to the identification numbers of the transgenic pigs.

FIG. **9** shows the results of Southern Blot analysis of transgenic pigs harboring the phytase transgene, wherein the porcine genomic DNA was double digested with Hind III and ApaL I. "M" denotes markers; "Pc" denotes positive control; and "Nc" denotes negative control. The numbers correspond to the identification numbers of the transgenic pigs.

## DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is directed to a novel promoter sequence of the porcine pancreatic amylase gene and uses thereof in the expression of recombinant genes and production of transgenic animals. The promoter of the present invention permits space and time-determined expression of transgenes in a transgenic animal.

In accordance with an embodiment of the present invention, there is provided an isolated porcine pancreatic amylase gene promoter.

As used herein, the term "isolated" refers to material, such as a nucleic acid, which is substantially or essentially free from components that normally accompany or interact with it as found in its naturally occurring environment. The isolated material optionally comprises material not found with the material in its natural environment.

As used herein, the term "promoter" refers to an untranslated DNA sequence upstream of a coding region that contains the binding site for an RNA polymerase and initiates transcription of the coding region. The promoter region may also include other elements that act as regulators of gene expression.

In a preferred embodiment, the promoter of the present invention has the nucleotide sequence of SEQ ID NO: 1.

In accordance with another embodiment of the present invention, there is provided a recombinant DNA construct comprising the porcine pancreatic amylase gene promoter of the present invention operatively linked to a nucleotide sequence of interest.

In a preferred embodiment, the recombinant DNA construct is an expression vector comprising an expression cassette. As used herein, the term "expression cassette" refers to a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements which permit transcription of a nucleotide sequence of interest in a host cell. The expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Typically, the expression cassette portion of an expression vector includes, among other sequences, a nucleotide sequence to be transcribed, and a promoter. In the present invention, the expression cassette can also be referred to as a "transgene" when used to produce transgenic animals.

As used herein, "operatively linked" includes reference to a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the second sequence. Generally, "operatively linked" means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame.

According to the present invention, the nucleotide sequence of interest under the control of the promoter can be any nucleic acid form. Correspondingly they can be coding nucleic acids or structural or functional nucleic acids. The term "coding nucleic acid" is understood to mean more particularly a nucleic acid coding for a peptide or protein. The peptide or protein can e.g. be a structural protein or a peptide or protein having enzymatic activity. A "structural nucleic acid" is more particularly understood to mean a nucleic acid leading to the formation of complexes, particularly with other molecules. It can inter alia be an rRNA and in particular an antisense nucleic acid. A "functional nucleic acid" is more particularly understood to mean a nucleic acid, which exerts a specific action on a system, particularly a biological system. Such a specific action can e.g. be the aiding or inhibiting of translation or transcription. An example of a functional nucleic acid is an antisense nucleic acid.

As used herein, the singular forms "a", "an", and "the" include plural referents unless the context clearly indicates otherwise.

In a preferred embodiment, the nucleotide sequence of interest under the control of the promoter is a coding nucleic acid. More preferably, the coding nucleic acid codes for a digestive enzyme, such as a phytase, a cellulase, a glucanase or a xylanase.

In accordance with a further embodiment of the present invention, there is provided a method for producing a transgenic animal comprising the steps of providing an animal embryo and introducing into the animal embryo a transgene comprising the porcine pancreatic amylase gene promoter of the present invention operatively linked to a nucleotide sequence of interest, thereby transforming the embryo with the transgene.

According to the present invention, the transgenic animal is preferably a mammal, more preferably livestock such as cattle, sheep or pigs. In a preferred embodiment, the transgenic animal of the present invention is a pig.

Generation of transgenic animals of the present invention is carried out conventionally by techniques well known in the art. There are a number of techniques that permit the introduction of genetic material (such as a transgene) into animals to be transformed, including the viral infection technique; the sperm mediated gene transfer (SMGT) technique; the embryonic stem cell technique; the nuclear transfer technique; and the pronuclear microinjection technique. Among them, the most commonly used technique is the pronuclear microinjection technique, which comprises direct injection of the transgene into the male pronucleus of fertilized eggs, resulting in the random integration into one locus of a varying number of copies, usually in a head to tail array. The injected eggs are then re-transferred into the uteri of pseudo-pregnant recipient mothers. Some of the resulting offspring may have one or several copies of the transgene integrated into their genomes, usually in one integration site. An advantage of the pronuclear microinjection technique is that the transgene would be stably integrated into the germline of transgenic animals so that it will be passed to their offspring.

According to a preferred embodiment of the present invention, the transgene is introduced into the embryo by pronuclear microinjection.

Preferably, prior to the introduction of the transgene into the embryo, it is removed from the vector portion by restriction enzyme digestion, for example by using restriction sites in the vector that flank the transgene. For cloning purposes, the transgene is generally inserted into an expression vector, such as the mammalian expression vector pCR® 3.1 (Invitrogen Corporation), prior to the introduction into the embryo. In general, the transgenic DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

In accordance with yet another embodiment of the present invention, there is provided a transgenic animal whose genome contains a transgene comprising a heterologous nucleotide sequence operatively linked to the promoter of the present invention. In a preferred embodiment, the transgenic animal is a pig.

As used herein, the term "heterologous" in reference to a nucleic acid is a nucleic acid that originates from a foreign species, or, if from the same species, is substantially engineered from its native form in composition and/or genomic locus by deliberate human intervention. A heterologous protein may originate from a foreign species or, if from the same species, is substantially engineered from its original form by deliberate human intervention.

Preferably, the transgenic animal is generated by introduction of a transgene into an embryo by pronuclear microinjection, insertion of the embryo into a surrogate mother, and allowing the embryo to develop to term.

The present invention is further illustrated by the following examples, which are provided for the purpose of demonstration rather than limitation.

## Example 1 Cloning of Porcine Pancreatic Amylase Gene Promoter

Darnis et al. has obtained the nearly full-length cDNA sequence of porcine pancreatic a-amylase gene (GenBank Accession No. AF064742) from a cDNA library constructed with the mRNA isolated from pancreatic tissues of pigs (Darnis et al., 1999, Biochem Biophys Acta. 1430:281-289; hereby incorporated herein by reference). The cDNA sequence has a length of 1,536 bp, and encodes a protein of 496 amino acids with a signal peptide of 15 amino acids. Based on the cDNA sequence, the promoter was cloned utilizing GenomeWalker™ Kit (Clontech Laboratories, Inc., Mountain View, Calif., USA) according to the manufacturer's instructions.

First, the genomic DNA isolated from porcine blood was digested at 37° C. by the four restriction enzymes (Dra I, EcoR V, Pvu II, and Stu I) provided in the kit. After 16 hours, the digested genomic DNA was precipitated by ethanol and resuspended. The resuspended digested genomic DNA was then ligated to the adaptor provided in the kit at 16° C. for 16 hours.

In addition to the two "adaptor primers" (AP1 and AP2) provided in the kit, three primers, GSP1, GSP2 and GSP3 were designed based on the sequence at the 5' end of the porcine pancreatic amylase gene (see Table 1). The first PCR was conducted utilizing the above-derived digested genomic DNA as a template and GSP3 and AP1 as primers. The second PCR was conducted utilizing the products of the first PCR as a template and GSP2 and AP2 as primers. The products of the second PCR were screened by gel electrophoresis, and possible fragments (i.e., those larger than 1 kb) were recovered from the gel and purified.

The selected fragments were cloned into pGEM®-T Easy Vectors (Promega Corporation, Madison, Wis., USA) and the formed vectors were named pGEM-T AMY. The clones were sequenced and two clones, numbered 412 and 1216, were selected.

TABLE 1

Primers Used in Genome Walking

| Primer | Sequence | Tm value (° C.) |
|---|---|---|
| AP1 | GTAATACGACTCACTATAGGGC (SEQ ID NO: 6) | 59 |
| AP2 | ACTATAGGGCACGCGTGGT (SEQ ID NO: 7) | 71 |
| GSP1 | GCTGAAAGCAGCAGAAACTTCAT (SEQ ID NO: 8) | 60.4 |
| GSP2 | ATGAACAATAGACGTTCGTCCAGACT (SEQ ID NO: 9) | 59.7 |
| GSP3 | TACCGCTCACATTCAAGAGCAATGTCA (SEQ ID NO: 10) | 62.3 |

## Example 2 Activity Test of Porcine Pancreatic Amylase Gene Promoter

To test the activity of the promoter cloned in Example 1, new primers (1216AMY-GFP-NotI-5' and 1216AMY-GFP-

EcoRV-3') (see Table 2) were designed in order to create an Not I restriction site at the 5' end and an EcoR V restriction site at the 3' end of the promoter by PCR. The PCR products were again cloned into pGEM®-T Easy Vectors and digested with the relevant restriction enzymes. The digested fragments were cloned into phrGFP vectors (Stratagene, La Jolla, Calif., USA) and the clones were sequenced. Two clones, 412 pAMY-phrGFP and 1216 pAMY-phrGFP, were obtained.

Rat pancreatic tumor cell line AR-42J (BCRC 60160) was purchased from the Bioresource Collection and Research Center of Food Industry Research and Development Institute, Hsinchu, Taiwan. The cells were cultured on a six-well culture plate, with each well containing $7\times10^4$ cells. The cultured cells were transfected with lipofectomine (Invitrogen Corporation, Carlsbad, Calif., USA) and either 412 pAMY-phrGFP or 1216 pAMY-phrGFP vectors. After culturing at 37° C. for 24 hours, green fluorescence was observed using a fluorescent microscope (FIG. **1**). These results showed that the cloned promoter was able to drive the expression of heterologous genes (in this case, the coding sequence for green fluorescence protein) in pancreatic cells.

TABLE 2

Primers Used in Constructing 1216pAMY-phrGFP, 1216pAMY-CEL and pAMY-PHY

| Primer | Sequence | Tm value (° C.) |
|---|---|---|
| 1216AMY-GFP-NotI-5' | GCGGCCGCCTGACATAAGCTGAA (SEQ ID NO: 11) | 79.5 |
| 1216AMY-GFP-EcoRV-3' | GATATCGGCCCAGCAGAACCCAA (SEQ ID NO: 12) | 76.4 |
| 1216AMY-NheI-5' | GCTAGCCTGACATAAGCTGAACCAA (SEQ ID NO: 13) | 71.8 |
| 1216AMY-BamHI-3' | GGATCCGGCCCAGCAGAACCCAA (SEQ ID NO: 14) | 82.0 |
| CEL-BamHI-5' | GGATCCATTATGAAACCCGAACCA (SEQ ID NO: 15) | 69.7 |
| CEL-XhoI-3' | CTCGAGTTATTCCTTTGGTTTTTC (SEQ ID NO: 16) | 66.6 |
| PHY-BamHI-5' | GGATCCCAGAGTGAGCCGGAGCT (SEQ ID NO: 17) | 74.1 |
| PHY-XhoI-3' | CTCGAGTTACAAACTGCACGCCGGTA (SEQ ID NO: 18) | 78.1 |

## Example 3 Preparation of Transgene Constructs for Microinjection

A cellulase gene (GenBank Accession No. AF053363) from *Piromyces rhizinflatus* and a phytase gene (GenBank Accession No. AF537219) from an *E. coli* strain isolated from bovine feces (ATCC 33965) were used to construct the transgenes. cDNAs of both genes were provided by Dr. Kuo-Joan Cheng of Institute of Bioagricultural Science, Academia Sinica, Taipei, Taiwan. The cDNAs of both genes were first amplified by PCR using primer pairs CEL-BamHI-5'+CEL-XhoI-3' (for the cellulase gene) and PHY-BamHI5'+PHY-XhoI-3' (for the phytase gene) (see Table 2) and cloned into pGEM®-T Easy Vectors. The promoter cloned in Example 1 was also amplified by PCR using primer pair 1216AMY-NheI-5'+1216AMY-BamHI-3' (see Table 2) and the PCR products were also cloned into pGEM®-T Easy Vectors.

The promoter (digested with Nhe I and BamH I) and either the cellulase or phytage gene (digested with BamH I and Xho I) were cloned into the mammalian expression vector pCR® 3.1 (Invitrogene Corporation) to form the vector pAMY-CEL or pAMY-PHY. After mass production of the vectors, a transgene (SEQ ID NO:2) containing the promoter, a signal peptide, a structural gene expressing a polypeptide having the sequence of SEQ ID NO:3 and a BGH poly A tail and a transgene (SEQ ID NO:4) containing the promoter, the signal peptide, a structural gene expressing a polypeptide having the sequence of SEQ ID NO:5, and a BGH poly A tail were excised from the vectors by restriction enzyme digestion. The excised transgenes were recovered trice with QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, Calif., USA) and subjected to CsCl density gradient centrifugation (CsCl, 1 gm/ml; ethylene bromide (EthBr), 24 μl; DNA, 100-200 μg, 0.02% Triton-X100) at 100,000 rpm for 6 hrs. After centrifugation, the band containing linear DNA was extracted under UV light and mixed with saturated butanol aqueous solution to remove EtBr. The DNA was then dialyzed with TE buffer (pH 8.0) for 24 hours to remove CsCl, precipitated with ethanol, and redissolved in TE buffer (pH 7.4). After the concentration and quality of the DNA were determined by gel electrophoresis and a spectrophotometer, the DNA was diluted to 3 ng/μl and stored under −20° C.

## Example 4 Production of Transgenic Mice

The ICR female mice used as embryo donors and recipients were purchased from National Taiwan University Animal Center (Taipei, Taiwan). The present experiment was conducted on 6 to 7 week old, sexually mature female mice.

Fertilized mouse embryos of the pronucleus stage were flushed out and recovered from the oviducts of superovulated female mice. Ten to twenty embryos to be microinjected were placed into a microdrop of M2 buffer in the center of a depression microscope slide. The microdrop was covered with heavy mineral oil to prevent evaporation of the buffer. Microinjection was performed using a differential interference contrast microscope (Axiovert 10, Zeiss, Germany) with Narashigi NT-8 micromanipulators (Narashigi, Japan). The holding pipette for stabilizing the embryo and the injection pipette for DNA injection were prepared as described in Wu et al., 1995, J. Chin. Soc. Anim. Sci. 24:181-189 (hereby incorporated herein by reference). About 2 pl of DNA solution containing 3 to 5 ng/μl of the transgene constructs prepared in Example 3 were injected into the male pronucleus of each embryo.

Embryos surviving the microinjection process as judged by morphological observation (i.e., those retaining dense deutoplasm and intact appearance) were implanted into recipient females made pseudo-pregnant by mating with vasectomized males. About 10 to 20 microinjected mouse embryos were transferred into each of the two oviducts of pseudopregnant females. Embryos were allowed to come to term and the newborn mice were analyzed for the presence of the transgenes by PCR as described below.

In the present study, 267 ICR mouse embryos were microinjected with the transgenes, among which 245 surviving embryos were implanted into the oviducts of recipient female mice (the loss rate for microinjected embryos was 8.2%). A total of 63 mouse pups were born, meaning that the survival rate for mouse embryos was 25.7%.

## Example 5 Analysis of Transgenic Mouse Genomic DNA

Among the 63 potential transgenic mouse pups obtained in Example 4, only 57 have successfully grown up to weaning

age. Using tissue samples removed from the tails of the 57 surviving pups, genomic DNA was obtained by the following procedures.

The tissue sample was cut into tiny pieces and mixed with a solution containing 630 μl of tissue lysis buffer (100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 200 mM NaCl), 70 μl of 10% SDS, and 35 μl of 100 mg/ml Proteinase K. The mixture was incubated in a 55° C. water bath for 12 to 16 hours, and then centrifuged at 12,000× rpm for 5 minutes. The supernatant from centrifugation was extracted twice with phenol/chloroform (1:1), and then centrifuged at 12,000× rpm again for 5 minutes to obtain two layers. DNA was precipitated from the upper layer with absolute ethanol, washed with 70% ethanol, and redissolved in autoclaved distilled and deionized $H_2O$.

PCR reactions on the genomic DNA samples obtained above were carried out conventionally. Primer pairs were designed based on the sequences of the porcine pancreatic amylase gene promoter, the junction between the promoter and the structural gene, the structural gene, and the BGH poly A tail (Table 3). To primarily examine if a potential transgenic mouse carries the desired transgene, the genomic DNA sample was subjected to PCR (reaction volume=20 μl) using either primer pair 1216-pF+CEL-R (for the cellulase gene) or 1216-pF+PHY-R (for the phytase gene). For the cellulase gene, the reaction conditions were: (1) 94° C., 2 mins; (2) 94° C., 30 secs; 65° C., 30 secs; and 72° C., 2 mins; 35 cycles; (3) 72° C., 7 mins; and (4) 4° C., termination. For the phytase gene, the reaction conditions were: (1) 94° C., 2 mins; (2) 94° C., 30 secs; 67° C., 30 secs; and 72° C., 2 mins; 35 cycles; (3) 72° C., 7 mins; and (4) 4° C., termination.

The results of the PCR analysis are shown in FIGS. **2** and **3**. As can be seen from the results, pups No. 4, 29, 33 and 48 carry the cellulase transgene (FIG. **2**), while pups No. 1, 20, 29, 33, 47 and 48 carry the phytase transgene (FIG. **3**), which means that pups No. 29, 33 and 48 carry both transgenes. The identities of the transgenes were further confirmed by PCR using primer pair 1216C-jF+1216C-jR or 1216P-jF+1216P-jR.

TABLE 3

Primers Used in Transgenic Animal DNA Analysis

| Primer | Sequence | Tm value (° C.) | PCR Product |
|---|---|---|---|
| 1216-pF | AGGGACCGAAGGAGAGTGTT (SEQ ID NO: 19) | 64.1 | 314 bp |
| 1216-pR | ACCATTCTTGCTCTGCTGTGA (SEQ ID NO: 20) | 64.6 | |
| 1216C-jF | ACCGTTGACAACCTCAGAGCA (SEQ ID NO: 21) | 66.8 | 404 bp |
| 1216C-jR | TCGTTCACCAAAGTGTCCAGA (SEQ ID NO: 22) | 65.2 | |
| 1216C-aF | CATTGTTGCAGCCTTACAA (SEQ ID NO: 23) | 59.0 | 262 bp |
| 1216C-aR | ACTCAGACAATGCGATGCA (SEQ ID NO: 24) | 60.8 | |
| 1216P-jF | ACCGTTGACAACCTCAGAGCAA (SEQ ID NO: 25) | 68.8 | 350 bp |
| 1216P-jR | ACGCTCGTCGACATCAGCAATA (SEQ ID NO: 26) | 70.0 | |
| 1216P-aF | TCGGCTAAGCGATAACAG (SEQ ID NO: 27) | 58.4 | 291 bp |
| 1216P-aR | ACAACAGATGGCTGGCAACT (SEQ ID NO: 28) | 64.1 | |
| CEL-R | GGATCCTTATTCCTTTGGTTTTTC (SEQ ID NO: 29) | 53.8 | |
| PHY-R | GGATCCTTACAAACTGCACGCCGG (SEQ ID NO: 30) | 64.5 | |
| CEL-Hyb-3' | TCCGTTCCATTCAACTGGTG (SEQ ID NO: 31) | 65.2 | |
| PHY-Hyb-3' | TCAGTCACGTTCGCGTTATCT (SEQ ID NO: 32) | 65.0 | |

Example 6 Production of Transgenic Pigs

Sexually mature crossbred gilts (more than 6 month old) were used in the present study as embryo donors and recipients. Superovulated donor gilts were artificially inseminated and fertilized embryos were surgically recovered from their oviducts. The embryos were centrifuged at 12,000× g for 10 minutes at 25° C. in order to show the pronuclei (the cytoplasm of porcine embryos was opaque with lipid and pronuclei were invisible before centrifugation).

Microinjection of the embryos was performed as described above in Example 4. Embryos surviving the microinjection process as judged by morphological observation were implanted into oestrous recipient gilts. About 10 to 20 microinjected porcine embryos were transferred into each of the two oviducts of recipient gilts. Embryos were allowed to come to term and the newborn pigs were analyzed for the presence of the transgenes by PCR and Southern blot as described below.

In the present study, 145 porcine embryos were microinjected with the transgenes, among which 138 surviving embryos were implanted into the oviducts of 6 recipient gilts. Among the 6 recipients, 2 were pregnant and gave birth to a total of 13 piglets.

Example 7 Analysis of Transgenic Pig Genomic DNA

Genomic DNA was obtained from tissue samples removed from the ears of the 13 potential transgenic piglets by the same procedures as described in Example 5. PCR was performed on the genomic DNA samples using one of the following primer pairs: 1216-pF+1216C-aR (for full-length cellulase transgene), 1216-pF+1216P-aR (for full-length phytase transgene), 1216C-jF+1216C-jR (for promoter-cellulase junction) and 1216P-jF+1216P-jR (for promoter-phytase junction) (see Table 3).

The results of the PCR analysis are shown in FIGS. **4** and **5**. As can be seen from the results, piglets No. 29-3, 29-7, 29-8, (FIG. **4**A) and 13-2 and 13-5 (FIG. **4**B) carry the cellulase transgene, while piglets No. 29-7 and 29-8 carry the phytase transgene (FIG. **5**), which means that piglets No. 29-7 and 29-8 carry both transgenes. Among the 5 transgenic piglets, piglet No. 13-2 died of bacterial infection 10 days after birth.

Southern blotting was performed on the genomic DNA of the 5 transgenic piglets in order to understand the level of integration of the transgenes, using PCR products of primer pairs 1216C-aF+1216C-aR, 1216P-aF+1216P-aR, CEL-BamHI-5'+CEL-Hyb-3' and PHY-BamHI-5'+PHY-Hyb-3' (see Tables 2 and 3) as probes. Recovered and purified PCR products were first denatured in boiling water and placed on ice. Radioactive labeling of the PCR products was carried out with Rediprime™ II DNA Labeling System (GE Healthcare UK Ltd, UK) according to the manufacturer's instructions. Southern Blotting was performed essentially as described in Koetsier et al., 1993, Biotechniques 15(2):260-2 (hereby incorporated herein by reference). The procedures employed are outlined below.

Genomic DNA of the 5 transgenic piglets digested with relevant restriction enzymes was subjected to 0.8% agarose gel electrophoresis (50V, 4-6 hrs) and depurination with 0.25 N HCl for 15 minutes. After the Bromophenol Blue dye turned yellow, the gel slab was incubated in a denaturing solution containing 1.5 M NaCl and 0.4 N NaOH. After blotting for 2.5 to 4 hours, the nylon membrane (Hybond™-N+, GE Healthcare UK Ltd) with DNA blots was placed into a 2×SSC solution for neutralizing the reaction. For pre-hybridization, the membrane was incubated in a pre-hybridization solution (2×SSC, 1% SDS, 0.5% fat-free milk powder and 0.75 mg/ml denatured salmon sperm DNA) at 66° C. for 2 to 5 hours. For hybridization, the membrane was moved into a hybridization solution (2×SSC, 1% SDS, 0.5% fat-free milk powder, 0.5 mg/ml denatured salmon sperm DNA and 10% dextran sulfate) containing the radioactive labeled probes, and incubated at 66° C. for 16 to 24 hours. After hybridization, the membrane was twice washed at 55° C. for 20 minutes with a solution containing 0.1% SDS and 0.1×SSC. Finally, the membrane was air dried and analyzeded by a Bio-imaging Analyzer (BAS-1500, FUJIFILM, Japan).

The results of the Southern Blot analysis are shown in FIGS. **6** to **9**. In FIG. **6**, the porcine genomic DNA was digested with Afl II and the probe was the PCR product of primer pair 1216C-aF+1216C-aR. The image shows that all of the 5 transgenic piglets carry the cellulase transgene, but integrated copy numbers may be larger in piglets No. 13-2 and 29-8 in view of the stronger hybridization signals for the two. In FIG. **7**, the porcine genomic DNA was double digested with Hind III and Hae II and the probe was the PCR product of primer pair CEL-BamHI-5'+CEL-Hyb-3'. The image shows that except for piglet No. 13-2 (whose genomic DNA was damaged), the other four piglets all have the 3,726-kb fragment same as the positive control, meaning that they all carry the cellulase transgene but with different integration levels. In FIG. **8**, the porcine genomic DNA was digested with Drd I and the probe was the PCR product of primer pair 1216P-aF+1216P-aR. The image shows that piglet No. 29-8 carries the phytase transgene, but the restriction digestion for piglet No. 29-7 seems insufficient. In FIG. **9**, the porcine genomic DNA was double digested with Hind III and ApaL I and the probe was the PCR product of primer pair PHY-BamHI-5'+PHY-Hyb-3'. As can be seen from the image, only piglet No. 29-8 has the 3,892-kb fragment same as the positive control. However, piglet No. 29-7 has a fragment near the 8 kb position, which is believed to represent the undigested phytase transgene.

It will be appreciated by those skilled in the art that changes could be made to the embodiments described above without departing from the broad inventive concept thereof. It is understood, therefore, that this invention is not limited to the particular embodiments disclosed, but it is intended to cover modifications within the spirit and scope of the present invention as defined by the appended claims.

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Lys Ile Val Tyr Pro Gly Ile Val Ala Ala Leu Gln Lys Gly Arg Gly
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Lys Pro Lys Glu

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ttacagacag ttagggcacc attgtgactg tgaattcagt tggctctaat tccgcctctg 2160

tcagtgaagg acttcagaaa taaaatctaa tcctacctaa acaatacatg attaagacct 2220

ttctgtagat aacatgccag atgtttcaaa acttgctgtt ccctcagtaa ggaaaacatt 2280

gtctgagaag gtcatttaga tagtattcct gggagatttt cgggatgttc ctcacctgtt 2340

tagtgtaatt atcaatagtt atttttggag tatgcattca cggtttgtgc tctaagtatt 2400

tattcatgtc aatatttgct ttgtaaaata tgcttcttgc aggattataa atacttgccg 2460

ggaagaccgt tgacaacctc agagcaaaat gaagttgttt ctgctgcttt cagccattgg 2520

gttctgctgg gccggatcc cag agt gag ccg gag ctg aag ctg gaa agt gtg 2572
Gln Ser Glu Pro Glu Leu Lys Leu Glu Ser Val
1 5 10

gtg att gtc agt cgt cat ggt gtg cgt gct cca acc aag gcc acg caa 2620
Val Ile Val Ser Arg His Gly Val Arg Ala Pro Thr Lys Ala Thr Gln
15 20 25

ctg atg cag gat gtc acc cca gac gca tgg cca acc tgg ccg gta aaa 2668
Leu Met Gln Asp Val Thr Pro Asp Ala Trp Pro Thr Trp Pro Val Lys
30 35 40

ctg ggt tgg ctg aca ccg cgc ggt ggt gag cta atc gcc tat ctc gga 2716
Leu Gly Trp Leu Thr Pro Arg Gly Gly Glu Leu Ile Ala Tyr Leu Gly
45 50 55

cat tac caa cgc cag cgt ctg gta gcc gac gga ttg ctg gcg aaa aag 2764
His Tyr Gln Arg Gln Arg Leu Val Ala Asp Gly Leu Leu Ala Lys Lys
60 65 70 75

ggc tgc ccg cag tct ggt cag gtc gcg att att gct gat gtc gac gag 2812
Gly Cys Pro Gln Ser Gly Gln Val Ala Ile Ile Ala Asp Val Asp Glu
80 85 90

cgt acc cgt aaa aca ggc gaa gcc ttc gcc gcc ggg ctg gca cct gac 2860
Arg Thr Arg Lys Thr Gly Glu Ala Phe Ala Ala Gly Leu Ala Pro Asp
95 100 105

tgt gca ata acc gta cat acc cag aca gat acg tcc agt ccc gat ccg 2908
Cys Ala Ile Thr Val His Thr Gln Thr Asp Thr Ser Ser Pro Asp Pro
110 115 120

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tta ttt aat cct cta aaa act ggc gtt tgc caa ctg gat aac gcg aac     2956
Leu Phe Asn Pro Leu Lys Thr Gly Val Cys Gln Leu Asp Asn Ala Asn
    125                 130                 135

gtg act gac gcg atc ctc agc agg gca gga ggg tta att gct gac ttt     3004
Val Thr Asp Ala Ile Leu Ser Arg Ala Gly Gly Leu Ile Ala Asp Phe
140                 145                 150                 155

acc ggg cat cgg caa acg gcg ttt cgc gaa ctg gaa cag gtg ctt aat     3052
Thr Gly His Arg Gln Thr Ala Phe Arg Glu Leu Glu Gln Val Leu Asn
                160                 165                 170

ttt ccg caa tca aac ttg tgc ctt aaa cgt gag aaa cag gac gaa agc     3100
Phe Pro Gln Ser Asn Leu Cys Leu Lys Arg Glu Lys Gln Asp Glu Ser
            175                 180                 185

tgt tca tta acg cag gca tta cca tcg gaa ctc aag gtg agc gcc gac     3148
Cys Ser Leu Thr Gln Ala Leu Pro Ser Glu Leu Lys Val Ser Ala Asp
        190                 195                 200

aat gtc tca tta acc ggt gcg gta agc ctc gca tca atg ctg acg gag     3196
Asn Val Ser Leu Thr Gly Ala Val Ser Leu Ala Ser Met Leu Thr Glu
    205                 210                 215

ata ttt ttc ttg caa caa gca cag gga atg ccg gag ccg ggg tgg gga     3244
Ile Phe Phe Leu Gln Gln Ala Gln Gly Met Pro Glu Pro Gly Trp Gly
220                 225                 230                 235

agg atc acc gat tca cac cag tgg aac acc ttg cta agt ttg cat aac     3292
Arg Ile Thr Asp Ser His Gln Trp Asn Thr Leu Leu Ser Leu His Asn
                240                 245                 250

gcg caa ttt tat ttg tta caa cgc acg cca gag gtt gcc cgc agc cgc     3340
Ala Gln Phe Tyr Leu Leu Gln Arg Thr Pro Glu Val Ala Arg Ser Arg
            255                 260                 265

gcc acc ccg tta tta gat ttg atc aag aca gcg ttg acg ccc cat cca     3388
Ala Thr Pro Leu Leu Asp Leu Ile Lys Thr Ala Leu Thr Pro His Pro
        270                 275                 280

ccg caa aaa cag gcg tat ggt gtg aca tta ccc act cca gtg ctg ttt     3436
Pro Gln Lys Gln Ala Tyr Gly Val Thr Leu Pro Thr Pro Val Leu Phe
    285                 290                 295

atc gcc gga cac gat act aat ctg gca aat ctc ggc ggc gca ctg gag     3484
Ile Ala Gly His Asp Thr Asn Leu Ala Asn Leu Gly Gly Ala Leu Glu
300                 305                 310                 315

ctc aac tgg acg ctt ccc ggt cag ccg gat aac acg ccg cca ggt ggt     3532
Leu Asn Trp Thr Leu Pro Gly Gln Pro Asp Asn Thr Pro Pro Gly Gly
                320                 325                 330

gaa ctg gtg ttt gaa cgc tgg cgt cgg cta agc gat aac agc cag tgg     3580
Glu Leu Val Phe Glu Arg Trp Arg Arg Leu Ser Asp Asn Ser Gln Trp
            335                 340                 345

att cag gtt tcg ctg gtc ttc cag act tta cag cag atg cgt gat aaa     3628
Ile Gln Val Ser Leu Val Phe Gln Thr Leu Gln Gln Met Arg Asp Lys
        350                 355                 360

acg ccg ctg tca tta aat acg ccg ccc gga gag gtg aaa ctg acc ctg     3676
Thr Pro Leu Ser Leu Asn Thr Pro Pro Gly Glu Val Lys Leu Thr Leu
    365                 370                 375

gca gga tgt gaa gag cga aat gcg cag ggc atg tgt tcg ttg gca ggt     3724
Ala Gly Cys Glu Glu Arg Asn Ala Gln Gly Met Cys Ser Leu Ala Gly
380                 385                 390                 395

ttt acg caa atc gtg aat gaa gca cgc ata ccg gcg tgc agt ttg taa     3772
Phe Thr Gln Ile Val Asn Glu Ala Arg Ile Pro Ala Cys Ser Leu
                400                 405                 410

ctcgagtcta gagggcccgt ttaaacccgc tgatcagcct cgactgtgcc ttctagttgc   3832

cagccatctg ttgtttgccc ctcccccgtg ccttccttga ccctggaagg tgccactccc   3892

actgtccttt cctaataaaa tgaggaaatt gcatcgcatt gtctgagtag gtgtcattct   3952

attctggggg gtggggtggg gcaggacagc aaggggggagg attgggaaga caatagcagg   4012
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catgctgggg a 4023

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<212> TYPE: PRT
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

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1 5 10 15

His Gly Val Arg Ala Pro Thr Lys Ala Thr Gln Leu Met Gln Asp Val
20 25 30

Thr Pro Asp Ala Trp Pro Thr Trp Pro Val Lys Leu Gly Trp Leu Thr
35 40 45

Pro Arg Gly Gly Glu Leu Ile Ala Tyr Leu Gly His Tyr Gln Arg Gln
50 55 60

Arg Leu Val Ala Asp Gly Leu Leu Ala Lys Lys Gly Cys Pro Gln Ser
65 70 75 80

Gly Gln Val Ala Ile Ile Ala Asp Val Asp Glu Arg Thr Arg Lys Thr
85 90 95

Gly Glu Ala Phe Ala Ala Gly Leu Ala Pro Asp Cys Ala Ile Thr Val
100 105 110

His Thr Gln Thr Asp Thr Ser Ser Pro Asp Pro Leu Phe Asn Pro Leu
115 120 125

Lys Thr Gly Val Cys Gln Leu Asp Asn Ala Asn Val Thr Asp Ala Ile
130 135 140

Leu Ser Arg Ala Gly Gly Leu Ile Ala Asp Phe Thr Gly His Arg Gln
145 150 155 160

Thr Ala Phe Arg Glu Leu Glu Gln Val Leu Asn Phe Pro Gln Ser Asn
165 170 175

Leu Cys Leu Lys Arg Glu Lys Gln Asp Glu Ser Cys Ser Leu Thr Gln
180 185 190

Ala Leu Pro Ser Glu Leu Lys Val Ser Ala Asp Asn Val Ser Leu Thr
195 200 205

Gly Ala Val Ser Leu Ala Ser Met Leu Thr Glu Ile Phe Phe Leu Gln
210 215 220

Gln Ala Gln Gly Met Pro Glu Pro Gly Trp Gly Arg Ile Thr Asp Ser
225 230 235 240

His Gln Trp Asn Thr Leu Leu Ser Leu His Asn Ala Gln Phe Tyr Leu
245 250 255

Leu Gln Arg Thr Pro Glu Val Ala Arg Ser Arg Ala Thr Pro Leu Leu
260 265 270

Asp Leu Ile Lys Thr Ala Leu Thr Pro His Pro Pro Gln Lys Gln Ala
275 280 285

Tyr Gly Val Thr Leu Pro Thr Pro Val Leu Phe Ile Ala Gly His Asp
290 295 300

Thr Asn Leu Ala Asn Leu Gly Gly Ala Leu Glu Leu Asn Trp Thr Leu
305 310 315 320

Pro Gly Gln Pro Asp Asn Thr Pro Pro Gly Gly Glu Leu Val Phe Glu
325 330 335

Arg Trp Arg Arg Leu Ser Asp Asn Ser Gln Trp Ile Gln Val Ser Leu
340 345 350

Val Phe Gln Thr Leu Gln Gln Met Arg Asp Lys Thr Pro Leu Ser Leu

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355 360 365

Asn Thr Pro Pro Gly Glu Val Lys Leu Thr Leu Ala Gly Cys Glu Glu
370 375 380

Arg Asn Ala Gln Gly Met Cys Ser Leu Ala Gly Phe Thr Gln Ile Val
385 390 395 400

Asn Glu Ala Arg Ile Pro Ala Cys Ser Leu
405 410

<210> SEQ ID NO 6
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<220> FEATURE:
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<213> ORGANISM: Artificial
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actatagggc acgcgtggt 19

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<220> FEATURE:
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atgaacaata gacgttcgtc cagact 26

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taccgctcac attcaagagc aatgtca 27

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gcggccgcct gacataagct gaa 23

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gatatcggcc cagcagaacc caa 23

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<213> ORGANISM: Artificial
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ggatccggcc cagcagaacc caa 23

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<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 15

ggatccatta tgaaacccga acca 24

<210> SEQ ID NO 16
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 16

ctcgagttat tcctttggtt tttc 24

<210> SEQ ID NO 17
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 17

ggatcccaga gtgagccgga gct 23

<210> SEQ ID NO 18
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial

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<220> FEATURE:
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<400> SEQUENCE: 18

ctcgagttac aaactgcacg ccggta 26

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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 19

agggaccgaa ggagagtgtt 20

<210> SEQ ID NO 20
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: primer

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<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 21

accgttgaca acctcagagc a 21

<210> SEQ ID NO 22
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<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 22

tcgttcacca aagtgtccag a 21

<210> SEQ ID NO 23
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 23

cattgttgca gccttacaa 19

<210> SEQ ID NO 24
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 24

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<210> SEQ ID NO 25
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<212> TYPE: DNA
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accgttgaca acctcagagc aa 22

<210> SEQ ID NO 26
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<212> TYPE: DNA
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<400> SEQUENCE: 26

acgctcgtcg acatcagcaa ta 22

<210> SEQ ID NO 27
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial
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<400> SEQUENCE: 27

tcggctaagc gataacag 18

<210> SEQ ID NO 28
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<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 28

acaacagatg gctggcaact 20

<210> SEQ ID NO 29
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 29

ggatccttat tcctttggtt tttc 24

<210> SEQ ID NO 30
<211> LENGTH: 24
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<220> FEATURE:
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<400> SEQUENCE: 30

ggatccttac aaactgcacg ccgg 24

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<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 31

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tccgttccat tcaactggtg 20

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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 32

tcagtcacgt tcgcgttatc t 21

We claim:

1. An isolated DNA promoter comprising the nucleotide sequence of SEQ ID NO:1.

2. A recombinant DNA construct comprising the promoter according to claim 1 operatively linked to a nucleotide sequence of interest.

3. The recombinant DNA construct according to claim 2, wherein the nucleotide sequence of interest is a coding sequence.

4. The recombinant DNA construct according to claim 3, wherein the nucleotide sequence of interest encodes a digestive enzyme.

5. The recombinant DNA construct according to claim 4, wherein the digestive enzyme is selected from the group consisting of a phytases, a cellulases, a glucanase and a xylanase.

6. The recombinant DNA construct according to claim 2, comprising the nucleotide sequence of SEQ ID NO: 2.

7. A method for producing a transgenic pig that exhibits enhanced digestion of cellulose, comprising the steps of (a) providing a pig embryo, (b) introducing into the pig embryo by pronuclear microinjection a transgene including a promoter comprising the nucleotide sequence of SEQ ID NO:1 operatively linked to the nucleotide sequence of SEQ ID NO:2, (c) inserting the pig embryo into a pseudopregnant pig, (d) allowing the pig embryo to develop to term, thereby producing a transgenic pig, and (e) identifying the transgenic pig from step (d) whose genome comprises the transgene.

8. A transgenic pig whose genome comprises a transgene including a promoter comprising the nucleotide sequence of SEQ ID NO:1 operatively linked to the nucleotide sequence of SEQ ID NO:2, wherein the transgenic pig exhibits enhanced digestion of cellulose.

9. The recombinant DNA construct according to claim 6, further comprising the nucleotide sequence of SEQ ID NO: 4.

10. The method according to claim 7, wherein the transgene further comprises the nucleotide sequence of SEQ ID NO:4.

11. The transgenic pig according to claim 8, wherein the transgene further comprises the nucleotide sequence of SEQ ID NO: 4.

* * * * *

# 中華民國專利證書

發明第 I 350851 號

發明名稱：新穎之豬胰澱粉酶基因啟動子及製備表現異源性消化酵素之基因轉殖動物之方法

專利權人：國立臺灣大學

發明人：鄭登貴、吳信志、徐啟真、林育聖、林之任、徐濟泰、鄭國展

專利權期間：自2011年10月21日至2026年 5 月16日止

上開發明業經專利權人依專利法之規定取得專利權

經濟部智慧財產局

局長 王美花

中華民國 100 年 10 月 21 日

注意：專利權人未依法繳納年費者，其專利

# 發明專利說明書

※申請案號：095117405　　※IPC 分類：C12N 15/09

## 一、發明名稱：

**新穎之豬胰澱粉酶基因啟動子及製備表現異源性消化酵素之基因轉殖動物之方法**

NOVEL PORCINE PANCREATIC AMYLASE GENE PROMOTER AND METHOD FOR PREPARING TRANSGENIC ANIMALS EXPRESSING HETEROLOGOUS DIGESTIVE ENZYMES

## 二、中文發明摘要：

本發明係關於新穎之啟動子及其驅動基因轉殖動物(特別是豬)中外源基因表現之用途。因此，本發明提供一種用於產製基因轉殖動物之方法，該基因轉殖動物帶有受本發明啟動子調節之異源性基因。

## 三、英文發明摘要：

The present invention relates to a novel promoter and its use in driving expression of foreign genes in transgenic animals (especially pigs). Accordingly, the present invention provides a method for producing transgenic animals harboring heterologous genes regulated by the promoter of the present invention.

## 四、指定代表圖：

(一)本案指定代表圖為： 第（　　）圖

(二)本代表圖之元件符號簡單說明：

本案若有化學式時，請揭示最能顯示發明特

徵的化學式：

## 六、 發明說明：

【發明所屬之技術領域】

[0001] 本發明大體上係關於異源性蛋白質在動物中之表現及基因轉殖動物之產製。

【先前技術】

[0002] 台灣畜牧業佔農業生產的第一位，其為台灣農民帶來優渥且穩定的收入。然而，家畜所產生之大量排泄物並未妥善處理，因而導致環境污染。根據台灣行政院農業委員會的統計資料，2002 年底豬隻在養頭數為 679.4 萬頭。假設每頭豬每天產生 6 公斤的排泄物，則每年將產生將近 1500 萬公噸的豬排泄物，加上家禽之排泄物。如此大量的排泄物所造成的環境污染已成為台灣畜牧業的主要問題。如何降低排泄物的產生又不影響農民收益是一個重要課題。

[0003] 除了上述環境議題外，自台灣於 2002 年加入世界貿易組織(WTO)後，台灣畜牧業必須與進口畜產品競爭。如何降低生產成本同時提高生產效率已成為另一重要課題。一般而言，飼料費用佔總生產成本的 60 至 70%。因此，降低生產成本的關鍵在於改善飼料利用率。有效的方式包括利用生物技術發產飼料添加劑以增進飼料消化率，以及培育可分解纖維素之家畜以減少排泄量。

[0004] 動物排泄物之質與量取決於該動物對飼料中各組成份之消化及利用率。一般而言，家畜本身不具有合成纖維素酶(cellulase)及植酸酶(phytase)等消化酵素之能力，因而不能有效消化飼料中之纖維素及植物性磷。此等無能力影響了 飼料利用率並造成飼料的浪費。此外，未消化之營養分由動物排出體外而成為環境污染源。為謀增進動物對飼料中營養分之利用率，一常見之方法係將微生物所量產之特定消化酵素添加於飼料中。近來已自真菌及細菌的 cDNA 庫中篩選出若干消化酵素之 cDNA，並以大腸桿菌量產該等酵素(Ye *et al.*,2001,Inter.J.Biochem.Biol.33 : 87-94 ; Zhang *et*

*al.*,1998,Biotechnol.Lett.20：1001-1005；以參考方式併入本文)。然而此方法生產成本昂貴，且所補充之消化酵素在進入動物的消化道後，常因遭受既有之消化酵素或胃酸破壞而無法進行其作用。

[0005] 自 80 年代以來，基因轉殖動物的產生不僅使吾人能夠在活體內研究基因功能，更提供了改良家畜基因遺傳之新途徑。在傳統育種方法中，往往需花費數十年或甚至百年以上方能改良家畜之基因遺傳。然而，利用基因轉型技術即可在單一世代中達到相同的改良。在 1985 年時，Hammer 等人首先發表了其於基因轉殖豬、綿羊與家兔之研究結果(Hammer *et al.*,1985,Nature 315：680-683；以參考方式併入本文)。其後，研究者們應用源自各種動物(包括牛、鼠、與人類)之生長素基因產生帶有異源性生長素基因之轉殖豬，冀能增加豬之瘦肉率並縮短其飼養期間(Pursel and Rexroad,1993,J.Anim.Sci.71：10-19；以參考方式併入本文)。例如，Pursel 等人將數種異源性生長素基因構築於小鼠重金屬硫蛋白-I(metallothionein-I)啟動子的控制下(Pursel *et al.*,1989,Science 244：1281-1288；以參考方式併入本文)，因此該異源 性基因之轉錄可藉由在飼料中添加微量之金屬鋅而有效予以誘發及調節。

[0006] 如業界所知，操縱動物(如豬)以改變及/或改良其表型特徵(如生產力及品質)需於動物組織中表現異源性基因。此種基因操縱因之取決於驅動及控制所需基因表現之手段，例如適當啟動子之可得性及使用，該等啟動子對動物有效且可調節基因表現以於基因轉殖動物中提供所求效果。較佳係有各種不同的啟動子供選擇，俾針對特定基因、構築體、細胞、組織、動物或環境選出最適合的啟動子。

[0007] 目前業界對大量表現啟動子，及表現模式由空間界定之啟動子仍有持續的需求。

【發明內容】

[0008] 在本發明中，一新穎之豬胰澱粉酶基因啟動子可作用地連接至一真菌纖維素酶基因或一細菌植酸酶基因以形成一表現卡匣(expression cassette)，並將之應用於產生基因轉殖小鼠及豬。

[0009] 因此，就一方面而言，本發明提供一種單離之豬胰澱粉酶基因啟動子。在一較佳具體實例中，該啟動子具有序列識別編號：1 中所載核苷酸序列。

[0010] 就另一方面而言，本發明提供一種重組 DNA 構築體，包含本發明之豬胰澱粉酶基因啟動子可作用地連接至一標的核苷酸序列。在一較佳具體實例中，該標的核苷酸序列編碼一消化酵素如植酸酶、纖維素酶、葡聚糖酶(glucanase)及木聚糖酶(xylanase)。

[0011] 就再一方面而言，本發明提供一種產製基因轉殖動物之方法，包含下列步驟：將一轉殖基因導入一動物胚胎中，該轉殖基因包含本發明之豬胰澱粉酶基因啟動子可作用地連接至一標的核苷酸序列，並藉此以該轉殖基因轉型該胚胎。

[0012] 就更另一方面而言，本發明提供一種基因轉殖動物，其基因組包含一轉殖基因，該轉殖基因包含一異源性核苷酸序列可作用地連接至本發明之啟動子。在一較佳具體實例中，該基因轉殖動物為豬。

【實施方式】

[0013] 如前所述，本發明係針對豬胰澱粉酶基因啟動子之新穎啟動子序列，及其用於表現重組基因及產製基因轉殖動物之用途。本發明之啟動子可在基因轉殖動物體內於特定空間及時間表現轉殖基因。

[0014] 依據本發明之一具體實例，係提供一種單離之豬胰澱粉酶基因啟動子。

[0015] 本文中所使用之「單離(的/之)」乙詞係形容一物質(如核酸)，其實質上或基本上不含一般在其自然發生環境中伴隨之或與之相互作用的成分。該單離之物質選擇性地包含在其自然環境中非共同存在之物質。

[0016] 本文中所使用之「啟動子」乙詞係指位於編碼區域上游的一段未轉譯 DNA 序列，其包含 RNA 聚合酶之結合位點並起始該編碼區域之轉錄。該啟動子區域亦可包含作為基因表現之調節子之其他元件。

[0017] 在一較佳具體實例中，本發明之啟動子具有以下序列：

CTGACATAAGCTGAACCAATGCCTTGCATAATACCTGCA
ATTTAGAGTCTATAAGTAAAAACCACTTATTGATCACA
TGAGCCATCGTGCTGTTTTTTTGCTAGGAATATTAACT
ATGAAATCTGCTCTTAATAAGGTTTATCCAGAATGACA
GTCATGTAAATCCTTATTTTTTATAACATTAATCCAAT
ATCACTTAATAACAACCCGGAGGTTAAAACCTGCCATAC
AGAGGAGTACATAACTATGGCTGGGAATATCAATATA
AGTTTCATAAAGGTATTTTTCCAACTGCATATGAAAGT
AGGAGTAGTTACTAGCTATTGAAGGGTGATACAAGAA
AGAAGAAAAGCCCTGGAAAGTCATGAAAGAATAAAAT
TGCTTGTCAAATACGCAAAATGTTTATTTTTTGCGGGA
GATGGATATTGGGGACTCTGCACTTGTGTTCCGCCCCTC
TAACAATTTGAAATATTGAACTCCTAACTCCCAATGGT
ATGCGATTAGGCTGTGGGGTCTTTGGGAACAACTTAGG
TCAAAGTGACATCATGAGAGTGGAGGCCCCATGATGGG
TTAGTGTCCTTGTACGAAGAGAAAGAGAATCAGGATCT
CTGAGCTCCACACTCCGTGAGGATACAAGAAGCTTGCTG
TCTGTGAACATGGAATGGGGCTTTCGCAAGACACTGGA
GCTGCTGATAGTGTAGTCTTGGGTTTCCCAGCCTCTAGA
AATGTGAGAAAGAAATATTTGTGCTAAGCCATCCAGCC
TATATGGCATTCTTGTTACAGCAGCTGGAACTGAATGA
GAAAAATAGGACACGGAGTATGTTCACGATGTGGGCTG
GAGGAGGGACCGAAGGAGAGTGTTGGGATTCACAGAGT
GCTCTCGGACCCCCTCCACAAAGCTAGTACTTCCTCACTT
TTCCTCATCTTAGTAAATGGTGTCATCAGATACCTGTTT
CCTCAATTTTTCTCTTTCCCCCAGTCTTCGGTGCTAATCT
ATCGATAAACCGATTGCTTCGCCACCTCTGAGATATATT
CTATCAGGGCCCTAGAGCAGCCACTTTCCTCTTTCGTGG
ACCACTACAAAAGCCTACCTGATCTCTTGGCCCCCAGTCG
TGTCCTCCTATAATCCGGTTTTTCACAGCAGAGCAAGAA
TGGTTTTCTTGGAAAGGAAATCAGAATCTCTTCACTCA
TCTTCTTTCAGCCTCAAAAGCCCTCTCTTTCCTTATGTTC
TACAAGGTTCTACATGATCTGGCCTACCTCTCTGATTTC
ATCTCATTTTACTCTTCCCTTTGTCACTCACACATGTTT

AGCTGCACTGATGTTGAAAGTTTGTTCAGTGTCACTTG
AGTATCCCACGGTTGTTCCTACCTTGGGCTTTTGCTATT
GCACTTTCCTCTATGGAGACTGCTTTTCCTCTGATCTTC
AAATAAGTGGGTCCTTCTACTCCTTCCAGTTCTGGCTGA
CAATCACTCCCTCTGAAACAGCTTTCCTGACTATTTCCA
GTCTAAAATATCCTGAAAAATTCAGTCCTTTTCCCTTT
AACTGCACCGTGGGTTCATGCTAGTTCTCACTGCTCTCT
TTAACTTAGTATCGTTGTTGTTATCATTCCATCTTGCT
ATATTTTCCTTACCTTCCCCTAGAATGTAGGCTGAGAAC
AAGAGTCTTGTCTGTCTTGTTCATCCTTGTATCCTGAGT
ATCATGCCGGCATTTAGCAAAAGCACTCGGCCACTACCT
GTTGGATGAATGGATTAGGTTTTTCCCACCTGTACGGT
TATGTCTTTACTAGGATTTCTTGTACCTTACGAAGGAA
AATAGATGTGGATTCATTAACTTAGTGTTTTAGCACAT
ATAAGGGACTTTTTGCTAGAAGGAGAAAAAAAAAAAGT
CCATTCTTTCCTGCTACAGCCAGTGCATTTTCACATGCG
TTAATGTAAGCGTGGGGAAAAAAAAATCTGACACCTA
AAGTCGTGGTCATTTCACTTCCGGATAACTTCCTAAATC
TTAGTGGAGAATCTCAAGTATCTAACAACTGGGGTAGG
AGGTACCAACTGAACTGAGTTGAATAACATGTGTCTTC
TTACAATGGAAACATTGCACGTGTTTACAGACAGTTAG
GGCACCATTGTGACTGTGAATTCAGTTGGCTCTAATTCC
GCCTCTGTCAGTGAAGGACTTCAGAAATAAAATCTAAT
CCTACCTAAACAATACATGATTAAGACCTTTCTGTAGA
TAACATGCCAGATGTTTCAAAACTTGCTGTTCCCTCAGT
AAGGAAAACATTGTCTGAGAAGGTCATTTAGATAGTA
TTCCTGGGAGATTTTCGGGATGTTCCTCACCTGTTTAGT
GTAATTATCAATAGTTATTTTTGGAGTATGCATTCACG
GTTTGTGCTCTAAGTATTTATTCATGTCAATATTTGCT
TTGTAAAATATGCTTCTTGCAGGATTATAAATACTTGC
CGGGAAGACCGTTGACAACCTCAGAGCAAAATGAAGTT
GTTTCTGCTGCTTTCAGCCATTGGGTTCTGCTGGGCC
(序列識別編號：1)

[0018] 依據本發明之一具體實例，係提供一種重組 DNA 構築體，

包含本發明之豬胰澱粉酶基因啟動子可作用地連接至一標的核苷酸序列。

[0019] 在一較佳具體實例中，該重組 DNA 構築體為一表現載體，包含一表現卡匣。本文中所使用之「表現卡匣」乙詞係指以重組方式或合成方式產生之核酸構築體，含有一系列之特定核酸元件俾使標的核苷酸序列得以於宿主細胞中轉錄。表現卡匣可併入質體、染色體、粒線體 DNA、原漿質 DNA、病毒或核酸片段中。典型上，一表現載體之表現卡匣部分包含一欲轉錄之核苷酸序列及一啟動子，及其他序列。在本發 明中，當用於產製基因轉殖動物時，表現卡匣亦可稱為「轉殖基因」。

[0020] 本文中所使用之「可作用地連接」乙詞係指啟動子與第二序列間之功能性連接，其中該啟動子序列起始並媒介該第二序列之轉錄。一般而言「可作用地連接」意指被連接之核酸序列係連續的，且當需要連接兩段蛋白質編碼區域時，其為連續且位於相同的編閱架構(reading frame)中。

[0021] 依據本發明，受啟動子控制之標的核苷酸序列可為任何核酸形式。對應而言其可為編碼核酸或結構性或功能性核酸。「編碼核酸」乙詞據瞭解係較特定地指編碼一肽或蛋白質之核酸。該肽/蛋白質可為例如結構性蛋白質，或具有酵素活性之肽/蛋白質。結構性核酸據瞭解係較特定地指導致複合物形成(特別是與其他分子)之核酸。其尤其可為一 rRNA 及特別是反義核酸。功能性核酸據瞭解係較特定地指對一系統(特別是生物系統)行使特定動作之核酸。該特定動作可為例如協助或抑制轉譯或轉錄。功能性核酸之一實例為反義核酸。

[0022] 在一較佳具體實例中，受啟動子控制之標的核苷酸序列為一編碼核酸。更佳者，該編碼核酸編碼一消化酵素如植酸酶、纖維素酶、葡聚糖酶及木聚糖酶。

[0023] 依據本發明之又一具體實例，係提供一種產製基因轉殖動物之方法，包含下列步驟：將一轉殖基因導入一動物胚胎中，該轉殖基因包含本發明之豬胰澱粉酶基因啟動子可作用地連接至一標的核苷酸序列，並藉此以該轉殖基因轉型該胚 胎。

[0024] 依據本發明，該基因轉殖動物較佳為不包含人類之單胃動

物，更佳為哺乳動物。在一較佳具體實例中，本發明之基因轉殖動物為豬或小鼠。

[0025] 本發明基因轉殖動物之產生係藉由業界熟知之技術以傳統方式進行。將遺傳物質(如轉殖基因)導入欲轉型動物體內之方法不只一種，包括病毒感染技術、精子媒介基因轉移(SMGT)技術、胚胎幹細胞技術、核移置技術、及原核顯微注射技術。其中最常用的技術為原核顯微注射技術，其包含將轉殖基因直接注射入受精卵之雄原核中，造成各種拷貝數隨機嵌插入一個位置(通常係以頭尾相接陣列的形式)。然後將經注射之卵再次移置入偽懷孕受胚母體之子宮中。所得子代中某些可能有一或數個拷貝的轉殖基因嵌插在其基因組中(通常在同一嵌插位置)。原核顯微注射技術的一個優點為轉殖基因可穩定嵌插於基因轉殖動物之生殖細胞系中，因而可傳承予其後代。

[0026] 依據本發明，轉殖基因較佳藉由原核顯微注射導入胚胎中。

[0027] 較佳者，在將轉殖基因導入胚胎前先以限制酵素切割(例如利用載體中轉殖基因兩側之限制位點)將之由載體部分分離。用於選殖時，通常在導入胚胎前先將轉殖基因插入一表現載體中，如哺乳動物表現載體 pCR®3.1(Invitrogen Corporation)。一般而言係藉由業界已知方法，將轉殖基因 DNA 序列插入適當之限制核酸內切酶位點。該等方法及其他 資訊應屬熟習本技藝者所知範圍。

[0028] 依據本發明之又另一具體實例，係提供一種基因轉殖動物，其基因組包含一轉殖基因，該轉殖基因包含一異源性核苷酸序列可作用地連接至本發明之啟動子。在一較佳具體實例中，該基因轉殖動物為豬。

[0029] 本文中所使用之「異源性」乙詞係用於形容一核酸為源自外來物種之核酸，或者在源自相同物種的情況下，因蓄意人為介入，而在組成及/或基因組位置方面將其自然形式實質地加工。異源性蛋白質可源自外來物種，或者在源自相同物種的情況下，因蓄意人為介入而將其自然形式實質地加工。

[0030] 較佳者，該基因轉殖動物的產生係以原核顯微注射將轉殖基因導入胚胎中，將該胚胎植入代理孕母體內，並使胚胎發育直至出生。

[0031] 本發明以下列實例進一步闡明，其係供示範目的而非限制。

[0032] Darnis 等人已由一以單離自豬胰臟組織之 mRNA 所建構之 cDNA 庫獲得豬胰 α-澱粉酶基因之近全長 cDNA 序列(GenBank 存取編號 AF064742)(Darnis *et al.*,1999,Biochem Biophys Acta.1430：281-289；以參考方式併入本文)。該 cDNA 序列長度為 1,536 bp，編碼一 496 個胺基酸之蛋白質，其具有 15 個胺基酸之訊息肽。基於該 cDNA 序列，利用 GenomeWalker™ 套組 (Clontech Laboratories,Inc.,Mountain View,CA,USA)依據製造商之說明選殖啟動子。

[0033] 首先，將由豬血中單離之基因組 DNA 以套組中提供之四種限制酵素(*Dra* I、*EcoR* V、*Pvu* II 及 *Stu* I)於 37℃下截切。16 小時後，以乙醇沉澱經截切之基因組 DNA 並使其回溶。然後於 16℃下，使該回溶之經截切基因組 DNA 與套組中提供之轉接子(adaptor)進行 16 小時之接合反應。

[0034] 除了套組中提供之兩個「轉接子引子」(AP1 及 AP2)外，另基於豬胰澱粉酶基因 5’端之序列設計三個引子 GSP1、GSP2 及 GSP3(參見表 1)。第一次 PCR 的進行係使用如上述所得之經截切基因組 DNA 作為模板及 GSP3 及 AP1 作為引子。第二次 PCR 的進行係使用第一次 PCR 的產物作為模板及 GSP2 及 AP2 作為引子。以凝膠電泳篩選第二次 PCR 的產物，由凝膠回收可能的片段(亦即大於 1kb 者)並純化之。

[0035] 將所選擇的片段選殖入 pGEM®-T 簡易載體(Promega Corporation,Madison,WI,USA)中，所形成之載體命名為 pGEM-T AMY。將選殖株定序並挑選出編號為 412 及 1216 的兩株。

[0036]

**表 1 用於基因組移行之引子**

| 引子 | 序列 | Tm 值 (℃) |
|---|---|---|
| AP1 | GTAATACGACTCACTATAGGGC（序列識別編號：6） | 59 |
| AP2 | ACTATAGGGCACGCGTGGT（序列識別編號：7） | 71 |
| GSP1 | GCTGAAAGCAGCAGAAACTTCAT（序列識別編號：8） | 60.4 |
| GSP2 | ATGAACAATAGACGTTCGTCCAGACT（序列識別編號：9） | 59.7 |
| GSP3 | TACCGCTCACATTCAAGAGCAATGTCA（序列識別編號：10） | 62.3 |

[0037] 為測試實例 1 中選殖之啟動子的活性，設計新的引子(1216AMY-GFP-NotI-5’及 1216AMY-GFP-EcoRV-3’)(參見表 2)俾用 PCR 於啟動子之 5’端創造一 *Not* I 限制位點，及於 3’端創造一 *EcoR* V 限制位點。重新將 PCR 產物選殖入 pGEM®-T 簡易載體中，並以相關限制酵素截切。將經截切之片段選殖入 phrGFP 載體(Stratagene,La Jolla,CA,USA)中並將選殖株定序，獲得 412 pAMY-phrGFP 及 1216 pAMY-phrGFP 兩個選殖株。

[0038] 大鼠胰臟腫瘤細胞株 AR-42J(BCRC 60160)係購自食品工業發展研究所之生物資源保存及研究中心(新竹，台灣)。將細胞培養於六孔培養盤上，各孔內含 $7\times10^4$ 個細胞。將培養之細胞以 Lipofectamine(Invitrogen Corporation,Carlsbad,CA,USA)及 412 pAMY-phrGFP 或 1216 pAMY-phrGFP 載體轉染。於 37℃下培養 24 小時後，使用螢光顯微鏡觀察綠色螢光(圖 1)。該等結果顯示所選殖之啟動子能夠在胰臟細胞中驅動異源性基因(此處為綠色螢光蛋白之編碼序列)之表現。

[0039] **表 2 用於構築 1216pAMY-phrGFP、1216pAMY-CEL 及 pAMY-PHY 之引子**

| 引子 | 序列 | Tm 值 (℃) |
|---|---|---|
| 1216AMY-GFP-NotI-5’ | GCGGCCGCCTGACATAAGCTGAA（序列識別編號：11） | 79.5 |
| 1216AMY-GFP-EcoRV-3’ | GATATCGGCCCAGCAGAACCCAA（序列識別編號：12） | 76.4 |
| 1216AMY-NheI-5’ | GCTAGCCTGACATAAGCTGAACCAA（序列識別編號：13） | 71.8 |
| 1216AMY-BamHI-3’ | GGATCCGGCCCAGCAGAACCCAA（序列識別編號：14） | 82.0 |
| CEL-BamHI-5’ | GGATCCATTATGAAACCCGAACCA（序列識別編號：15） | 69.7 |
| CEL-XhoI-3’ | CTCGAGTTATTCCTTTGGTTTTTC（序列識別編號：16） | 66.6 |
| PHY-BamHI-5’ | GGATCCCAGAGTGAGCCGGAGCT（序列識別編號：17） | 74.1 |
| PHY-XhoI-3’ | CTCGAGTTACAAACTGCACGCCGGTA（序列識別編號：18） | 78.1 |

[0040] 使用源自 *Neocallimastix patriciarum* 之纖維素酶基因(GenBank 存取編號 AF053363)及源自一單離自牛糞之大腸桿菌株(ATCC 33965)之植酸酶基因(GenBank 存取編號 AF537219)構築轉殖基因。兩個基因之 cDNA 皆由中央研究院生物農業科學研究所(台北，台灣)鄭國展博士所提供。首先以 PCR 擴增兩個基因之 cDNA，使用引子對 CEL-BamHI-5’+CEL-XhoI-3’(用於纖維素酶基因)及 PHY-BamHI5’+PHY-XhoI-3’(用於植酸酶基因)(參見表 2)，並選殖入

pGEM®-T 簡易載體中。於實例 1 中選殖之啟動子亦以 PCR 使用引子對1216AMY-NheI-5’ +1216AMY-BamHI-3’(參見表 2)予以擴增，且亦將 PCR 產物選殖入 pGEM®-T 簡易載體中。

[0041] 將啟動子(以 *Nhe*I 及 *BamH*I 截切)及纖維素酶或植酸酶基因(以 *BamH*I 及 *Xho*I 截切)選殖入哺乳動物表現載體 pCR®3.1(Invitrogen Corporation)中，以形成載體 pAMY-CEL 或 pAMY-PHY。量產該等載體後，以限制酵素截切將含有啟動子、訊息肽、結構性基因及 BGH 聚 A 尾端之轉殖基因(序列識別編號：3 及 4)由載體切出。切出之轉殖基因以 QIAquick 凝膠萃取套組(Qiagen Inc.,Valencia,CA,USA)回收兩次， 並於 100,000 rpm 進行 6 小時之 CsCl 密度梯度離心(CsCl，1 gm/ml；EthBr，24 μl；DNA，100-200 μg；0.02% Triton-X100)。離心後於 UV 光下取出含有線性 DNA 之亮帶，並與飽和丁醇水溶液混合以移除 EtBr。然後以 TE 緩衝液(pH 8.0)將 DNA 透析 24 小時俾移除 CsCl，以乙醇沉澱之，並回溶於 TE 緩衝液(pH 7.4)中。以凝膠電泳及分光光度計測定 DNA 之濃度及品質後，將 DNA 稀釋至 3 ng/μl 並貯存於-20℃下。

[0042] 用作胚胎接受者及供應者之 ICR 母小鼠係購自國立台灣大學動物中心(台北，台灣)。本實驗係以 6 至 7 週齡之性成熟母小鼠進行。

[0043] 由超級排卵母小鼠之輸卵管中沖洗回收原核階段之受精小鼠胚胎。將 10 至 20 個欲顯微注射之胚胎置於凹槽玻片中央之一小滴 M2 緩衝液中。以重級礦物油覆蓋該小滴以防止緩衝液蒸發。使用分光干擾倒立顯微鏡(Axiovert 10，Zeiss，德國)與 Narashigi NT-8 顯微操作器(Narashigi，日本)進行顯微注射。固定胚胎用之固定針管及注射 DNA 用之注射針管係如 Wu *et al.*,1995,J.Chin.Soc.Anim.sci.24：181-189(以參考方式併入本文)中所述般製備。將約 2 pl 之 DNA 溶液(含 3 至 5 ng/μl 實例 3 中製備之轉殖基因構築體)注射入各胚胎之雄原核中。

[0044] 由外觀形態判斷經顯微注射過程後仍存活之胚胎(亦即保有緻密卵黃質及完整外觀者)，並將之植入受胚母體(其已藉由與切除輸精管之雄性交配而造成假懷孕)中。每隻假懷 孕雌性之兩側輸卵管內各置入約 10 至 20 個經顯微注射之小鼠胚胎。使胚胎發育直至出生，如下述般以 PCR 分析新生小鼠是

否帶有轉殖基因。

[0045] 在本研究中，共對 267 個 ICR 小鼠胚胎顯微注射轉殖基因，將其中 245 個存活胚胎植入受胚母小鼠之輸卵管中(胚胎經顯微注射之損失率為 8.2%)。共 63 隻仔小鼠出生，表示小鼠胚胎之存活率為 25.7%。

[0046] 於實例 4 所得之 63 隻可能的基因轉殖仔小鼠中，只有 57 隻成功成長至離乳年齡。使用取自該 57 隻存活仔鼠尾部之組織樣本，藉由下列步驟獲取基因組 DNA。

[0047] 將組織樣本切成小塊，並與含 630 μl 組織溶離緩衝液(100 mM Tris-HCl(pH8.5)、5 mM EDTA、200 mM NaCl)、70 μl 10% SDS 及 35 μl 100 mg/ml 蛋白酶 K 之溶液混合。將混合物置於 55℃ 水浴中反應 12 至 16 小時，然後於 12,000 x rpm 離心 5 分鐘。離心所得上清液以酚/氯仿(1：1)萃取兩次，然後再次於 12,000 x rpm 離心 5 分鐘以分成二層。以無水乙醇由上層沉澱 DNA，以 70% 乙醇清洗之，並回溶於滅菌二次水中。

[0048] 針對上述所得基因組 DNA 樣本之 PCR 反應係以傳統方式進行。基於豬胰澱粉酶基因啟動子、啟動子與結構性基因間之連接點、結構性基因及 BGH 聚 A 尾端之序列設計引子對(表 3)。為了初步檢測可能的基因轉殖小鼠是否帶有所求轉殖基因，使用引子對 1216-pF+CEL-R(用於纖維素酶基因)或 1216-pF+PHY-R(用於植酸酶基因)對基因組 DNA 樣本進行 PCR(反應體積=20 μl)。對纖維素酶基因之反應條件為：(1)94℃，2 分鐘；(2)94℃，30 秒；65℃，30 秒；及 72℃，2 分鐘；35 個循環；(3)72℃，7 分鐘；及(4)4℃，終止反應。對植酸酶基因之反應條件為：(1)94℃，2 分鐘；(2)94℃，30 秒；67℃，30 秒；及 72℃，2 分鐘；35 個循環；(3)72℃，7 分鐘；及(4)4℃，終止反應。

[0049] PCR 分析之結果示於圖 2 及 3。如該等結果所示，第 4、29、33 及 48 號仔鼠帶有纖維素酶轉殖基因，而第 1、20、29、33、47 及 48 號仔鼠帶有植酸酶轉殖基因，表示第 29、33 及 48 號仔鼠帶有雙轉殖基因。轉殖基因的身分進一步使用引子對 1216C-jF+1216C-jR 或 1216P-jF+1216P-jR 以 PCR 確認。

[0050]

表 3　用於基因轉殖動物 DNA 分析之引子

| 引子 | 序列 | Tm 值 (℃) | PCR 產物 |
|---|---|---|---|
| 1216-pF | AGGGACCGAAGGAGAGTGTT（序列識別編號：19） | 64.1 | 314 bp |
| 1216-pR | ACCATTCTTGCTCTGCTGTGA（序列識別編號：20） | 64.6 | |
| 1216C-jF | ACCGTTGACAACCTCAGAGCA（序列識別編號：21） | 66.8 | 404 bp |
| 1216C-jR | TCGTTCACCAAAGTGTCCAGA（序列識別編號：22） | 65.2 | |
| 1216C-aF | CATTGTTGCAGCCTTACAA（序列識別編號：23） | 59.0 | 262 bp |
| 1216C-aR | ACTCAGACAATGCGATGCA（序列識別編號：24） | 60.8 | |
| 1216P-jF | ACCGTTGACAACCTCAGAGCAA（序列識別編號：25） | 68.8 | 350 bp |
| 1216P-jR | ACGCTCGTCGACATCAGCAATA（序列識別編號：26） | 70.0 | |
| 1216P-aF | TCGGCTAAGCGATAACAG（序列識別編號：27） | 58.4 | 291 bp |
| 1216P-aR | ACAACAGATGGCTGGCAACT（序列識別編號：28） | 64.1 | |
| CEL-R | GGATCCTTATTCCTTTGGTTTTTC（序列識別編號：29） | 53.8 | |
| PHY-R | GGATCCTTACAAACTGCACGCCGG（序列識別編號：30） | 64.5 | |
| CEL-Hyb-3' | TCCGTTCCATTCAACTGGTG（序列識別編號：31） | 65.2 | |
| PHY-Hyb-3' | TCAGTCACGTTCGCGTTATCT（序列識別編號：32） | 65.0 | |

[0051] 本研究中使用性成熟之雜交新母豬(六月齡以上)作為胚胎供應者及接受者。對超級排卵之供胚新母豬進行人工授精，並以外科手術由其輸卵管回收受精胚胎。將胚胎於 25℃下於 12,000 x g 離心 10 分鐘俾顯現出原核(豬胚胎的細胞質因脂質而不透明，在離心前看不見原核)。

[0052] 如以上實例 4 中所述般進行胚胎之顯微注射。由外觀形態判斷經顯微注射程序後仍存活之胚胎，並將之植入發情期之受胚新母豬中。每隻受胚新母豬之兩側輸卵管內各置入約 10 至 20 個經顯微注射之豬胚胎。使胚胎發育直至出生，如下述般以 PCR 及南方吸漬法分析新生小豬是否帶有轉殖基因。

[0053] 在本研究中，共對 145 個豬胚胎顯微注射轉殖基因，將其中 138 個存活胚胎植入 6 隻受胚新母豬之輸卵管中。在 6 隻接受者中，有 2 隻懷孕並生下共 13 隻小豬。

[0054] 藉由與實例 5 中所述相同程序，由 13 隻可能的基因轉殖小豬耳朵取得組織樣本而得到基因組 DNA。使用下列引子對對基因組 DNA 樣本進行 PCR：1216-pF+1216C-aR(用於全長纖維

素酶轉殖基因)、1216-pF+1216P-aR(用於全長植酸酶轉殖基因)、1216C-jF+1216C-jR(用於啟動子-纖維素酶連接點)及1216P-jF+1216P-jR(用於啟動子-植酸酶連接點)(參見表3)。

[0055] PCR 分析之結果示於圖 4 及 5。如該等結果所示。第 29-3、29-7、29-8、13-2 及 13-5 號小豬帶有纖維素酶轉殖基因，而第 29-7 及 19-8 號小豬帶有植酸酶轉殖基因，表示第 29-7 及 19-8 號小豬帶有雙轉殖基因。在 5 隻基因轉殖小豬中，第 13-2 號小豬在出生 10 天後死於細菌感染。

[0056] 對 5 隻基因轉殖小豬之基因組 DNA 進行南方吸漬分析，俾瞭解轉殖基因的嵌插程度，使用引子對 1216C-aF+1216C-aR、1216P-aF+1216P-aR 、 CEL-BamHI-5 ’ +CEL-Hyb-3 ’ 及 PHY-BamHI-5’+PHY-Hyb-3’(參見表 2 及 3)之 PCR 產物作為探針。首先將回收及純化之 PCR 產物於沸水中變性並置於冰上。以 Rediprime™ II DNA 標定系統(GE Healthcare UK Ltd,UK)依據製造商之說明對 PCR 產物進行放射性標定。南方吸漬法基本上係如 Koetsier *et al.*,1993,Biotechniques 15(2)：260-2(以參考方式併入本文)中所述般進行。所用程序略述如下。

[0057] 對經相關限制酵素截切之5隻基因轉殖小豬基因組DNA進行0.8%洋菜膠電泳(50V，4 至 6 小時)，並以 0.25 N HCl 進行 15 分鐘之去嘌呤反應。當溴酚藍染劑轉為黃色後，將膠體浸泡於含 1.5 M NaCl 及 0.4 N NaOH 之變性溶液中。經 2.5 至 4 小時之轉漬後，將帶有 DNA 漬跡之尼龍膜(Hybond™-N⁺,GE Healthcare UK Ltd)置於 2 x SSC 溶液中以中和反應。前雜交反應係於 66℃下將膜浸泡於一前雜交溶液(2 x SSC、1% SDS、0.5%脫脂奶粉及 0.75 mg/ml 變性鮭魚精子 DNA)中 2 至 5 小時。雜交反應係將膜移至含放射性標定探針之雜交溶液(2 x SSC、1% SDS、0.5%脫脂奶粉、0.5 mg/ml 變性鮭魚精子 DNA 及 10%葡聚糖硫酸鹽)中，並於 66℃下浸泡 16 至 24 小時。雜交反應後，於 55℃下以一含 0.1% SDS 及 0.1 x SSC 之溶液將膜清洗 20 分鐘兩次。最後，將膜風乾並以生物影像分析器(BAS-1500，FUJIFILM，日本)分析之。

[0058] 南方吸漬分析之結果示於圖 6 至 9 中。在圖 6 中，豬基因組 DNA 係經 *Afl* II 截切且探針為引子對 1216C-aF+1216C-aR 之

PCR 產物。該影像顯示 5 隻基因轉殖小豬全都帶有纖維素酶轉殖基因，但第 13-2 及 29-8 號小豬之較強雜交訊號顯示該兩者之嵌插拷貝數可能較大。在圖 7 中，豬基因組 DNA 係經 *Hind* III 及 *Hae* II 截切且探針為引子對 CEL-BamHI-5’+CEL-Hyb-3’之 PCR 產物。該影像顯示除第 13-2 號小豬(其基因組 DNA 受損)外，其他四隻小豬均具有與正對照組相同之 3,726-kb 片段，表示其均帶有不同嵌插程度之纖維素酶轉殖基因。在圖 8 中，豬基因組 DNA 係經 *Drd* I 截切且探針為引子對 1216P-aF+1216P-aR 之 PCR 產物。該影像顯示第 29-8 號小豬帶有植酸酶轉殖基因，但第 29-7 號小豬之限制酵素截切似乎不足。在圖 9 中，豬基因組 DNA 係經 *Hind* III 及 *ApaL* I 雙截切且探針為引子對 PHY-BamHI-5’+PHY-Hyb-3’之 PCR 產物。如該影像所示，只有第 29-8 號小豬具有與正對照組相同之 3,892-kb 片段。然而第 29-7 號小豬在接近 8 kb 的位置具有一片段，相信其代表未截切之植酸酶轉殖基因。

[0059] 熟習本技藝者當瞭解可針對上述具體實例加以改變而不背離其廣泛之發明概念。因此，應瞭解本發明並不限於所揭示之特定具體實例，而是意欲涵蓋本發明(其係以後附申請專利範圍界定)精神及範圍內之修飾。

【圖式簡單說明】

[0060] 上述發明內容及實施方式與所附圖式一併閱讀將更增瞭解。為達闡明本發明之目的，圖式中所示具體實例為目前較佳者。然而，應瞭解本發明並不限於所示之確切配置及手段。

[0061] 在圖式中：

[0062] 圖 1 顯示經 412 pAMY-phrGFP 或 1216 pAMY-phrGFP 載體轉染之 AR-42J 大鼠胰臟腫瘤細胞之螢光顯微影像。A1 小圖為經 1216 pAMY-phrGFP 轉染之 AR-42J 細胞在亮視野下之影像。A2 小圖為經 1216 pAMY-phrGFP 轉染之 AR-42J 細胞在暗視野下之影像。B1 小圖為經 412 pAMY-phrGFP 轉染之 AR-42J 細胞在亮視野下之影像。B2 小圖為經 412 pAMY-phrGFP 轉染之 AR-42J 細胞在暗視野下之影像。

[0063] 圖 2 顯示帶有纖維素酶轉殖基因之基因轉殖小鼠之 PCR 確認結果。「M」表示分子量標記；「+」表示正對照組；「-」

表示負對照組；及「W」表示野生型。數字係對應基因轉殖小鼠之識別編號。

[0064] 圖 3 顯示帶有植酸酶轉殖基因之基因轉殖小鼠之 PCR 確認結果。「M」表示分子量標記；「+」表示正對照組；「-」表示負對照組；及「W」表示野生型。數字係對應基因轉殖小鼠之識別編號。

[0065] 圖 4A 及 4B 顯示帶有纖維素酶轉殖基因之基因轉殖豬之 PCR 確認結果。「M」表示分子量標記；「+」表示正對照組；「-」表示負對照組；及「W」表示野生型。數字係對應基因轉殖豬之識別編號。

[0066] 圖 5 顯示帶有植酸酶轉殖基因之基因轉殖豬之 PCR 確認結果。「M」表示分子量標記；「+」表示正對照組；「-」表示負對照組；及「W」表示野生型。數字係對應基因轉殖豬之識別編號。

[0067] 圖 6 顯示帶有纖維素酶轉殖基因之基因轉殖豬之南方吸漬分析結果，其中豬基因組 DNA 係以 *Afl* II 截切。「M」表示分子量標記；「Pc」表示正對照組；及「Nc」表示負對照組。數字係對應基因轉殖豬之識別編號。

[0068] 圖 7 顯示帶有纖維素酶轉殖基因之基因轉殖豬之南方吸漬分析結果，其中豬基因組 DNA 係以 *Hind* III 及 *Hae* II 雙截切。「M」表示分子量標記；「Pc」表示正對照組；及「Nc」表示負對照組。數字係對應基因轉殖豬之識別編號。

[0069] 圖 8 顯示帶有植酸酶轉殖基因之基因轉殖豬之南方吸漬分析結果，其中豬基因組 DNA 係以 *Drd* I 截切。「M」表示分子量標記；「Pc」表示正對照組；及「Nc」表示負對照組。數字係對應基因轉殖豬之識別編號。

[0070] 圖 9 顯示帶有植酸酶轉殖基因之基因轉殖豬之南方吸漬分析結果，其中豬基因組 DNA 係以 *Hind* III 及 *ApaL* I 雙截切。「M」表示分子量標記；「Pc」表示正對照組；及「Nc」表示負對照組。數字係對應基因轉殖豬之識別編號。

【主要元件符號說明】

【序列式】

# 七、 申請專利範圍：

1.一種單離之 DNA 啟動子，包含序列識別編號：1 之核苷酸序列。

2.一種重組 DNA 構築體，包含申請專利範圍第 1 項之啟動子可作用地連接至一標的核苷酸序列。

3.根據申請專利範圍第 2 項之重組 DNA 構築體，其中該標的核苷酸序列為一編碼序列。

4.根據申請專利範圍第 3 項之重組 DNA 構築體，其中該標的核苷酸序列編碼一消化酵素。

5.根據申請專利範圍第 4 項之重組 DNA 構築體，其中該消化酵素係選自由植酸酶、纖維素酶、葡聚糖酶(glucanase)及木聚醣酶(xylanase)組成之群。

6.根據申請專利範圍第 2 項之重組 DNA 構築體，包含序列識別編號：2 之核苷酸序列。

7.根據申請專利範圍第 2 項之重組 DNA 構築體，包含序列識別編號：4 之核苷酸序列。

8.一種產製基因轉殖動物之方法，包含下列步驟：將一轉殖基因導入一動物胚胎中，該轉殖基因包含申請專利範圍第 1 項之啟動子可作用地連接至一標的核苷酸序列，並藉此以該轉殖基因轉型該胚胎，其中該基因轉殖動物為豬或小鼠。

9.根據申請專利範圍第 8 項之方法，其中該標的核苷酸序列編碼一消化酵素。

10.根據申請專利範圍第 9 項之方法，其中該消化酵素係選自由植酸酶、纖維素酶、葡聚糖酶及木聚醣酶組成之群。

11.根據申請專利範圍第 8 項之方法，其中將轉殖基因導入動物胚胎中係藉由原核顯微注射而達成。

12.根據申請專利範圍第 11 項之方法，進一步包含下列步驟：將該胚胎植入一代理孕母體內，並使胚胎發育直至出生。

## 八、 圖式：

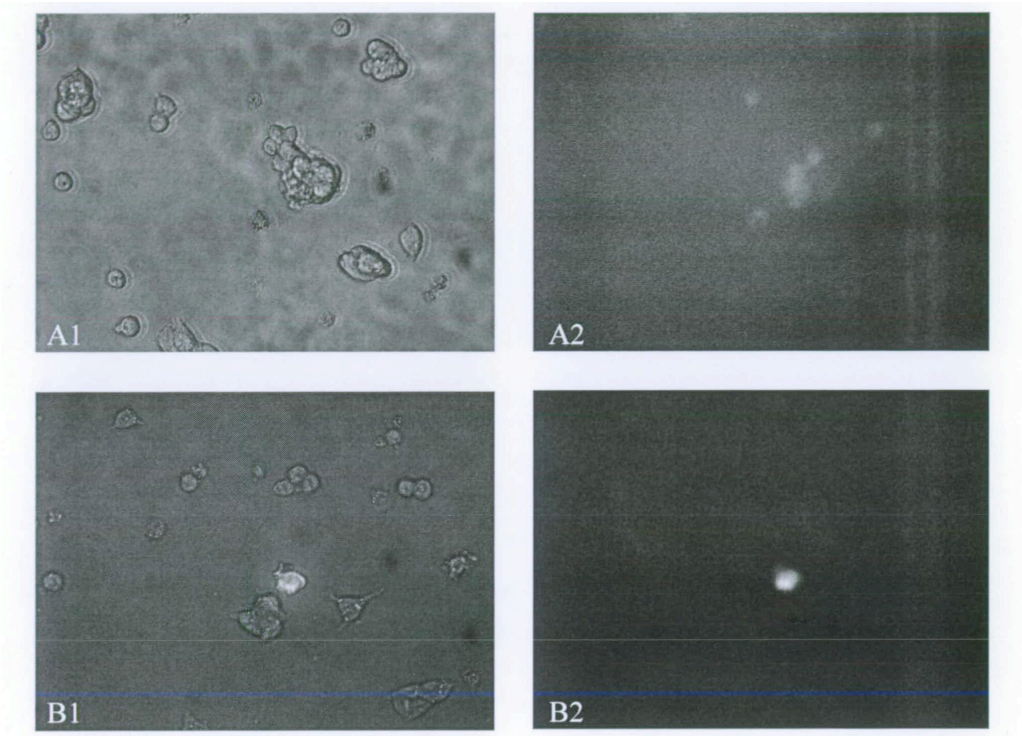


圖 1

圖 1

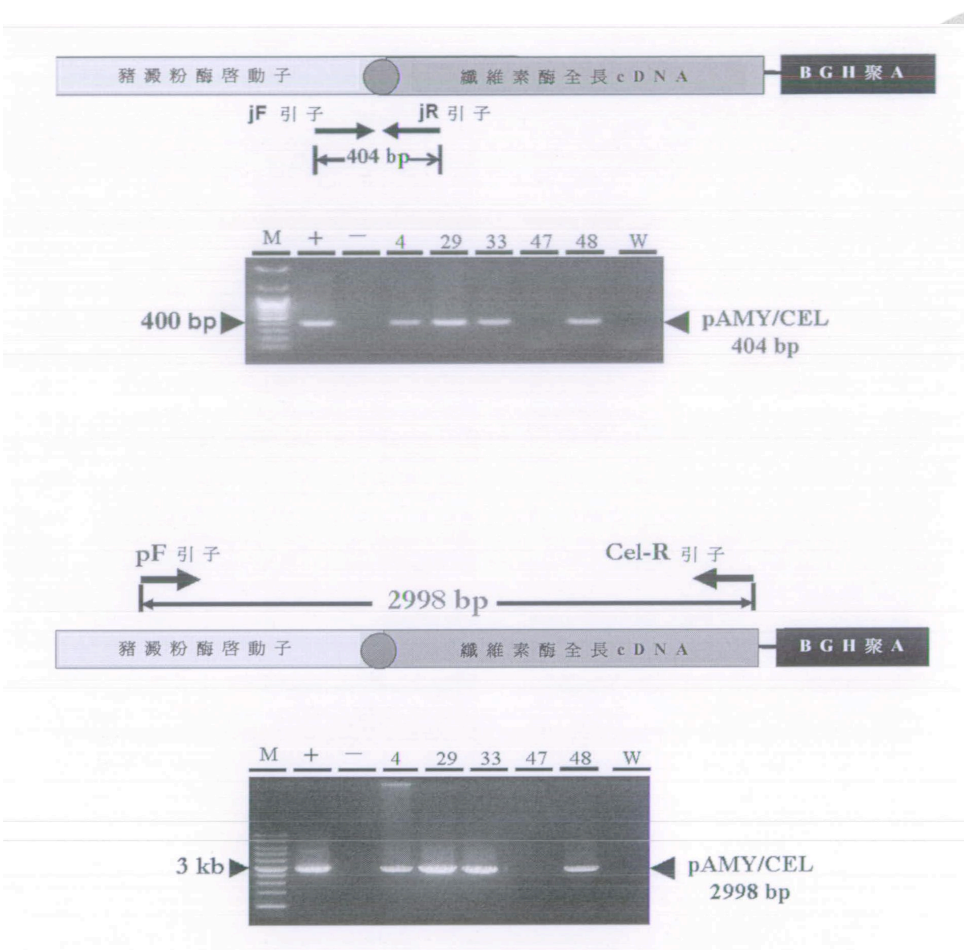
猪澱粉酶啓動子
纖維素酶全長cDNA
BGH聚A
jF 引子
jR 引子
404 bp
M + − 4 29 33 47 48 W
400 bp
pAMY/CEL
404 bp
pF 引子
Cel-R 引子
2998 bp
猪澱粉酶啓動子
纖維素酶全長cDNA
BGH聚A
M + − 4 29 33 47 48 W
3 kb
pAMY/CEL
2998 bp

圖 2

圖 2

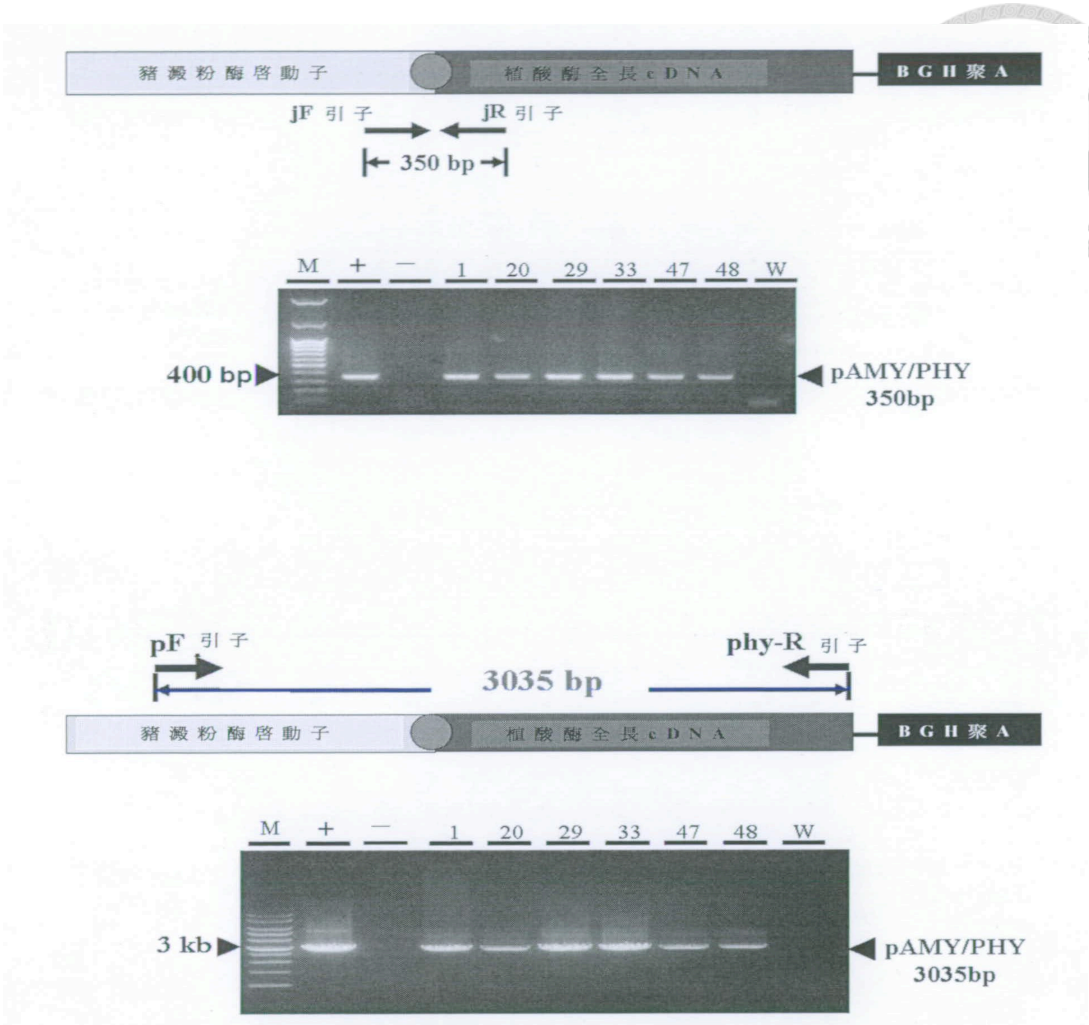
豬澱粉酶啓動子
植酸酶全長cDNA
BGH聚A
jF 引子
jR 引子
350 bp
M + — 1 20 29 33 47 48 W
400 bp
pAMY/PHY
350bp
pF 引子
phy-R 引子
3035 bp
豬澱粉酶啓動子
植酸酶全長cDNA
BGH聚A
M + — 1 20 29 33 47 48 W
3 kb
pAMY/PHY
3035bp

圖 3

圖 3

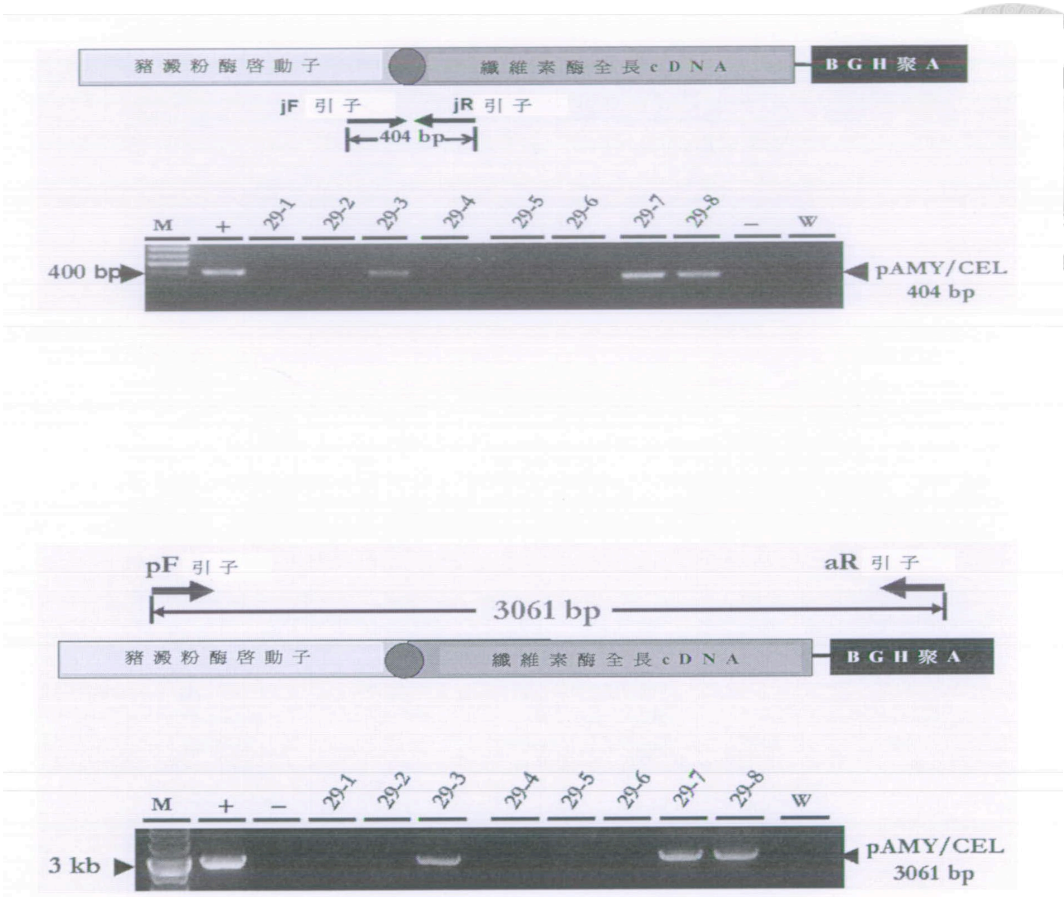
豬澱粉酶啓動子
纖維素酶全長cDNA
BGH聚A
jF 引子
jR 引子
404 bp
M
+
29-1
29-2
29-3
29-4
29-5
29-6
29-7
29-8
–
W
400 bp
pAMY/CEL
404 bp
pF 引子
aR 引子
3061 bp
豬澱粉酶啓動子
纖維素酶全長cDNA
BGH聚A
M
+
–
29-1
29-2
29-3
29-4
29-5
29-6
29-7
29-8
W
3 kb
pAMY/CEL
3061 bp

圖 4A

圖 4A

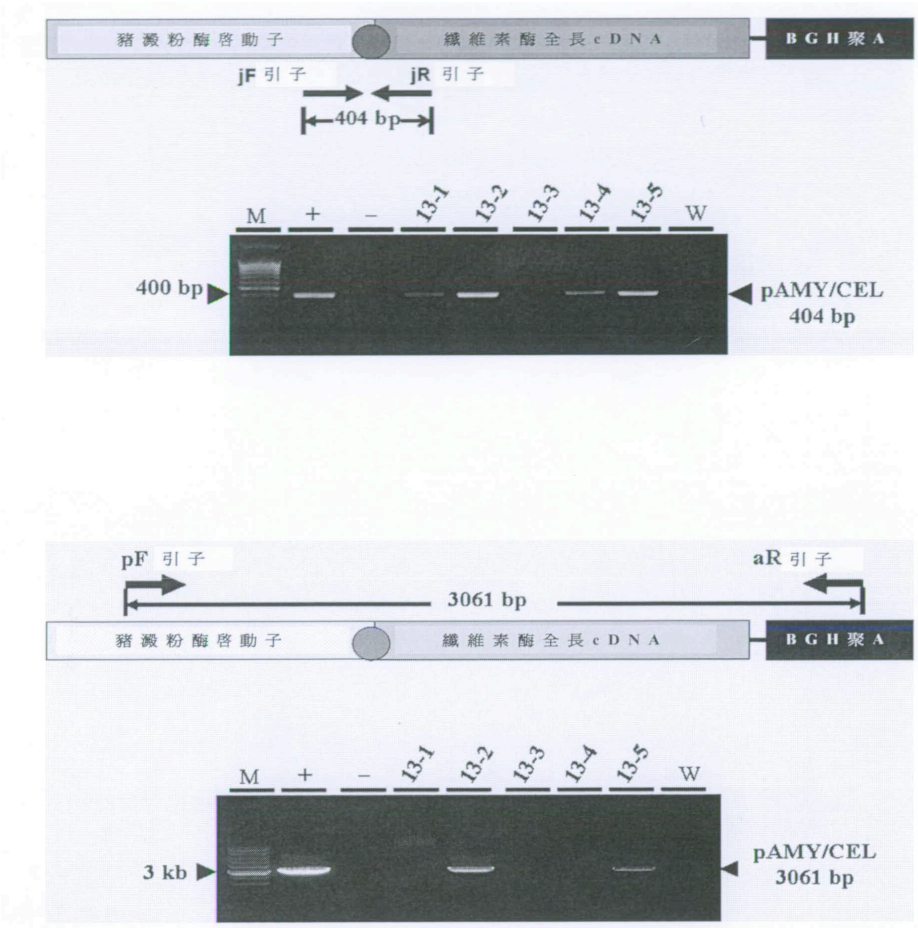
豬澱粉酶啓動子
纖維素酶全長cDNA
BGH聚A
jF 引子
jR 引子
404 bp
M
+
–
13-1
13-2
13-3
13-4
13-5
W
400 bp
pAMY/CEL
404 bp
pF 引子
aR 引子
3061 bp
豬澱粉酶啓動子
纖維素酶全長cDNA
BGH聚A
M
+
–
13-1
13-2
13-3
13-4
13-5
W
3 kb
pAMY/CEL
3061 bp

圖 4B

圖 4B

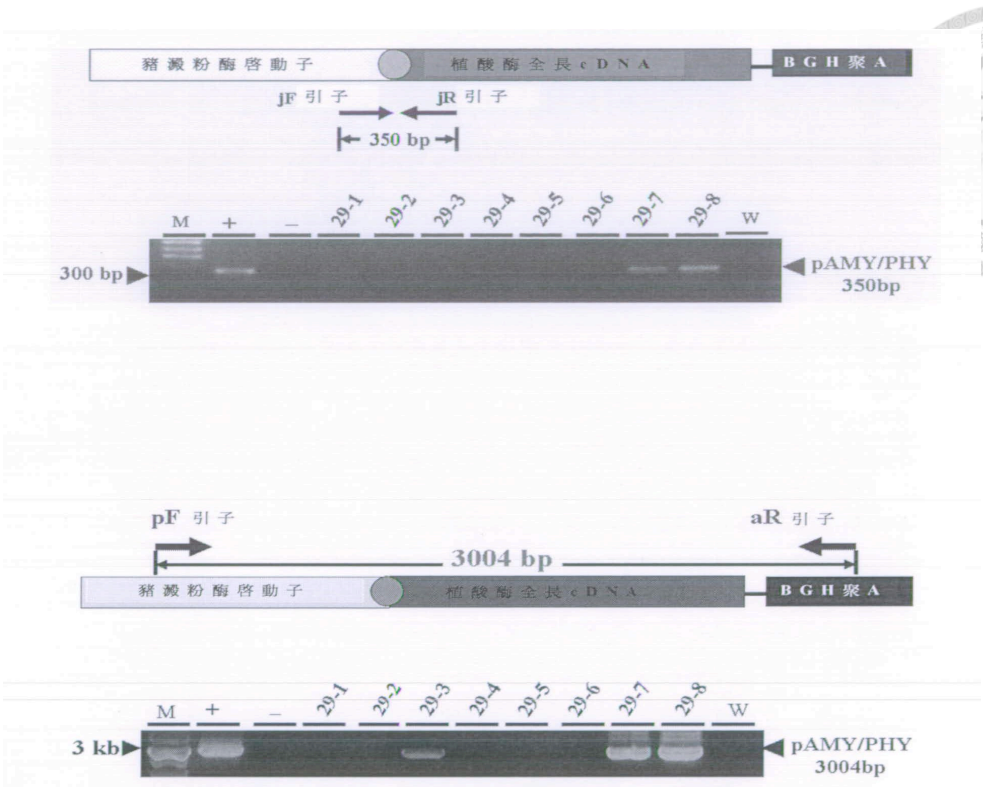
豬澱粉酶啓動子
植酸酶全長cDNA
BGH聚A
jF 引子
jR 引子
350 bp
M
+
–
29-1
29-2
29-3
29-4
29-5
29-6
29-7
29-8
W
300 bp
pAMY/PHY
350bp
pF 引子
aR 引子
3004 bp
豬澱粉酶啓動子
植酸酶全長cDNA
BGH聚A
M
+
–
29-1
29-2
29-3
29-4
29-5
29-6
29-7
29-8
W
3 kb
pAMY/PHY
3004bp

圖 5

圖 5

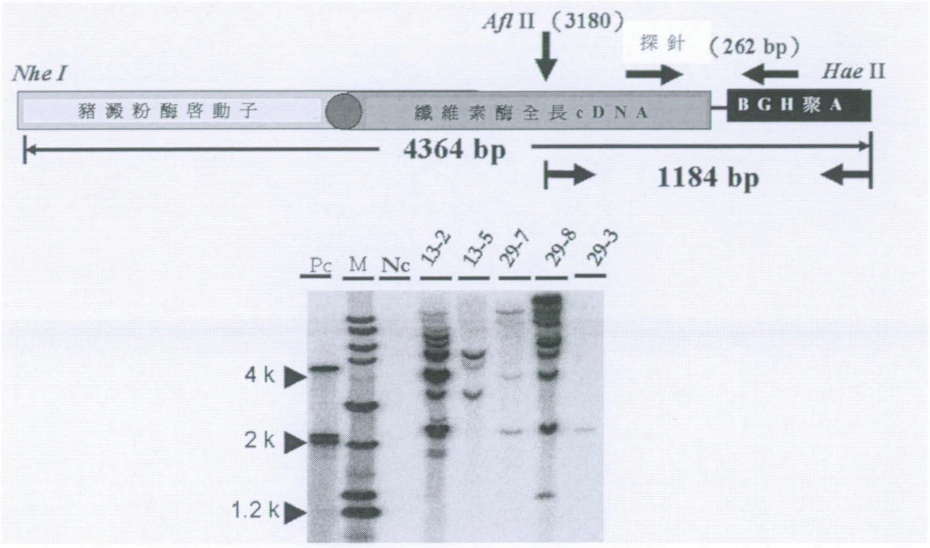
AflII (3180)
探針
(262 bp)
NheI
HaeII
猪澱粉酶啓動子
纖維素酶全長cDNA
BGH聚A
4364 bp
1184 bp
Pc
M
Nc
13-2
13-5
29-7
29-8
29-3
4 k
2 k
1.2 k

圖 6

圖 6

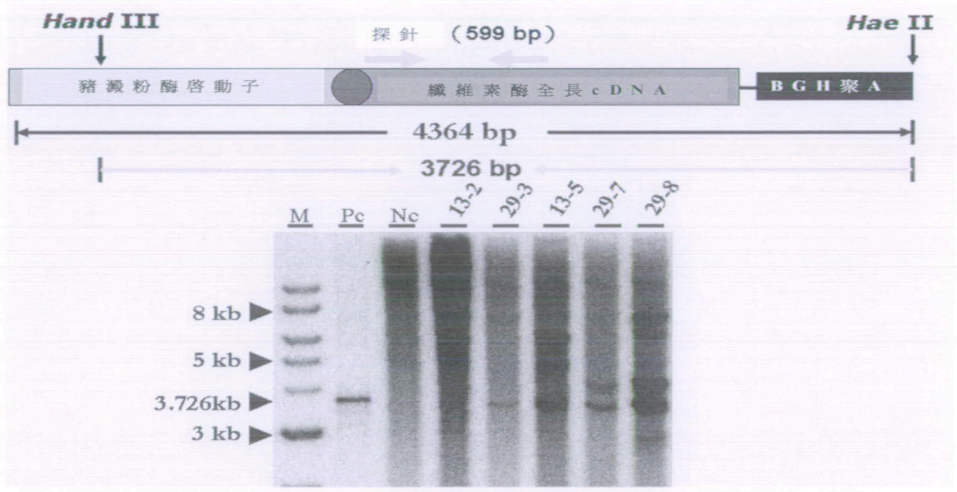
Hand III
探針 (599 bp)
Hae II
豬澱粉酶啓動子
纖維素酶全長cDNA
BGH聚A
4364 bp
3726 bp
M
Pc
Nc
13-2
29-3
13-5
29-7
29-8
8 kb
5 kb
3.726kb
3 kb

圖 7

圖 7

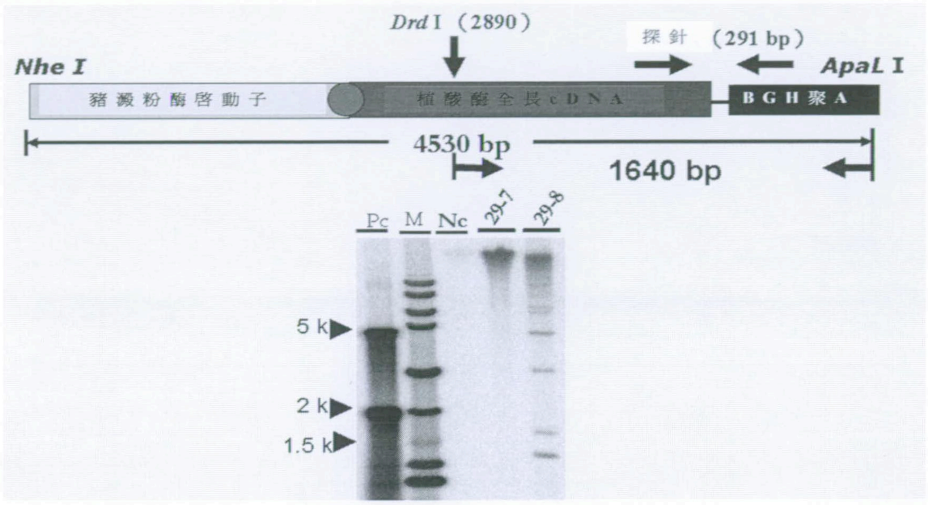
DrdI (2890)
探針 (291 bp)
NheI
ApaLI
豬澱粉酶啓動子
植酸酶全長cDNA
BGH聚A
4530 bp
1640 bp
Pc
M
Nc
29-7
29-8
5 k
2 k
1.5 k

圖 8

圖 8

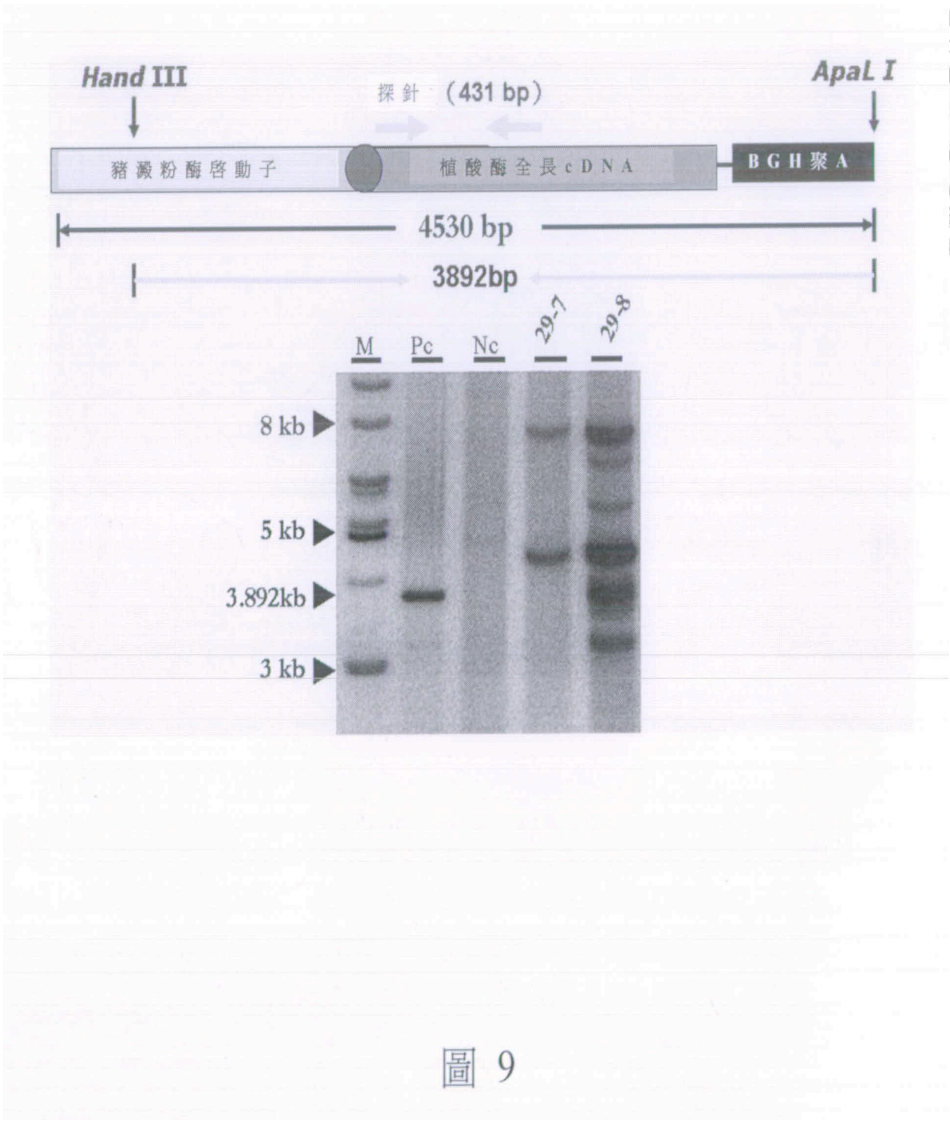
Hand III
探針 (431 bp)
ApaL I
豬澱粉酶啓動子
植酸酶全長cDNA
BGH聚A
4530 bp
3892bp
M
Pc
Nc
29-7
29-8
8 kb
5 kb
3.892kb
3 kb

圖 9

圖 9