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Full Length Research Paper

Generation of transgenic mice producing fungal xylanase in the saliva as a model for improving feed digestibility

Dewu Liu[1], Mao Zhang[1], Zicong Li[1], Hui Xu[1], Gengyuan Cai[2] and Zhenfang Wu[1]*

[1]College of Animal Science, South China Agricultural University, Guangzhou, 510642, People's Republic of China.
[2]Institute of Animal Science, Guangdong Academy of Agricultural Sciences, Guangzhou, 510640, People's Republic of China.

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The cell wall of animal feed grains contain a high proportion of xylan. Monogastric farm animals lack xylan-hydrolyzing enzymes in their gastro-intestinal tract. The indigestible dietary xylan impairs normal digestive function of single-stomached livestock by preventing efficient breakdown and assimilation of feed nutrients. The digestive capability of simple-stomached animals could be improved by providing them with a foreign xylanase gene for secretion of the enzyme into their digestive tract. In this study, we produced transgenic mice carrying an *Aspergillus niger* xylanse gene controlled by a salivary gland-specific regulatory element. The fungal xylanase is expressed specifically in the submandibular gland and secreted in the saliva of transgenic mice. The xylanase concentration in the saliva of transgenic mice reached 0.29 ± 0.03 U/ml. Digestibility of nutrients tends to increase in transgenic mice, although it does not differ significantly from that in wild-type controls. To our knowledge, this is the first demonstration of the production of fungal xylanase in the saliva of simple-stomached animals. Results from the present study encourage further investigation of employing transgenic technology to enhance the digestive capability of monogastric agriculture animals by introducing enzyme able to degrade dietary xylan into the digestive tract.

Key words: Transgenic mice, xylanase, digestibility, saliva.

INTRODUCTION

Xylan is a major structural polysaccharide in plant cell wall (Collins et al. 2005). Animal feed cereals, including barley, wheat, rye and oats that contain high xylan. Xylan is not digested in single-stomached agricultural animals, such as swine and chicken, because they do not synthesize the enzymes necessary for degrading the dietary xylan. Indigestible feed xylan forms viscous gels together with other grain nonstarch polysaccharides in the gastro-intestinal tract inhibiting the breakdown and absorption of feed nutrients (Kim et al., 2005; McNab and Smithard, 1992). Therefore, the presence of xylan in feed grains can markedly reduce feed digestibility and increase environmental pollution with excessive excretion of unabsorbed nutrients.

Supplementation of cereal-based animal feed with xylanase has been shown to improve nutrient digestibility of monogastric animals by enhancing the hydrolysis of anti-nutritional dietary xylan (Esmaeilipour et al., 2011; He et al., 2010; Kim et al., 2008; Nortey et al., 2007; Pirgozliev et al., 2010). Enzyme supplementation, however, increases the cost of feed. Furthermore, many animal feeds during the production process are subjected to heat treatments which usually cause loss of enzyme activity (Walsh et al., 1993).

An alternative method to enzyme supplementation is to produce transgenic animals that can secret xylanase into their digestive tract to breakdown the dietary xylan. A previous study has used this strategy to express bacterial xylanase in the exocrine pancreas of transgenic mice, but without data showing whether the enzyme is secreted into the digestive tract and whether the enzyme has

*Corresponding author. E-mail: zili@hawaii.edu. Tel: +86 20 85284869. Fax: +86 20 85280369.

effects on the nutrient digestibility of transgenic mice (Fontes et al., 1999).

Salivary glands are also potential bioreactors to express exogenous digestive enzymes, since a single-stomached animal, such as a pig, can secret up to 15 L of saliva per day (Golovan et al., 2001b). The secreted saliva contains various components that enter the stomach and participate in the digestion of diet. Recently, transgenic mice as well as transgenic pigs producing bacterial phytase in the salivary glands with secretion of the enzyme into saliva were generated for phosphorus pollution control (Golovan et al., 2001a; Golovan et al., 2001b; Yin et al., 2006).

To test the hypothesis that transgenic expression of xylanase in simple-stomached animals can improve feed digestibility, we produced transgenic mouse model expressing *Aspergillus niger* xylanase under the control of a salivary glands-specific promoter. Characterization of the transgenic mice showed that they expressed xylanase in the submandibular gland and secreted the enzyme into the saliva at a concentration of 0.29 ± 0.03 U/ml.

MATERIALS AND METHODS

Activity analysis of xylanase secreted by *A. niger* strain GIM3.452

The liquid medium containing 30 g of sucrose, 3 g of $NaNO_3$, 0.01 g of $MgSO_4 \cdot 7H_2O$, 1 g of K_2HPO_4, 1000 ml of distilled water, pH=6.8, was autoclaved at 121°C for 30 min and cooled. *A. niger* was inoculated in a medium and cultured for 6 days in a shaker at 200 rpm at 30°C. Xylanase activity in the supernatant medium was measured as previously described (Lu et al., 2003). One unit of xylanase activity (abbreviated as U) was defined as the amount of enzyme that liberated 1 µmol of reducing sugar (xylose) from the substrate solution per minute under our assay condition.

Cloning and *in vitro* eukaryotic expression of xylanase genes from *A. niger* strain GIM3.452

Total RNA was extracted from *A. niger* strain GIM3.452 (from Guangdong Institute of Microbiology Culture Collection Center, China) using RNAiso Plus kit (Takara, Dalian, China) and reverse-transcribed into cDNA. According to the sequence of xynA (GenBank Accession No: XM001389959), and xynB (GenBank Accession No: DQ174549), two sets of primers were designed to amplify the cDNA sequence of xynA and xynB. The sequence of the primers are as follows, xynAF(5'-GAGCCCATTGAACCCCGT-3'), xynAR(5'-CTAGAGAGCAT-TTGCGAT-3'), xynBF(5'-TCGACCCCGAGCTCGACC-3'), xynBR (5'-TCACTGA-ACAGTGATGGA-3'). Then the xylanase gene and the signal peptide of porcine parotid secretory protein (Yin et al., 2003), was fused by overlap extension PCR. The fusion gene was inserted into the plasmid pcDNA6 (Invitrogen, USA) to generate recombinant plasmids p-xynA and p-xynB. Using Lipofectamine ™ 2000 (Invitrogen), these two plasmids were transfected into pig kidney cells (PK15). Three days after transfection, cell culture medium was collected and xylanase activity was measured as described earlier.

Construction of transgene plasmid

A forward primer (5-TTGGCGCGCCATGTTTCAACTTTGGAA-3) and a reverse primer (5-ATGGCGCGCCTCACTGAACAGTGATGGA-3) containing an *Asc* I site (underlined) were designed to amplify the fusion gene carrying the signal peptide and the xynB gene, using the plasmid p-xynB as the PCR template. The PCR product was digested with *Asc*I, purified and ligated into the *Asc*I sites of plasmid pPSPBGP-Neo derived from plasmid pPAB (Yin et al., 2006), to generate the plasmid pPSP-xynB. The identity of pPSP-xynB plasmid was verified by PCR, restriction analysis and DNA sequencing.

Production of transgenic mice

The plasmid pPSP-xynB was linearized with *Not*I and purified. The purified transgene fragment was dissolved in buffer containing 7.5 mM Tris, 0.2 mM ethylenediaminetetraacetic acid (EDTA) and the concentration was adjusted to 150 ng/µL. Transgenic mice (FVB strain) were produced by pronuclear microinjection. The experimental protocol for this study was approved by the University's institutional animal care and use Committee.

Identification of transgenic mice by PCR and Southern blot

Genomic DNA was isolated from the tail of founder mice using Tissue DNA kit (Omega, USA). Transgene DNA was detected by PCR amplification using a forward primer K3 (5-TCGACCCCGAGCTCGACC-3) and a reverse primer K4 (5-TAGAA-GGCACAGTCGAGG-3), and β-actin was used as an internal control. For Southern blot analysis, 10 µl of DNA was digested with *Xba*I, fractionated in a 0.7% agarose gel, and transferred to a nylon membrane (Amersham Pharmacia Biotech, USA). The membrane was hybridized with xynB gene probe (567 bp) labeled with DIG-High Prime DNA Labeling and Detection Starter KitII (Roche). After hybridization, the membrane was incubated for 30 min in blocking solution and incubated for 30 min in Anti-Digoxigenin-AP antibody solution. The membrane was exposed for 5 to 20 min after incubation with 1 ml of CSPD ready-to-use, and picture was captured by an imaging system.

Analysis of xylanase activity in the saliva

Following the previous report (Yin et al., 2006), six-week old transgenic and wild-type founder mice were given intraperitoneal injection of pilocarpine to stimulate saliva secretion. Once salivation became visible; the salivary fluid was aspirated using a micropipette fitted with a bent disposable tip and collected into a 1.5 ml Eppendorf tube, which was kept on ice throughout the procedure. Between 200 and 300 µL of saliva was collected. Xylanase activity in the saliva was measured as described previously.

Analysis of feed digestibility

Seven-week old transgenic and wild-type founder mice were individually caged and fed with standard mouse feed containing 40% (wt/wt) of corn, 16.7% (wt/wt) of wheat flour, 18.3 (wt/wt) of soybean meal, 18.3% (wt/wt) of bran, 4.2% (wt/wt) of fish meal, 0.8% (wt/wt) of soya bean oil and 0.4% (wt/wt) of titanium dioxide, which was used as an indigestible marker. Fecal samples were collected from transgenic and wild-type mice once a day for 15 days. Fecal samples from the same mouse were mixed. Titanium dioxide content in the fecal samples and feed samples was

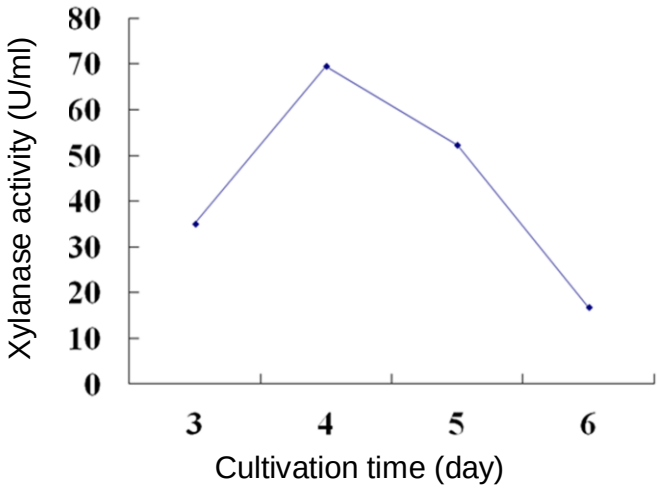

Figure 1. The activity of xylanase secreted by *A. niger*.

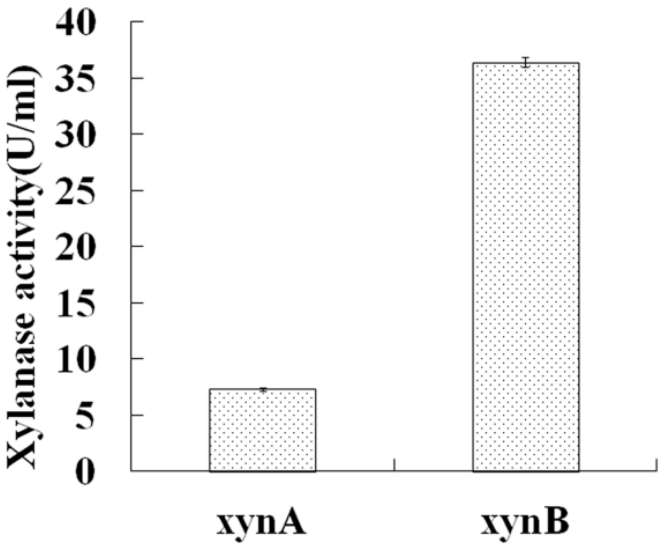

Figure 2. The activity of two xylanases expressed in pig kidney cells.

determined following the protocol described by Short et al. (1996). Dry matter content was determined using standard procedures (ambient pressure drying method). Gross energy was determined using an adiabatic bomb calorimeter standardized with benzoic acid. Nitrogen content was determined by Kjeldahl method (National Institute of Standards, China). Content of crude protein was calculated by total nitrogen content × 6.25. The apparent nutrient digestibility was calculated by the following formula:

Nutrient digestibility = (1-Titanium dioxide content in feed /titanium dioxide content in feces × nutrient content in feces /nutrient content in feed) × 100%.

RT-PCR analysis of xylanase transgene expression

Total RNA was isolated from various tissues of euthanized 6-month old transgenic founder mice using the Trizol reagent (Takara) following the manufacturer's instructions. Isolated RNA was reverse-transcribed into cDNA followed by treatment with DNase to prevent genomic DNA contamination. The primer set of xynBF and xynBR, as described earlier, was used to detect xynB transgene mRNA expression.

Data analysis

Data were analyzed by SAS 9.2 program (SAS Institute, Cary, NC), and the two samples T-test for means program was used for mean comparisons. Data are present as mean ± standard error of mean (SEM). Significant difference of means between two different groups was determined at $P < 0.05$.

RESULTS

Activity analysis of xylanase secreted by *A. niger* strain GIM3.452

To determine the activity of xylanases secreted by *A. niger*, we cultured the strain GIM3.452 of *A. niger* for six days, and measured the activity of xylanases secreted into supernatant medium by the fungi. As shown in Figure 1, the xylanase activity reached 69.5 U/ml on the fourth day of cultivation, and then decreased gradually to 16.8 U/ml on the sixth day of cultivation. The high xylanase activity found in the culture medium of *A. niger* strain GIM3.452 suggests that its genome contains genes encoding highly active xylanases.

Cloning and *in vitro* eukaryotic expression of xylanase genes from *A. niger* strain GIM3.452

A. niger carries multiple xylanase-coding genes (Pel et al., 2007). We cloned two xylanase genes, including xynA (927 bp) and xynB (567 bp), from the GIM3.452 strain by RT-PCR. The enzyme encoded by xynB has been demonstrated to possess high resistance to the acidic conditions in animal gastrointestinal tract (Deng, et al. 2006). xynA is a newly identified xylanase-coding gene from *A. niger* (Pel et al., 2007). Analysis of the amino acid sequences of two cloned xylanase genes displayed that they are over 99% homologous to the reported xynA from strain CBS 513.88 (GenBank Accession No: XM001389959), and xynB from strain CGMCC 1067 (GenBank Accession No: DQ174549).

To compare the activity between xynA and xynB, these two cloned genes were expressed in pig kidney cells (PK15 cells) by transfection with plasmids containing the cloned xynA or xynB gene. These two genes were fused with the signal peptide (SP) of pig parotid secretory protein and driven by a CMV promoter. Three days after transfection, the activity of xynA and xynB secreted into the supernatant medium was measured as 7.33±0.12 and 36.44±0.42 U/ml, respectively (Figure 2). This result suggests that xynB probably has a higher activity than xynA in pig cells.

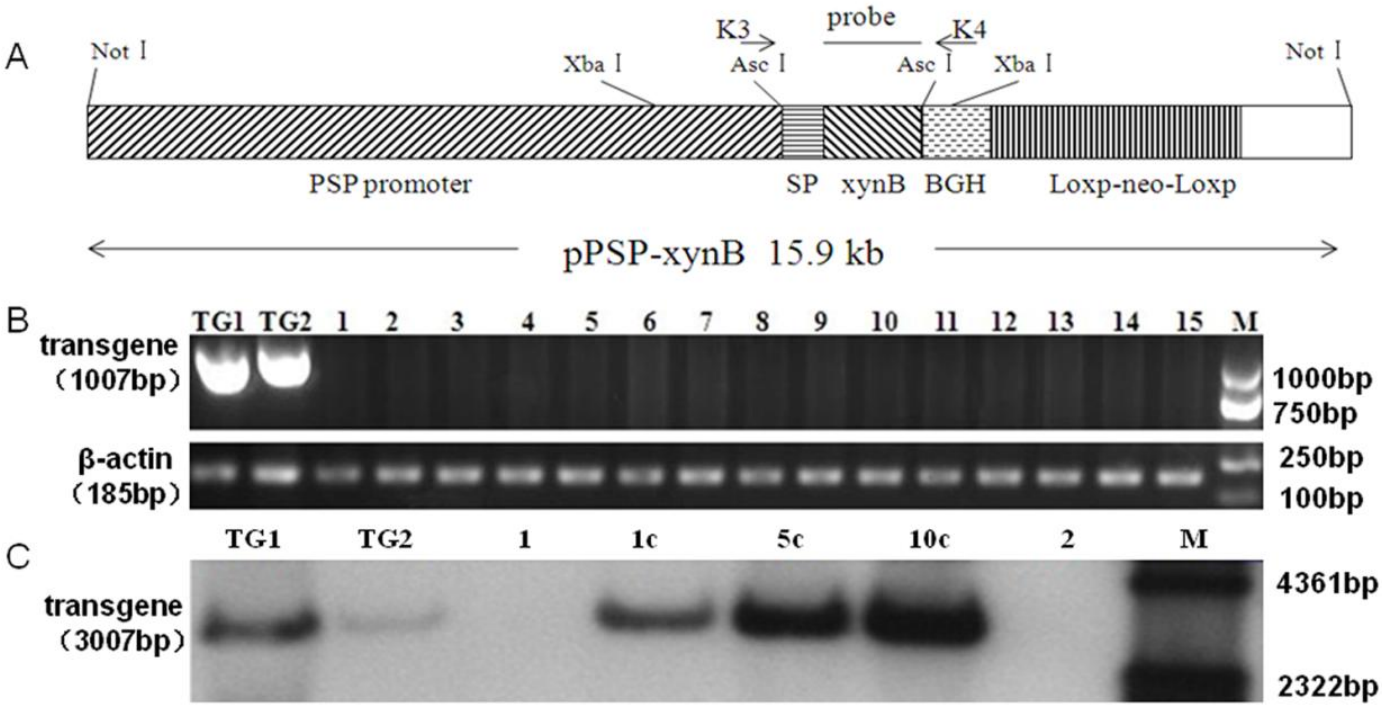

Figure 3. Production of transgenic mice carrying the xylanase B (xynB) gene from *A. niger*. **(A)** The map of linearized transgene plasmid pPSP-xynB. PSP (porcine parotid secretory protein promoter). SP, signal peptide of porcine parotid secretory protein; BGH, bovine growth hormone polyA; Loxp-neo-Loxp, CMV promoter-controlled neomycin-resistant gene flanked by loxp sites; NotI, XbaI and AscI are restriction enzyme sites; K3 and K4, position of primers that are used to identify the transgenic mice by PCR); probe, position of probe that is used to identify the transgenic mice by Southern blot. **(B)** Identification of transgenic mice by PCR. TG1 and TG2 are two transgenic founder mice; 1 to15 are fifteen wild-type founder mice; M, marke; β-actin, endogenous control. **(C)** Identification of transgenic mice by Southern blot. TG1 and TG2 are the same two transgenic founder mice identified by PCR; 1 and 2 are two wild-type founder mice identified by PCR; 1c, 5c and 10c, positive controls containing 1 copy, 5 copies and 10 copies of transgene, respectively; M, marker.

Production of transgenic mice carrying xynB transgene

The cloned xynB gene from *A. niger* strain GIM3.452 was inserted downstream of a salivary glands-specific promoter, the porcine PSP promoter, to obtain the transgene plasmid pPSP-xynB (Figure 3A). The linearized transgene plasmid pPSP-xynB was microinjected into the male pronucleus of mouse zygotes to generate transgenic mice. In total, we obtained 17 founder mice, two (1 male + 1 female) of them were identified as transgenic mice by PCR (Figure 3B) and Southern blot (Figure 3C). The copy number of transgene inserted into the genome of two transgenic founder mice seems to be less than 5 (Figure 3C). Two transgenic founder mice were viable. Unfortunately, they were infertile, since they could not produce offspring even after mating over 10 times with fertile wild-type male or female mice.

Xylanase was secreted into saliva by transgenic mice

Saliva samples from 6-week old transgenic (n=2) and wild-type (n=3) founder mice were collected and examined for xylanase secretion. Xylanase was detected in the saliva of transgenic mice at a concentration of 0.29 ± 0.03 U/ml, while no detectable xylanase was found in the saliva of wild-type mice.

Feed nutrient digestibility in transgenic and wild-type mice

The nutritional potential of salivary xylanase was investigated by analyzing the dry matter digestibility, gross energy digestibility and crude protein digestibility of growing transgenic (TG, n=2) and wild-type (WT, n=15) founder mice fed with a normal diet containing 4% of xylan. No statistically significant difference (P>0.05) in the dry matter digestibility (WT vs. TG = 61.76±0.51% vs. 61.48±0.34%), gross energy digestibility (WT vs. TG = 68.79±0.51% vs. 70.31±0.26%) and crude protein digestibility (WT vs. TG = 66.31±0.45% vs. 71.10±4.56%) was found between wild-type and transgenic founder mice, although the gross energy digestibility and crude protein digestibility tended to increase in the transgenic

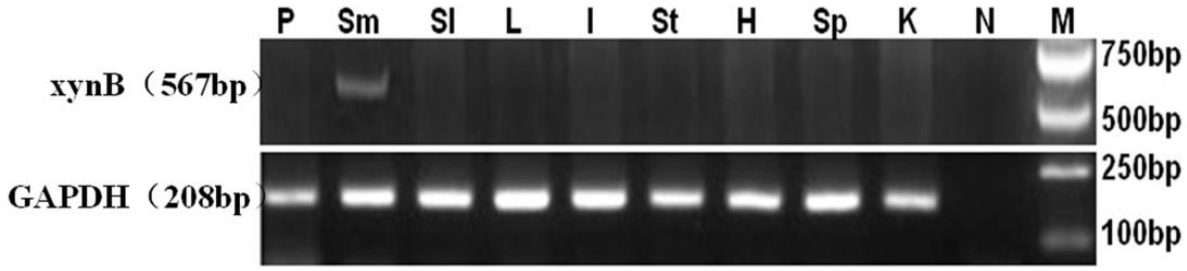

Figure 4. RT-PCR analysis of xynB transgene expression in different tissues of transgenic mice. P, parotid gland; Sm, submandibular gland; Sl, sublingual gland; L, liver; I, intestinal; St, stomach; H, heart; Sp, spleen; K, kidney; N, negative control, use water as template for RT-PCR amplification; GAPDH, endogenous control.

found mice.

xynB transgene was specifically expressed in a salivary gland of transgenic mice

To investigate the expression specificity of xynB transgene in transgenic mice, two transgenic founder mice were euthanized at the age of 6 months. Total RNA was isolated from various tissues of transgenic mice for RT-PCR analysis. The result show that xynB transgene was exclusively expressed in one of the salivary glands, the submandibular gland, in transgenic mice (Figure 4).

DISCUSSION

In this study, we tested an approach to increase feed digestibility by inserting a fungal xylanase gene into the genome of simple-stomached animals for secretion of the enzyme in the saliva. Using transgenic mice as models, we demonstrated that the fungal xylanase can be specifically expressed in a salivary gland and secreted into the saliva. To our knowledge, this is the first work that successfully produced *A. niger* xylanase in the saliva of monogastric animals by transgenic techniques.

However, the transgenic mice producing salivary xylanase only showed a trend of increase, but not a statistically significant improvement, in feed digestibility. Since the number of transgenic mice produced and analyzed in the present study was small, further investigation is needed to determine whether salivary glands-specific expression of foreign xylanase gene in monogastric animals can enhance the digestive capability.

To produce xylanase in the saliva of transgenic mice, we used porcine parotid secretory protein (PSP) promoter to control the expression of xynB transgene. This porcine PSP promoter is highly active in the parotid gland, as well as the submandibular gland in pigs (Yin et al., 2004) and it can drive expression of a bacterial phytase gene in the salivary glands of transgenic mice (Yin et al. 2006). In the present study, expression of xynB transgene was only detected in the submandibular gland of transgenic mice, which was consistent with the tissue specificity of the porcine PSP promoter. The xylanase concentration detected in the transgenic mice was an average of 0.29 ± 0.03 U/ml, which seems low. However, if for a pig, which can produce up to 15000 ml of saliva per day (Golovan, et al. 2001b), xylanase secretion in the saliva could reach 4350 units per day. Given that the daily feed intake of a growing pig is between 1.5 to 3.0 kg, and supplementation of xylanase only at a concentration of 500 U/kg of feed, already significantly improved the digestive capability of pigs (He et al., 2010), the level of synthesized salivary xylanase should have a significant effect on the increase of feed digestibility of pigs.

In summary, the present study shows that fungal xylanase can be expressed with tissue specificity in a salivary gland, and secreted into the saliva of transgenic mice using the promoter and signal peptide of porcine parotid secretory protein. The nutrient digestibility of the transgenic mice tends to be higher, although it does not statistically differ from that of wild-type controls. These results encourage further studies on using transgenic xylanase for improving the feed digestibility of simple-stomached farm animals.

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