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Cellulose Digestibility Increased with CelD Transgenic Pigs

Park, J. K.†, Y. K. Lee, P. Y. Lee, S. W. Kim, S. J. Kim, H. G. Lee, J. H. Han, H. K. Chung, C. G. Park, Y. H. Chung, C. H. Lee[1], H. T. Lee[2] and W. K. Chang

Division of Animal Biotechnology, National Livestock Research Institute, RDA

ABSTRACT

This study was performed to test the cellulose digestibility using the transgenic pigs harboring cellulose degradation gene D (CelD). After delivered offsprings between normal pig and transgenic swine, DNA was isolated from piglets tail for PCR analysis. In first generation, five out of 65 piglets showed CelD positive. Unfortunately, four CelD-positive pigs were died during growing, but one survived pig was used as a transgenic founder to produce F_1 descendents. Among 3 F_1 transgenic pigs produced, one died and the remaining two pigs were used to test the fiber digest efficiency. An assorted feed was composite of 5% fiber with other ingredients. The feed of 3 kg per day was provided to the pigs including transgenic founders and littermate controls. The manure quantity was measured daily for a month, and all manures were dried for three days to analysis nitrogen, phosphate and fiber concentrations. The fiber digestion efficiencies of the transgenic F_1 pigs showed approximately 10% higher than those of control pigs. Fiber digestion was not greatly improved in transgenic pigs as it had been expected approximately 30%. Nitrogen concentration of transgenic pig's manure was slowly decreased compare to the control pigs. Because there were only two transgenic pigs tested, a large number of transgenic pigs may be necessary to obtain more reliable data. Breeding of animals to obtain sufficient transgenic pigs subjected for a further study is on progress.

Taken together, this study demonstrated successful production of transgenic pigs with increase of cellulose digestibility in the porcine feed.

(Key words : CelD, Transgneic pig, Digestibility, Fiber, Pancreas)

Ⅰ. INTRODUCTION

A single stomach, especially in pigs, are known to have extremely low rate of fiber digestion efficiency and this is because they do not produce the digestive enzymes for fibers, which enable clastic action of plant cell wall. Cellulose degradation gene D (CelD) consists of an N-terminal signal peptide, a family 48 catalytic domain of glycosyl hydrolase, and a dockerin domain. N-terminal amino acid sequence analysis of the *C. josui* cellulosomal proteins indicates that both CipA (or scaffoldin) and CelD are major components of the cellulosome. Multienzyme complexes having high activity against crystalline cellulose, known as a cellulosome,

† Correspinding author : J. K. Park, Animal Biotechnology Division, National Livestock Research Institute., E-mail : parkjk@rda.go.kr

[1] Department of Animal Science, Korea National Agricultural College, RDA.

[2] Animal Resources Research Center, Konkuk University.

have been identified and characterized in cellulolytic clostridia such as *Clostridium cellulolyticum* (Belaich et al., 1997), *Clostridium cellulovorans* (Doi et al., 1994), and *Clostridium thermocellum* (Bayer et al., 1996; Beguin and Lemaire, 1996) and in anaerobic cellulolytic fungi such as *Neocallimastix patriciarum* and *Piromyces* sp. (Teunissen et al., 1993). More than 10 cellulase genes of *Clostridium themocellum* were revealed, and among these 5 kinds of genes were reported such as Cel-A, -B, -C, -D and E (Beguin et al., 1987). Endoglucanases is required in order to hydrolysis cellulose (Beguin et al., 1990), and in the constructed Cel D genes, hGH genome sequences is included in the part of intron. The researches upon the efficiency of transgenic method through these genes were reported that introns increase transcriptional efficiency in accordance to the above DNA construct (Brinster et al., 1988). It has been assumed that secretion of cellulose enzymes enabling clastic action of plant cell wall in pancreas may result improvement in the digestive efficiency at least to 10~30%, and it is expected that efficiency of using inferior feed rather than the concentrate may increase greatly. The digestive enzymes will be composed in "acinar cell" of pancreas, packaged in zymogen granule, and again be secreted to duodenum by the operation of a hormone called cholecystokinin (CCK). It was assumed that in order to make the secretion of digestive enzymes in pancreas possible (Padfield et al, 2000), high revelation of the CelD gene expression in acinar cell of the external secreting gland of pancreas by activating serine protease, one of the nine idioblasts, in pancreas as the rat elastase I (rEI) enhancer would be appropriate.

Many genetic modifications in swine were one of our interests in agriculture and human medicine. The purpose of the present study was to characterize the transgenic pigs possessing fiber clastic enzymes, to make use of the fibers as high-energy source as well as to produce environment-friendly animals in accordance to the decrease of biomass production rate as nitrogen.

Ⅱ. MATERIALS AND METHODS

1. Generation of Transgenic Pigs

Piglets were bred each other by natural mating after their sexual maturation over 10 month. Two transgenic and control pigs were management in same metabolic cage to collected the manure. To analysis the ingredient, pig's manure each collected for 3days were mixed after grinding and analyzed eight time. The method of analysis was conducted according to National Livestock Research Institute procedure.

2. Recombinant Cellulose Degradation Gene

The gene construct was prepared from CelD gene of *Clostridium thermocellum* (Beguin et al., 1987) with rat elastase I promoter, human growth hormone intron and the 3' fragment containing human growth hormone polyadenylation signal in plasmid Bluescript SK(+). The 3.0 kb fragment (rEl-CelD) was isolated from plasmid Bluescript SK(+) vector by Sal I digestion and EcoR I, and was used for DNA microinjection (Fig. 3).

3. Isolation of Genomic DNA

Genomic DNA from porcine tails were extracted as described by Hogan *et al.* (1986). Porcine tail was cut with the dissecting scissors and placed in a 1.5 ㎖ microcentrifuge tube containing 0.7 ㎖ of 50 mM Tris-Cl (pH 8.0), 100 mM EDTA, 0.5% SDS, 20 ㎍/ml Proteinase K, and the tubes were incubated at 55℃ overnight in a shaking incubator. After incubation with 0.7 ㎖ of phenol, 0.1 M Tris (pH 8.0) was added and the tubes were shaken vigorously for 10 min, so that two phases are com-

pletely mixed. The aqueous phase was separated by centrifugation for 3 min and transferred to a fresh tube. 0.7 ml of phenol/chloroform (1:1) was added, and the tubes were shaken vigorously. After centrifugation the aqueous phase was transferred to a fresh tube. Following a second phenol/chloroform (1:1) extraction, 70 μl of 3 M sodium acetate (pH 7.0) and 0.7 ml of 100% ethanol were added and shaken to mix throughly. The DNA pellet was ethanol-precipitated by centrifugation in a microcentrifuge tube for 2 min and ethanol supernatant was discarded as much as possible. the DNA was washed to remove traces of SDS and phenol with 1 ml of 70% ethanol. after centrifugation the DNA pellet dried at room temperature and dissolved in 100 μl of TE overnight. The concentration of DNA was approximately calculated by electrophoresis through a 0.1% agarose gel.

4. Transgene Analysis

Genomic DNA extracted from porcine tail was subjected to PCR. Amplification for the transgene was performed by 30 cycles for 1 min each at 94℃, at 58℃ and at 72℃. PCR products were applied individually on the wells of 1% agarose gel and visualized under ultraviolet light after staining with ethidium bromide. Oligonucletides used for PCR was that forward primer was 5'-TGC TGA TAA GAG CCG TAT AAA G-3', and reverse primer was 5'- ATT TGC ATC TAT AGT CGC CTT T-3'. This primer pair predicted a DNA fragment of 600 bp. The *ø174 /Hae III* digested pUC18 DNA fragments are used as size marker. The samples that were positive for the presence of the transgene by the PCR analysis were confirmed by a southern analysis using DIG System (BM, the Germany). Approximately 600 bp of intercept from PCR product was used as a probe.

5. Analysis of the Crude Fiber Contents in Excreta

In order to analyze the efficiency of transgenic pig's digesting crude fibers, specially manufactured feed was supplied to animals. Components of feed are presented in Table 1, and 5.37% of the crude fiber with dried grass which is higher than the general concentrate 4% , has been assorted.

Ⅲ. RESULTS

1. Production and Analysis of F_1 Piglets

The 12 heads of F_1 piglets were produced by naturally crossing normal and transgenic pigs (F_0). The PCR results obtained from 12 heads of F_1 is shown in Fig. 1. Among the 12 heads of $F_{1,3}$ were identified as transgenic pigs. Lane 1, 2 and 6 were positive bands to inserted the transgene as Cel D.

The F1 piglets which was confirmed as the transgenic pig was used to test the fiber digest efficiency rate when the feed is ingested. Seven male and five females were born by natural mating between transgenic female (F_0) and normal male (control). Out of the 12 piglets of $F_{1,3}$ heads were identified as the transgenic pig. Overall transgenic rate was 25.0% as shown in Table 2. This result is higher than the transgenic rate shown in transgenic founders (7.69%). One female died as soon as

Table 1. Feed composition for digestion ability test in transgenic pigs **(unit : %)**

Moisture	Crude protein	Crude fat	Crude fiber*	Crude ash	Ca	P
13.07	15.43	5.26	5.37	4.45	0.76	0.43

*Percentage (5.37%) of crude fiber was higher than an assorted feed (4%) of pig.

delivered and the confirmed transgenic F_1 male and femal piglets were used for testing fiber digestion efficiency when the feed is ingested.

2. Southern Blotting Analysis

DNA was extracted from the tail of offspring and analyzed by Southern blot using non-isotopic DIG system. The used probe was approximately 600 bp of intercept resulted from Cel D gene internal sequence by PCR amplify. Lanes 1 and 4 shows each two bands originated from Cel D gene. In lane 1, fainter bands of about 5.0 kb and 3.0 kb, and 1.2 kb and 0.8 kb fragments from lane 4 were shown in Fig. 2. Negative control pig's genomic DNA were cut with two endonucleases (*Bgl* II and *Hinf* I), but did not detected any fragments. So the present study are interested in defining the copy number and the orientation of a transgene that has no sequence homology with any endogenous gene.

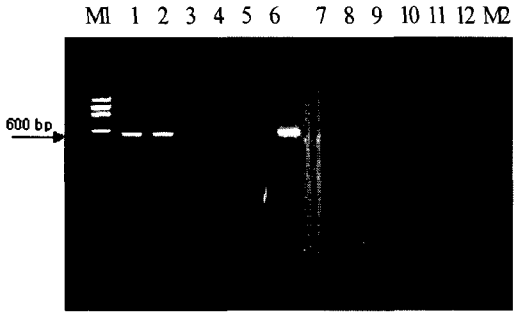

Fig. 1. PCR analysis for the identification of transgenic F_1 piglets. Agarose gel electrophoresis shows PCR products obtained from 12 piglets. Lanes 1, 2 and 6 represent detection of the transgene. M1: size marker with øX174/Hae III, M2: size marker with pBR322/Hae III.

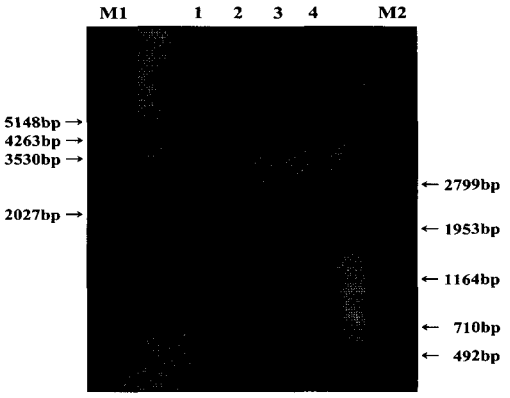

Fig. 2. Southern blotting analysis of transgenic founders using DIG system. A 600 bp of Cel D gene internal sequences were amplified by PCR and were used as probe to detect transgenic piglets. In lane 1, 5 kb and 3 kb fragment shows Cel D insertion. In lane 4, 1.2 kb and 0.8 kb fragment bears Cel D. Lane 2 and 3, which were nagative control pig's genomic DNA cut with each endonucleases did not show any Cel D signals. M1; DNA molecular marker III (Roche), Lane 1; Cel D *Bgl* II cut, Lane 2; negative *Bgl* II cut, Lane 3; negative *Hinf* I cut, Lane 4; Cel D *Hinf* I cut, M2; Molecular weight marker VII (Roche).

Table 2. Production of F1 piglets following natural mating using transgenic female (F0) and normal male pig

TG pig ID	Control pig ID	Total piglets produced		No. transgenic pigs (%)	
		Females	Males	Females	Males
3-95 (♀)	0-75 (♀♂)	5	7	2 (40.0)	1 (14.3)
Total		12		3 (25.0)	

3. Digestion Ability Test of F_1 Piglets

The result of the excrement analysis after supplying specially manufactured feed to examine the fiber digestion ability in F_1 piglet is summarized in Table 3. To analyze the excrement along with the animal growth, it measurements were taken in eight different time points. The result shows that in average, the fiber contents were 8.38% in control pigs, and 7.68% in transgenic pigs, representing a lowered fiber contents in transgenic pigs. This could be due to an absorption of more fiber in transgenic pigs. Excreta composition analysis of F_1 piglets is also shown as a graphical data in Fig. 3. Crude fiber concentration in the excreta of transgenic pigs were decrease approximately 10% than the control pigs, and nitrogen content was also slowly down. However, expected results, down the fiber content of about 20~30%, could not be fined in this study.

Table 3. The results of excreta composition analysis from F_1 TG and control pigs

No. analysis	Samples*	Composition of porcine excreta			
		Moisture	N	P	Crude fiber
1st time	Control	6.93	4.19	2.63	7.27
	TG	6.67	4.15	2.46	7.27
2nd time	Control	5.06	4.19	2.76	7.45
	TG	5.17	4.08	3.55	6.99
3rd time	Control	7.68	4.23	2.30	8.54
	TG	7.45	4.03	2.43	7.84
4th time	Control	4.43	4.48	0.83	11.45
	TG	5.03	4.23	0.88	8.83
5th time	Control	6.18	4.54	2.47	8.70
	TG	5.36	4.18	2.42	9.65
6th time	Control	5.92	4.43	2.40	7.43
	TG	5.39	4.38	2.40	6.88
7th time	Control	6.89	4.11	2.60	8.17
	TG	8.94	4.32	2.87	6.98
8th time	Control	7.91	4.22	2.92	8.05
	TG	7.14	4.39	3.02	6.97
Average	Control	6.38	4.30	2.36	8.38
	TG	6.39	4.22	2.50	7.68

*Each samples were collected from two control and transgenic pigs. N, nitrogen; P, phosphate; TG, transgenic pig.

Ⅳ. DISCUSSION

The results presented here indicate that transgenic pig expressing the cellulose degradation gene D (CelD) in pancreas and expressed control of the rat elastase-1 promoter display pancreatic acinar cell for growing. The pancreatic acinar cell is to secrete a complex mixture of digestive enzymes (Williams et al., 1997). The strong cellulolytic activity of the clostridial cellulosome systems may be ascribed to the function of the cellulose-binding domain of the scaffolding proteins and/or to the ordered structure of the cellulosomes, because the activity of the dissocisted catalytic components was shown to be only 25 to 30% of the acivity of the intact cellulosome from *C. thermocellum* (Morage et al., 1996). Accordingly, this study was try to produce the transgenic animal to secreted cellulase in por-

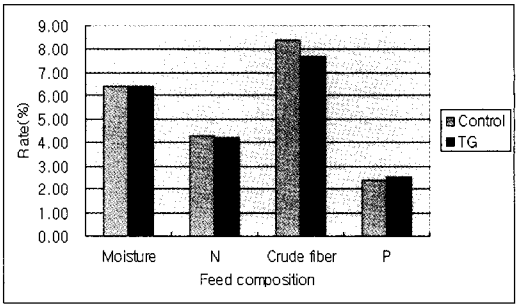

Fig. 3. Comparison of excreta composition between transgenic and control pigs. N, nitrogen; P, phosphate; TG, transgenic pig.

cine pancreas. The transgenic pigs introduced the CelD gene by microinjection (Park et al., 2002) was produced. As a results, we bred one female transgenic pig(F_0) and two F_1 pigs for increase number of transgenic pigs. In our study, efficiency of producing transgenic pigs was 7.69% in first generation and 25.0% in second generation and it was similar than other reported results in efficiency of first generation transgenic pig production. However, the transmit efficiency of transgene in second generation was higher than it's first generation. The ratio of production of offspring to the DNA-injected embryos is 10% (Pursel et al., 1990) or less. Out of those piglets, transgenic offspring are obtained at under 10%, except for the results reported by Nottle et al., 2001. The efficiency of producing transgenic animals is an important factor in transgenic technology (Uchida et al., 2001). Although numerous groups have succeeded in making transgenic pigs, there efficiencies of transgenic pig production by DNA microinjection are also about 1% (Hammer et al., 1985; Martin and Pinkert, 1994). The analysis result of the excrement after supplying the specially manufactured feed to examine the fiber digest ability of F_1 piglet. The result showed that the content of the fiber (7.68%) in the transgenic pig excrement was a little lower than the control pig (8.38%). We can not compare this results because no case to success of transgenic pig production with CelD gene.

In conclusion, from the current study, transgenic pigs were produced by microinjection method. These pigs showed an improvement in their digestion rate, and it is expected for these pigs to enhance the feed efficiency and to improve the productivity. Also, production of transgenic pigs harboring cellulose degradation gene D is a new and effective approach for decreasing the cost of pig production and for reducing nitrogen pollution in livestock industry in future.

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