Meat Science 86 (2010) 436–439

Contents lists available at ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci

Identification of suitable reference genes for gene expression analysis of pork meat quality and analysis of candidate genes associated with the trait drip loss

Jean McBryan [a], Ruth M. Hamill [a], Grace Davey [b], Peadar Lawlor [c], Anne Maria Mullen [a,*]

[a] Teagasc Ashtown Food Research Centre, Ashtown, Dublin 15, Ireland
[b] Martin Ryan Institute, National University of Ireland Galway, Ireland
[c] Pig Development Unit, Teagasc Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

ARTICLE INFO

Article history:
Received 18 September 2009
Received in revised form 21 April 2010
Accepted 14 May 2010

Keywords:
Longissimus
Water holding capacity
Reference genes
Real-time quantitative PCR
Calpastatin
RPL4

ABSTRACT

The aim of this study was to identify a set of stably expressed endogenous control genes for quantitative PCR analysis of mRNA expression in the porcine *LTL* muscle and to subsequently perform expression analysis of potential candidate genes associated with drip loss. Expression stability of seven commonly used reference genes was examined in $n=60$ pigs from three independent populations of different genetic backgrounds. The genes examined were: *ACTB*, *ATP5G1*, *B2M*, *GPX1*, *RPL4*, *TBP* and *YWHAZ*. GeNorm analysis of expression stability identified *B2M*, *RPL4* and *TBP* as consistently stable in each breed examined. Analysis of meat samples divergent for water holding capacity identified positive and negative associations between drip loss and gene expression using *B2M*, *RPL4* and *TBP* as endogenous controls. Specifically, expression of *COL1A1* increased significantly with increasing drip loss while expression of *CAST* decreased significantly with increasing drip loss. This study therefore indicates the use of *B2M*, *RPL4* and *TBP* as suitable endogenous controls for gene expression analysis of the porcine *LTL* muscle. Further study is recommended to identify the detailed roles of *COL1A1* and *CAST* with respect to the development of drip loss.

1. Introduction

Real-time quantitative PCR (qPCR) is an efficient method for quantifying gene expression and a useful tool for studying the molecular basis of variation in meat quality (Plastow et al., 2005). An essential part of qPCR is normalisation of the data using stably expressed reference genes as endogenous controls. This helps to control for the many technical variables such as variation in starting material, cDNA synthesis, presence of inhibitors etc. (Nolan, Hands, & Bustin, 2006). To date, several studies have assessed the stability of certain reference genes in various types of porcine tissue (Nygard, Jorgensen, Cirera, & Fredholm, 2007; Svobodova, Bilek, & Knoll, 2008). For example, certain reference genes have been recommended specifically for comparison of backfat and longissimus muscle while different reference genes have been recommended for comparison of porcine embryonic tissue at different stages of development (Erkens et al., 2006; Kuijk et al., 2007). However, the expression of reference genes is known to vary between tissue types and under certain conditions (Silver, Best, Jiang, & Thein, 2006), therefore it is important to select stable reference genes suitable to the specific samples and conditions being examined.

The longissimus muscle, or loin, can be considered the most economically important cut in a pork carcass (Marcoux, Pomar, Faucitano, & Brodeur, 2007). Studies examining the transcriptome of the porcine longissimus dorsi muscle have identified gene expression changes associated with meat quality traits such as tenderness (Lobjois et al., 2008) and drip loss (Ponsuksili et al., 2008). The majority of identified gene expression changes are quite small (1.5 or 2 fold expression changes associated with extremes of a trait) and confirmation of the significance of expression fold changes by qPCR has been shown to be dependent on the choice of reference genes for normalisation (Lobjois et al., 2008). The aim of this study was to identify reference genes that are stably expressed within the longissimus thoracis et lumborum (LTL) muscle of 60 animals of different genetic backgrounds, representing 3 different pig breeds, and to confirm the suitability of these genes as endogenous controls by identifying differentially expressed genes associated with the meat quality trait of drip loss.

2. Materials and methods

2.1. Animal sampling and drip loss measurement

25 Large White × Large White/Landrace gilts, 19 Duroc × Large White/Landrace gilts and 16 Pietrain × Large White/Landrace gilts were slaughtered under controlled conditions in a pilot-scale abattoir at Teagasc Ashtown Food Research Centre, Dublin over 7 sampling dates between April 2007 and August 2008. For each

* Corresponding author.
E-mail address: anne.mullen@teagasc.ie (A.M. Mullen).

0309-1740/$ – see front matter
doi:10.1016/j.meatsci.2010.05.030

animal, longissimus tissue was removed immediately after exsanguination, finely chopped under RNAse free conditions and preserved in RNALater® (Ambion) within 10 min post-slaughter. Drip loss was measured using the hanging bag method (Honikel, 1998). Samples of a standard size (4 cm × 4 cm × 2 cm) were cut and weighed at 24 h *post mortem*. Samples of similar weight and dimensions were suspended in plastic bags at 4 °C and were re-weighed at 72 h *post mortem*. Drip loss was calculated as the percentage of weight lost over the 48 h period.

2.2. RNA isolation and cDNA synthesis

RNA was isolated from muscle tissue using a Qiagen RNeasy® Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's instructions, including a DNase treatment step. 1 μg of total RNA was used for cDNA synthesis using random primers and Superscript® III reverse transcriptase (Invitrogen Life Technologies).

2.3. Quantitative real-time PCR

Real-time PCR was performed with Power SYBR® Green on the 7500 system (Applied Biosystems). Primer sequences are listed in Table 1. Cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s. Reactions were carried out in triplicate for each cDNA sample. Melting curve analysis confirmed that only one PCR product was amplified per reaction. Relative expression values were determined by comparison to a standard curve using serial dilutions of a pool of cDNA.

2.4. Data analysis

Relative stability was analysed using GeNorm which calculates the gene expression stability measure 'M' as the average pairwise variation for that gene with all other tested reference genes (Vandesompele et al., 2002). The mean drip loss ± standard deviation for all Large White animals was 4.0% ± 2.1. Eight animals with low drip loss and eight animals with high drip loss were selected. The mean drip loss of high and low drip categories was at least one standard deviation away from the mean drip loss of all Large White animals. An unpaired *t*-test was used to compare gene expression values between high and low drip groups.

3. Results

The expression of seven potential reference genes was measured by quantitative PCR analysis in *LTL* muscle of 25 animals of Large White origin. The seven genes (*B2M*, *RPL4*, *TBP*, *ATP5G1*, *YWHAZ*, *ACTB* and *GPX1*) are commonly used as endogenous controls and were selected for their involvement in a variety of cellular functions in order to minimise the risk of coregulation. Gene symbols, full names, gene ontology functions and primer details are shown in Table 1. The stability of expression of each gene was determined using GeNorm software to calculate a stability measure or *M* value. *B2M*, *RPL4* and *TBP* had the lowest *M* values, indicating that they were the most stably expressed genes from the panel (Table 2).

The expression of all seven genes was also assessed in two independent populations of animals: 19 animals of Duroc origin and 16 animals of Pietrain origin. *B2M*, *RPL4* and *TBP* were ranked within the top 4 most stably expressed genes in both of these populations with *M* values ≤ 0.41 (Table 2). These results suggest stable expression of *B2M*, *RPL4* and *TBP* in multiple pig breeds. Consistently, *GPX1* and *ACTB* were found to be the least stably expressed of the genes examined.

To assess if *B2M*, *RPL4* and *TBP* are suitable endogenous controls for the identification of gene expression changes associated with meat quality traits, the expression of two genes of interest to water holding capacity was examined in the Large White animal population: the collagen *COL1A1* is a component of the extracellular matrix; *CAST* is an inhibitor of the calpain family of protease enzymes. Water holding capacity is a measure of the ability of meat to bind water, which is of economic significance in relation to yield and is also significant in relation to quality attributes such as texture and colour (Fischer, 2007). Drip loss is the percentage weight lost under the force of gravity by muscle/meat in a defined time over the *post mortem* period due to release of water and proteins during meat ageing (Honikel, 1998). Drip loss (Honikel, 1998) is a measure used to predict water holding capacity as high drip loss is associated with low water holding capacity (see Jennen et al., 2007 for review). Within the Large White population, 8 samples were identified as having low drip loss (Mean ± standard deviation, 1.9% ± 0.5), and 8 had high drip loss (6.9% ± 1.6). Expression of *COL1A1*, normalised to the expression of *B2M*, *RPL4* and *TBP*, showed a significant positive correlation with drip loss in these animals (1.6 fold change, *p*-value 0.0168) (Fig. 1). Expression of *CAST*, also normalised to the expression of *B2M*, *RPL4* and *TBP*, showed a significant negative correlation with drip loss in these animals (1.3 fold change, *p*-value

Table 1
Selected candidate genes and primer details. Details of gene symbol, name, location and function are provided, together with sequence accession number, primer sequences and PCR product size for each of the seven potential reference genes and two genes of interest to drip loss. Location and function are based on gene ontology classifications. Fwd: forward primer; rev: reverse primer; bp: base pairs.

Gene symbol	Gene name	Location	Function	Accession number	Primers	Product size (bp)
B2M	Beta 2 microglobulin	Golgi apparatus	Immune response	NM_213978	Fwd: AAACGGAAAGCCAAATTACC Rev: ATCCACAGCGTTAGGAGTGA	178 bp
RPL4	Ribosomal protein L4	Ribosome	Translational elongation	DQ845176	Fwd: AGAGATCCAAAGAGCCCTCCGC Rev: GCCTGGCGAAGAATGGTGTTTC	144 bp
TBP	TATA box binding protein	Nucleus	Regulation of transcription	DQ845178	Fwd: TTAATGGTGGTGTTGTGGACGGC Rev: CCAAATAGCAGCACAGTACGAGCAA	168 bp
ATP5G1	ATP synthase, H+ transporting, mitochondrial F0 complex	Mitochondrion	Transporter	DQ629147	Fwd: GGAACCCATCTTTGAAGCAG Rev: AGGATAAGGAAGGCGACCAT	103 bp
YWHAZ	Tyrosine 3-monooxygenase /tryptophan 5 monooxygenase activation protein	Cytoplasm, nucleus	Protein binding, signal transduction	XM_001927228	Fwd: ATGCAACCAACACATCCTATC Rev: GCATTATTAGCGTGCTGTCTT	178 bp
ACTB	Beta actin	Cytoskeleton	Binding	DQ845171	Fwd: AAGGAGAAGCTGTGCTACGTCGCC Rev: GTTGCCGATGGTGATGACCTGG	150 bp
GPX1	Glutathione peroxidase 1	Cytoplasm, mitochondrion	Enzyme activity	NM_214201	Fwd: TGCTTGGCAGTTACAGTGCT Rev: GGGATTTTCCTGGACATCAG	95 bp
COL1A1	Collagen, type 1, alpha 1	Extracellular matrix	Structural	AF201723	Fwd: CCAGTCACCTGCGTACAGAA Rev: ACGTCATCGCACAACACATT	110 bp
CAST	Calpastatin	Unknown	Endopeptidase inhibitor	NM_214067	Fwd: GTGCCTCCTCCAGACACTTC Rev: CTCGGTTTTCTTCCCATCAG	108 bp