Identification Of Porcine Candidate Genes For Water-Holding Capacity Using QTL-region-specific BAC-arrays

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Introduction

Meat quality relates nutritional and sensory properties and can be measured by means of technological indicators such as pH, conductivity, colour, drip loss, cooking loss, thaw loss or shear force. In particular, variation in water-holding capacity (WHC) is a major concern for meat industry and consumers. Linkage studies led to the identification of QTL for WHC on various porcine chromosomes (SSC) (Hu *et al.*, 2005 (PigQTLdb)). In a F2 population (DUPI) based on Duroc and Piétrain we identified QTL for traits related to WHC on SSC 2, 3, 4, 5, 6, and 18 (Liu *et al.*, 2007, 2008). In addition to this approach based on genetic linkage maps, function driven holistic expression profiling and the integration of both have a great potential towards elucidating the expression and inheritance of complex traits including WHC. We have previously identified functional and regulatory networks and candidate genes for WHC by microarray expression analysis and eQTL detection (Ponsuksili *et al.*, 2008a,b, 2009, 2010). As a complementary approach of integrating map-based QTL analysis and expression-profiling to identify candidate genes for traits related to meat quality, we used BAC arrays representing the previously identified QTL regions on SSC5 and SSC18 (Liu *et al.*, 2007; Cox *et al.*, 2002). Using the region-specific BAC arrays we listed positional functional candidate genes for WHC. In this respect an advantage of the region-specific BAC-arrays is that they are not limited to annotated genes covered by commercially available microarrays. However, as a proof of principle and in order to demonstrate the suitability of using QTL-region-specific BAC arrays to identify candidate genes in a relatively large region of interest we focus here on the gene *GLI3* that was correspondingly detected by microarray and BAC array analyses. We address here the three-way relationship of polymorphism, differential expression of *GLI3* and phenotypic variation in WHC.

Material and methods

Construction of SSC18 QTL-region-specific BAC Array and expression analysis. A contig of 68 BAC clones was constructed covering the WHC QTL region on SSC 18 flanked by markers *SJ061* and *SY31* (homologous to a 6 Mb interval on human chromosome 7 (HSA7:40-46 Mb, build 36)). BAC clones were selected by exploiting publicly available BAC end sequence information and BLASTN searches. BAC arrays were prepared by spotting DNA of each BAC clone in duplicate onto nylon membranes together with positive (*NRAP*, *RPL3, HPRT1* PCR fragments*)* and negative *(E.coli* DNA*)* controls. Hybridisation

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experiments were conducted depending on the phenotype (high vs. low drip loss) and depending on the genotype at the QTL on SSC 18 (*QQ* vs. *Qq* vs. *qq*). For the later animals were selected to build groups of four animals for each of the QTL genotypes that were estimated using QTL express (http://qtl.cap.ed.ac.uk/). For phenotype-dependent hybridisation six discordant sib pairs of the F2 generation of the DUPI resource populations (average WHC in DUPI: 1.92±0.94% drip, high 4.14±0.77 vs. low 0.9±0.77 % drip, $p<0.0001$) were selected. Targets prepared from *M. longissimus dorsi* (MLD) total RNA samples and radioactively labelled were hybridized in duplicate onto BAC arrays. After scanning using a Storm phosphorimager (GE Healthcare) the BAC arrays were stripped and re-hybridized using BAC vector fragments. Spot intensities were evaluated using the software Image Quant TL (GE Healthcare). The background correction was made with the "Spot edge average" method and the normalization was done against the overall mean spot intensity. Intensities were finally expressed relative to normalized spot intensities obtained after BAC vector hybridization. Differential expression was evaluated by T-test and „Significance Analysis of Microarray, SAM" (Tusher *et al*., 2001). BAC with significantly ($p<0.05$) different intensities were scored as `positive´. In silico comparative analyses were conducted to identify genes covered by the respective BAC clones. The differential expression of the genes was validated by quantitative real-time RT-PCR (qPCR) using the same animals as used for the BAC array analysis.

Analyses of GLI3. Porcine EST and sequences of the BAC clone CH242-195B10 were aligned in order to derive primers for comparative sequencing of animals of breeds German Landrace (DL), Piétrain, Duroc, Wild boar and F2 DUPI animals for detection of polymorphisms. Transcript abundance was obtained by qPCR of MLD of 70 DUPI animals. DUPI animals (n=128) were genotyped at an exonic SNP by pyrosequencing. Furthermore, animals of commercial herds of the breed DL (n=280) and the crossbreed PiF1 (n=331) were genotyped at an intronic SNP using PCR-RFLP. At slaughter meat quality data were collected according to guidelines of the Zentralverband der Deutschen Schweineproduktion e.V. (ZDS, 2005). The following pork quality traits were analyzed: meat color, drip loss , thawing loss, cooking loss, pH at 45 min. p.m. (pH1), pH at 24 hour p.m. (pH24), conductivity at 45 min. p.m. (CON1 and IMP1), conductivity at 24 p.m. (CON24 and IMP24) and shear force (SHEAR). The association between genotypic and phenotypic variation was evaluated using general mixed models (PROC Mixed, SAS v. 9.2; SAS Inc., Cary, NC, USA). The analyses were done separately within the DUPI populations and the commercial herds, respectively. For DUPI the model included the fixed effects of genotype, sire, sex and slaughterhouse and the random effects of dam and slaughter date and the covariate weight at slaughter. The model used in the commercial herds included the fixed effects of genotype of *RYR1* and *GLI3*, breed, farm, and sex and the random effects of sire, dam and slaughter date. Moreover the interaction of *RYR1* and *GLI3* genotype and the covariate of weight at slaughter were used.

Results and discussion

Expression analysis using QTL-region-specific BAC arrays. According to the current comparative map the BAC contig covered 68 annotated genes. The QTL-dependent BAC array expression analyses revealed six `positive´ BACs. By exploiting the publicly available

BAC end sequence information the `positive´ BACs were anchored to the homologous human regions that contain eight genes (Table 1). For the phenotype dependent analysis T-tests and SAM correspondingly revealed two BACs with significantly ($p<0.05$) different intensities containing five genes (Table 1). The BAC array analysis were compared to our previous Affymetrix GeneChip analysis (Ponsuksili *et al.*, 2008a,b) and differential expression of eight genes was validated by qPCR (Table 1). The BAC array analysis covered 33 genes that were not represented by the commercial microarrays. For one gene (*NUDCD3)* out of six genes not present on Affymetrix arrays differential expression was shown. For three genes non-regulation (*IGFBP3*, *C7orf10*) or regulation (*STK17A*) was confirmed. For *CAMK2B* qPCR revealed differential expression that was not shown by Affymetrix arrays.
The BAC array analyses enabled identifying BACs containing differentially regulated transcripts not covered by Affymetrix GeneChips. The comparison with the Affymetrix GeneChip experiment showed no results that were incompatible - the analyses supported each other for one gene. There were no indications of false positive results. In terms of sensitivity the analysis indicated that the BAC arrays enabled detecting BAC containing genes that were regulated at fold changes ≥1.5.

Table 1: BACs with significant different spot intensities depending on WHC (A) or QTL genotypes (B), genes covered and expression according to microarrays and qPCR

A	BAC name	Gene name	Microarray	qPCR
	CH242-145I13	*GCK*	not expressed	no data
		YKT6	no data	no data
		CAMK2B	no regulation	regulation (p=0.06)
		NUDCD3	no data	regulation (p=0.02)
	CH242-195B10	*GLI3*	regulation	regulation (p=0.04)

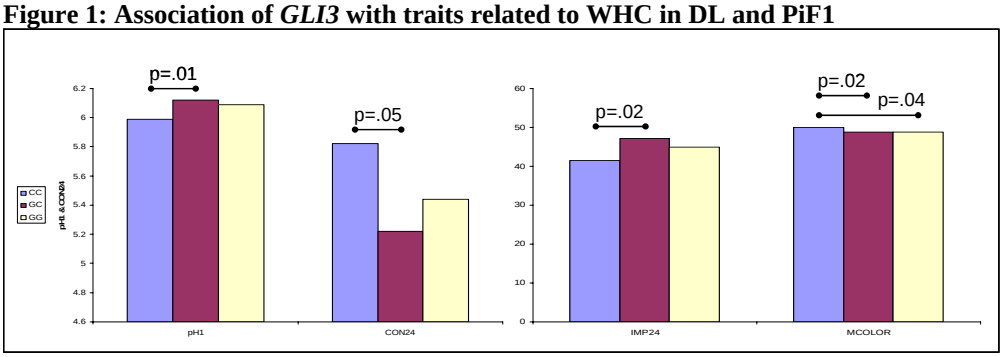
B	BAC name[#]	Gene name	Microarray	qPCR
	CH242-7F5	*STK17A*	regulation	regulation (p=0.02)
		HECW1	no data	no regulation
	CH242-195B10	*GLI3*	regulation	regulation (p=0.02)
	CH242-201C7*	*IGFBP3*	no regulation	no regulation
	CH242-38G17	*C7orf10*	no regulation	no regulation

#other two BACs do not contain any annotated genes;
*contains also the non-annotated loci *LOC100129619*, *LOC442304*, *LOC222052* that were not analyzed

Candidate gene analysis. The BAC clone CH242-195B10 was found showing intensity differences in comparisons depending on the phenotype and the genotype; this clone contained the *GLI3* gene, as evidenced by PCR. A probe set representing *GLI3* also showed significant correlation to WHC in the Affymetrix GeneChip analyses (Ponsuksili *et al.*, 2008a,b). Differential expression of *GLI3* was also confirmed by qPCR. The GLI3 protein is a zinc finger transcription factor mediating sonic hedgehog signalling pathway (SHH) and modulating the transcription of downstream genes including myogenic regulatory factor (MRF) Myf5. Accordingly *GLI3* is also functionally important in muscle development and regeneration. In mice association with body weight was shown (Martin *et al.*, 2007). In order to further promote of QTL-region-specific BAC arrays as tools providing an experimental link between the QTL linkage map, the physical map and differential expression for identification of positional functional candidate genes *GLI3* was selected for subsequent

analysis. Comparative sequencing revealed two SNPs. Genotyping DUPI animals at the exonic SNP revealed association with traits related to WHC, namely pH24 (p=0.03) and thaw loss (p=0.04). Moreover, the SNP was significantly associated with expression of *GLI3* (p=0.01). In DL (minor allele G, freq 0.46) and PiF1 (minor allele C, freq 0.35) association was evident for the *GLI3* genotype with pH1, CON24, IMP24, and MCOLOR (Figure 1).

Figure 1: Association of *GLI3* with traits related to WHC in DL and PiF1



Conclusion

QTL-region-specific BAC arrays proved to be suitable to complement genome-wide microarray analysis for detection of functional positional candidate genes. By demonstrating the three-way relationship between polymorphisms of *GLI3*, its trait-dependent expression and phenotypic variation in traits related to WHC we promote *GLI3* as a functional positional candidate gene.

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