

Porcine *RPL28* Gene, Differentially Expressed in the Backfat Tissue Between Meishan, Large White Pigs and Their F1 Hybrids

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Abstract: The mRNA differential display technique was performed to investigate the differences in gene expression in the backfat tissues between Meishan, Large White pigs and their F1 hybrids. One novel gene that was differentially expressed was identified through both semi-quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Quantitative reverse transcriptase Polymerase Chain Reaction (Q-PCR) and it was highly expressed in F1 hybrids, Meishan x Large White and Large White x Meishan compared to their parents. The full-length cDNA was then cloned using the Rapid Amplification of cDNA Ends (RACE) Method. The nucleotide sequence of the gene is not homologous to any of the known porcine genes. The sequence prediction analysis revealed that the open reading frame of this gene encodes a protein of 137 amino acids has high homology with the RPL 28 of seven species-cattle (97%), human (97%), mouse (95%) and rat (97%) so that it can be defined as the porcine *RPL28* gene. Tissue expression profile analysis indicated that the swine *RPL28* gene is differentially expressed in detected tissues. The experiment suggested that the swine *RPL28* gene might play role in the formation of heterosis.

Key words: RPL28, differential expression, pig, heterosis, backfat, China

INTRODUCTION

In agriculture, the use of heterosis in part animals and crop plants has achieved great success and is considered essential to meet the world's food needs (Duvick, 1999; Phillips, 1999; Nassimi *et al.*, 2006). The hypothesis of Dominance, Superdominance and Epistasis has also been brought forward to explain the phenomenon of heterosis for a century.

However, the molecular mechanism of heterosis or hybrid vigor remains to be revealed. The development in QTL (Quantitative Trait Loci) mapping method has made a large advancement on studying the relationships between heterosis and the modes of QTL interactions on plants (Lu *et al.*, 2003; Syed and Chen, 2004; Farooq and Azam, 2002; Mirshamsi *et al.*, 2008). The heterosis is in fact the external exhibition of gene expression and regulation in the heterozygote (Zhu, 2000). So, the study on heterosis in expressional level becomes more meaningful. A lot of studies on heterosis in this level have been done both in plants (Romagnoli *et al.*, 1990;

Tsaftaris, 1995; Xiong *et al.*, 1998) and in animals (Liu *et al.*, 2004; Wang *et al.*, 2004). They have found that certain genes are actually differentially expressed between hybrids and parents and may be related to the formation of heterosis (Wu *et al.*, 2001; Xie *et al.*, 2003).

The mRNA differential display technique is a worldwide method to either identify gene differential expression or to discover novel genes that are involved in important biological pathways (Liang *et al.*, 1993; Liang and Pardee, 1992). The principle of differential display is the systematic amplification of the 3' termini of mRNAs by using anchored oligo-dT primers in combination with 5' arbitrary primers. The isolation of the PCR products by gel electrophoresis and their direct comparison allows the identification of differentially expressed genes. Here we report the identification of a differentially expressed gene *RPL28* encoding ribosomal protein L28 in the F1 hybrids, Meishan x Large White (MS x LW) and Large White x Meishan (LW x MS) and their parents, Meishan (M) and Large White pigs (LW).

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MATERIALS AND METHODS

Animals: Purebreds, LW and MS and their F1 hybrids LW x MS and MS x LW were from the Pig Jingpin Plant of Huazhong Agricultural University and were bred under the same conditions.

Sample collection and total RNA extraction: Samples of backfats were collected from pigs at slaughter and immediately stored in liquid nitrogen. Total RNA was extracted using TRIzol Reagent (Invotrigen, USA). After that, total RNA was treated with RNase-free DNase I (Promega, USA) to destroy potential contaminating genomic DNA. Total RNA integrity was monitored by denaturing 1% agarose gel electrophoresis.

Differential display: About 2 µg of total RNA pool derived from 6 pigs was obtained as template for reverse transcription. About 0.5 µg of the oligo (dT) 15 primer (Promega, USA) per mg of the mRNA sample was added to a total volume of 15 µL with DEPC treated ddH₂O. The tube was spun briefly and heated to 70°C for 5 min to denature the template and then cooled immediately on ice/water. The subsequent steps of RT were carried out according to the user manual of M-MLV reverse transcriptase (Promega, USA).

The DD-PCR reaction system was 25 µL in total containing: 1.0 µL cDNA (about 1 µg), 0.5 µL 10 mM mixed dNTPs, 2.5 µL 10× Taq DNA polymerase buffer, 1.5 µL 25 mM MgCl₂, 2.0 µL, 10 µM anchor and arbitrary primers each, 2.0 units of Taq DNA polymerase (1 U/1 µL) and 13.5 µL sterile water. The PCR was done as follows: 94°C for 5 min, 40°C for 5 min, 72°C for 5 min, 3 cycles of 94°C for 2 min, 40°C for 2 min, 72°C for 5 min followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min and then 72°C extension for 10 min. After that the PCR products were isolated on 8% non-denaturing polyacrylamide gel (39:1) following the method described by Liu *et al.* (2004) and then were visualized by silver staining.

Re-amplification, cloning and sequence analysis: The bands of interest were excised from the gel placed in 40 µL ddH₂O, mashed with a tip and then boiled for 10 min. After that, 4 µL upper liquid was used for re-amplification, the bands were re-amplified as follows: 0.5 µL 10 mM mixed dNTPs, 2.5 µL 10× Taq DNA polymerase buffer, 1.5 µL 25 mM MgCl₂, 0.5 µL 10 µM anchor and arbitrary primers each, 1.0 units of Taq DNA polymerase (1 U/1 µL) and 14.5 µL sterile water. The PCR annealing temperature was 60°C. The bands of true

positive were only cloned using pMD-18T cloning kit (TaKaRa, Japan) and sequenced by Huanuo Company (Shanghai, China). After that the obtained sequences were compared with those available in GenBank using BLAST.

Semi-quantitative RT-PCR: On the basis of the sequences, the forward and reverse specific primers were synthesized to identify these ESTs. Semi-quantitative RT-PCR were performed using about 100 ng of cDNA which was same as that of DDRT-PCR, 5 pmoles each specific primers, 200 µM of each dNTP, 1 unit of Taq polymerase and 1×Taq polymerase buffer in a 25 µL volume. The PCR program initially started with a 94°C denaturation for 4 min followed by 20-36 cycles of 94°C for 1 min, ta°C (ta: annealing temperature) for 1 min, 72°C for 1 min.

Exponential amplification range for each gene was tested on the adjusted cDNA. 1.25, 2.5 and 5 pmoles of GAPDH primers were added, respectively when 20 cycles were remaining in the specified gene's exponential amplification range. The PCR samples were electrophoresed on 2% agarose/EtBr gels. Densitometry values were measured using the BandScan Software (www.Glyko.com). RT-PCR values are presented as a ratio of the specified gene's signal in the selected exponential amplification cycle divided by the GAPDH signal. Each sample was repeated eight times in duplicate PCR. The standard deviation for each error bar was calculated according to the eight densitometry values.

SYBR green real-time RT-PCR analysis of RPL28 expression: Real-time relative quantitative RT-PCR was performed denaturing at 95°C for 2 min, amplifying for 45 cycles at 95°C for 30 sec, annealing and extension at 58°C for 30 sec and 72°C for 18 sec, respectively using an MJ option 2 detection system (MJ Research, USA). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primers are: 5'-CGTCCCTGAGACACGATGGT-3' and 5'-GCCTGGACTGTG-C CGTGGAAT-3' and RPL28 primers are 5'-CCGCAACTCCTTCCGCTAC-3' and 5'-TTGATGGTGGTCCGCACGT-3'.

For spatial expression analysis, total RNAs were also isolated from various tissues including backfat tissue, longissimus dorsi, heart, liver, spleen, lung, kidney, stomach, uterus, ovary and small intestine of Meishan pigs. Each sample was repeated four times and the comparative C_t(^{ΔΔC}) value method (Livak and Schmittgen, 2001) was used to compute relative quantification. Expression levels were considered not detectable when the C_t value of the targeted gene exceeded 35 in the sample tissue.

RESULTS AND DISCUSSION

Identification of EST39, an up-regulated gene, in F1 hybrids: One band, designated as EST39 and visualized only in the F1 Large White x Meishan and Meishan x Large White hybrids was isolated from the differential display gel and re-amplified. Semi-quantitative RT-PCR analysis showed that the expression level of EST39 in backfat was higher in the F1 hybrids than in their parents (Fig. 1).

Cloning and analysis of porcine RPL28 gene: The differentially expressed ESTS36 shares 91% sequence identity with the human RPL28 gene. ESTS36 composes of classic polyadenylation signal, AATAA and poly (A). Researchers obtained a 513 bp full-length porcine RPL28 cDNA by 5' RACE-PCR (Fig. 2). Porcine RPL28 gene contains an open reading frame of 137 nucleotides. Researchers inferred that the ATG codon at nucleotide (nt) residue 43-45 is the true start site of translation because it begins the longest reading frame and is preceded by one in-frame stop codon in the 5' Untranslated Region (UTR).

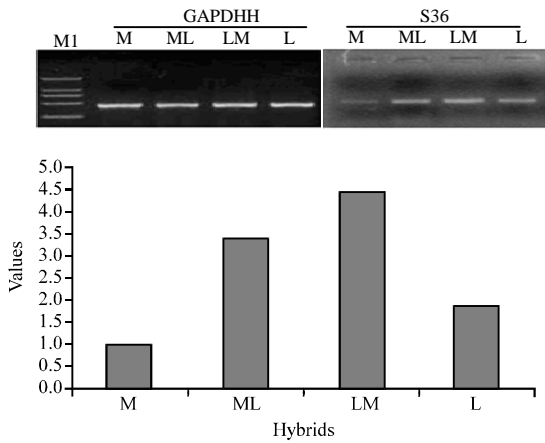


Fig. 1: Identification of *ESTS36* as up-regulated gene in backfat of F1 hybrids compared with their parents

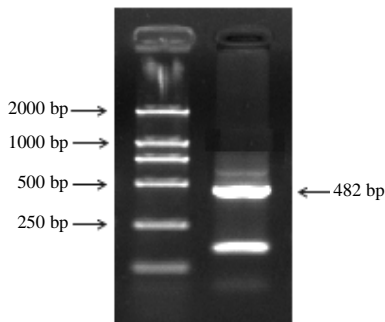


Fig. 2: RACE-PCR amplification of 5' end of porcine RPL28

Analysis of the RPL28 deduced protein sequence: The porcine transcripts coded for a 383 amino acid peptide with theoretical pI/MW of 11.97/15.7 kDa (Fig. 3). The protein of RPL28 gene localized to the cell nucleus by online PSORT Version II U prediction. RPL28 which has not signal peptide sequence is a kind of non-secreted protein by Signal P 3.0 Server analysis.

The phylogenetic tree, constructed using the porcine RPL28 amino acids and other protein sequences of vertebrate isocitrate dehydrogenases available in the GenBank database (Fig. 4) showed two features: the evolutionary relationship between porcine RPL28 and cattle, dog and human's is closer than others; the origin of *Xenopus laevis* RPL28 is earliest followed by zebra fish and mammalian which is consistent with the evolutionary process from amphibian to mammalian.

Expression profile of porcine RPL28: Real-time analysis was performed to further reveal the differential expression

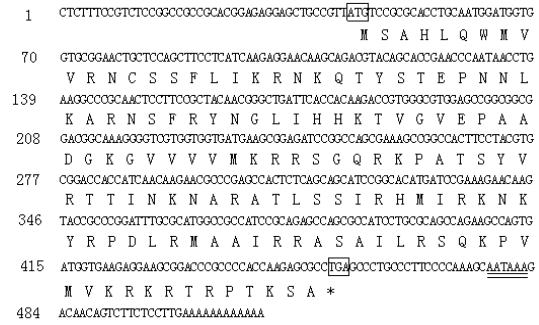


Fig. 3: Full-length cDNA and predicted protein sequence of porcine RPL28 gene. Initiator codon (ATG) and the stop codon TGA were shown in blank box and asterisk, respectively. The canonical AATAAA polyadenylation signal was double-underlined

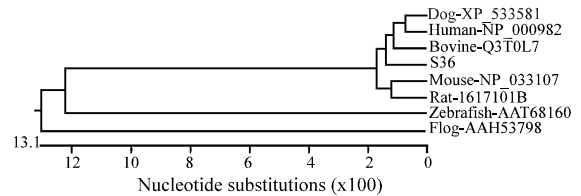


Fig. 4: Phylogenetic relationship of RPL28, the sequences used here are derived from GenBank Database expect for S36 and their accession numbers are followed by the name of species. The horizontal branch lengths are proportional to the estimated divergence of the sequence from the branch point. The vertical branch lengths are arbitrary

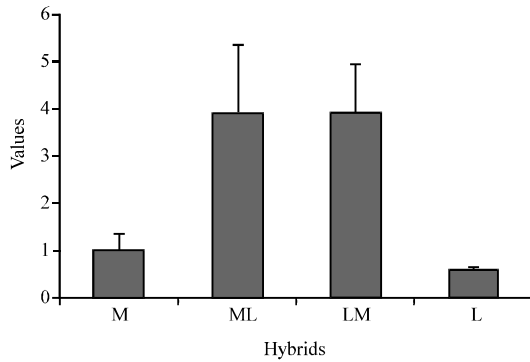


Fig. 5: The expression patterns of *RPL28* gene revealed by real-time PCR, the values were normalized to housekeeping GAPDH expression. The value of Meishan pigs were set to 1

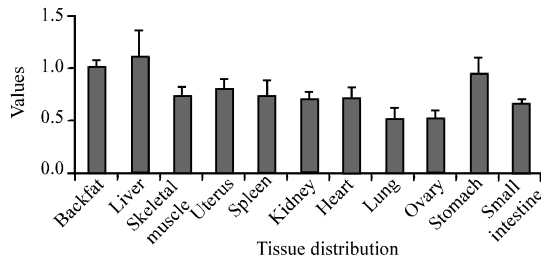


Fig. 6: The tissue distribution of *RPL28* gene, the value of backfat sample was set to 1

of RPL28 between F1 hybrids and their parents. GAPDH was used to normalize the expression level of RPL28. The relative quantitative results showed that RPL28 mRNA in backfat was up-regulated in F1 hybrids as compared with their parents (Fig. 5). Researchers also determined the spatial expression of ACL in various porcine tissues (Fig. 6). The highest level of porcine ACL mRNA expression was observed in the liver followed by the backfat, stomach, uterus, skeletal muscle, spleen cardiac muscle, kidney, small intestine, ovary and lung.

As one of organelles, ribosome plays important role in the synthesis of protein. It was composed of 4 ribosomal RNA (rRNA) and 80 Ribosomal Proteins (RP). Ribosomes are divided into two subunits. The smaller subunit binds to the mRNA while the larger subunit binds to the tRNA and the amino acids. When a ribosome finishes reading an mRNA, these two subunits split apart. The functional research on RP has become one of hotspots due to the revelation of the role of RP on human disease. Some research found that RP not only participated the synthesis of protein but also functioned as endonuclease. For example, RPS3 play role as endonuclease both in mammal and drosophila. Human RPL22 can format RNP complex in combination with EB

virus. A little is known about *RPL28* gene except for isolation from human and mouse. The full-length of mouse RPL28 cDNA is 555 bp encoding 137 amino acids.

CONCLUSION

In the present study, the *RPL* gene expression was up-regulated at backfat of F1 hybrids and their parents indicating ribosomal protein may be involved in the formation of porcine heterosis.

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