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Review

Quality pork genes and meat production

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Abstract

Functional genomics, including analysis of the transcriptome and proteome, provides new opportunities for understanding the molecular processes in muscle and how these influence its conversion to meat. The Quality Pork Genes project was established to identify genes associated with variation in different aspects of raw material (muscle) quality and to then develop genetic tools that could be utilized to improve this quality. DNA polymorphisms identified in the porcine *PRKAG3* and *CAST* genes illustrate the impact that such tools can have in improving meat quality. The resources developed in Quality Pork Genes provide the basis for identifying more of these tools.

Keywords: Pig; Pork; Meat quality; Muscle genes; Functional genomics

Contents

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1. Introduction

Pig breeding companies are now paying more attention to meat quality and are including quality traits as an integral part of selection programmes. This has been in part driven by consolidation and integration in the meat processing industry and the retail sector together with an increasing consumer awareness of food quality. In addition, the development of the field of genomics has also stimulated interest in breeding for meat quality as this "trait" constitutes the classic case, where DNA marker based selection is at its most efficient: where the trait cannot be measured on the selection candidate and can only be measured at high costs on its relatives postmortem. Once a DNA marker (a polymorphism, see below) has been shown to be associated with variation in the target trait, then it can be used to genetically type young animals for pre-selection before performance testing. This is a distinct advantage over sib slaughter schemes which are increasingly difficult and expensive to implement (see Knap, Sosnicki, Klont, & Lacoste, 2002). Even so sib slaughter schemes will continue to play a role, both for the identification of new markers and for monitoring breeding lines in order to optimize the breeding direction. The advantage of incorporating markers into selection programmes can be sustained when new markers are identified to replace older markers that begin to reach fixation. The database builds up over time to provide a very useful resource for this purpose or further validation of DNA markers identified in experimental populations or to test candidate genes.

In the last decade DNA tests have been developed that allow much more effective eradication of undesirable alleles of the major genes negatively impacting pork quality, such as the *Hal* gene (HAL–1843™, as licensed from the Innovations Foundation, Toronto, Canada, owner of the trademark, Fujii et al., 1991) and the *RN* gene (Milan et al., 2000) which are associated with pale, soft and exudative meat and processed ham yield, respectively.

Breed differences in meat quality traits are large (Sellier, 1998) and commercially relevant. Duroc, Hampshire and Berkshire lines are commonly marketed as "meat quality lines" and several industry lines have been based on these breeds. However, significant variation in meat quality related traits is also present within breeds (e.g. Gil et al., 2003). The discovery of the *Hal* and *RN* genes also encouraged researchers to consider single gene effects as an alternative to breed effects. As indicated above, once DNA markers for meat quality are identified they can be used in a wide range of breeds by changing allele frequencies through selection and/or introgression.

Recent examples of these marker effects include polymorphism in the genes for calpastatin (*CAST*) and *PRKAG3* that are associated with quantitative variation in tenderness (*CAST*) and pH and colour (*PRKAG3*) (Ciobanu et al., 2001; Ciobanu, Lonergan, et al., 2002; Ciobanu, Bastiaansen, et al., 2004a; Ciobanu, Lonergan, Bastiaansen, et al., 2004b). Future success for the industry will require the production of consistent and predictable high product quality to ensure customer satisfaction. The target should be to combine efficient growth with the best possible meat quality or alternatively the aim can be described as optimizing meat quality with the lowest cost production.

To date DNA markers have been identified using two basic approaches: quantitative trait loci (QTL) mapping or the "candidate gene approach". The first utilizes specific genetic designs (for example three generation families based on divergent breed crosses such as Chinese Meishan or Wild Boar and Large White) to find the location of QTL on the genetic map. Several QTL studies have addressed pork quality traits (summarized in Bidanel & Rothschild, 2002) and they provide the starting point for the identification of individual genes (or

markers) influencing these traits (the positional candidate gene approach). Indeed, the RN^- mutation, identified initially by a mapping approach was then elucidated using positional cloning and a "BAC contig" constructed for that region of the genome (a physical representation of the DNA sequence from the QTL region) (Milan et al., 2000).

It is now possible to add functional genomics to the range of options available for understanding the molecular basis of pork quality. The objective of this study was to develop the resources required to do undertake this approach. The QualityPorkGENES project (www.qualityporkgenes.com) was initiated in order to create a unique phenotypic resource that could be exploited through the application of new functional genomics tools that determine differences in the transcriptome and proteome of muscle and relate this to the different aspects of meat quality. This approach has the potential advantage of generating information, in parallel, on multiple genes and gene products which, in turn provides the opportunity to identify pathways and interacting genes (see Maltin & Plastow, 2004). In this way the efficiency of marker detection is increased as well as providing insight into epistatic effects that can further the understanding of the genetic component of meat quality.

2. Materials and methods

2.1. Animals and samples

Five closed populations (breeding lines) were chosen for the project. These were based on Large White, Landrace, Duroc, Piétrain and Meishan breeds (although in the latter case a Meishan/Large White crossbred line was used) in order to represent a significant proportion of European pig production. The Piétrain line was normal for the "Halothane" gene. In addition, initial characterization of lines (at IRTA Monells, Spain) indicated that these lines would provide significant variation both within and between populations. Animals were reared under the same environment and production regime in a breeding nucleus farm in France. Data were collected on farm for growth, ultrasonic backfat and basal stress hormone levels. At approximately 140 days of age the animals were transported in batches of around 25 to overnight lairage at a research abbatoir in Spain (IRTA Monells). Animals were harvested after CO_2 stunning and carcass, meat quality, biochemical and sensory data were collected for 100 animals per line (in 22 batches). Urine samples were taken after transport on arrival at lairage and the next morning before they were moved to the CO_2 chamber. Immediately after harvest loin (*Longissimus thoracis*) and ham (*Semimembranosus* – SM) muscles were collected and samples prepared for fibre type and proteome analysis (after mounting and freezing in liquid nitrogen) and RNA isolation (RNAlater, Ambion Inc.).

2.2. Phenotypic traits

Measurements of fat depths at 45 min postmortem (p.m.) were made using the Fat-O-Meat'er equipment at 60 mm from the mid-line at the level of the last rib (LRFOM). At 24 h p.m., the surface area of the eye of the *Longissimus thoracis* (AREALT) was measured between the 3rd and 4th last rib level. This was done by making a transversal cut at this point and taking a digital image. This image was used to calculate the AREALT using a specific program (Pomar, Rivest, Jean dit Bailleul, & Marcoux, 2001). Each left half carcass was cut and dissected following the method of Walstra and Merkus (1995). In order to have more commercial cuts some parts were jointed as hind hand plus leg (ham), loin minus subcutaneous fat of the loin (loin) and belly plus to ventral part of the belly (belly). Estimated carcass lean content (PLEAN) was calculated using the Spanish official equation (Gispert & Diestre, 1994).

The left side of each carcass was used to assess meat quality. Muscle pH was measured using a Crison portable meter equipped with a xerolyt electrode in the *Longissimus thoracis* muscle (LT) at 45 min ($pH_{45}LT$) and 24 h (pH_uLT) p.m. Colour measurements were taken 24 h p.m. on the exposed cut surface of the LT at the last rib level, using a Spectrophotometer Minolta C2002 in the CIELAB space (CIE, 1976). Drip loss was determined in the muscle LT according to the method of Honikel (1998).

The metabolic traits of the muscle were determined by measuring the lactate dehydrogenase (LDH) activity according to Ansay (1974) and the isocitrate dehydrogenase (ICDH) activity according to Briand, Talmant, Briand, Monin, and Durand (1981). Enzyme activities are expressed as μmol NADH per minute per g of muscle (LDH) and as nmol NADPH per minute per g of muscle (ICDH). The percentage of slow myosin heavy chain (MHC-I) in the muscle was determined with a specific MHC-I monoclonal antibody by the ELISA technique (Picard, Leger, & Robelin, 1994).

Cathepsin B activity was assayed with *N*-CBZ-L-arginyl-L-arginine 7-amido-4-methylcoumarin (Z-Arg-Arg-NHMec) (Etherington & Wardale, 1982). One unit of activity was defined as the amount of enzyme hydrolysing 1 nmol of substrate per min at 37 °C. Activities were given in enzyme units per mg of extracted protein. The protein concentration of the enzyme extracts was measured according to Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as standard.

Intramuscular fat (IMF) content was determined by a NIT (Near Infrared Transmittance) apparatus. Fat was extracted by the method of Folch, Lees, and Stanley (1957) and aliquots of the extracts were collected to perform the analysis of fatty acids and triglycerides. Fatty acids were determined by GC-FID as methyl esters (FAMES) by the method described by AOCS. A capillary column coated with cyanopropylpolysiloxane was used (SGE, BPX-70) 30 m × 250 μm, width film 0.25 μm. Triglycerides were determined by HPLC with RI detector.

Sensory characterization of the samples was performed through a Quantitative Descriptive Analysis (QDA) using 6 selected trained assessors including texture descriptors in cooked meat. Samples of LT (1.5 cm thick) were stored at 4 °C during 24 h and afterwards frozen. Twenty-four hours before the evaluation, the samples were thawed at 4 °C. They were cooked in an electric oven at 110 °C until reaching an internal temperature of 65 °C and then cut and evaluated. The samples were quantified for hardness and juiciness using a 10 point scoring scale, where 0 means 'absence or minimum intensity' and 10 means 'very strong or maximum intensity'.

Texture Profile Analysis (TPA) was undertaken in sample cubes of 1.5 cm × 2 cm × 2 cm obtained from an adjacent slice of that used for sensory analysis. Samples were cooked as described in sensory analysis. All the measurements were carried out using a Texture Analyser TA.TX2 (Stable Micro systems Ltd) with a 25-kg load cell and using a crosshead speed of 5 mm/s.

The statistical analysis of the phenotypic traits was performed using the MIXED procedure of SAS (1999). The effect of the genetic type was included in the model as a fixed effect. The slaughter day (batch) was included as a block effect and the carcass weight as a covariate if it was significant. Family relationships were accounted for by means of the inclusion of sire within genetic line as a random effect. Significant differences ($p < 0.05$) between least squares means were obtained with the Tukey test. For the sensory analysis, GLM procedure was used. Genetic line and session were in the model as fixed effects. Significant differences between means were obtained with the Tukey test.

2.3. Proteomics

Loin muscle samples from each animal for the five breeds were subjected to two dimensional gel electrophoresis (2DGE) over the p*I* range of 3–10 and molecular weight range of 10–200 kDa, enabling the separation of more than 750 spots per gel. Image analysis (Phoretix Evolution) was used to generate a master proteome map representing the proteomes of the five divergent porcine breeds. Areas of saturation and poor resolution were removed digitally prior to analysis.

2.4. Preparation of cDNA libraries and Real-time PCR

Total RNA and mRNA were prepared from muscle samples preserved in RNAlater (Ambion Inc.). RNA was analysed for quality by Northern blot analysis and visualization using the Agilent BioAnalyser. The RNA was then used for the construction of suppression subtractive hybridisation (SSH) porcine muscle cDNA libraries from animals within and between divergent genotypes and for the construction of a full-length representative pig muscle cDNA library.

Suppression subtractive hybridisation was performed using the PCR-select cDNA Subtraction kit (BD Biosciences Clontech UK). Eight SSH libraries were constructed based on specific contrasts available in the project (breed, muscle type, meat quality and stress). A standard full-length pig muscle cDNA library was prepared to facilitate the isolation of full-length cDNA for candidate genes to be identified from the SSH libraries, microarray screening and proteomic analysis. The full-length cDNA library was constructed using the λ Zap Express cDNA synthesis/Gigapack cloning kit (Stratagene Cloning Systems) using 5 μg of mRNA pooled from LT and SM muscle from all five pig lines, and different slaughter days. The quality of this library was assessed by titration of library size and cDNA fragment length analysis using PCR with the M13 forward and reverse primers.

The expression pattern of the candidate gene *PRKAG3* was analysed by quantitative real-time polymerase chain reaction (PCR) using either the lightcycler system (Roche) or the MX3000P 96 well real-time PCR System (Stratagene), and the Quantitect SYBR green one-step RT-PCR kit (Qiagen). A PCR fragment of 118 bp was generated using primers located at 310–428 bp in the gene sequence (Genbank AF214520), sense: PRKAG3LCF2: (5′)CAGTCCAGGCCAGTTGCTGA GTC antisense: PRKAG3LCR2: (5′)GCTCTGTTGGG GGGTTGTCCAC. Total RNAs were selected from LT and SM muscle from Landrace and Meishan animals that had been selected as showing variation in meat quality on the basis of pH_u and LMinolta colour values and from randomly chosen Piétrain animals. A typical experiment analysed expression of *PRKAG3* in RNA from at least eight different animals (up to 14). Samples were analysed in triplicate and each experiment was repeated on two or three separate occasions. Expression level was inferred from the cycle threshold (Ct) value, or Ct values were converted to copy number per μl using a standard curve of cRNA standards in vitro transcribed from cloned cDNA products. Results were analysed statistically using ANOVA with Tukey post hoc analysis.

2.5. Microarray construction

Microarrays have been constructed using purified PCR products generated from cDNA clones. These clones were derived from the SSH libraries plus additional muscle expressed candidate genes. All PCRs were prepared in a sodium phosphate/SDS spotting buffer using a 384 well format prior to printing. The products were spotted onto Corning GAPS 2™ slides along with Cy3 and Cy5 orientation spots and control elements, using a 48×100-μm pin tool and a BioRobotics Microspot 2500 microarrayer. For the prototype array every element was spotted in duplicate using a 17×18 subarray, giving a total of 14,688 elements per slide. The second generation slides have been spotted in triplicate using a 21×21 subarray allowing a total of 21168 elements per slide.

Post spotting, slides were baked for 2 h at 80 °C. They were then blocked in 1% BSA (Molecular Biology grade, Sigma Aldrich B 2518), 0.1% SDS in 3× SSC at 55 °C for 1 h. This was immediately followed by denaturation in a 95-°C water bath for 2 min, rinsing in isopropanol, spinning dry, and storage in dark, dry conditions at RT prior to hybridization.

2.6. Microarray hybridization

Twenty μg of each RNA sample selected for hybridization to the slides was labelled with either Cy3 or Cy5 using an indirect method. RT-PCR (SuperScript II, Invitrogen) with a polyT primer was used to generate complex cDNA in the presence of aminoallyl dUTP (Sigma). The reaction was followed by a post-PCR coupling to the reactive CyDye (Amersham Pharmacia) in the presence of sodium bicarbonate/hydroxylamine buffer. Samples were then purified using a Qiagen PCR Purification Kit column prior to precipitation in the presence of 10 μg of pig genomic DNA plus 500 ng of cloned pig SINE repeat element. The resulting pellet was resuspended in 100 μl of formamide hybridization buffer (Sigma) along with 1 μl of yeast tRNA (Sigma) and 1 μl of poly dA (Amersham Pharmacia Biotech). Hybridization mixes were applied to the slides, and incubated at 42 °C for 18 h before washing in an SSC series to a final concentration of 0.1 X. Slides were scanned on an Axon 4100 scanner, at 10 μm resolution, and images captured with the GenePix software.

2.7. Image analysis

All images were analysed for the pixel intensities in each of the two channels (Cy3 and Cy5) using BlueFuse (BlueGnome Ltd.). Background signal was removed from the spot signals, and replicate data fused to produce single values, with a high confidence probability, for each clone represented on the array.

2.8. Microarray experimental design and analysis

Animals for the first experiments (investigating water holding capacity and intramuscular fat content) were selected on the basis of a factor analysis, where the first four factors were descriptive of (1) fatness versus leanness (2) water holding capacity (pH, LMinolta, driploss) (3) intramuscular fat and (4) percentage loin. The lines with the greatest variance for each factor were identified: the Meishan-factor (1), Large White and Piétrain-factor (2), Duroc and Meishan-factor (3) and Landrace-factor (4). Animals were ranked on their individual factor scores and the top ten and bottom ten animals for each factor were selected within the lines of interest. Outliers, animals with residual values more than three standard deviations from their respective within-line means, were removed.

For each factor comparison, cDNA samples prepared from animals with high or low scores from at least two different breeds were hybridized to the prototype array using a loop design. At each point within the loop one high scoring sample is compared against one low. Each sample occurs twice within the loop with an intrinsic dye swap. (Fig. 1(a)) (Kerr & Churchill, 2001a, 2001b).

A second experiment will be undertaken to enable the relationship between gene expression and tenderness and sensorial traits to be established. One hundred individual animals, 20 animals from each line, will be arrayed. Samples will be compared against a common pooled control sample (reference design: Fig. 1(b)) for this second expression experiment.

For both microarray experiments the data will be analysed using microarray ANOVA (Kerr, Martin, & Churchill, 2000). ANOVA methods can be used to normalize the microarray data, for example by taking account of variation between slides, and also to provide estimates of changes in gene expression that are corrected for potential confounding effects, such as slaughter batch.

3. Results and discussion

3.1. Carcass, biochemical and meat quality traits

More than 150,000 datapoints have been collected to describe the phenotypic characteristics of the animals in this study. These can be assigned to 358 measured and 62 derived traits for the following groups; performance (7), carcass (20), cutting (84), meat quality (20), biochemical (e.g., enzyme activity) (16), endocrine (18), sensory (13), texture (12), fatty acid content (198) and fibre type (32).

Table 1 shows the least squares mean values and standard error of the parameters of carcass quality and also

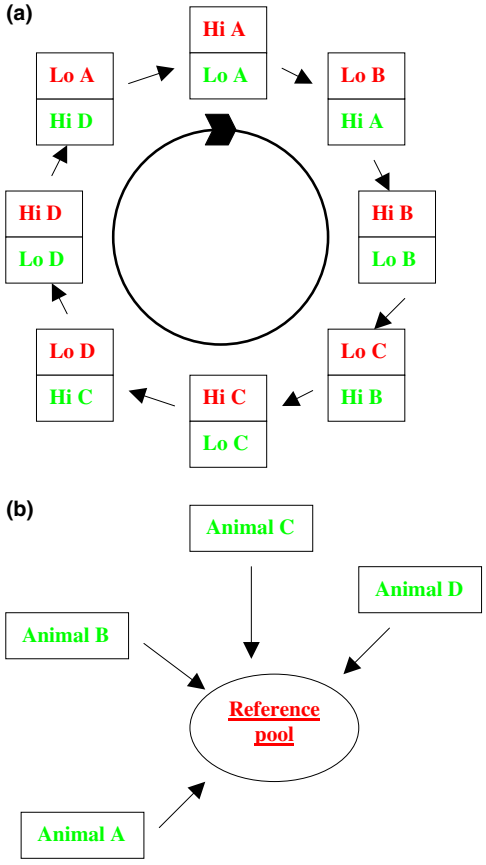

Fig. 1. (a) Loop design: all samples appear twice within the loop, with an intrinsic dye swap. Samples are labelled red (Cy5) or green (Cy3) and each pairwise comparison includes samples from one high and one low scoring animal. (b) Reference design: each test sample is compared against a common reference. For QPG the reference will be a pool of RNA from LT muscles, taken from approximately 100 animals and equally representing all five breeds. The reference will be labelled uniformly for all slides. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the differences between lines. The Meishan based line showed lowest value for carcass weight together with the Piétrain based line and the Duroc based line the highest value, although not significantly different from the Landrace and Large White based lines. In relation to the carcass quality, the Piétrain line had the leanest carcass, showing – with significant differences – the least fat depth and, accordingly, the highest muscular area (57.64 cm^2) and lean content in the carcass (60.40%). The fattest line was the Meishan line, showing 21.96 mm of LRFOM and 48.02% of lean content. The other lines were intermediate. These results were in accordance with Gil et al. (2003).

Meat quality parameters are shown in Table 2. It can be seen that the Landrace based line presented lower pH_{45} and the higher L^* value than the Large White based line, indicating lighter meat. None of the lines presented PSE meat. Ultimate pH was significantly higher in the Duroc and Piétrain lines with respect to the Landrace. Drip losses were significantly lower in Duroc than in Landrace line, the other lines being intermediate.

The activities of the enzymes LDH and ICDH and the glycolytic ratio LDH/ICDH were determined to assess the metabolic traits of the muscles (Table 3). The within line variability for the LDH was between 19.1% and 25.5%. This variable showed no significant differences between the five lines studied. The within line variability for ICDH activity and for the ratio LDH/ICDH were in the range 26.3–39.5%. ICDH differed significantly between lines: The Duroc and Piétrain lines presented higher activity than the Large White and Meishan lines. In accordance with this, the glycolytic ratio for the Duroc and Piétrain lines was lower than for the Meishan line, with the Landrace and Large White lines having intermediate values. The percentage of MHCI did not differ between lines. The within line variability of this variable was between 32.9% and 41.3%. The values obtained for these variables were similar to those reported before for different porcine genetic lines or crosses (Gil et al., 2003; Henckel, Oksbjerg, Erlandsen, Barton-Gade, & Bejerholm, 1997; Petersen, Henckel, Oksbjerg, & Sorensen, 1998). Cathepsin B activity also showed a high variability in all the lines (between 40.9% in the Large White line and 48.1% in the Piétrain line). The Duroc based line had higher proteolytic activity than the Meishan line, and the other lines showed values in between. Hernández, Zomeño, Ariño, and Blasco (2004) also report differences in cathepsin B activity between different porcine lines.

Table 4 shows the means and standard deviations for IMF, fatty acids and triglycerides in LT muscle. IMF showed a high within line variability. The highest values

Table 1
Least squares means (lsm) and standard error (se) of carcass characteristics depending on the genetic line*

	n	Landrace		Large White		Duroc		Piétrain		Meishan	
		lsm	se	lsm	se	lsm	se	lsm	se	lsm	se
Carcass weight (kg)	500	89.70^{ab}	0.71	90.74^{ab}	0.68	91.63^{a}	0.72	88.59^{bc}	0.78	85.70^{c}	0.83
Fat depth at the last rib measured with FOM (mm)	499	13.60^{c}	0.38	13.11^{cd}	0.36	15.92^{b}	0.40	11.66^{d}	0.43	21.96^{a}	0.48
Area of LT muscle (cm^2)	500	48.02^{b}	0.69	46.94^{b}	0.65	48.59^{b}	0.72	57.64^{a}	0.79	41.30^{c}	0.87
Estimated lean with FOM (%)	500	57.18^{b}	0.39	57.56^{b}	0.37	55.48^{c}	0.40	60.40^{a}	0.44	48.02^{d}	0.48

* Values in a row with a common superscript are not significantly different ($P > 0.05$).

Table 2
Least squares means (lsm) and standard error (se) of meat quality parameters measured in the *Longissimus thoracis* muscle depending on the genetic line*

	n	Landrace		Large White		Duroc		Piétrain		Meishan	
		lsm	se	lsm	se	lsm	se	lsm	se	lsm	se
pH45	500	6.49^{b}	0.02	6.62^{a}	0.02	6.57^{ab}	0.02	6.55^{ab}	0.02	6.58^{ab}	0.03
pHu	500	5.59^{b}	0.01	5.63^{ab}	0.01	5.64^{a}	0.01	5.64^{a}	0.01	5.59^{ab}	0.01
L^* (lightness)	500	48.39^{a}	0.30	46.40^{b}	0.29	46.15^{b}	0.30	46.66^{b}	0.32	47.27^{ab}	0.33
a^* (redness)	500	2.75^{c}	0.11	2.92^{bc}	0.11	2.97^{abc}	0.11	3.36^{ab}	0.12	3.39^{a}	0.13
b^* (yellowness)	500	4.32^{a}	0.15	3.66^{b}	0.14	3.44^{b}	0.15	3.92^{ab}	0.16	4.26^{a}	0.16
Drip loss	461	3.28^{a}	0.14	2.92^{ab}	0.13	2.49^{b}	0.14	2.87^{ab}	0.15	2.90^{ab}	0.17

* Values in a row with a common superscript are not significantly different ($P > 0.05$).

Table 3
Least squares means (lsm) and standard error (se) of biochemical parameters measured in the *Longissimus thoracis* muscle depending on the genetic line*

	n	Landrace		Large White		Duroc		Piétrain		Meishan	
		lsm	se	lsm	se	lsm	se	lsm	se	lsm	se
Lactate dehydrogenase (LDH) (μmol NADH min^{-1} g^{-1})	249	3135.66	75.34	3016.87	73.84	3051.47	76.47	3294.89	74.71	3139.31	76.37
Isocitrate dehydrogenase (ICDH) (nmol NADPH min^{-1} g^{-1})	249	1.32^{ab}	0.06	1.30^{b}	0.06	1.54^{a}	0.06	1.55^{a}	0.07	1.23^{b}	0.07
Ratio (LDH/ICDH) $\times 10^{-3}$ (μmol $nmol^{-1}$)	249	2.50^{ab}	0.11	2.48^{ab}	0.11	2.20^{b}	0.12	2.32^{b}	0.12	2.80^{a}	0.12
Myosin Heavy Chain-I (%)	248	7.33	0.41	7.29	0.40	8.31	0.42	8.41	0.41	6.91	0.42
Cathepsin B (units mg $protein^{-1}$)	244	0.034^{ab}	0.002	0.032^{bc}	0.002	0.041^{a}	0.002	0.033^{bc}	0.002	0.026^{c}	0.002

* Values in a row with a common superscript are not significantly different ($P > 0.05$).

Table 4
Least squares means (lsm) and standard error (se) of intramuscular fat, fatty acids and triglycerides of the *Longissimus thoracis* muscle depending on the genetic line*

	n	Landrace		Large White		Duroc		Piétrain		Meishan	
		lsm	se	lsm	se	lsm	se	lsm	se	lsm	se
Intramuscular fat (%)	498	1.09^{b}	0.07	1.00^{b}	0.07	1.81^{a}	0.08	1.21^{b}	0.08	1.90^{a}	0.09
Stearic acid (%)	247	12.21^{b}	0.14	12.31^{b}	0.14	13.40^{a}	0.15	11.92^{b}	0.16	12.17^{b}	0.17
Linoleic acid (%)	247	13.80^{a}	0.45	13.84^{a}	0.43	10.51^{b}	0.47	13.18^{a}	0.51	9.34^{b}	0.56
Dioleyl-steaoryl-glycerol (%)	93	5.32	0.19	5.13	0.19	5.83	0.20	5.83	0.20	5.62	0.20
Disteaoryl-oleyl-glycerol (%)	93	1.58	0.14	1.12	0.15	1.43	0.15	1.15	0.16	1.08	0.16

* Values in a row with a common superscript are not significantly different ($P > 0.05$).

of IMF were found in the Duroc and Meishan lines. The other lines have similar values although it is interesting the leanest line, Piétrain, tends to have a higher value than Landrace and Large White. Regarding the fatty acids, the percentage of stearic was higher in the Duroc line – that also presented a high IMF content – than in the other four lines. The Meishan line although having a high percentage of IMF, did not show a percentage of stearic acid as high as the Duroc line. It is interesting to note that the within line variability for this acid was quite low (5.8–7.7%). The percentage of linoleic acid was significantly higher in the Landrace, Large White and Piétrain lines than in the Duroc and Meishan lines. These data, together with the values of stearic acid, would suggest that the metabolism for the C18 acids was different between lines, which could be related with the genetics and, thus, with specific genes directly or indirectly associated with lipid metabolism. With respect to the minority triglycerides, dioleyl-steaoryl-glycerol (SOO) and disteaoryl-oleyl-glycerol (SSO) that are important because they are related with the consistency of the fat, there were not significant differences between lines. In the case of SSO this may be due to the high within line variability. The Landrace and Duroc lines show the highest values for SSO and this could relate to greater fat firmness in these lines.

Table 5 shows the least square means and the significant differences per genetic line for hardness and juiciness sensory attributes. Hardness was lower in Meishan, Duroc and Piétrain lines and higher in Landrace and

Table 5
Least squares means (lsmeans) of the sensory characteristics of the Longissimus thoracis muscle depending on the genetic line*

	n	Landrace	Large White	Duroc	Piétrain	Meishan	RMSE[a]
		lsmeans	lsmeans	lsmeans	lsmeans	lsmeans	
Hardness	250	5.3[a]	5.1[a]	4.6[b]	4.6[b]	4.2[b]	0.8508
Juiciness	250	2.5[b]	2.6[b]	3.1[a]	3.1[a]	3[a]	0.6222

[a] RMSE: Root mean standard error.
* Values in a row with a common superscript are not significantly different ($P > 0.05$).

Table 6
Means and standard deviations (std) of the instrumental texture analysis of the *Longissimus thoracis* muscle depending on the genetic line[a]

	n	Landrace		Large White		Duroc		Piétrain		Meishan	
		mean	std	mean	std	mean	std	mean	std	mean	std
Hardness	247	15.36[a]	2.57	15.36[a]	3.13	15.12[a]	2.59	13.86[b]	2.51	15.09[ab]	2.08

[a] Values in a row with a common superscript are not significantly different ($P > 0.05$).

Large White lines. Regarding juiciness it seems that the lean lines were those with the lowest score for juiciness.

Table 6 shows the average values (means) and the significant differences per genetic line (Tukey test) for hardness from instrumental texture analysis (TPA test). Regarding this parameter obtained from the TPA curve, it seemed that the Piétrain line was different from the rest with the exception of the Meishan line. Hardness was evaluated differently by the instrumental test than by the sensory panel. The conditions in the mouth during chewing possibly could explain the different assessment of the sensorial hardness compared with the instrumental evaluation. Further analysis is needed regarding relations or correlations with other variables in order to interpret these results.

3.2. Stress hormone analysis

Urine was sampled when spontaneously voided in the farm (basal conditions), when the animals arrived in the lairage area (after approximately 10 h of transportation in a lorry) and the next morning before slaughter. Levels of cortisol and catecholamines (adrenaline and noradrenaline) were measured in these urine samples as described (Hay & Mormède, 1997a, 1997b). Blood was sampled during exsanguination, and creatine kinase activity, cortisol, glucose, lactate and free fatty acid (FFA) levels were measured in serum. A strong relationship between the urinary concentrations of cortisol in basal conditions and after transportation stress, were found indicating that genetic factors primarily influence the intrinsic activity/reactivity of the corticotropic axis. Across genetic types, concentrations of cortisol in urine were also correlated with carcass fat content, except for Piétrain pigs that combine a very low adiposity together with high urinary cortisol levels. This result confirms previous data demonstrating the genetic linkage between the hypothalamic-pituitary adrenal axis activity and fat accumulation, with *Cbg*, encoding corticosteroid-binding globulin, the carrier of cortisol in plasma, as a strong candidate gene (Désautés et al., 2002; Ousova et al., 2004). A correlation between cortisol levels measured in urine collected after slaughter and adiposity was also demonstrated in a segregating F2 intercross between Large White and Duroc pigs (Foury et al., 2005). Since urinary cortisol levels are directly related to cortisol secretion by the adrenal gland, the present data, together with available literature, suggest that adrenal secretory activity is another candidate for genetic polymorphism that could influence carcass composition. It will be interesting to see if this pathway is identified in the transcriptome analysis to be undertaken in the next stage of the project.

Secretion of catabolic hormones (cortisol and catecholamines) was the lowest in Large White pigs that also showed the lowest levels of energy mobilisation under slaughter stress (low levels of plasma lactate and FFA). Low levels of plasma glucose in Meishan and Duroc pigs suggest that their metabolism is more oriented towards energy storage, in accordance with the higher carcass fat content. Altogether these measures also showed that in the present experiments animals experienced very low levels of stress before and at the time of slaughter, so that the phenotypic and molecular data can be considered as representing 'basal' values with a minimal influence of pre-slaughter stress, that is a main component of meat quality, in interaction with genetic factors (Mormède et al., 2004; Sellier & Monin, 1994).

3.3. Longissimus thoracis proteome

Proteomics technology is becoming increasingly popular in the identification of gene products to aid in determining meat quality. Recent studies have utilised two dimensional gel electrophoresis (2DGE) to investigate changes in the *Longissimus thoracis* (LT) muscle

proteome over 72 h postmortem in relation to meat quality (Lametsch & Bendixen, 2001; Lametsch et al., 2003; Lametsch, Roepstorff, & Bendixen, 2002; Morzel et al., 2004). The degradation of specific structural and metabolic proteins was demonstrated that varied significantly with aging time. In contrast, the current study presents the first major investigation comparing the proteome of the LT muscle for five divergent porcine breeds at the time of slaughter. This will enable us to detect breed differences in perimortem muscle metabolism in relation with organoleptic characteristics and phenotype.

A master proteome map was constructed for conditions yielding nearly 800 spots (a typical gel is shown in Fig. 2). The identity of a number of key muscle proteins was confirmed after excision and analysis by MALDI-TOF mass spectrometry and their expression is comparable with previous studies (Lametsch & Bendixen, 2001; Lametsch et al., 2002; Yan et al., 2001). Initial comparison of the proteome of the five breeds has identified 21 spots that significantly differ ($p < 0.01$; data not shown). These proteins are now being identified using MALDI-TOF mass spectrometry. Any novel proteins will be characterised by Electrospray mass spectrometry and comparison with translated sequences from the cDNA libraries generated within the project.

3.4. cDNA libraries

One of the main aims of the project is the analysis of the transcriptome of muscle at the time of slaughter and to relate this to differences observed in the phenotypic characteristics of the muscle. In order to do this, specific cDNA libraries were prepared from both loin and ham muscle to provide the basis for the construction of microarray tools that could be used for this analysis. The quality of the SSH cDNA libraries was assessed by careful analysis of each step during the subtraction process and by hybridisation of subtracted cDNAs to known housekeeping genes that should decrease in representation during the subtraction process (see Fig. 3 for examples). Furthermore approx 500 clones from each of the eight SSH libraries have now been sequenced using the M13 reverse primer, and this analysis has revealed a low-level of sequence redundancy, another indicator of the quality of the SSH libraries. This analysis also suggests that these libraries may prove a useful resource for the isolation and characterization of candidate genes associated with meat quality as each library forms a unique population of cDNAs. A standard full-length pig muscle cDNA library was prepared to facilitate the isolation of full-length cDNA for candidate genes to be identified from the SSH libraries, microarray screening

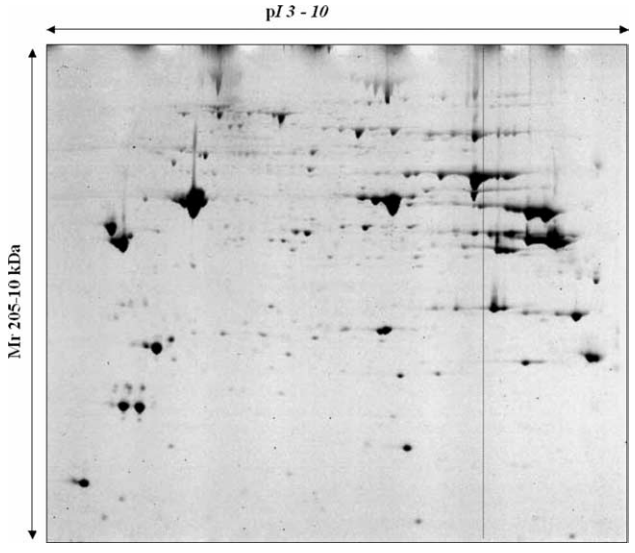

Fig. 2. A typical two dimensional gel electropherogram used for proteomic analysis. The p*I* range (3–8.5) used for analysis, which excludes areas of saturation and poor resolution, is shown to the left of the dotted line.

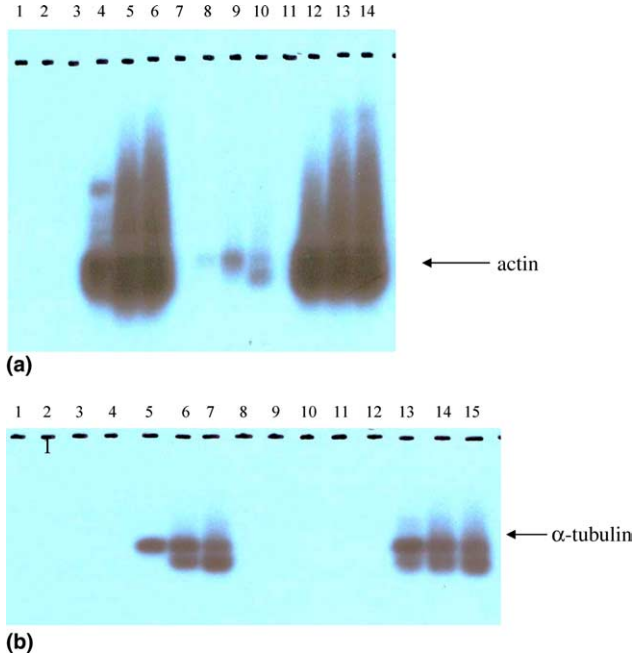

Fig. 3. Analysis of the quality of SSH libraries – reduction of the abundance of housekeeping genes in subtracted cDNA libraries. (a) SSH 3 and 4 hybridisation to actin. Lanes 1–3 SSH3 secondary PCR subtracted cDNA cycle 10, 12 and 14, respectively. Lanes 4–6 secondary PCR unsubtracted cDNA cycle 10, 12 and 14, respectively. Lanes 7 and 11 blank lanes. Lanes 8–10 SSH4 secondary PCR subtracted cDNA cycle 10, 12 and 14, respectively. Lane 12–14 secondary PCR unsubtracted cDNA cycle 10, 12 and 14, respectively. (b) SSH 3 and 4 hybridisation to α-tubulin Lane 1–3 SSH3 secondary PCR subtracted cDNA cycle 10, 12 and 14, respectively. Lane 5–7 secondary PCR unsubtracted cDNA cycle 10, 12 and 14, respectively. Lane 4, 8, 12 blank lanes. Lane 9–11 SSH4 secondary PCR subtracted cDNA cycle 10, 12 and 14, respectively. Lane 13–15 secondary PCR unsubtracted cDNA cycle 10, 12 and 14, respectively.

and proteomics analysis. The quality of this library was assessed by titration of library size and cDNA fragment length analysis. The results obtained showed that the size of the cDNA library (4.8×10^5 pfu) was above the bench-mark figure of 10^5 cDNA clones required to have a good probability that all rare mRNA transcripts would be represented in the library. Analysis of the size of cDNA inserts from random clones showed the largest cDNA insert to be 3.3 kb in length, with the majority of clones >0.7 kb and an average insert size of approximately 1.4 kb. The quality of the libraries has also been investigated by DNA sequence analysis. Using contig assembly followed by BLAST to NR and EST databases to assign tentative gene identities to the SSH library clones, over 900 separate genes are detected, with many genes represented by different non-overlapping sequences.

3.5. Microarray development

The SSH libraries have formed the basis of the first microarrays constructed for initial transcriptional profiling using muscle mRNA from animals selected on the basis of variation in key aspects of quality such as pH and colour, drip loss, loin area or marbling. The rationale behind using these clones is that they have already been taken through a pre-selection process, and therefore should be enriched for those transcripts with differential expression profiles at the extremities of commercially important phenotypic traits. Additional clones for candidate genes were selected from the literature or from prior knowledge, and genes defined as muscle specific or muscle variant (based on the human HUGe index analysis of skeletal muscle, Hsiao et al., 2001) were also included on the array. Every element occurs more than once within the microarray grid design.

This prototype array has been verified by hybridization with samples from pooled LT against pooled SM (Fig. 4). Genes that showed up or down regulation in LT were verified from the known characteristics of the muscle types. In addition, genes enriched in LT by SSH were up-regulated in LT on the array, giving greater confidence that the SSH libraries will provide a valuable resource of potential candidate genes for the phenotypes compared in the subtraction process. This will be particularly important where changes at the transcriptional level are more subtle than can be detected with an array-based approach. The first large-scale microarray experiments are now being undertaken using this array focusing on the relationship between variation in gene expression and the water holding capacity of meat (measured by drip loss, pH and colour) and its intramuscular fat content.

The second-generation microarray now under construction will contain fewer individual elements, with some of the redundancy removed from the gene set,

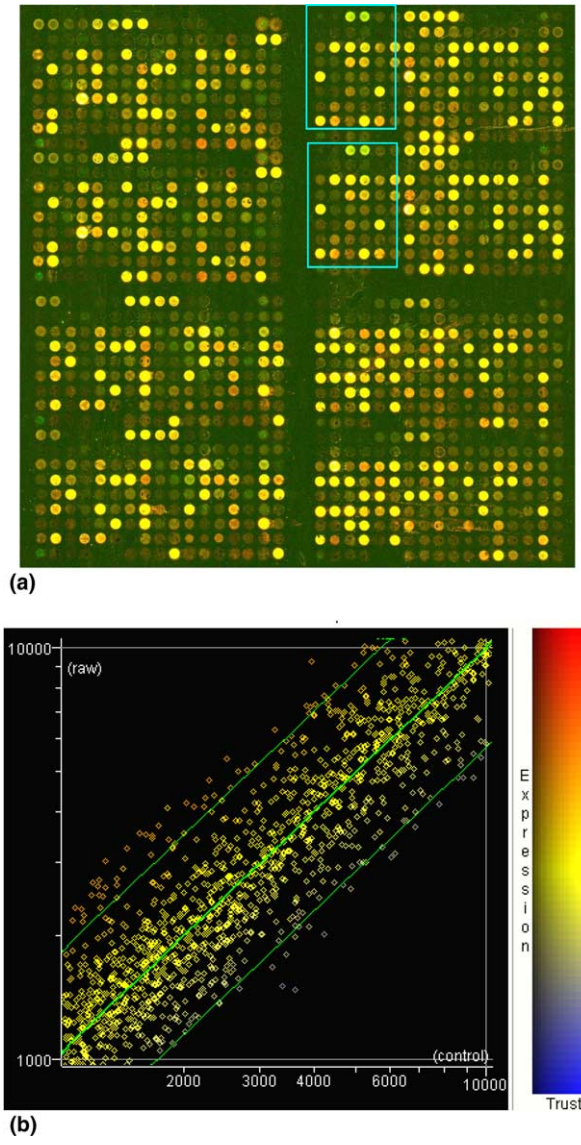

Fig. 4. (a) Example of a section of a microarray hybridized with LT (Cy3) and SM (Cy5). The boxes highlight duplication of elements within the subarray. Most elements are expressed equally, giving shades of yellow, but a few genes are upregulated in LT (green) or SM (red). (b) Following extraction of pixel intensity data from LT v SM experiments, ratio information has been entered into GeneSpring (Silicon Genetics). This shows a section of the output with the 1:1 ratio line and flanking 1.75-fold up or down regulated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

but additional clones from the most unique SSH libraries and further candidates supplementing it, giving approximately 7000 different elements. The reduction in clone number will allow a triplicate printing of all the elements: this means that the internal validation of data by comparison of pixel values from replicate elements on a slide will not be compromised by loss of information from one replicate. This can occur owing to background or hybridization anomalies, and with a

duplicate system, singleton data is often discarded owing to lack of confirmation of the intensity ratios.

This second-generation array will be used to assess the correlation of gene expression results with phenotypic measurements of tenderness and sensorial traits, together with other detailed measurements such as fatty acid composition. The five batches of animals selected for this experiment have detailed phenotypic measurements and also proteomics results. One hundred individual animals, 20 animals from each line, will be arrayed and compared against a common pooled control sample in a reference design (Fig. 1(b)). By using a common reference, data from each animal can be compared against any other animal in the experiment. In addition, some samples from the first series of loop designs occur within the second experiment. As both arrays contain a core common set of elements, these animals are potentially useful for the comparison of data between the experimental designs. In the first experiment genes which show significant differential expression between high and low scoring animals will be identified. In the second experiment the objective will be to find significant correlations between phenotypic trait values and the normalized residual gene expression values, after correction for dye, array and batch effects.

3.6. New tools for understanding variation in muscle phenotype

The project was established in order to exploit new technology developments that allow parallel analysis of gene expression (the transcriptome and proteome). The ultimate aim being to identify variation in the genes which contribute to variation in meat quality. The assumption was that some of these genes will be expressed in muscle at the time of slaughter and that variation in expression levels would be associated with variation in quality parameters of meat derived from this muscle. When these genes are identified the resources developed in the project (e.g., cDNA libraries) will enable further analysis of them and also the search for variation in the sequence of the genes. Once sequence polymorphism is identified this can then be tested for association with variation in the traits of interest. The project database allows for simultaneous analysis of effects on related or secondary traits including aspects of performance and also the yield of some cuts.

This approach is illustrated by the results obtained for the "RN" gene, *PRKAG3* (see Ciobanu et al., 2001; Milan et al., 2000), and the protease inhibitor calpastatin, *CAST* (see Ciobanu et al., 2002; Ciobanu, Bastiaansen, et al., 2004a; Ciobanu, Lonergan, et al., 2004b). Variation in the gene sequence has been identified that explains significant variation in the pH and colour or the tenderness of meat. Simple DNA screening tests can then be developed to select for animals having

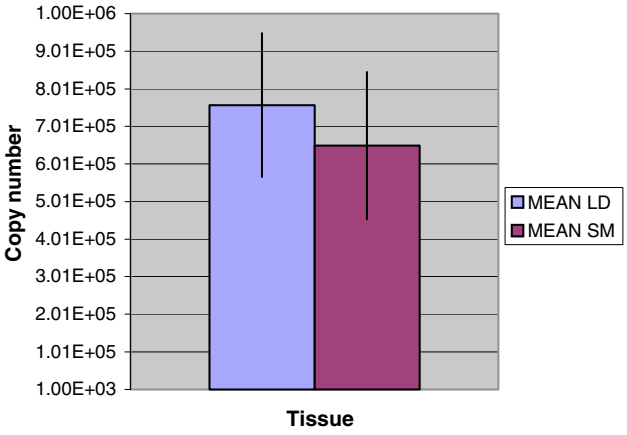

Fig. 5. Differential expression of *PRKAG3* in Loin (LT) and Ham (SM) muscle samples from Piétrain animals as determined by real-time PCR.

the preferred genotype. One interesting observation made with respect to the effect of variation in *PRKAG3* was that the impact on pH and colour seemed to be more pronounced in ham than loin (Ciobanu et al., 2001). One explanation may be that this difference relates to a difference in the levels of expression in these two muscles. The resources developed in this project allowed this hypothesis to be tested. Initial real time PCR analysis of samples from Landrace and Meishan lines revealed a significant line difference in the expression level of *PRKAG3*, with expression been higher in Landrace (results not shown). Furthermore the expression level of *PRKAG3* in LT muscle was greater than SM ($p = 0.06$) and this disparity was more evident in the Meishan line than the Landrace line. In a second analysis RNA was selected from LT and SM muscle from randomly chosen Piétrain animals. The expression level of *PRKAG3* in this line was again greater in loin samples than in ham (mean LT versus SM copy number $p = 0.041$) although there was also substantial individual variation in expression of this gene (Fig. 5). The next step will be to see if *PRKAG3* genotype can explain some of this individual variation. The expression results suggest that the *PRKAG3* gene may be expressed differentially between muscles and this may play a role in the variation of metabolism (and ultimately meat quality) between muscles.

4. Conclusions

DNA markers for meat quality traits have already been identified and are being used by pig breeding companies as part of genetic improvement programmes (see Knap et al., 2002). The Quality Pork Genes project has established a phenotypic database and collection of RNA/DNA samples that will enable the search for

new genes responsible for variation in pork meat quality, including the interaction with stress. It is anticipated that this unique resource will result in the identification of markers (which may be metabolic, biochemical, and neuroendocrine as well as genetic markers) associated with variation in quality. These markers will then be delivered for further research and/or validation of their utility in breeding and selection or screening, in order to provide the basis for the development of new tools to improve raw material quality.

The project database and samples together with the new research tools (e.g., muscle specific cDNA microarrays), represent important resources that can be used to advance research in muscle physiology and meat quality. Ultimately, these tools will play a role in delivering consistently better product quality to the consumer.

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