A novel porcine gene, *USP7*, differentially expressed in the *musculus longissimus* from Wujin and Large White pigs

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ABSTRACT: The mRNA differential display technique was performed to investigate differences in the gene expression in the *musculus longissimus* from Wujin and Large White pigs. A fragment of one differentially expressed gene was isolated and sequenced. A complete cDNA sequence was obtained using the rapid amplification of cDNA ends (RACE) method. The sequence prediction analysis revealed that the open reading frame of this gene encodes a protein of 1 103 amino acids, which is homologous with the ubiquitin specific peptidase 7 (*USP7*) of four species, rat (identity 98%), human (98%), mouse (98%) and chicken (95%), so it can be defined as the porcine *USP7* gene. The differences in the *USP7* gene expression in muscles from Wujin and Large White pigs were confirmed using semi-quantitative RT-PCR. The gene expression analysis in eight tissues of a Wujin × Large White cross showed that *USP7* was expressed in all the tissues, except for fat.

Keywords: pig; mRNA differential display; RACE; *USP7* gene

The mRNA differential display, first described by Liang and Pardee (1992), is an expeditious and efficient method for identification and characterization of altered gene expression in different cell types. It was statistically shown that 80–120 primer combinations would be sufficient to cover all the transcript populations in the cell (Liang et al., 1993). This technique possesses the following advantages over other similar techniques: it is based on simple and established methods, more than two samples can be compared simultaneously and only a small amount of starting material is needed (Yamazaki and Saito, 2002).

Chinese indigenous pig breeds such as Wujin, Erhualian and Tongcheng often have valuable traits such as disease resistance, high fertility, good maternal qualities, unique product qualities, longevity and adaptation to harsh conditions. High growth rate and lean meat content are typical of European pig breeds such as Large White, Landrace and Duroc (Pan et al., 1993). The phenotypic variation within a species is dependent to a great extent on gene expression differences. In the porcine skeletal muscles there are differences among breeds as well as among different developmental stages (e.g. Lin and Hsu, 2005; Bílek et al., 2008). The detection of the gene expression differences between the Chinese indigenous pig breeds and the European pig breeds or finding the differentially expressed genes between these two types of pig breeds may serve as a basis for understanding molecular mechanisms of these phenotypic differences.

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The present study applied the mRNA differential display technique to isolate the differentially expressed genes in the *musculus longissimus* from one Chinese indigenous pig breed – Wujin and one European pig breed – Large White.

MATERIAL AND METHODS

Sample collection, RNA isolation and first-strand cDNA synthesis

The *musculus longissimus* samples were collected from 120-day-old Large White (4 males and 4 females) and Wujin (4 males and 4 females) pigs for mRNA differential display and semi-quantitative RT-PCR identification. The tissues including spleen, small intestine, heart, liver, ovary, lung, muscle (*musculus longissimus*), fat and kidney were collected from one adult Wujin × Large White cross for the later tissue expression profile analysis. The tissues were immediately frozen in liquid nitrogen and stored at –80°C. The total RNA was extracted from the tissues using the Total RNA Extraction Kit (Gibco, Grand Island, New York, USA). Before the first-strand cDNA synthesis, DNase I treatment of the total RNA was performed. First-strand cDNA synthesis was conducted by RNA reverse transcription as previously described (Liu et al., 2004).

Differential display

The differential display PCR amplification of each reverse transcription product was carried out with ten arbitrary primers and nine oligo (dT) primers as previously described (Liu et al., 2004).

The PCR products were then separated on 8% non-denaturing polyacrylamide gel and displayed using the silver staining described previously (Liu et al., 2006). The differentially expressed gene band was extracted from the gel and used as a template for the re-amplification which was performed with the corresponding oligo(dT) primer and the arbitrary primers used in the mRNA differential display (Liu et al., 2006). The re-amplification products were then cloned into PMD18-T vector (TaKaRa, Dalian, China) and sequenced bidirectionally by the commercial fluorometric method. At least five independent clones were sequenced for each PCR product.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed for porcine *USP7* gene identification and expression profile analysis as described earlier (Liu and Xiong, 2007). To eliminate the effect of cDNA concentration, we repeated the RT-PCR five times using 100, 200, 300, 400, and 500 ng cDNA as templates, respectively. The housekeeping gene, *beta-actin* (DQ845171), was selected as an internal control. The primers used were: 5'-TGCTGTCCCTGTACGCCTCTG-3' (forward primer 1) and 5'-ATGTCCCGCACGATCTCCC-3' (reverse primer 1). The PCR product was 220-bp long. The following gene specific primers were used to perform semi-quantitative RT-PCR for the porcine *USP7* gene identification and expression profile analysis: 5'– CCGGCCATGCTAGACAAC–3' (forward primer 2) and 5'–TATCGCCATCCATCAGCT–3' (reverse primer2). The PCR product was 391-bp long and it was amplified in a total volume of 25 µl containing: 2 µl cDNA (100–500 ng), 5 pmoles of each oligonucleotide primer (forward primer 1 and 2, reverse primer 1 and 2), 2.5 µl 2 mmol/l mixed dNTPs, 2.5 µl 10 × *Taq* DNA polymerase buffer, 2.5 µl 25 mmol/l $MgCl_2$, 1.0 unit of *Taq* DNA polymerase and sterile water. The PCR program started with a denaturation at 94°C for 4 min, followed by 25 cyc-les of 94°C/50 s, 55°C/50 s, 72°C/50 s, and extension at 72°C for 10 min.

The quantification of the PCR products was carried out with the use of Glyco Band-Scan software (PROZYME®, San Leandro, California) and the ratio of *USP7*/*beta-actin* was calculated using the common EXCEL program. Significance of the differences in ratios of *USP7*/*beta-actin* was analysed by the least-squares method (GLM procedure, SAS version 8.0).

5'- and 3'-RACE

5'- and 3'-RACE were performed using the BD SMART™ RACE cDNA Amplification Kit (BD Sciences, San Jose, CA, USA), according to the producer's instructions. The gene-specific primers (GSPs) for porcine *USP7* gene were: 5'-RACE GSP: 5'- CGCCATCCATCAGCTCATCAAGGGC-3'; 3'-RACE GSP:5'- CAGATTCGGTTGTGGCCCATGCAGG-3'.

RACE touchdown PCRs were carried out with 5 cycles of 94°C 30 s and 72°C 3 min, followed by

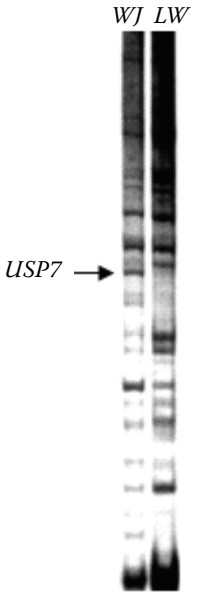

Figure 1. The differential expression analysis of cDNA from the *musculus longissimus* in Wujin (WJ) and Large White (LW) breeds (8% polyacrylamide gel stained with silver nitrate); the arrow indicates the cDNA of the *USP7* gene

5 cycles of 94°C 30 s, 70°C 30 s and 72°C 3 min, finally with 30 cycles of 94°C 30 s, 68°C 30 s, 72°C 3 min to terminate the reaction. The RACE PCR products were then cloned into PMD18-T vector (TaKaRa, Dalian, China) and sequenced bidirectionally by the commercial fluorometric method. At least five independent clones were sequenced for each PCR product.

Sequence analysis

The cDNA sequence prediction was conducted using GenScan software (http://genes.mit.edu/GENSCAN.html). The protein prediction and analysis were performed using the Conserved Domain Architecture Retrieval Tool of BLAST at the National Centre for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST) and the ClustalW software (http://www.ebi.ac.uk/clustalw).

RESULTS AND DISCUSSION

mRNA differential display

From the mRNA differential display, one band, later identified as the *USP7* gene, was found to be moderately expressed in the *musculus longissimus* of Wujin pigs, while it was barely visible in the pattern of the longissimus muscle of Large White pigs (Figure 1).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was then carried out using the gene specific primers (forward primer 1 and 2, reverse primer 1 and 2) and the results are presented in Figure 2.

Semi-quantitative RT-PCR results indicated that the *USP7* gene was not almost expressed in the

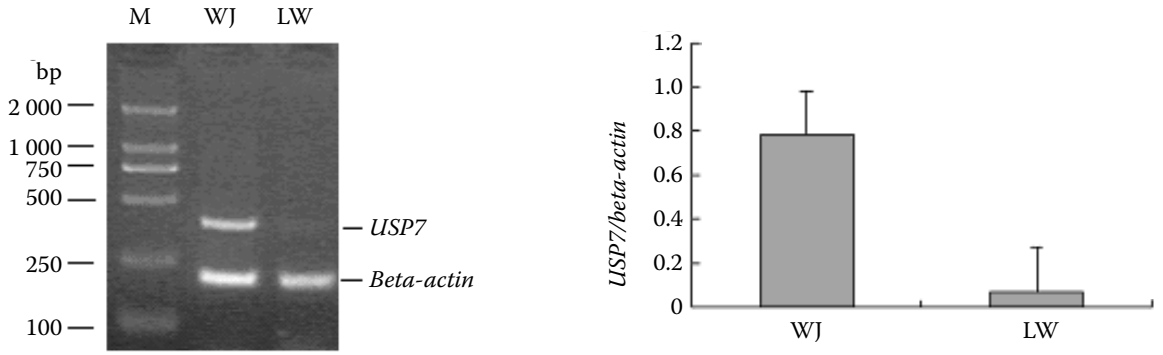

Figure 2. The semi-quantitative RT-PCR analysis of *USP7* gene on 1% agarose gel stained with ethidium bromide; error bars indicate standard deviations (n = 5) of relative *USP7* mRNA expression levels to *beta-actin*; WJ – Wujin; LW – Large White; the *USP7/beta-actin* ratios are the averages of four semi-quantitative RT-PCRs using 100, 200, 300, 400 and 500 ng cDNA as templates; the signals of the PCR products were measured by Glyco Band-Scan software, version 4.50 (same as in Figure 3); M – DL2000 marker; 1 – spleen; 2 – small intestine; 3 – heart; 4 – liver; 5 – ovary; 6 – lung; 7 – muscle; 8 – fat; 9 – kidney

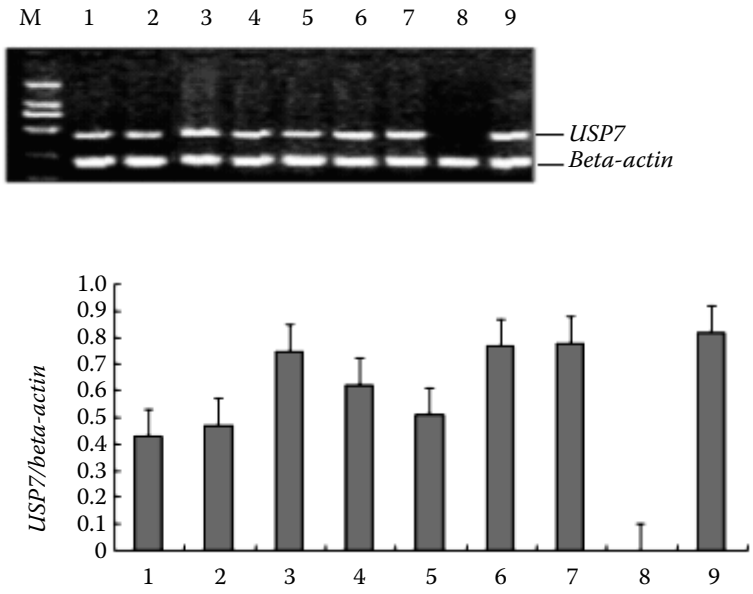

Figure 3. The tissue expression profile analysis of the porcine *USP7* gene on 1% agarose gel stained with ethidium bromide; error bars indicate standard deviations (n = 5) of relative *USP7* mRNA expression levels to *beta-actin*

longissimus muscle of Large White pigs and it was moderately expressed in the *musculus longissimus* of Wujin pigs. This also coincided with the result of mRNA differential display.

5'- and 3'-RACE

Using 5'-RACE, one PCR product of ~2.3 kb was obtained. The 3'-RACE product was ~2.2 kb long. The products were then cloned to *T*-vector and sequenced. Taken together, a 4 216-bp cDNA complete sequence was finally obtained.

Sequence analysis

The nucleotide sequence analysis using the BLAST software at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) revealed that this gene was not homologous to any of the known porcine genes and after its identification it was then deposited into the GenBank database (accession No. FJ167680). An open reading frame encoding 1 103 amino acids was found in the 4 216-bp cDNA sequence. In the predicted results, probability of exon was 0.974, poly-A signal was from 3 824 bp to 3 829 bp (consensus: AATAAA). Further BLAST analysis of this protein revealed that this protein is homologous to the ubiquitin specific peptidase 7 (*USP7*) proteins of four species – rat (accession No. NP 001019961; identity 98%), human (NP 003461; 98%), mouse (NP 001003918; 98%) and chicken (NP 989802; 95%).

Tissue expression profile

The semi-quantitative RT-PCR analysis of the tissue expression profile was carried out using the tissue cDNAs of one adult Wujin × Large White cross as the templates. The tissue expression analysis indicated that the porcine *USP7* gene is moderately expressed in muscle, heart, spleen, liver, ovary, lung, small intestine and kidney but hardly expressed in fat (Figure 3).

USP7 is a ubiquitin-specific protease and plays an important role in interspecies interactions between organisms, protein deubiquitination and ubiquitin-dependent protein catabolic process (D'Andrea and Pellman, 1998; Hong et al., 2002; Li et al., 2002). The porcine *USP7* gene has not been reported before now.

From the results obtained above, we concluded that the *USP7* gene was differentially expressed in the *musculus longissimus* from Wujin and Large White pigs. Wujin is a fat-type pig breed, comprising much more body fat than lean meat or muscle. On the other hand, Large White is a typical lean-type pig breed, presenting the opposite phenotype. The meaning of this difference in the expression levels is not known at present. The gene is expressed also in other tissues studied, except for fat.

From the alignment analysis it could be seen that porcine *USP7* protein is very similar to the *USP7* proteins of other species (mouse, rat, human, chicken). This indicates that the *USP7* gene is highly conserved.

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