

## Molecular characterization, sequence variation and association with fat deposition traits of *ACOX1* gene in pigs\*

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### ABSTRACT

Preoxisomal acyl-CoA oxidase 1 (ACOX1) catalyses the beta-oxidation of very long chain fatty acids and thus plays an essential role in fatty acid degradation. The objective of this study was to clone a gene encoding ACOX1 and to characterize its association with fat deposition in pigs. The complete coding sequence was 1986bp and encoded a protein of 661 amino acids including the carboxyl-terminal sequence (Ser-Lys-Leu) known as a minimal peroxisome-targeting signal. The predicted porcine ACOX1 amino acid sequence shared 88, 84, 81, 86 and 78% identity to that of human, mouse, rat, bovine and chicken counterparts, respectively. The A/C polymorphism in intron 9 was detected by PCR-*Pst*I-RFLP and the allele frequencies of this polymorphism were significantly different between Chinese fatty type pig breeds (Meishan, Erhualian, Tongcheng and Qiping) and western commercial pig breeds (Landrace, Large White and Duroc). The association analysis between the A/C polymorphism and carcass traits in 298 Large White × Meishan F<sub>2</sub> offspring showed that the single genotype polymorphism (SNP) genotype was significantly associated with traits related to fat deposition such as backfat thickness and carcass fat percentage. The *ACOX1* gene was widely expressed in the tissues examined by semi-quantitative reverse transcription (RT) PCR, with the highly abundant in liver, kidney and fat. The present study offered preliminary evidence to further determine the role of the *ACOX1* gene in pig fat deposition.

**KEY WORDS:** acyl-CoA oxidase 1, palmitoyl, peroxisomal (ACOX1), SNP, fat deposition, pig

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## INTRODUCTION

Peroxisomal beta-oxidation of fatty acids is catalysed by enzymes that are immunologically distinct from the analogous mitochondrial enzymes. Peroxisomal acyl-CoA oxidase 1 (*ACOX1*) is the first and rate-limiting enzyme in the peroxisomal beta-oxidation pathway catalysing the desaturation of acyl-CoAs to 2-*trans*-enoyl-CoAs, thereby producing H<sub>2</sub>O<sub>2</sub> (Fournier et al., 1994; Li et al., 2000). Fan et al. (1998) found that homozygous *ACOX1*-null mice were viable, but growth-retarded and infertile. Expression of the *ACOX1* gene was significantly increased in male mice fed a high-fat diet, compared with a low-fat diet (Kim et al., 2004). The human *ACOX1* gene was located at 17q23-qter by PCR analysis of human/rodent hybrid DNAs (Moghrabi et al., 1995). The porcine *ACOX1* gene was physically mapped to SSC12p11-2/3p13 using somatic cell hybrid mapping and was most closely linked to *SW2494* (LOD=5.32) by radiation hybrid mapping analysis (Cirera et al., 2003), where significant quantitative trait loci (QTL) affecting average daily gain, birth weight, backfat thickness and fatty acid composition have been reported in several experiments (Casas-Carrillo et al., 1997; Knott et al., 1998; Clop et al., 2003; Yue et al., 2003). Thus, the *ACOX1* gene is a potential positional candidate gene for traits related to fat deposition, given its role in the metabolism of fatty acids.

The aim of this study was to examine *ACOX1* as a candidate gene for fat deposition and carcass traits. To achieve this target, we characterized the nucleotide sequence and transcription profiles of the pig *ACOX1*, performed screening of *ACOX1* for polymorphisms, and examined associations with carcass traits in a Large White × Meishan pig cross.

## MATERIAL AND METHODS

### *Amplification and sequencing of the pig ACOX1 mRNA*

Total RNA was isolated from *longissimus dorsi* muscle samples of Landrace pigs using Trizol reagent (Invitrogen, USA). One microgram of treated total RNA was used to synthesize the first-strand cDNA using superscript reverse transcriptase and oligo (dT) primer (Promega, USA) following the manufacturer's instructions.

To obtain *ACOX1* cDNA, expressed sequence tags (ESTs) database mining was performed with BLASTN using the obtained sequence information of the mouse *ACOX1* gene (NM\_015729). Several partially porcine ESTs highly identical to the *ACOX1* sequence (CB285697, BC288205, BI403700, BE232949, BI184671) were selected. From those EST sequences, primer pairs AC-1 and AC-

2 were designed using Primer 5.0 software (Table 1). PCR was performed in a 25  $\mu$ l reaction mixture containing: 1 $\times$  PCR buffer, 1.5 mM MgCl<sub>2</sub>, 150  $\mu$ M of each dNTP, 0.25  $\mu$ mol of each PCR primer, 2 units of *Taq* DNA polymerase (Biostar International, Toronto, ON, Canada), and 2  $\mu$ l cDNA derived from the *longissimus dorsi* muscle. PCR was run in GeneAmp PCR system 9600 thermocycler (Perkin-Elmer Co., USA) with the following cycling conditions: 95°C initial denaturation for 4 min, 35 cycles of 95°C denaturation for 50 s, optimal temperature annealing for 50 s, and 72°C extension for 1 min. A final extension was performed at 72°C for 10 min.

Table 1. Primers used for amplification of *ACO1* gene

Primer symbol	Primer sequence (5'-3') forward and reverse sequence	Amplified region	T <sub>m</sub> , °C	Size, bp
AC-1	F: CAGGACCTACAGAAGGAGCGA R: TCGTCGTCAGGACACTTCAGAG	Exon 2~14	63	1996
AC-2	F: TGCCTCTGCTGGCTGTCAC R: GGATTTCATCTGGGTCTGCGA	Exon 1~2	61	276
AC-3	F: TGCCTATGCCTTCCAGTTTGT R: CATTAGCCGTCAGGATGTGA	Intron 8	63	994
AC-4	F: CGGGCACGGCTATTCTCAC R: CTGGCTGGGCAGGTCATTC	Intron 9	64	753
AC-5	F: TGGAAATCAGTCAGAACTCAGG R: GAAAATCAAATGCGTCAACC	Intron 12	58	293
AC-6	F: GGAAATGAACCCGACCAGTA R: TGCGTCTCAGGAAGCAGTAAG	Intron 9	61	419
<i>G3PDH</i>	F: ACCACAGTCCATGCCATCAC R: TCCACCACCCTGTTGCTGTA		59	480

The PCR products were fractionated on a 1.0% (W/V) agarose gel, and selected bands were purified using a gel extraction kit (Sangon, Shanghai, China). The purified PCR products were ligated into the pGEM-T vector (Promega, USA) and transformed into DH5 $\alpha$  competent cells (Lingfei Company, Wuhan, China). Cloned PCR products were sequenced by Sangon Company (Shanghai, China).

### Bioinformatics analysis

Nucleotide BLAST programs at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) were used for sequence homology searches in the public database. The ORF finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) was used to deduce the amino acid sequences. The deduced amino acid sequence was analysed at the ExPASy Molecular Biology Server (<http://au.expasy.org/>). The instability index was calculated according to the method of Guruprasad et al. (1990). The protein hydrophilicity plot was produced employing the Kyte-Doolittle analysis (<http://>

[www.vivo.colostate.edu/molkit/hydropathy/index.html](http://www.vivo.colostate.edu/molkit/hydropathy/index.html)). The prediction of protein localization sites in cells was performed at PSORT WWW Server (<http://psort.ims.u-tokyo.ac.jp/form2.html>). Amino acid sequences were aligned using Clustal W, version 1.8 multiple alignment program. Identical amino acids are enclosed by black and conservative by gray boxes using the BOXSHADE 3.21 program ([http://www.ch.embnnet.org/software/BOX\\_form.html](http://www.ch.embnnet.org/software/BOX_form.html)).

#### *Polymorphism discovery and PCR-RFLP genotyping*

To screen for polymorphisms in the *ACOXI* gene, partial genomic sequences of *ACOXI* were obtained from genomic pools of several pig breeds (Large White, Landrace, Tongcheng, Qingpin, Meishan, Erhualian) using primer pairs AC-3, AC-4 and AC-5. The genomic pools consisted of DNA samples of five individuals per breed. PCR amplifications were performed on a GeneAmp PCR system 9600 thermocycler (Perkin-Elmer Co., USA) in a final reaction volume of 25  $\mu$ l consisting of approximately 50 ng of genomic DNA, 1.5 mM MgCl<sub>2</sub>, 150  $\mu$ M of each dNTP, 0.25  $\mu$ mol of each primer and 1.5 units of *Taq* polymerase in a 1 $\times$  PCR buffer. After an initial denaturation step at 95 °C for 5 min, the reaction mixture was subjected to the following PCR conditions: 35 cycles of 94°C for 45 s, optimal temperature for 45 s, and 72°C for 45 s, plus a final extension at 72°C for 10 min. PCR products were purified with a gel extraction kit (Sangon, Shanghai, China) and bidirectionally sequenced to identify polymorphisms in the *ACOXI* gene.

The PCR-*Pst*I-restriction fragment length polymorphism (RFLP) analysis was used to verify the presence of the A/C polymorphism identified at position 374 (the fragments amplified by primer pair AC-4). In order to improve the efficiency of detection, primer pair AC-6 was designed according to the sequence obtained using primer pair AC-4. The PCR was performed using 50 ng of genomic DNA in a final volume of 25  $\mu$ l. The thermal profile was as follows: 95°C for 3 min, 35 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 45 s, with a final extension at 72°C for 10 min. Amplification products (8.5  $\mu$ l) were digested with 0.5  $\mu$ l of *Pst*I restriction enzyme (10 U/ $\mu$ l) in a 10  $\mu$ l volume, and the genotypes were determined by analysis of the digestion products on 2.5% agarose gels stained with ethidium bromide. Allele frequencies for PCR-RFLP were studied in three commercial pig breeds (Large White, Landrace, Duroc) and four Chinese indigenous pig breeds (Meishan, Erhualian, Qingping, Tongcheng pigs).

#### *mRNA expression of the ACOXI gene*

Total RNA extraction and cDNA synthesis were carried out as described above using spleen, lung, liver, kidney, small intestine, muscle, heart, fat and stomach

samples. The samples were collected from three LargeWhite  $\times$  Meishan crossbred pigs and pooled. Primer pair AC-1 (Table 1) was used for reverse transcription (RT) PCR. The porcine housekeeping gene (*G3PDH*) specific primers (Table 1) were used for amplification as an internal control. PCR products were separated by electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining. To validate the results, the RT-PCR was repeated. Electrophoresis band intensities of the PCR products were quantified using BandsScan software. Mean *ACOX1* mRNA expression levels normalized against the corresponding *G3PDH* were presented.

#### *Animal material, traits measurement and association studies*

The population used in the association analysis consisted of an  $F_2$  population involving Large White and Meishan cross pigs (Zuo et al., 2005; Dai et al., 2006). The pedigree was formed by 298  $F_2$ , 54  $F_1$  and 21 grandparent animals in 37 families. The average weight at slaughter was  $87.0 \pm 7.07$  kg. The  $F_2$  pigs were slaughtered at 2000 and 2003 following a common protocol (Xiong and Deng, 1999). The fat deposition and carcass traits were as follows: dressing percentage (DP, %), carcass length (CL, cm), skin percentage (SP, %), bone percentage (BP, %), fat percentage of carcass (FP, %), lean meat percentage (LMP, %), ratio of lean to fat (RLF), backfat thickness at shoulder (BFT1, cm), backfat thickness at 6-7th rib (BFT2, cm), backfat thickness at thorax-waist (BFT3, cm), backfat thickness at buttock (BFT4, cm), average backfat thickness (ABT, cm), leaf and caul fat percentage (LCFP, %), loin eye height (LEH, cm) and loin eye area (LEA,  $\text{cm}^2$ ).

The association between genotypes and traits was performed with the GLM procedure of SAS version 8.0 software package (SAS Institute, Cary, NC.). The statistical model was assumed to be:

$$T_{ijkl} = \mu + s_i + y_j + g_k + f_l + b_{ijkl}X_{ijkl} + e_{ijkl}$$

where:  $T_{ijkl}$  is the observed values of a given trait;  $\mu$  is the overall mean;  $s_i$  is effect of sex ( $i=1$  for male or 2 for female),  $y_j$  is the effect of year ( $j=1$  for year 2000 or 2 for year 2003),  $g_k$  is the effect of genotype ( $k=AA, AB$  and  $BB$ ),  $f_l$  is the effect of family ( $l=37$ );  $b_{ijkl}$  is the regression coefficient of the slaughter weight,  $X_{ijkl}$  is the slaughter weight, and  $e_{ijkl}$  is the random residual.

Both additive and dominance effects were estimated using the REG procedure of SAS version 8.0, where the contrast coefficients for the additive effect were denoted as -1, 0 and 1 for  $AA, AB$  and  $BB$ , respectively, and the contrast coefficients for the dominance effect was denoted as 1, -1 and 1 for  $AA, AB$  and  $BB$ , respectively (Liu, 1998).

## RESULTS AND DISCUSSION

The AC-1 and AC-2 RT-PCR products were cloned into vector pGEM-T and sequenced. Sequencing results showed that the sizes of the AC-1 and AC-2 PCR products were 1996 bp and 276 bp, respectively. These two overlapping cDNA were assembled into one 2022 bp cDNA and deposited into the Genbank database under the accession number DQ842227. The composite pig *AOCX1* cDNA contained 20 bp of the 5'-UTR region, 1986 bp of coding sequence and 16 bp of the 3'-UTR region. The pig *ACOX1* mRNA coded for a protein of 661 amino acids with a calculated molecular weight of 74829.5 Da and theoretical pI of 7.13. The protein had a total of 70 negatively charged residues and 69 positively charged residues. The calculated instability index was 44.57 classifying the protein as unstable with an estimated half-life of 30 h in mammalian reticulocytes *in vitro*. The porcine *ACOX1* polypeptide sequence exhibited 88, 84, 81, 86 and 78% identity to that of human, mouse, rat, bovine and chicken counterparts, respectively.

Kyte-Doolittle hydrophobicity analysis revealed that the porcine *ACOX1* protein was mainly hydrophilic. The protein had no potential signal peptide suggesting that it might be non-secretory. The predicted protein localization site in cells was peroxisome. The peroxisomal acyl Co-enzymeA oxidase (branched chain) 72-kDa subunit was cleaved into the 50 and 22-kDa subunits after import into peroxisomes (Baumgart et al., 1996a,b). The region of the acyl-CoA oxidase 72-kDa subunit cleaved (proteolytic cleavage between Val468 and Ala469) (Miyazawa et al., 1987) was also conserved in the *ACOX1* protein of all six species (Figure 1). The consensus sequence for a Ser/Thr dehydratase pyridoxal-phosphate attachment site (LVDINSLDSLTEAYKLRAARLVEI) was present in the *ACOX1* of the six species (Figure 1). The C-terminus of pig *AOCX1*, like other species counterparts, contained an SKL motif. This tripeptide has been shown to be a C-terminal peroxisomal import signal present in several peroxisomal matrix proteins (Figure 1).

According to the exon/intron organization of the human *ACOX1* gene, three PCR primer pairs (AC-3, 4 and 5) were designed to amplify introns 8, 9 and 12 of genomic pools representing commercial pig breeds and Chinese indigenous pig breeds. The PCR products were bidirectionally sequenced. Sequencing results showed that the sizes of the AC-3, AC-4 and AC-5 PCR products were 994 bp, 746 bp and 300 bp for Meishan pigs, respectively. The above gapped sequences of different pig breeds have been deposited into the GenBank database under the accession numbers: DQ842229 (exon 8-9, 9-10, 12-13 of Landrace), DQ842230 (exon 8-9, 9-10 of Large White), DQ842231 (exon 8-9, 9-10 of Meishan), DQ842226 (exon 8-9 of Erhualian), DQ842232 (exon 9-10, 12-13 of Tongcheng) and DQ842228 (exon 9-10, 12-13 of Qingping). The

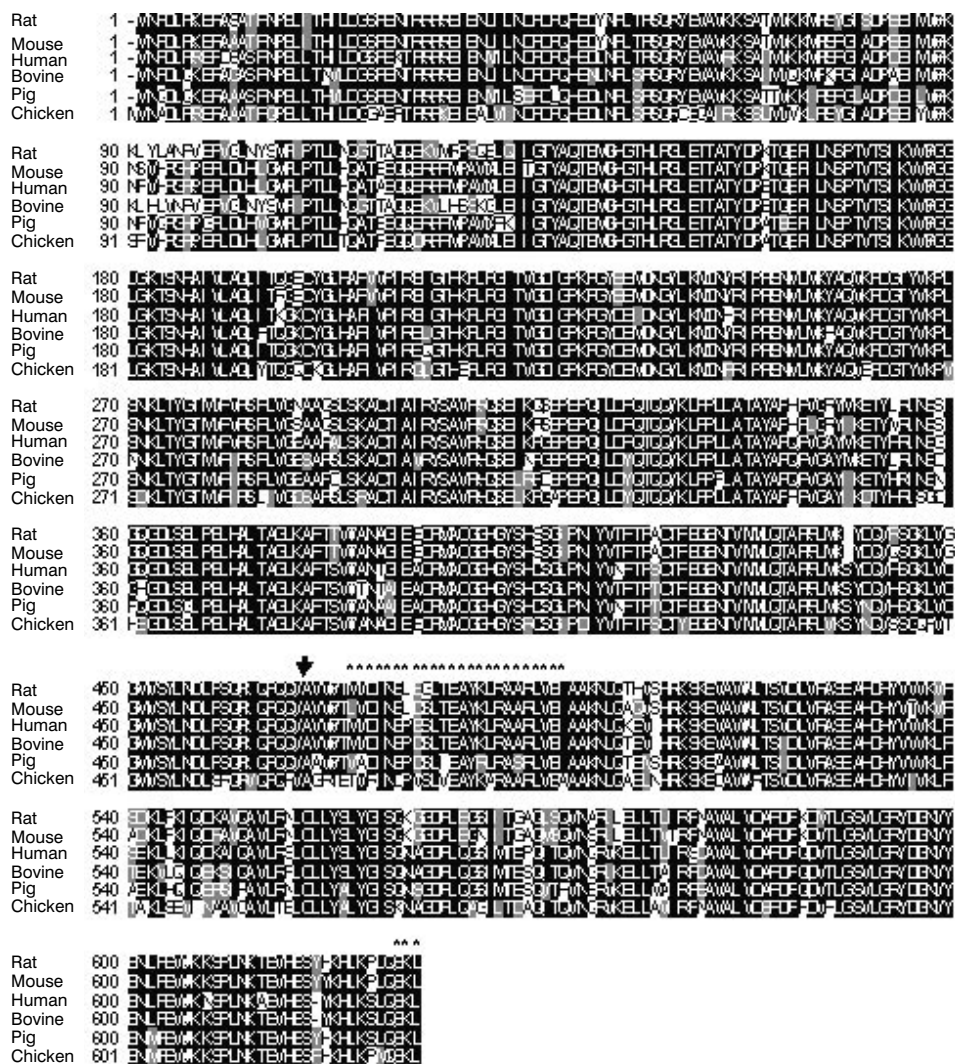


Figure 1. Amino acid sequence comparison between the porcine ACOX1 protein and its counterparts from six species. Sequence gaps introduced to optimize the alignment are indicated by dashes. Identical amino acid residues are shown in grey while similar amino acid residues are shown in light grey. The GeneBank accession numbers are as follows: bovine ACOX1 (NM\_001035289), human ACOX1 (NM\_004035), mouse ACOX1 (NM\_015729), rat ACOX1 (NM\_017340), chicken ACOX1 (NM\_001006205). The position of the acyl-CoA oxidase 72-kDa subunit cleaved (proteolytic cleavage between Val468 and Ala469) is indicated by an arrow. The conserved Ser/Thr dehydratase pyridoxal-phosphate attachment site and C-terminal peroxisomal import signal are indicated by the above asterisk

locations of splice donor/acceptor sites in the three introns followed the consensus GT/AG rule (Breathnach et al., 1978). Using the RepeatMasker, we searched

the nucleotide sequence of the three genomic fragments for repetitive sequences. Three SINES (short interspersed nucleotide elements) were identified. Two SINES were located in intron 8 and one SINE appeared in intron 9. By comparing the sequences of the pig breeds studied in the pooled samples mentioned above, a total of 44 putative polymorphic sites including five deletions or insertions were identified in this 2040-bp sequence.

A single nucleotide mutation (C/A) at position 374 within one SINE sequence of intron 9 led to the *Pst*I restriction enzyme site (CTGCA↓G) change and was further detected by *Pst*I restriction enzyme cleavage. There were two *Pst*I sites within the 419-bp fragment amplified by primer pair AC-6, which were located at the position of 103 bp and 156 bp, respectively. Only the *Pst*I site at 156 bp was polymorphic. Because the 53 bp band was too weak to be shown in the agarose gel, the two allele forms were identified as: A (316 bp and 103 bp) and B (263 bp and 103 bp) (Figure 2). The existence of the *Pst*I-RFLP was confirmed by additional investigations of 130 unrelated individuals representing seven pig breeds. Allele frequencies for the

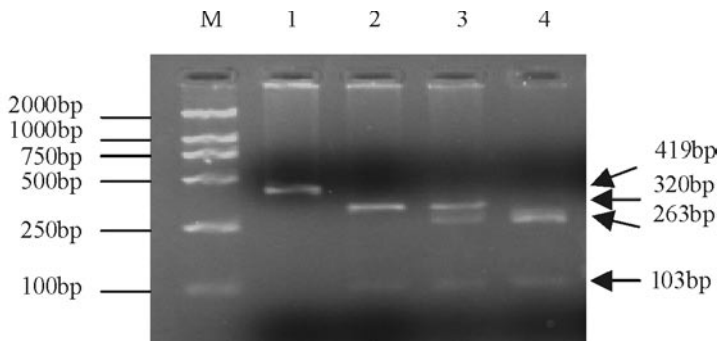


Figure 2. Agarose gel electrophoresis (2.5%) showing the *Pst*I-RFLP results of porcine *ACOX1* gene. Lane M: DNA molecular marker DL2000 (TaKaRa, Dalian, China); Lane 1: undigested PCR products, 419bp; Lane 2: genotype *AA*, 320bp + 103bp; Lane 3: genotype *AB*, 320bp + 263bp + 103bp; Lane 4: genotype *BB*, 263bp + 103bp

Table 2. Distribution of *Pst*I-RFLP genotype and allele frequencies in seven pig breeds

Breed	n	Genotype			Allele frequency	
		<i>AA</i>	<i>AB</i>	<i>BB</i>	<i>A</i>	<i>B</i>
Large White	15	15	0	0	1	0
Landrace	21	21	0	0	1	0
Duroc	18	18	0	0	1	0
Tongcheng	21	0	2	19	0.05	0.95
Qingpin	18	0	2	16	0.06	0.94
Meishan	22	0	0	22	0	1
Erhualian	15	0	0	15	0	1



*PstI* polymorphism are listed in Table 2. Allele *B* was exclusively present in Meishan and Erhualian pigs and predominant in Qingping and Tongcheng pigs, whereas only allele *A* was found in Large White, Landrace and Duroc pigs. The extreme distribution of allele frequencies between Chinese fatty type pigs and commercial lean type pigs may be due to the long-term selection.

The porcine *ACOXI* mRNA, detected by the cDNA was widely distributed in all the tissues, with the highest level in kidney, then in liver, fat, stomach, lung, spleen, small intestine, heart and the lowest in muscle (Figures 3 and 4). In mouse and rat, the *AOCXI* mRNA was detected in all tissues examined by northern blot analysis and quantitative RT-PCR, with the highest level in liver and

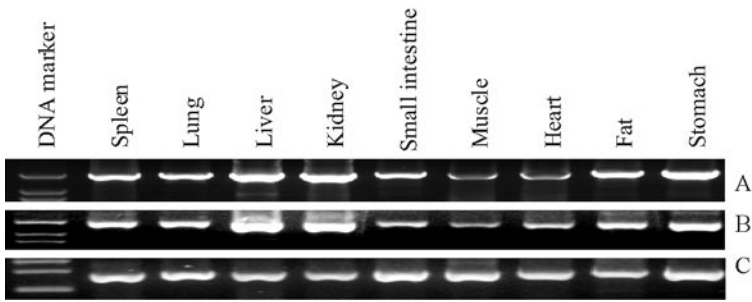


Figure 3. Reverse transcription (RT) PCR tissue expression analysis of porcine *ACOXI* in spleen, lung, liver, kidney, small intestine, muscle, heart, fat and stomach. A and B show RT-PCR amplification results of porcine *ACOXI* gene, 1996bp; C shows the control PCR products with *G3PDH* specific primer, 480bp; DNA marker: DNA molecular marker DL2000 (TaKaRa, Dalian, China)

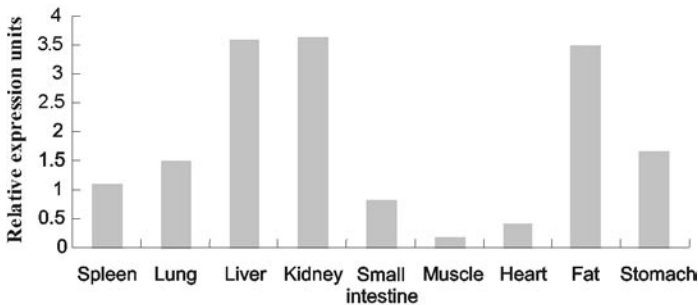


Figure 4. Relative mRNA expression ratio of the porcine *ACOXI* gene in nine tissues. Bar show the mean expression amount of mRNA of three pigs as the ratio of the band intensity of each PCR product to the corresponding *G3PDH* PCR product

kidney tissue (Miyazawa et al., 1987; Nöhammer et al., 2000). The results obtained here are in agreement with the reports in mouse and rat although the different method to examine the expression was used. The pattern of porcine *AOCXI* cDNA exhibiting the highly abundance in fat is consistent with the important role in lipid metabolism. Our results also show that acyl-CoA oxidase is an evolutionary conserved enzyme with a wide expression pattern.

#### *Association studies with backfat thickness and carcass traits*

The *PstI* PCR-RFLP was genotyped in the three-generation Meishan by Large White reference family. The distribution of genotypic and allelic frequencies in the resource population is shown in Table 3. The results of significant association between *ACOXI* genotypes and carcass traits are given in Table 4. Statistically significant associations with fat percentage (FP), lean meat percentage (LMP), backfat thickness at shoulder (BFT1), at thorax-waist (BFT2), and average backfat thickness (ABT) were found. Pigs with genotype *AB* had

Table 3. Distribution of genotypic and allelic frequencies in the resource population

Generation	n	Genotype			Allele frequency	
		<i>AA</i>	<i>AB</i>	<i>BB</i>	<i>A</i>	<i>B</i>
F <sub>0</sub>	21	6	0	15	0.29	0.71
F <sub>1</sub>	54	0	54	0	0.50	0.50
F <sub>2</sub>	298	72	159	67	0.51	0.49

Table 4. Significant association of *ACOXI PstI*-RFLP genotypes with carcass traits<sup>1</sup>

Trait	<i>ACOXI-PstI</i> -RFLP genotype, mean±SE			Genetic effects, mean±SE	
	genotype <i>AA</i> n=72	genotype <i>AB</i> n=159	genotype <i>BB</i> n=67	additive	dominance
FP, %	22.701 ± 0.528 <sup>ab</sup>	21.390 ± 0.353 <sup>a</sup>	22.066 ± 0.544 <sup>b</sup>	-0.309 ± 0.378	0.492 ± 0.260*
LMP, %	53.722 ± 0.433 <sup>A</sup>	55.221 ± 0.290 <sup>B</sup>	54.602 ± 0.445 <sup>AB</sup>	0.438 ± 0.310	-0.532 ± 0.213*
BFT1, cm	3.652 ± 0.085 <sup>a</sup>	3.408 ± 0.057 <sup>b</sup>	3.523 ± 0.087 <sup>ab</sup>	-0.061 ± 0.061	0.087 ± 0.042*
BFT2, cm	2.888 ± 0.068 <sup>A</sup>	2.661 ± 0.046 <sup>B</sup>	2.779 ± 0.07 <sup>AB</sup>	-0.051 ± 0.049	0.084 ± 0.034*
ABT, cm	2.555 ± 0.064 <sup>a</sup>	2.374 ± 0.043 <sup>b</sup>	2.439 ± 0.066 <sup>ab</sup>	-0.048 ± 0.046	0.062 ± 0.031*

<sup>1</sup> data in the table are least square means ± standard error. Values in each row with different lower case superscripts are significantly different at P<0.05, and with capital superscripts are different at P<0.01. Negative values of the additive effects denote a decrease of the trait due to the B allele, \* P<0.05

significantly lower fat percentage and backfat thickness, but higher lean meat percentage as compared those with genotype *AA* or *BB* (Table 4). It is possible that this polymorphism indirectly affects production traits by being in linkage disequilibrium with another polymorphism that directly influences the quantitative traits analysed. This hypothesis is supported by the fact that the *ACOXI* gene is very close linked to the marker *SW2494* on chromosome 12 (Cirera et al., 2003). An

quantitative trait loci (QTL) at a significance level for fat depth at approximately 10th rib has been mapped to marker *S0143* of pig chromosome 12 in a Wild boar × Meishan family (Yue et al., 2003), while the marker *S0143* (6.6 cM) is close linked with the marker *SW2494* (13.4 cM) on the USDA linkage map. To better evaluate the presence of the effect on fat deposition, investigating the association in more pig populations is necessary.

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