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Polymorphism in the \textit{ELOVL6} Gene Is Associated with a Major QTL Effect on Fatty Acid Composition in Pigs

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Abstract

Background: The \textit{ELOVL} fatty acid elongase 6 (\textit{ELOVL6}), the only elongase related to \textit{de novo} lipogenesis, catalyzes the rate-limiting step in the elongation cycle by controlling the fatty acid balance in mammals. It is located on pig chromosome 8 (SSC8) in a region where a QTL affecting palmitic, and palmitoleic acid composition was previously detected, using an Iberian x Landrace intercross. The main goal of this work was to fine-map the QTL and to evaluate the \textit{ELOVL6} gene as a positional candidate gene affecting the percentages of palmitic and palmitoleic fatty acids in pigs.

Methodology and Principal Findings: The combination of a haplotype-based approach and single-marker analysis allowed us to identify the main, associated interval for the QTL, in which the \textit{ELOVL6} gene was identified and selected as a positional candidate gene. A polymorphism in the promoter region of \textit{ELOVL6}, \textit{ELOVL6c}.-533C>T, was highly associated with the percentage of palmitic and palmitoleic acids in muscle and backfat. Significant differences in \textit{ELOVL6} gene expression were observed in backfat when animals were classified by the \textit{ELOVL6}.c.633C>T genotype. Accordingly, animals carrying the allele associated with a decrease in \textit{ELOVL6} gene expression presented an increase in C16:0 and C16:1(n-7) fatty acid content and a decrease of elongation activity ratios in muscle and backfat. Furthermore, a SNP genome-wide association study with \textit{ELOVL6} relative expression levels in backfat showed the strongest effect on the SSC8 region in which the \textit{ELOVL6} gene is located. Finally, different potential genomic regions associated with \textit{ELOVL6} gene expression were also identified by GWAS in liver and muscle, suggesting a differential tissue regulation of the \textit{ELOVL6} gene.

Conclusions and Significance: Our results suggest \textit{ELOVL6} as a potential causal gene for the QTL analyzed and, subsequently, for controlling the overall balance of fatty acid composition in pigs.

Introduction

Food fatty acid (FA) composition is a critical aspect in human health and it is also relevant for meat quality. It determines important sensorial and technological aspects of meat due to the variability in the melting point of fatty acids. Thus, variation in fatty acids has an important effect on flavor, muscle color and firmness or softness of the fat in meat [1]. Meat fat is primarily composed of monounsaturated fatty acid (MUFA) and saturated fatty acid (SFA). Oleic acid is the most abundant and nutritionally relevant FA, followed by palmitic and stearic acids [2,3]. The highest rate of \textit{de novo} synthesis of these FAs occurs in liver and adipose tissue, which converts the excess of glucose into FAs for storage and transport [4]. During \textit{de novo} synthesis of FAs, palmitic acid (C16:0) produced by cytoplasmic acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) is transferred to endoplasmic reticulum membranes, where FA elongase and desaturase enzymes catalyze the conversion of saturated FAs into monounsaturated FAs, such as palmitoleic acid (C16:1(n-7)) or oleic acid (C18:1(n-9)) [5,6]. Accordingly, FA elongase activity has an important role in regulating the synthesis of \textit{de novo}-derived MUFA and establishing the balance among C16:1(n-7), C18:1(n-7) and C18:1(n-9) [6].

In 2003, Clop et al. identified a QTL on porcine chromosome 8 (SSC8) with significant effects on C16:0 and C16:1(n-7) contents and a suggestive effect on C18:1(n-9) detected in backfat, using an Iberian x Landrace F2 intercross (IBMAP) [7]. Previous studies in our group evaluated the \textit{MTTP} gene as a positional candidate gene for this QTL fatty acid composition detected on SSC8 [8]. A mutation in the lipid transfer region of the \textit{MTTP} protein (p.Phe840Leu) was associated with fatty acid composition of porcine fat and with the \textit{MTTP} lipid transfer activity measured.
with an *in vitro* assay. Furthermore, two QTL regions in 62 and 92 cM on SSC8, related with C16:0 and C16:1(n-7) fatty acid content in *Langissimus dorsi* muscle, respectively, were detected in a Chinese cross between Duroc and Erhualian [9]. More recently, a Genome-Wide Association Study (GWAS), performed on *Langissimus dorsi* muscle fatty acid composition from an Iberian x Landrace backcross population, detected this QTL between positions 92.1 Mb-96.7 Mb on SSC8 (according to Sscrofa 9.61 genome assembly) at 10 Mb from the *MTTP* gene [10]. This QTL was also identified using backfat fatty acid composition at positions 89 cM (C16:0) and 91 cM (C16:1(n-7)) (Muñoz et al., manuscript in preparation). In this region, a relevant gene for fatty acid metabolism has been located: ELOVL, fatty acid elongase 6 (ELOVL6). The ELOVL6 gene is a member of the elongation-of-very-long-chain-fatty-acid gene family (ELOVL) of condensing enzymes that perform the first and rate-limiting step in the elongation cycle in mammals [11]. These enzymes use malonyl-CoA as the 2-carbon donor to initialize the elongation process. To generalize, FA elongases can be divided into two major groups: a) enzymes involved in the elongation of saturated and monounsaturated very-long-chain fatty acids (*ELOVL1, 3, 6 and 7*) and b) enzymes which are elongases of polyunsaturated fatty acids (*ELOVL2, 4 and 5*) [12,13]. The *ELOVL fatty acid elongase 6* (ELOVL6) gene (also known as LCE and FACE) is the only elongase involved in *de novo* lipogenesis, which catalyzes the elongation of long-chain saturated and monounsaturated FAs with 12-16 carbons to C18, but it does not possess activity beyond C18 [11]. Analysis of ELOVL6-deficient mice demonstrated that ELOVL6 plays a crucial role in the overall fatty acid composition balance [5], and alterations in this composition have important effects on *de novo* lipogenesis and fatty acid oxidation [5]. The clear relationship between ELOVL6 function and the QTL phenotype makes this gene a promising positional and functional candidate gene for the traits analyzed.

In the present study, a refined localization of the QTL affecting C16:0 and C16:1(n-7) FA in muscle and the evaluation of the porcine ELOVL6 gene as candidate gene for this QTL was carried out in an Iberian x Landrace backcross population. DNA sequencing, gene expression analyses and association studies were performed to evaluate the involvement of this gene in C16:0 and C16:1(n-7) FA contents. In this article, we present different evidence that supports the role of ELOVL6 gene polymorphism in the determination of muscle fatty acid composition in pigs.

**Materials and Methods**

**Animal samples**

Animals used in this study belong to the IBMAP cross, a population generated by crossing three Iberian (Guadalyeras line) boars with 31 Landrace sows [14], and containing several generations and backcrosses. The ELOVL6 sequencing and gene expression analyses were carried out in animals from a backcross (BC1_LD) generated by crossing five F1 (Iberian x Landrace) boars with 26 Landrace sows and producing 144 backcrossed animals. All animals were maintained under intensive conditions and feeding was *ad libitum* with a cereal-based commercial diet. Animal care and procedures were performed following national and institutional guidelines for the Good Experimental Practices and approved by the Ethical Committee of the Institution (IRTA-Institut de Recerca i Tecnologia Agroalimentaries). Animals were slaughtered at an average age of 179.8±2.3 days, and samples of liver, muscle (*Langissimus dorsi*) and adipose tissue (backfat) were collected, snap-frozen in liquid nitrogen and stored at −80°C until analyzed. Genomic DNA was obtained from blood samples of all animals by the phenol-chloroform method, as described elsewhere. Composition of fatty acid with 12 to 22 carbons was determined in muscle [10] and backfat (Muñoz et al., manuscript in preparation) using a protocol based on gas chromatography of methyl esters [15].

**Linkage map and haplotype reconstruction**

A total of 439 animals, including the founder populations, were genotyped with the Porcine SNP60K BeadChip [16]. CRI-MAP version 2.503, developed by Evans and Maddox [http://www. animalgenome.org/bioinfo/tools/share/crimap], was used to build the linkage map using the genotype information of SSC8. In addition, previously detected polymorphisms in the *MTTP* and *FABP2* genes were also included in the analysis [8,17]. Raw data had a high genotyping quality (call rate >0.99) and, after selecting SNPs with MAF >5%, markers with genotyping and mapping errors were excluded by using the “Chrompic” option of CRI-MAP and R scripts developed by our group. Finally, we recalculated the genetic distances, employing the “Fixed” option, and 2,565 SNPs were retained for subsequent analyses (Table S1). Haplotypes were reconstructed using DualPHASE software [18], which exploits population (linkage disequilibrium) and family information (Mendelian segregation and linkage) in a Hidden Markov Model setting.

**Chromosome 8 association and fine-mapping analyses**

GWAS for the intramuscular profile of palmitic and palmitoleic acids was performed with a mixed model [19,20] accounting for additive effects associated with each marker (see below) by using Qxpak 5.0 [21]:

\[ y_{ijkm} = \beta_{0} + \beta_{1}x_{i} + \beta_{2}b_{j} + \beta_{3}c_{i,k} + \beta_{4}d_{k} + u_{i} + e_{ijkm}, \]

in which \( y_{ijkm} \) is the l-th individual record, sex (two levels) and batch (five levels) are fixed effects, \( \beta \) is a covariate coefficient with \( \epsilon \) being carcass weight, \( \lambda \) is a \(-1, 0, +1\) indicator variable depending on the l-th individual genotype for the k-th SNP, \( a_{k} \) represents the additive effect associated with SNP, \( u_{i} \) represents the infinitesimal genetic effect treated as random and distributed as \( N(0, \sigma_{u}^{2}) \), \( A_{c} \) is a numerator of the kinship matrix and \( e_{ijkm} \) is the residual. The same model was carried out for studying the association of polymorphisms detected in the ELOVL6 gene with palmitic and palmitoleic acid profiles in muscle and backfat.

QTL fine-mapping was performed by simultaneously exploiting linkage and linkage disequilibrium (LD) using a haplotype-based approach [18] and following the mixed model:

\[ y = Xb + Z_d h + Z_u u + \epsilon, \]

in which \( h \) is a vector of fixed effects (sex and batch), \( b \) is the vector of random QTL effects corresponding to the K cluster defined by the Hidden State (HS), \( u \) is the vector of random individual polygenic effects and \( \epsilon \) is the vector of individual error. The genome-wide significance was determined using the R-package *qvalue* [22], and the cut-off of the significant association was set at \( q\text{-value} \leq 0.05 \).

In order to estimate the LD between the SNPs located within the candidate region, a LD analysis was performed using the genotype and phases information from DualPHASE software. The
LD estimated for each pair of SNPs was visualized using the “LDheatmap 0.9” R package [23].

**RNA isolation and cDNA synthesis**

Total RNA was obtained from liver, muscle and backfat tissues using the RiboPure™ Isolation of High Quality Total RNA (Ambion®), following the manufacturer’s recommendations. RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop products) and checked for purity and integrity in a Bioanalyzer-2100 (Agilent Technologies). The isolated RNA was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) and random hexamers in a total volume of 20 μl containing 1 μg (liver and muscle) or 0.3 μg (backfat) of total RNA, following the manufacturer’s instructions.

**Amplification and sequencing of the pig ELOVL6 coding region and proximal promoter**

The proximal promoter and the entire coding region of the ELOVL6 gene was amplified and sequenced in twenty samples from the BC1_LD. Primers (Table S2) to amplify two overlapping fragments of 688 bp and 499 bp, including the complete coding region, were designed from the human GenBank NM_024090.2 sequence, assuming conservation across species. The proximal promoter region was amplified for the Sus scrofa breed mixed chromosome 8 sequence (GenBank:NW_003610943) available at the Scrofa10.2 database (primers in Table S2) and assuming conservation with the human and mouse ELOVL6 promoters [24]. A total of 1046 bp of the ELOVL6 promoter and exon 1 were sequenced in two overlapping fragments of 604 bp and 650 bp. Primers were designed using the software PRIMER3 [25] and were validated using the software PRIMER EXPRESS™ (Applied Biosystems). PCR products were purified using the ExoSAP-IT® method and sequenced with a Big Dye Terminator v.1.1 Cycle Sequencing Kit in an ABI 3730 analyzer (Applied Biosystems).

**Gene expression quantification**

A total of 110 animals of the BC1_LD backcross were selected to perform gene expression quantification in liver, backfat and muscle. PCR primers were designed using PRIMER EXPRESS™ software (Applied Biosystems) and are shown in Table S2. Primers for amplification of ELOVL6 mRNA were designed from the available sequence (GenBank:XM_003537048) covering exons 3–4 to amplify a 103-bp-long fragment. Three genes frequently used as references in RT-qPCR experiments were analyzed as endogenous controls: β-2 microglobulin (β2M); Hypoxanthine phosphoribosyltransferase1 (HPRT1) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [27,28]. All reference genes were tested using the software GeNorm [29], and the two best endogenous controls for all tissues were β2M and HPRT1. PCR amplification was performed in triplicate in a 20 μl final volume containing 2 μl of cDNA sample, diluted 1:20 in DEPC-treated H2O from liver and muscle samples, and 1:5 from backfat samples. For gene amplification, FastStart Universal SYBR Green Master (RoX; Roche Applied Biosystems) was used. Primers were used at 900 nM for the ELOVL6 gene and 600 nM for both reference genes, except from HPRT1 in the muscle study (900 nM). PCR amplification was run on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using 96-well optical plates under the following conditions: 10 min at 95°C, 40 cycles of 15 sec at 95°C and 1 min at 60°C. A dissociation curve was drawn for each primer pair to assess that there was no primer-dimer formation.

To quantify and normalize the relative quantification (RQ) data, the 2⁻ΔΔCt method [30] was applied using a sample with low expression as a calibrator. Comparison of mean values between genotypes was made using a linear procedure of R software, which employs a single stratum analysis of variance considering sex and batch as fixed effects. Differences were considered statistically significant at a p-value of < 0.05.

**Genotyping**

BC1_LD backcross animals (N = 144) were genotyped with the Porcine SNP60 BeadChip (Illumina) using the Infinium HD Assay Ultra protocol (Illumina). Raw data had a high genotyping quality (call rate >0.99) and was visualized and analyzed with the GenomeStudio software (Illumina). For subsequent data analysis, a subset of 54,996 SNPs was selected by removing the SNPs with a minor allele frequency <5%, those with missing genotypes >5% and the duplicated SNPs in the Scrofa 10.2 assembly.

The SNPs ELOVL6:c.-533C>T, ELOVL6:c.-480C>T, ELOVL6:c.-333C>T and ELOVL6:c.-480C>C>T and were genotyped using the KASP SNP genotyping system platform [http://www.kbioscience.co.uk/ reagents/KASP/KASP.html]. A total of 160 animals were genotyped, 125 of those belonging to BC1_LD and the rest being parental animals of the IBMAP cross (F0 and F1).

**GWAS of gene expression**

Association analyses of RT-qPCR expression data of ELOVL6 mRNA in liver, backfat and muscle, and whole-genome SNP genotypes, were carried out with Qxpak 5.0 software. The position of the SNPs was based on the Sus scrofa 10.2 genome assembly [http://www.animalgenome.org/repository/pig/]. For GWAS analysis, the previously described model [1], without correcting for carcass weight, was used. The infinitesimal effect allows us to adjust the data for family information and, thus, to correct the inter-chromosomal linkage disequilibrium effect. In this analysis, each SNP was tested individually to check the association. Chromosome X was analyzed using the same models, but including a dosage compensation parameter [31]. The R package q-value [22] was used to calculate the FDR-based q-value to measure the statistical significance at the genome-wide level for association studies. The cut-off of significant association at the whole genome level was set at q-value<0.1. This significance threshold is likely too stringent due to the linkage association among SNP genotypes. Gene annotation for 2 Mb genomic intervals around the most significant SNPs was based on the Porcine Fatty Acid Composition and Porcine Fatty Acid Composition.
a cut-off at a chromosome-wise level lower than q-value < 0.05 were selected.

Results

Linkage and haplotype reconstruction

The length of the linkage map on SSC8 was 131.2 cM and the ratio between the genetic and the physical distance was 0.89 cM/Mb, similar to that previously reported [32]. Genotypes from a total of 2,565 SNPs of the Porcine SNP60 BeadChip (Illumina) were employed to reconstruct the haplotypes through Dual-PHASE software. Previous studies showed that the estimation of the phenotypic effect of haplotype clusters is a good approximation to identify the functionally relevant ones, as well as to reduce the confidence interval for the fine mapping QTL [18,33]. In this study, a method based on Hidden Markov Models that simultaneously phases and sorts haplotypes using linkage and LD information for haplotype reconstruction was employed. A total of twenty haplotype clusters (K = 20) were used for fine mapping, as described below.

Fine mapping and gene annotation

A combination of the haplotype-based approach and GWAS for the intramuscular profile of palmitic and palmitoleic acids was performed in 144 BC1_LD individuals and 2,565 SNPs. It is worth noting that, for both traits, the two strategies showed the highest association at the same position (Figure 1). For instance, the GWAS profile corresponding to palmitic acid was maximized at 119,727,822–119,887,525 bp (p-value = 6.19 × 10^{-69}) and the profile score from the haplotype-based analyses showed the maximum association signal at position 117,824,360–119,887,525 bp (p-value = 3.57 × 10^{-67}) (Figure 1A). For palmitoleic acid, the GWAS profile was maximized at 119,851,321–120,104,023 bp (p-value = 4.23 × 10^{-69}) and the profile scores from the haplotype-based analyses were maximized at position 117,824,360–119,727,822 bp (p-value = 1.09 × 10^{-65}) (Figure 1B). In general, the association signal obtained by GWAS was higher than were curves obtained with the haplotype-based approach. However, it should be noted that the haplotype-based approach allowed us to simultaneously exploit linkage analysis and LD (LDLA). In addition, although both strategies were modeled by a LDLA approach, HS was treated as additive random effects, whereas in GWAS a single-marker regression analysis was performed and the SNP alleles were treated as additive fixed effects.

According to the fine mapping data, the region comprised between 117–121 Mb was annotated using Biomart software in the Ensembl Sscrofa10.2 dataset [www.ensembl.org]. A total of 21 genes were located in this region, but only two were clearly related to fatty acid metabolism: ELOVL6 (at position 120,119,244 bp) and PLA2G12A (at position 120,966,787 bp). The coincidence between the biological function of ELOVL6 and the observed QTL effect on fatty acid composition on SSC8 strengthens the interest of the ELOVL6 gene as the positional candidate gene for this QTL.

Identification of polymorphisms in the porcine ELOVL6 gene

To characterize the porcine ELOVL6 gene, a 1,046-bp long fragment of the ELOVL6 promoter and exon 1 was amplified from genomic DNA and sequenced, assuming conservation with the human and mouse genes. In addition, the entire coding region of the ELOVL6 gene was amplified and sequenced. The alignment and analysis of these sequences allowed for the identification of eight polymorphisms (Table 1): one synonymous polymorphism in exon 4 and seven nucleotide substitutions in the promoter region. The SNPs located in the promoter were arranged in three haplotypes, which can be distinguished by genotyping the ELOVL6:c.-533C>T and ELOVL6:c.-406C>T polymorphisms (relative to the transcription start site, TSS, of the GeneBank:NW_008610943). Hence, these two tag polymorphisms and the ELOVL6:c.416C>T SNP in exon 4 (GeneBank:AB529461) were genotyped in parental and BC1_LD animals. Regarding the IBMAP founders, the ELOVL6:c.-533C allele and ELOVL6:c.416T allele were fixed in Iberian boars. The allele frequencies for these two SNPs were 0.25 for F1 Landrace sows and 0.78 and 0.72 for the BC1_LD Landrace sows, respectively. In contrast, ELOVL6:c.-406C>T SNP was not fixed in the Iberian founders, and therefore it was less informative. Both ELOVL6:c.-533C>T and ELOVL6:c.416C>T polymorphisms segregated in the BC1_LD animals with frequencies of 0.63 for allele C and 0.60 for allele T, respectively. Linkage disequilibrium analysis revealed that the three ELOVL6 polymorphisms were in strong LD (D’ = 0.99) with three of the most significant SNPs (SIRI1000509, INRA0030422 and H3GA0025321) identified in both GWAS and fine mapping analyses (Figure S1).

To assess if polymorphisms in the promoter region could affect ELOVL6 expression through the disruption of transcription factor-binding sites, a computer-assisted identification of potential cis-acting DNA-sequence motifs was carried out. As has been previously described in mouse liver, the ELOVL6 gene is regulated by SREBP-1 [4,24,34,35]. SREBP-1 presents dual DNA sequence specificity, binding to both E-box and SRE motifs [36]. Four SREBP binding sites were identified in the pig ELOVL6 promoter, three SRE elements in positions −27 to −17, −460 to −449 and −532 to −524 (Figure 2) and one E-box in position −341 to −330 (Figure 2A), relative to the TSS of the GenBank sequence NW_003610943, similar to those observed in the mouse promoter (Figure 2B) [24]. Also, other candidate transcription factors, with biological relevance, have elements in this promoter, such as MLX (at position −339 to −322), which belongs to the family of basic helix-loop-helix leucine zipper (bHLH-Zip) and induces ELOVL6 gene expression by glucose in mice [6], HNF4α at position −719 to −694) or KLF10 at position −577 to −372 (Figure 2). However, none of our polymorphisms changed these binding sites. Interestingly, two consecutive SNPs forming a haplotype at positions −533 (ELOVL6:c.-533C>T) and −534 (ELOVL6:c.-534C>T) were identified in the core binding site of the estrogen-related receptor alpha gene (ESRR-α), generating a multi-nucleotide polymorphism. Furthermore, the ELOVL6:c.-406C>T polymorphism was also located in a potential SP1 binding site (Figure 2).

Association of ELOVL6 polymorphisms with C16:0 and C16:1(n-7) composition in muscle and backfat

An association analysis with the SSC8 genotypes from 2,565 SNPs of the Porcine SNP60 BeadChip (Illumina) and three ELOVL6 SNPs in 125 BC1-LD animals was performed using the additive model (1). In this analysis, the ELOVL6:c.-533C>T polymorphism showed the highest association with the percentage of palmitic acid (p-value = 1.38×10^{-67}; α(estimated additive effect) = 0.742; see Figure S2A) and palmitoleic acid (p-value = 1.23 × 10^{-69}; α = 0.253; see Figure S2B) content in muscle. Also, a relevant association was observed between ELOVL6:c.416C>T polymorphism with palmitic, and palmitoleic acid content in muscle (p-value (C16:0) = 1.11 × 10^{-43}; α(C16:0) = 0.530; p-value (C16:1(n-7)) = 6.98 × 10^{-67}; α(C16:1(n-7)) = 0.214; see Figure S2). In backfat, the ELOVL6:c.-533C>T polymorphism was the most significantly associated one with...
palmitic acid content (p-value = 2 x 10^{-15}; \alpha = 0.976). In addition, ELOVL6:c.416C>T SNP showed a high association with palmitic acid content (p-value = 6.27 x 10^{-13}; \alpha = 0.859) (data not shown).

Analyzing the palmitoleic acid content in backfat, the most significantly associated SNP was H3GA0025290 (113,528,768 bp; \alpha = 0.182, p-value = 8.54 x 10^{-10}). ELOVL6 polymorphisms ELOVL6:c.-533C>T and ELOVL6:c.166C>T also showed a significant association with palmitoleic content: p-value = 6.14 x 10^{-09} (\alpha = 0.166) and p-value = 6.95 x 10^{-08} (\alpha = 0.151), respectively (data not shown). The clear association of the ELOVL6:c.-533C>T polymorphism with the percentage of both fatty acids in muscle and backfat yields new evidence to continue studying ELOVL6 as a candidate gene for SSC8 QTL.

**Effect of the ELOVL6: c.-533C>T polymorphism on gene expression and fatty acid composition**

The association of the ELOVL6: c.-533C>T polymorphism with the percentages of C16:0 and C16:1(n-7) suggests a role of this mutation in the regulation of ELOVL6 gene expression and, subsequently, in fatty acid metabolism. Thus, the expression profile of the pig ELOVL6 gene, in liver, backfat and muscle, organs particularly important in fatty acid metabolism, was studied by RT-qPCR in 110 BC1_LD animals. In accordance with previous results in mouse and rat, in which high ELOVL6 expression was found in tissues with active lipogenesis [4,35,37], the highest expression was found in backfat tissue, followed by liver and muscle. Clear differences in ELOVL6 expression were observed among samples in all tissues, with a highly significant effect of sex in liver (p-value = 6.5 x 10^{-13}), backfat (p-value = 3.4 x 10^{-14}) and muscle (p-value = 3.4 x 10^{-15}), where ELOVL6 gene expression was higher in females than in males.
In addition, the correlation between the ELOVL6 expression levels across the three tissues was analyzed, but no clear associations were observed among tissues. This result suggests that the mechanisms controlling ELOVL6 expression are different in backfat, liver and muscle tissues [35].

When animals were classified according to the ELOVL6:c.-533C>T genotypes, no significant variations were found between genotypes when liver and muscle samples were analyzed (Figure 3C–D). Nevertheless, different levels of expression between genotypes were obtained in backfat samples (p-value = 8.7 × 10^{-05}; Figure 3B), where animals with the CC genotype showed a significantly lower expression, as compared to animals with the other two genotypes. Interestingly, when only individuals with a known allele origin for the ELOVL6:c.-533C>T polymorphism were analyzed, the Iberian allele C decreased the expression, in comparison with the Landrace allele T (p-value = 4.6 × 10^{-03}). Accordingly, CC homozygous individuals showed a higher percentage of C16:0 in muscle (p-value = 3.61 × 10^{-05}) and backfat.
(p-value = 1.83 × 10^{-09}), in comparison with TT individuals (Figure 4A). Similar results were obtained for C16:1(n-7) in both tissues, with CC animals presenting a higher relative content of this fatty acid (p-value (muscle) = 7.1 × 10^{-09} and p-value (backfat) = 1.47 × 10^{-09}; Figure 4B). These data suggest a substrate accumulation in individuals with the C allele due to a hypothetical deficiency of the ELOVL6 gene (Figure 3A). In agreement with these data, a decrease of C18:0 content was also observed in backfat (p-value = 5 × 10^{-05}) in animals with the C allele, but such differences were not present in muscle (data not shown). Although non-significant differences were observed in the 18-carbon fatty acid content in muscle and backfat (except for the C18:0 in backfat), a significant decrease in elongation activity ratios (C18:0/C16:0 and C18:1(n-7)/C16:1(n-7)) were observed in both tissues in animals with the CC genotype (Figure 4C–D).

Genome-wide association studies of ELOVL6 gene expression

Taking into account that differences in ELOVL6 gene expression were observed among tissues and animals, GWASs using the RQ expression data of the three tissues and the genotypes of 54,998 SNPs distributed across the pig genome were carried out to find new, potential genomic regions associated with ELOVL6 gene expression. The promoter ELOVL6 SNPs (ELOVL6:c.-533C>T and ELOVL6:c.-480C>T) and the protein-coding region SNP (ELOVL6:c.416C>T) genotyped in this work, which are not included in the porcine SNP60 Bead-Chip, were incorporated into the study. First, backfat analysis of ELOVL6 gene expression showed three relevant regions in chromosomes SSC2, SSC4 and SSC8 which were significant at a chromosome-wise level (Figure S3A). Interestingly, the most significant peak was localized in SSC3 inside the QTL region, very close to the ELOVL6 gene (ALGA0049135; 117,548,144 bp; p-value = 2.74 × 10^{-06}) (Figure 5C). High association was also obtained with the ELOVL6:c.-533C>T polymorphism (p-value = 2.05 × 10^{-05}), whereas the other two ELOVL6 polymorphisms were not significantly associated. Gene annotation of the other two regions was performed to find potential trans-acting genetic variants modulating ELOVL6 gene expression. In SSC2, a significant region was found between positions 9.3 Mb and 9.6 Mb (DIAS0000337; 9,736,754 bp; p-value = 1.4 × 10^{-05}), in which several genes related to lipid metabolism were identified (Figure 5A). The most interesting ones were the estrogen-related receptor alpha (ESRRα), three genes which are members of the fatty acid desaturase family (FADS1, FADS2 and FADS3), the carnitine palmitoyltransferase 1A (CPT1A) and the nuclear receptor subfamily 1, group H, member 3 (NR1H3). Finally, the most significant region of SSC4 was found between positions 36 Mb and 44 Mb (ASGA0088888; 40,318,092 bp; p-value = 1.4 × 10^{-05}), where the Kruppel-like factor 10 (KLF10) gene was annotated (Figure 5B). In liver, three candidate chromosomal regions were significantly associated with ELOVL6 gene expression at a chromosomal level on SSC4, SSC5 and SSC9 (Figure S3B). The most significant region in SSC4 showed two peaks at the 30 Mb–35 Mb and 60 Mb–67 Mb regions (Figure 6). Gene annotation of both regions allowed us to identify several genes, which may be related to ELOVL6 expression. In SSC5, the most significant SNPs were ALGA0025162 (60,844,160 bp; p-value = 2.93 × 10^{-06}) and ALGA0024413 (34,206,333 bp; p-value = 3.58 × 10^{-06}). Proximal to

Figure 3. Association of ELOVL6: c.-533C>T genotypes on gene expression in backfat. A SNP genome-wide association study was performed with ELOVL6 relative expression levels measured by RT-qPCR in 110 samples from backfat, liver and muscle. Data include: Schematic representation of the elongation pathway of 16-carbon fatty acid (A), ELOVL6 expression levels in backfat (B), liver (C) and muscle (D). Data represent means ± SEM. Values with different superscript letters (a, b and c) indicate significant differences between groups (p-value < 0.05), as determined by a single stratum analysis of variance considering sex and batch as fixed effects.

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ALGA0025162 was located the hepatocyte nuclear factor 4 gamma (HNF4\gamma) and members 4 and 5 of the fatty acid binding protein family (FABP4 and FABP5) (Figure 6). SNP ALGA0024413 was near the significant region detected in backfat analysis, suggesting a co-effect in both tissues by the porcine KLF10 gene (Figure 6). No relevant genes were found in the 71 Mb to 79 Mb region of SSC5. In SSC9, the significant region was located in the 65 Mb–71 Mb interval, in which the acyl-CoA dehydrogenase 8 (ACAD8) was located. Finally, muscle ELOVL6 gene expression was associated with three regions in SSC6, SSC8 and SSC12 (Figure S3). The most significant one was situated in SSC6 between positions 18 Mb and 26 Mb (ALGA0034806; 19,862,636 bp; p-value = 4.02 × 10^{-6}), where the general transcription factor SET domain containing 6 (SETD6) was identified (data not shown). In SSC8 and SSC12, a significant region was found in intervals 15 Mb–19 Mb and 10 Mb–14 Mb, respectively (data not shown). Nevertheless, no relevant genes were identified using the current porcine gene annotation information. The significance threshold was likely too stringent owing to the linkage dependence among the SNPs included in the analysis and, thus, other suggestive SNP peaks may also contain relevant genes.

Discussion

The QTL affecting palmitic and palmitoleic acid contents on SSC8 was previously identified and the porcine MTTP gene was analyzed as a positional candidate gene [8]. These studies were performed using a reduced number of microsatellite markers and, as a consequence, the confidence interval had several Mb. The improvements in the porcine genome and the use of the SNP data from the Illumina 60 K porcine chip allowed us to make a better estimation of the QTL position by GWAS and haplotype-based approaches. GWAS studies maximized the QTL peak at 10 Mb from the MTTP gene, in the region where the ELOVL6 gene was located. Although the ELOVL6 gene has been selected as a new functional and positional candidate gene, a lower effect of the MTTP gene cannot be ruled out.

Despite the crucial role of genes such as ELOVL6 and members of the SCD family in determining the balance among C16:0, C16:1(n-7), C18:1(n-7) and C18:1(n-9) [5,6,38], the information regarding these genes in pigs is sparse. In this study, we characterized the porcine ELOVL6 gene and we presented several pieces of evidence confirming that this lipogenic enzyme is highly associated with fatty acid composition in pigs. Among the eight polymorphisms found in the porcine ELOVL6 gene, the ELOVL6:c.-533C>T polymorphism was clearly associated with C16:0 and C16:1(n-7) composition in muscle and backfat. An increase of C16s' fatty acid percentage in animals with the C allele, in comparison with animals carrying only the T alleles, was observed. In accordance with the function of ELOVL6 (Figure 3A), which elongates C16 to C18 fatty acids [5], a lower ELOVL6 gene expression was found in the backfat of animals with the Iberian allele. The lower ELOVL6 gene expression was associated with the accumulation of C16:0 and C16:1(n-7) in muscle and backfat, as has been previously described in mammalian cells by modulating ELOVL6 activity with siRNA [5,6]. Similar results were obtained using mice deficient for ELOVL6 [5], where an increase of C16 fatty acids and a decrease of C18 fatty acids was observed in ELOVL6\null mice. In agreement with these studies, the percentage of C18:0 showed a decrease in backfat, but no differences were observed in C18:1(n-7).

The relevance of adipose tissue in overall fatty acid synthesis in pigs must be considered for the interpretation of the present results. Liver and adipose tissue are the principal organs for fatty acid synthesis in pigs. Therefore, the QTL detected in SSC6, SSC8 and SSC12, which are associated with ELOVL6 expression, may have a direct impact on the accumulation of C16:0 and C16:1(n-7) in backfat.
implicated in de novo lipogenesis, although their contribution differs across species. In ruminants, such as cow and sheep, both liver and adipose tissue appear to be important sites of synthesis [39], while in mouse and rat adipose tissue accounts for at least 50% of the newly synthesized fatty acids [40]. Pig adipose tissue seems to be responsible for a greater contribution to overall fatty acid synthesis than does liver [41], as has been similarly observed in humans [42,43]. In agreement with this, the expression of ELOVL6, a gene involved in de novo lipogenesis, was higher in adipose tissue than in the liver and muscle of 110 BC1_LD animals. Furthermore, the effect of SNP ELOVL6:c.-533C>T in ELOVL6 expression was only significant in adipose tissue, suggesting that this polymorphism may have an influence in adipose fatty acid synthesis and, subsequently, in body fatty acid composition. In fact, adipose tissue is the major source of circulating free fatty acids (FFAs) and, together with the liver, supplies fatty acids to muscle [44]. In mice, the concentration of muscle palmitoleate is a direct reflection of adipose FFAs [44]. As in pig the contribution of adipose tissue in fatty acid synthesis is higher than in liver, we could hypothesize that the composition of fatty acids in muscles closely resembles that observed in adipose tissue [45,46]. High and moderate positive phenotypic correlations between backfat and muscle were found for C16 and C16:1(n-7) composition (r C16:0 = 0.72, p-value = 2.2 x 10^{-16} and r C16:1(n-7) = 0.43, p-value = 5.13 x 10^{-06}, respectively), supporting our hypothesis. Furthermore, a high correlation for palmitoleic fatty acid was not expected because another genomic region with a strong effect on this fatty acid in muscle, but not in backfat, was identified in SSC4 [10].

Despite the strong association (p-value = 2.05 x 10^{-05}) between the ELOVL6:c.-533C>T polymorphism and backfat ELOVL6 gene expression, SSC3 SNP ALGA0049135 (117,548,144 bp) was more significantly associated (p-value = 2.74 x 10^{-06}). However, ELOVL6:c.-533C>T showed a higher additive effect (a = 0.174), in comparison with SNPs ALGA0049135 (a = 0.154). Hence, further investigation is required to validate the ELOVL6:c.-533C>T polymorphism as the causal mutation or to identify new genetic variants in this QTL region modulating ELOVL6 gene transcription that could better explain the QTL underlying phenotypic variation in C16 and C16:1(n-7).

Another interesting region associated with ELOVL6 expression in backfat was observed in SSC2, in which the ESRR-α gene was identified. ESRR-α codes for a transcriptional regulator which
binds to an ERR-α response element (ERRE) containing a single-consensus half-site 5'-TNAAGGTCA-3' and regulates a variety of genes related to fatty acid metabolism [48]. Interestingly, this transcription factor is regulated by estrogens, the primary female sex hormones. Thus, the higher ELOVL6 gene expression observed in females may be explained by the increase of ESRR-α activity, due to the high levels of estrogens in females. Furthermore, ERRE was present in the ELOVL6 promoter and included two polymorphic positions in the core binding element (Figure 2), one of which was ELOVL6:c.-533C>T SNP, reinforcing this polymorphism as a candidate mutation to explain the differences in ELOVL6 mRNA observed among animals. Additionally, the ESRR-α binding site overlapped one SRE motif (-532 to -524 bp), suggesting that the two polymorphisms identified in this region (ELOVL6:c.-533C>T and ELOVL6:c.-534C>T) may have an important role in selecting which transcription factor (ESRR-α or SREBP1) binds to its corresponding element. Further studies are needed to determine both the effect of the two polymorphisms to ESRR-α or SREBP1 binding and how the selection of the transcription factor can affect ELOVL6 gene expression. In liver, a second significant peak was also obtained in SSC4, in which the HNF4c, FABP4 and FABP5 genes were identified. The porcine HNF4c gene is a member of the hepatocyte nuclear receptor superfamily, which is highly homologous to HNF4α, suggesting that it may have a similar function in the regulation of hepatic genes [49]. The protein structure of HNF4c revealed that fatty acids bind to its ligand binding pocket, acting as a regulatory molecule of HNF4c [50]. In spite of the presence of the HNF4c binding site in the ELOVL6 promoter, the main relationship described between both genes is that deficiencies in ELOVL6 gene expression deplete the newly synthesized fatty acids, which are coactivators of the HNF4c gene, producing a decrease in HNF4c activity [5]. Interestingly, preliminary results in our lab indicate higher expression levels of HNF4c in liver than in backfat (data not shown). These data point towards HNF4c as a regulator of ELOVL6 gene expression in liver and suggest that the polymorphism proximal to or within the HNF4c gene partially determines the differences in liver ELOVL6 gene expression. On the other hand, FABP5 is a protein that binds and transports long-chain fatty acids into the nucleus [51], where they can act as transcription factors on lipogenic genes, such as elongases [44]. Association analysis with muscle ELOVL6 gene expression data allowed us to identify significant regions, but only general transcription factors were found. Data obtained suggest a minimal elongation activity in muscle, and probably the difference in mRNA levels between animals was caused by the intramuscular adipocytes, as was observed in previous pig studies [52]. Apart from the potential relevance of all of these genes located in significant regions in regulating ELOVL6 gene expression, we cannot discard the involvement of other genes located in non-significant regions but with biological relevance in the regulation of ELOVL6 expression, such as SPI and SREBP genes. In the present study, we have identified four SREBP binding sites in the pig ELOVL6 promoter, but none of these cis-acting motifs was affected by ELOVL6 polymorphisms. However, SREBP has been described as a weak transcriptional activator that requires interaction with additional regulators like NF-Y and SPI to activate the transcription of genes involved in fatty acid metabolism [4,53]. Interestingly, a SNP disrupting a potential SPI binding site has been identified in the ELOVL6 promoter, not.

Figure 6. Significant region obtained in GWAS for liver gene expression. Association analysis between the liver ELOVL6 expression level and SNP genotypes for SSC4. Positions in Mb are relative to Sscrofa10.2 assembly of the pig genome. Vertical, dashed lines indicate the location of positional candidate genes. Horizontal, dashed lines mark the chromosome-wide significance level (FDR-based q-value<0.1).

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discarding the involvement of this mutation in the differences of gene expression observed among tissues. Taking into account the regulatory networks necessary for transcriptional activation, further investigation is required to determine the role of these mutations in the ELOVL6 expression together with the implication of tissue-specific factors and epigenetic modifications.

Finally, the results provided in the present study are both helpful for the understanding of molecular mechanisms governing important economical traits like meat quality, but also to improve the knowledge of human diseases related to obesity, including diabetes and metabolic syndrome. Fatty acid composition has been highly associated with insulin sensitivity, especially the ratio of C18 to C16 fatty acids, which is controlled by ELOVL6 activity [5]. The accumulation of C16 fatty acids, observed in our study, has been related to protection against hepatic lipotoxicity and insulin resistance [5]. Palmitoleic acid, segregated by adipose tissue, greatly strengthens the insulin-signaling pathway, avoiding tissue insulin resistance and obesity-related diseases [44].

Conclusions

In this work, the interval for the C16:0 and C16:1(n-7) QTL in SSC8 has been reduced, allowing for the identification of ELOVL6 as a positional candidate gene. The characterization of the coding and proximal promoter regions of the porcine ELOVL6 gene allowed for the identification of several mutations, especially the ELOVL6:-533C>T polymorphism strongly associated with muscle and backfat percentages of palmitic and palmitoleic acids. Interestingly, this SNP was also related to ELOVL6 expression levels in backfat and fatty acid content and elongation activity ratios in muscle and backfat. Thus, the ELOVL6:-533C>T polymorphism is a candidate causal mutation to explain the variation in palmitic and palmitoleic acid content observed in an Iberian x Landrace cross. Hence, this work provides the first report of the importance of the porcine ELOVL6 gene in the metabolism of fatty acids and, subsequently, in meat quality traits in pigs, but further functional studies in model organisms and validation in independent pig populations are required to confirm this causal mutation.

Supporting Information

Table S1 List of SNPs for SSC8 linkage map and haplotype reconstruction.

(XLSX)

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