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Identification and characterization of a differentially expressed protein (CAPZB) in skeletal muscle between Meishan and Large White pigs

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ABSTRACT

Actin capping protein beta (CAPZB) protein was identified with considerable differences in the longissimus dorsi muscle between Large White and Meishan pigs using proteomics approach. However, in pigs, the information on CAPZB is very limited. In this study, we cloned and characterized the porcine actin capping protein beta (CAPZB) gene. In addition, we present two novel porcine CAPZB splice variants CAPZB1 and CAPZB2. CAPZB1 was expressed in all twenty tissues. However, CAPZB2 was predominantly expressed in the skeletal muscle and heart. In addition, the two isoforms had different expression profiles during the skeletal muscle development and between breeds. Moreover, the SNP T394G was identified in the coding region of the CAPZB gene, which was significantly associated with the carcass traits including the LFW, CFW, SFT and LEA. Data presented in our study suggests that the CAPZB gene may be a candidate gene of meat production trait and provides useful information for further studies on its roles in porcine skeletal muscle.

1. Introduction

Actin capping proteins (CapZ) are a heterodimeric protein composed of α- and β-subunits that are present in a wide variety of tissues and organisms (Cooper and Sept, 2008). There are three α-subunit isoforms encoded by three genes (CapZα1, CapZα2, and CapZα3), and three β-isoforms, produced by alternate splicing of mRNA from one CAPZB gene in eukaryotic cells (Barron-Casella et al., 1995; Schafer et al., 1994). In mammalian cells, depletion of capping protein (CP) caused loss of lamellipodia elongation and explosive formation of filopodia (Mejillano et al., 2004). The CP mutations in Drosophila could affect the actin organization, bristle morphology, and viability (Hopmann et al., 1996). The CP mutant cells in budding yeast and Dictyostelium had an abnormal actin distribution, including the loss of actin cables and a heterogeneous size distribution (Amatruda et al., 1990, 1992; Hug et al., 1992). This protein is one of the key components that regulate the dynamic properties and organization of actin filaments, and regulates the growth of the actin filament at the barbed end (Hernandez-Valladares et al., 2010; Littlefield et al., 2001; Schafer et al., 1993).

The porcine CAPZB gene was mapped to SSC 6q2.3–q2.6 within known QTL (quantitative trait locus) for carcass traits and meat quality traits (Mohrmann et al., 2006; Yang et al., 2008). In this region, there are abundant evidences for QTL affecting backfat thickness and loin eye area on pig chromosome 6 (SSC6) in several pig resource populations (Kim et al., 2005; Liu et al., 2007; Varona et al., 2002). Moreover, previous studies indicated that CAPZB was a significantly trait-associated gene that provides a link between important pathways affecting pig meat quality traits using principal component analysis combined with microarray expression data (Ponsuksili et al., 2009, 2010). In our recent study, proteomics analyses was performed in the porcine longissimus dorsi muscle of different developmental stages. Significant expression difference was found between breeds and stages for CAPZB protein (Xu et al., 2009, 2012). Therefore, we focus on the porcine CAPZB which could be a candidate gene of porcine carcass and meat quality traits.

In this study, we cloned and characterized the porcine actin capping protein beta (CAPZB) gene. We observed two alternative splicing events of the CAPZB in porcine fetal and adult different tissues. To further understand their roles in muscle development, expression differences

Abbreviations: CAPZB, actin capping protein beta protein; LFW, leaf fat weight; CFW, caul fat weight; SFT, shoulder fat thickness; LEA, loin eye area; CDS, coding sequence; PPIA, peptidylprolyl isomerase A; ORF, open reading frame; BAC, bacterial artificial chromosome; SSCP, single strand conformation polymorphism.

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were detected in skeletal muscle from different developmental stages in between the indigenous Meishan pig and the Western breed, Large White. Considering its important function in the skeletal muscle development, we conducted the polymorphisms determination and association analysis in our Large White × Meishan pig resource family with meat quality and carcass traits.

2. Materials and methods

2.1. Animal and tissue

All animal work was conducted according to the guidelines for the care and use of experimental animals established by the Ministry of Agriculture of China. Sample collection was approved by the ethics committee of Sichuan Agricultural University. Large White and Meishan sows (7 sows for each breed) were artificially inseminated with semen from the boars of the corresponding breed. Four developmental stages (65 dpc, and three postnatal stages of 3, 60 and 120 days, four pigs in each stage), total 16 female Large White and Meishan pigs were derived respectively. For prenatal ages, 4 sows were slaughtered at 65 days after insemination, and fetuses were collected. The longissimus dorsi muscle was dissected from all the fetuses and 4 female fetuses from different pregnancy sows were used as the experimental samples. For postnatal ages, 4 sows (from different litters) per time point were slaughtered at 3, 60 and 120 days and longissimus dorsi muscle from the same area was collected. Adult Meishan pigs (females, 2-year-old, n = 2; males, 2-year-old, n = 2) heart, liver, spleen, lung, kidney, stomach, longissimus dorsi muscle, subcutaneous adipose tissue, ovary, uterus, testis, brain, and embryo [females, 65 day post-conception (dpc), n = 4] heart, liver, spleen, lung, kidney, stomach, longissimus dorsi muscle and brain were also collected, respectively, for spatial expression analysis. All tissues were collected within 30 min after slaughter and immediately frozen in liquid nitrogen then stored at −80 °C until RNA and protein extraction.

2.2. In silico cloning of porcine CAPZB gene

The cDNA sequences of mouse CAPZB (GenBank accession no. NM_001037761.2) were compared to all sequences available in the pig EST databases using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast). We selected the porcine ESTs that shared more than 85% sequence identity to the corresponding human cDNA to assemble the porcine CAPZB gene using the DNA Star program (Madison, WI, USA). Gene-specific primer pairs (CDS1-F and CDS1-R, Table S2) were designed to amplify the full-length coding region of porcine CAPZB.

NCBI's online ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and DNASTAR were employed to predict open reading frames for translated peptide products. Furthermore, ClustalW (http://www.ebi.ac.uk/clustalw/) was used for multiple sequence alignment and shaded using BOXSHADE 3.21 (http://www.ch.embnet.org/).

2.3. RNA isolation and cDNA synthesis

Total RNAs were isolated from pig tissues with Trizol reagent and treated with RNase-free DNase I (Takara) to remove contaminating genomic DNA. Nucleic acid concentrations were measured at 260 nm with a BioPhotometer (Eppendorf, Germany). Purity of the total RNA was determined by the A260/280 and A260/230 ratio and its integrity was tested by electrophoresis using 1% formaldehyde denaturing agarose gel. The first strand cDNAs were synthesized using AMV reverse transcriptase (Promega, Madison, WI, USA) in a 50 μL reaction mixture according to the manufacturer's instruction. Briefly, a mixture of 2 μg total RNA and 5 μL oligo (dT) was incubated at 70 °C for 5 min to break the RNA secondary structure. The mixture was then chilled on ice for at least 2 min and then 10 μL 5× RT buffer, 5 μL dNTPs (10 mM each), 50 U RNase inhibitor and 200 U AMV reverse transcriptase were added for a total volume of 50 μL. The RT mix was incubated at 42 °C for 60 min. Finally, the reverse transcriptase was inactivated by 5 min incubation at 90 °C.

2.4. Reverse transcription-PCR

Reverse transcription (RT)-PCR was used to amplify individual transcripts of CAPZB from the cDNAs of different adult and embryo tissues of porcine. Two microliters of the resulting single-stranded cDNA was amplified in 30 or 35 cycles with specific primer pairs (CAPZB-VF and CAPZB-VR, Table S1). PCR cycling conditions were as follows: 95 °C initial denaturation for 4 min, 30 cycles of 95 °C denaturation for 40 s, 60 °C annealing for 40 s, and 72 °C extension for 20 s. A final extension was performed at 72 °C for 7 min. The PCR fragments were purified and directly sequenced to confirm the correct amplification of the three genes. Beta-actin was used as an endogenous reference gene.

2.5. Quantitative real time RT-PCR analysis

Real-time RT-PCR was used to quantify the expression level of pig CAPZB1 and CAPZB2 gene at four developmental stages of two breeds using ABI 7300 real-time PCR thermal cycle instrument (ABI, USA), according to the supplied protocol. Each real-time PCR (in 25 μL) reaction contained 12.5 μL SYBR® Green Real time PCR Master Mixture (contains ROX Dye. Toyobo, Jap), 0.25 μM primers (CAPZB1-DLF and CAPZB1-DLR for CAPZB1; CAPZB2-DLF and CAPZB2-DLR for CAPZB2; Table S1) and 1 μL normalized template cDNA. The cycling conditions consisted of an initial, single cycle for 3 min at 95 °C followed by 40 cycles of cycling consisting of 20 s at 94 °C, 20 s at 60 °C or 64 °C, 15 s at 72 °C, and final extension for 5 min. The specificity of PCR products was confirmed by melting curve analysis. All PCR amplifications were performed in triplicate for each RNA sample. Gene expression levels were quantified relatively to the expression of PPIA by employing an optimized comparative Ct ($2-\Delta\Delta Ct$) value method. The values were first normalized to the expression of PPIA and then compared relatively to the expression of CAPZB1 at 120 days in Meishan pigs or CAPZB2 at 65 dpc in Large White pigs, which was set as 1. In our previous study, we explored commonly ten used reference genes in porcine skeletal muscle using SYBR green qPCR. We used both geNorm and NormFinder to analyze the expression stability and found that PPIA was the most suitable internal control for porcine skeletal muscle and between breeds (Feng et al., 2010). So, in this study, we used PPIA as the housekeeping gene to detect the mRNA expression level of CAPZB gene in porcine skeletal muscle developmental stages between breeds.

One-way ANOVA was employed using SPSS version 13.0 to compare the difference of gene expression in different developmental stages and Duncan's new multiple rang test was used to analyze statistical significance. While Paired-Sample *T* Test was carried out to identify the expression differences at each stage between the two breeds.

2.6. Western blotting

Total protein was extracted in preparation buffer [7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% IPG Buffer pH3–10, 10 mL proteinase inhibitor cocktail (BBI, Kitchener, Canada)]. Then incubated for 30 min at room temperature with occasional vortex, and centrifuged at 20,000 *g* for 30 min at 4. The supernatant was collected and stored at −80 °C until analysis. Protein concentrations were determined using the Bradford protein assay. 20 μg of total protein extract from different skeletal muscle samples was separated by 10% SDS-PAGE and subsequently electro transferred to PVDF membrane, and incubated a rabbit polyclonal anti-CAPZB antibody (ab96618, Abcam) and anti-alpha Tubulin antibody (ab15246) at a 1:1000 dilution at 4 °C overnight. Immunoblots were developed using horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz, CA) at a 1:5000 dilution, followed by detection with enhanced chemiluminescence. The antibody was validated using