An Expression QTL of Closely Linked Candidate Genes Affects pH of Meat in Chickens

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ABSTRACT Genetical genomics has been suggested as a powerful approach to study the genotype–phenotype gap. However, the relatively low power of these experiments (usually related to the high cost) has hindered fulfillment of its promise, especially for loci (QTL) of moderate effects.One strategy with which to overcome the issue is to use a targeted approach. It has two clear advantages: (i) it reduces the problem to a simple comparison between different genotypic groups at the QTL and (ii) it is a good starting point from which to investigate downstream effects of the QTL. In this study, from 698 F2 birds used for QTL mapping, gene expression profiles of 24 birds with divergent homozygous QTL genotypes were investigated. The targeted QTL was on chromosome 1 and affected initial pH of breast muscle. The biological mechanisms controlling this trait can be similar to those affecting malignant hyperthermia or muscle fatigue in humans. The gene expression study identified 10 strong local signals that were markedly more significant compared to any genes on the rest of the genome. The differentially expressed genes all mapped to a region <1 Mb, suggesting a remarkable reduction of the QTL interval. These results, combined with analysis of downstream effect of the QTL using gene network analysis, suggest that the QTL is controlling pH by governing oxidative stress. The results were reproducible with use of as few as four microarrays on pooled samples (with lower significance level). The results demonstrate that this cost-effective approach is promising for characterization of QTL.

EXISTING genetic variation provides a valuable resource for dissection of the genetic architecture of complex traits. Despite rapid developments in genetic and genomic tools, fine dissection of complex traits has remained challenging (Ron and Weller 2007). Many genetic studies have successfully mapped and identified chromosomal regions controlling these traits, but very few succeeded in further characterization and identification of genes and causative mutations. The extent to which protein-coding changes *vs.* regulatory changes contribute to the variation in complex traits is not clear. However, much of the naturally occurring variation in complex traits is believed to be partially controlled by regulatory elements. Changes in these regulatory elements, either at a single nucleotide level or more complex structural changes in the region, may underlie variations in gene expression (Guryev *et al.* 2008). Since its formal description (Jansen and Nap 2001), genetical genomics, the combined use of genetic mapping and expression profile (or other genomic information) in segregating populations, has shown to have great potential in addressing the issue. However, the successes to date do not match the original promise. One of the main reasons is the low power of the experiments linked to the low sample size due to high cost of such experiments, especially the genomic part of the study (De Koning and Haley 2005). One of the solutions proposed to addressing the high cost is the application of targeted approaches: focus on one or a few QTL rather than the whole genome. Instead of using the whole segregating population for the microarray experiment, one can select only individuals with alternative homozygote genotypes at the targeted QTL (QQ *vs.* qq). When all individuals

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Arrays have been submitted to the GEO database at NCBI as series GSE47800.
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are homozygous, the increase in power is equivalent to doubling the number of individuals. Some examples of the sample sizes required to detect eQTL of a given magnitude can be found in De Koning *et al.* (2007, section *Power, Precision, and Population Size*). Another strong aspect of the approach is its ability to identify downstream effects of the QTL. Selected individuals are expected to be a random mosaic for most of the genome except for the target region. In other words, selected individuals will carry a random sample of alleles (Q or q) at all loci across the genome from each parent but at the targeted position (or region), where they carry either Q (group 1) or q (group 2) from both parents.

Regarding chicken meat, the initial pH or pH 15 (pH 15 min post-mortem) affects water-holding capacity, drip loss, and processing meat yield, which are economically important traits (Berri *et al.* 2005). Little information on the genetic control of this trait in the chicken is available. However, the role of ryanodine-receptor calcium release channel (*RYR1*) in other meat-type animals such as pork is well documented (Fujii *et al.* 1991). Mutations in *RYR1* in pork cause uncontrolled muscle contraction triggered by the anesthetic Halothane at slaughter time. The *RYR1* is also called Halothane gene or Malignant Hyperthermia (MH) gene. MH is also a life-threatening disorder in human triggered by halogenated anesthetics or caffeine. It appears that the trait in the chicken is more tightly controlled, causing less severe problems.

We previously identified a QTL on chicken chromosome 1 affecting pH 15 (Nadaf *et al.* 2007). The QTL effect is moderate; however, this is the most significant QTL affecting initial pH identified in the chicken. The aim of the study is to better characterize the QTL and to identify candidate genes and mechanisms contributing to the effect of QTL on initial pH of chicken breast meat. The second aim of the study is to show the feasibility and efficiency of the approach for fine mapping QTL of moderate effect in practice.

Materials and Methods

Throughout this article, for gene expression analysis "significant" refers to q-values < 0.05 and "marginally significant" refers to P-values not adjusted for multiple testing. In addition, all of the genome coordinates are based on the chicken genome release of May 2006 (galGal3). We mean by "local" all the positions within 1-Mb region around the QTL unless stated otherwise. The word "cluster" (class) was used for the MCL clusters.

QTL mapping and selection of animals for the gene expression study

The targeted QTL was first identified in 2007 using 17 microsatellite markers on the chicken chromosome 1 (Nadaf *et al.* 2007). Subsequently, markers were added in the QTL regions (a total of 28 on GGA1) and QTL mapping was repeated using 698 birds for some metabolic traits, as presented in Nadaf *et al.* (2009). Here, using the same birds and the 28 markers, QTL mapping was performed by regression of phenotypes on line-origin additive coefficients (Haley *et al.* 1994) as implemented in GridQTL (Seaton *et al.* 2006) and detailed previously in Nadaf *et al.* (2009). The chromosome-wise significance level was estimated by 10000 permutations. The confidence interval was estimated using the two-LOD drop-off method (Mangin and Goffinet 1997). The genotype, phenotype, and marker information are available as GridQTL input files in Supporting Information, File S1.

The F2 population was created by crossing two selection lines: a low-growth (LG) line and a high-growth (HG) line. A total of 24 F2 birds were selected to be homozygous for the markers flanking the QTL. They were called qq if they were homozygous for the LG line alleles and they were called QQ if they were homozygous for the HG line alleles. Accordingly, the 24 selected F2 birds are expected to be a random mosaic for most of the genome except for the QTL region. Our previous finding showed that the HG line had a higher pH compared to LG chickens. It was the same for the targeted QTL, where the QTL allele originating from HG line increased the pH (Nadaf *et al.* 2007). As expected, QQ genotypes had a higher pH and this was confirmed to be the same for the 12 pairs of selected animals.

Gene expression analyses

The microarray data were submitted to the GEO database (accession no. GSE47800). Agilent 60-mer oligonucleotide microarrays were used for the gene-expression study. Each array includes 42,034 probes. RNA, prepared from *Pectoralis major* muscle harvested at 9 weeks of age, was hybridized using the gene expression hybridization kit according to the manufacturer's protocols (Agilent Technologies). The slides were scanned using an Axon 4200 AL scanner set at 5-μm scanning. Image files were analyzed using the Agilent Feature extraction software v. 9.5.3.1. The data were generated by Ark-Genomics (Talbot *et al.* 2012). Details of the characteristics of the microarrays can be found in Li *et al.* (2008). To compare the 24 selected birds for the two alternative homozygous genotypes at the QTL, 12 arrays were used in a dye-balanced design in a way that on each array one QQ and one qq sample were hybridized and then the red/green color were randomly assigned such that 6 red and 6 green were assigned to each genotype group. In choosing the 12 comparisons, an effort was made to make the comparison within the same full-sib family ($n = 18$, in 8 different families) or half-sib family ($n = 6$, in 2 different families). Another microarray experiment was conducted using 4 arrays of pooled samples of 3 individuals, comparing alternative homozygous genotypes, as in the first experiment (see Figure S1). While the pools include the same birds, the pairing of the pooled samples is different. All of the statistical analyses were conducted separately for the first and the second experiment. However, for the gene network analysis, all normalized gene expression values were analyzed jointly.

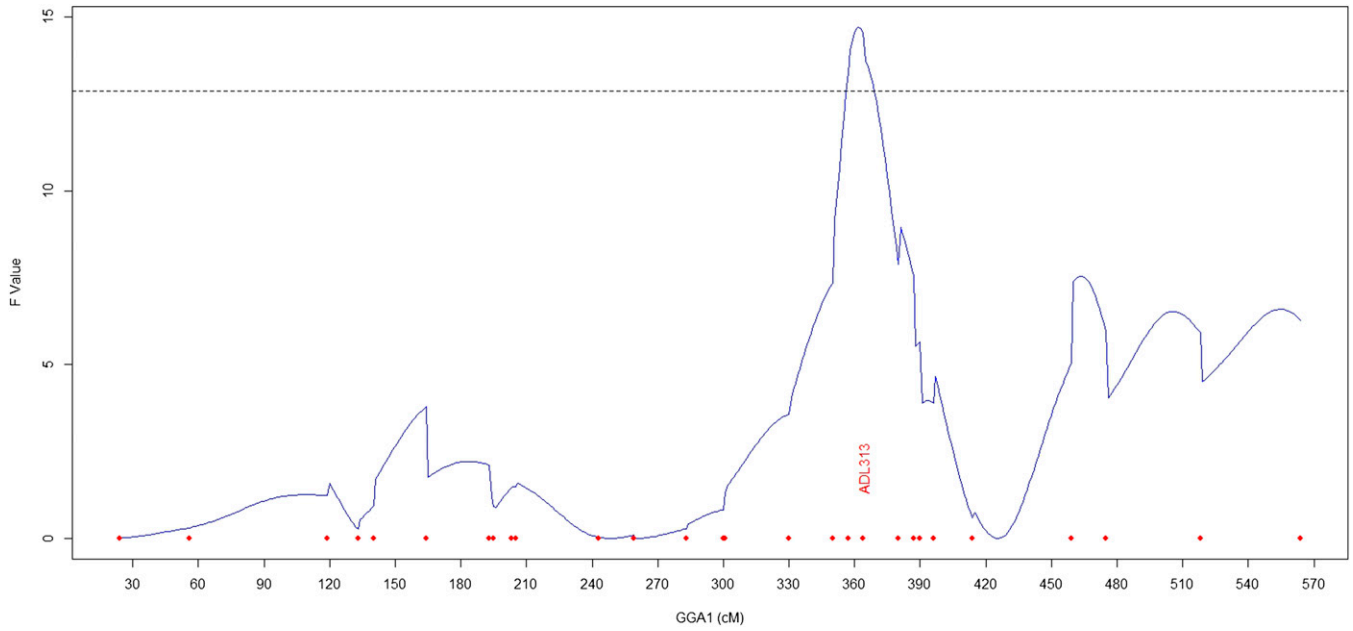

Figure 1 QTL profile on chromosome 1 (GGA1). The dashed line indicates chromosome-wise significance 1% threshold.

Normalization

Global loess normalization was used to adjust the expression differences for intensity dependencies (Smyth and Speed 2003). The assumption of the loess normalization is that the majority of the probes are not differentially expressed, which was true in this study. We did not perform additional background correction on the extracted expression values. To do so, we suppressed the default of the function normalizeWithinArrays using the bc.method option (File S2). A mixed model was used to correct the data for dye (fixed effect) and array (random effect) using SAS mixed model process (SAS 1999). The raw and normalized microarray data were submitted to the GEO database (accession no. GSE47800). The normalized data were used to assess the significance of the two genotype groups (with no additional explanatory effect).

Differential expression analysis

The microarray design was a two-color experiment with 12 biological replicates (12 arrays). The arrays were weighted based on their quality. Empirical array quality weights were estimated from within-array normalized data (Ritchie *et al.* 2006). Then, the estimated weights ($n = 12$) were used in the differential expression analysis. The differential expression analysis estimated the moderated *t*-statistics for all probes (Smyth 2005; see File S2 for the details). The moderated *t*-statistics are similar to ordinary *t*-statistics except that the standard errors are shrunk toward a moderated value, across all genes. To control the false discovery rate (FDR), *q*-values were obtained from *P*-values as explained in Benjamini and Hochberg (1995). File S2 is the R script that describes different steps of the gene expression analysis.

Coexpression and gene network analyses

For gene network analyses, the probes were filtered in different steps. First all the probes that were marginally significant ($P < 0.05$) were selected ($n = 2057$). To find the coexpressed genes, only highly correlated probes (with at least one other probe) were kept ($r \geq 0.9$, $n = 558$), where r is the Pearson correlation across all 16 arrays. The probes were then clustered using Markov cluster (MCL) algorithm (Van Dongen 2000). MCL clusters the probes based on the similarity of gene expression patterns across all arrays. The most important parameter to the algorithm is inflation. It affects the granularity of the clustering and higher values will result in a lower number of members (probes) in each classes and a higher number of total classes. We used Biolayout (Theocharidis *et al.* 2009) to perform the analysis. The default for the inflation value in Biolayout is 3. Because in this study the difference in phenotype of the two genotypes is due only to one QTL of moderate effect, to obtain a reasonable number of probes in each cluster the inflation value should be lower. Here we used half of the default value (inflation = 1.5).

Investigation on the enrichment of functional categories based on Gene Ontology (GO) was conducted for each of the clusters that have reasonable number of members ($n > 30$). To perform the analysis, DAVID (Huang *et al.* 2009) was used with the default values for components of the GO annotation including biological process (BP), cellular component (CC), and molecular function (MF).

Real-time RT–PCR assay

Total RNA was extracted from *P. major* muscle of 12 qq and 11 QQ individuals (the same as those used for microarray

Table 1 Top probes from differential expression analysis of breast muscle for chickens with alternative QTL genotypes for a QTL affecting muscle pH after slaughter

Probe name	log FC[a]	Ave Exp[b]	*t*-value[c]	*P*-value	Adj *P*-value[d]	Gene symbol	Within the 204k interval[e]
A_87_P016951	−2.307	3.449	−16.51	2.87E-10	7.19E-06	NA	Yes
A_87_P014348	−1.115	1.125	−16.28	3.42E-10	7.19E-06	ACOT9	Yes
A_87_P030344	−1.302	−0.489	−12.64	8.27E-09	0.000116	PRDX4	Yes
A_87_P034725	1.478	−0.244	11.03	4.38E-08	0.00046	NA	No (chr1:121537871–121537850)
A_87_P014256	0.812	1.753	10.28	1.02E-07	0.00086	NA	Yes
A_87_P011383	0.662	2.578	9.25	3.57E-07	0.0025	KLH15	Yes
A_87_P032384	2.574	0.599	8.44	1.03E-06	0.0062	NA	Yes
A_87_P006189	0.812	0.958	7.22	5.78E-06	0.0304	NA	No (chr1:127451013–127450954)
A_87_P025536	0.754	−0.174	7.04	7.56E-06	0.0353	APOO	Yes
A_87_P034683	2.21	4.085	6.89	9.55E-06	0.0401	PRDX4	Yes
A_87_P028458	−0.593	−0.41	−6.53	1.68E-05	0.0596	PRDX4	Yes
A_87_P036477	0.527	0.496	6.52	1.70E-05	0.0596	KLHL15	Yes
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A_87_P021966	0.753	6.056	5.61	7.62E-05	0.17	PRDX4	Yes

[a] Log of fold change.
[b] Average expression.
[c] The negative value means lower expression in the qq genotype.
[d] Adjusted *P*-value.
[e] Is the probe located on chromosome 1 between 121675396 and 121879556 bp (about 204 kb, genome assembly galGal3, May 2006)? See Table S1 for position and more annotations of these probes.

analysis; one bird lacked sufficient material for this assay) and real-time RT–PCR was performed using cDNA synthesized as previously described (Sibut *et al.* 2008). Primers were designed for each gene so that the amplicon contains the probe spotted on microarray (Table S2) and purchased from Eurogentec (Angers, France). PCR was run in an ABI PRISM 7000 apparatus (Applied Biosystems, Courtaboeuf, France) programmed to conduct one cycle (95° for 10 min) and 40 cycles (15 sec at 95° and 1 min at 60°). The specificity of the PCR reactions was assessed by analyzing the melting curves of the products and size and sequence verification of the amplicon. PCR runs for each sample were performed in triplicate. Each PCR run included a no-template control (H_2O) and triplicates of control, *i.e.*, a pool of all 23 cDNA samples. The calculation of absolute mRNA levels was based on the PCR efficiency and the threshold cycle deviation of an unknown cDNA *vs.* the control cDNA according to the equation proposed by Pfaffl(2001). The level of 18S rRNA was chosen as reference and confirmed to be stable across genotypes. Absolute mRNA levels were corrected for 18S rRNA to give relative levels. The ratio of gene expression determined by real-time RT–PCR was compared to the ratio (expressed as log FC) obtained using microarray analysis (Table 1)

Results

QTL mapping and animal selection

We mapped the QTL, using 28 markers covering 540 cM on chromosome 1 for pH 15 (Figure 1). The QTL was located at 362 cM and flanking markers were ADL148, *ADL313*, and GCT005 at 357, 364, and 380 cM, respectively. The closest marker to the QTL was *ADL313* located at 121766076–121766223 bp (genome assembly galGal3, May 2006). The QTL 95% confidence interval was approximated to be 52 cM (340–392) using two-LOD drop-off method (Mangin and Goffinet 1997). The QTL explained 2.1% of phenotypic variance. Further analyses showed that the additive effect of the QTL in females was about three times the effect in males (additive effect of 0.06 ± 0.016 *vs.* 0.02 ± 0.015 in females and males, respectively). Accordingly, alternative homozygote females for the QTL (12 from each genotype) were selected for the expression analysis. The two groups of animals (QQ and qq) had a mean adjusted pH 15 value of 0.13 and −0.13, respectively (*P*-value = 0.0008), which is about 1.4 standard deviation of the trait.

Microarray differential expression analysis

Statistical analysis was conducted on the 42,034 oligonucleotide probes on the Agilent 44k chip (all predicted chicken genes). After adjustment for multiple testing, 10 probes were significant at FDR < 0.05 (Table 1). The differentially expressed probes were exclusively seen in the QTL region (Figure 2). Eight of the 10 probes were located on chromosome 1 between 121675396 and 121879556 bp (about 204 kb) and 2 were flanking the region starting at 127451013 or 121537871 and ranking eighth and fourth in the top list, respectively. The two flanking probes were not within known genes. Differentially expressed probes in the region of 204 kb targeted four known genes: *PRDX4* (4 probes, ranked in the top list from third to eighteenth), *KLH15* (2 probes, ranked sixth and twelth), *ACOT9* (ranked second) and *APOO* (ranked ninth). Figure 3 shows the position of genes and probes in the Ensembl Genome browser (Flicek *et al.* 2012). In the figure, up- and downregulated probes in qq genotype are shown in red and green, respectively. Absolute *t*-values were also added to show the relative level of significance. Figure 3 also shows the *ADL313* marker that was the closest marker to the QTL.

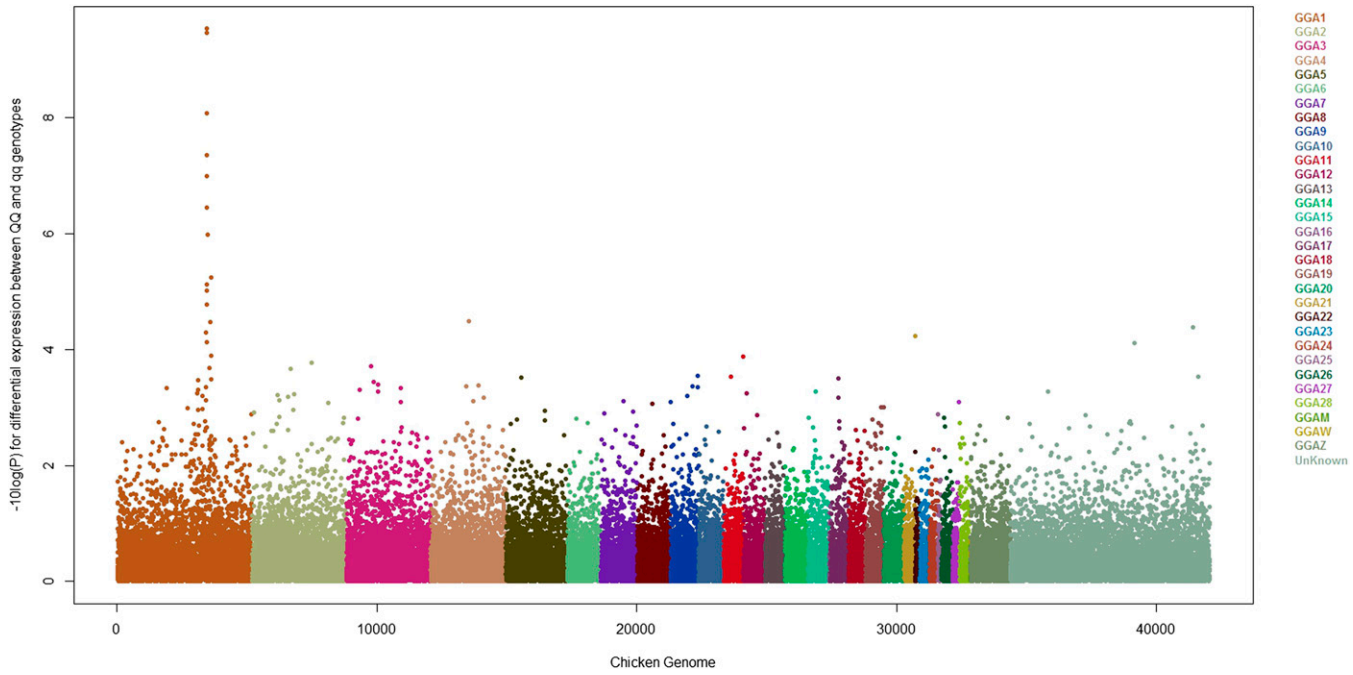

Figure 2 Manhattan plot of differential expression statistic –log10 (*P*-value) against the chicken genome. The signals are highly enriched at the QTL position on GGA1 (chromosome 1). Different colors indicate different chromosomes.

Similar results were obtained using four arrays of pooled samples (Figure S2), where they shared 4 of the top 10 differentially expressed probes and the six other probes were marginally significant ($P < 0.01$).

The mRNA levels of four genes (*KLHL15*, *APOO*, *PRDX4*, *ACOT9*), found to be differentially expressed on the microarray, were further quantified by RT–PCR in the same qq and QQ birds. Overexpression in qq compared to QQ was confirmed by real-time RT–PCR for *KLHL15* (log FC = 0.549, $P = 0.059$) and *APOO* (log FC = 0.665, $P = 0.056$). Real-time PCR analyses also showed differential expression between qq and QQ for *PRDX4* ($P = 0.003$) and *ACOT9* ($P = 0.017$) but ratios were reversed (log FC of −1.966 and 0.748 for *PRDX4* and *ACOT9*, respectively) compared to those observed on the microarray (Table 1).

Coexpression and functional analyses

A total of 2057 marginally significant probes ($P < 0.05$) were used for these analyses, of which the expression of 558 was highly correlated ($r \geq 0.90$). A total of 28 coexpressed groups were constructed on the basis of their correlations using BioLayout Express (Theocharidis *et al.* 2009) (Figure 4A). Two of the 28 groups of coexpressed genes comprised 78% of the selected probes (438 probes) including all top significant ones. MCL clustering constructed 57 clusters with similar pattern of expression across all 16 arrays.

On average, the connectivity of the network (number of connections between probes in classes or clusters) was not high (4.2, which was not unexpected for a phenotypic difference due to one moderate QTL). However the maximum connections were 32 and 30 and corresponded to phospholipase A2 (*PLA2G7* on chromosome 3) and catalase (*CAT* on chromosome 5). Figure 4B shows a higher resolution of the part of the network that includes a cluster (in purple) with four highly significant local probes (including probes within the genes *ACOT9* and *PRDX4*) and the biggest cluster (cluster in green with phospholipase A2 as the hub molecule).

Further functional analysis using DAVID, for the list of all probes in the two biggest clusters (cluster 1, $n = 80$ and cluster 2, $n = 31$), separately or both combined with the top significant list ($n = 10$), did not lead to any significant results. One of the main reasons was that the majority of the probes (~60%) did not have functional annotation. The list of all probes used for gene network analysis along with the DAVID annotation and MCL clustering information can be found in File S3.

Discussion

Characteristics of founder lines and the targeted region

The two lines used in this study were the result of >20 generations of selection on growth rate. The difference of the two lines for body weight at the time of slaughter is about threefold. Obviously this difference in body weight can be linked to several other biological changes such as growth, metabolism, and behavioral traits. Previously, QTL were identified in the F2 population for several growth and metabolism traits and some of them were near (but do not colocalize) the region targeted in this study (see Nadaf *et al.* 2009, Figure S1). In this study, we compare the expression

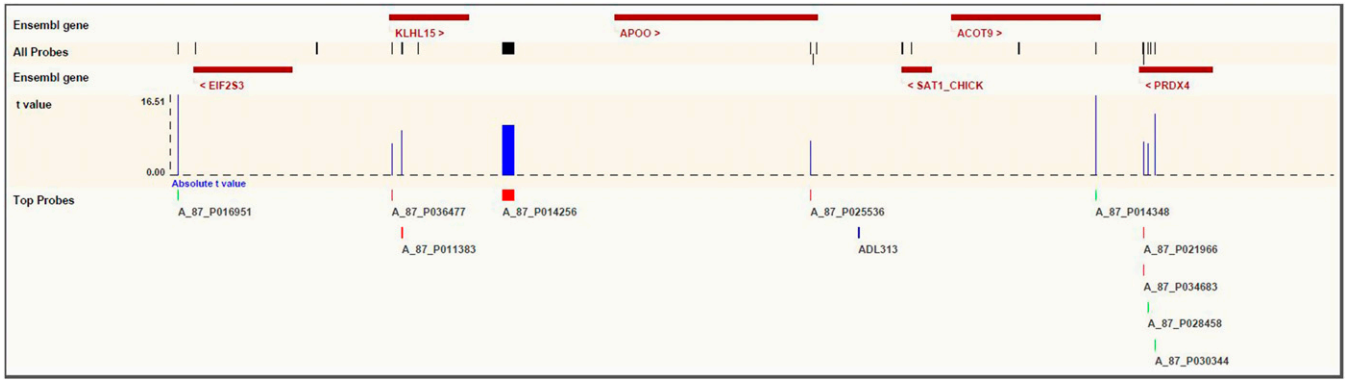

Figure 3 Position of genes and probes in Ensembl Genome browser (Flicek *et al.* 2012). Up- and downregulated probes (whose names start with A_87_P) in qq genotype are shown in red and green, respectively. Absolute *t*-values show the relative level of significance. The *ADL313* marker is the closest microsatellite marker from the QTL mapping results.

profile of the two segments of chromosome 1, coming from HG or LG chicken lines, at the QTL controlling breast meat pH 15. These chromosomal segments in an F2 population are relatively large, although in the chicken the rate of recombination is about threefold higher compared to mammals (in the chicken, 1 Mbp on physical map is about 3 cM on genetic map). This study revealed that this region on chromosome 1 harbors several differentially expressed probes. All of these probes are positional candidates for the QTL and some of them are also functionally linked to pH variation. However, these genes may affect traits other than pH. The potentially affected traits are expected to be related to muscle physiology but they are not limited to them. Indeed, behavioral traits can lead to important variation in muscle metabolism (Debut *et al.* 2005) and behavioral measurements on the pre-slaughter shackle line. A previous study clearly showed that LG birds were less active than HG birds (Nadaf *et al.* 2007). One hypothesis for local differentially expressed probes might be that they do not reflect actual expression differences in mRNA level but they show hybridization differences due to sequence polymorphisms in microarray probes region. However, the 60-mer oligonucleotides used in this study were shown to be robust against four or fewer SNP polymorphisms in the probe region (Hughes *et al.* 2001). In addition, the fact that two lines were divergently selected from a single population further reduces the probability of different hybridization efficiencies between genotypes.

Potential candidates for expression quantitative trait gene (eQTG)

The targeted QTL affected meat pH 15. This trait can be affected by activity of the birds and the stress that they experience just before death. Two measurements of bird's activity at the slaughter line are the percentage of straightening up (SU) and duration of wing flapping (WF) between hanging and electrical stunning (Debut *et al.* 2005). In HG and LG lines, it was previously shown that these measurements were highly correlated to pH 15 (-0.58, -0.79 in HG line and -0.54, -0.55 in LG line for SU and WF, respectively; Nadaf *et al.* 2007). The biological process contributing to these correlations can be similar to those generating fatigue in muscle. Muscle exercise can lead to acidosis and calcium dysregulation by affecting ryanodine-sensitive calcium channel. Reactive oxygen species (ROS) can be accumulated within exercising muscle and promote fatigue. In contrast, antioxidants such as reduced glutathione can prevent it (Ferreira and Reid 2008). Glutathione can aid peroxidase reaction by reducing oxidized *PRDX4* (one of the local differentially expressed gene) (Leyens *et al.* 2003). It has been shown that administration of reduced glutathione in mice can increase their swimming endurance (Novelli *et al.* 1991). Protein phosphatase 2A (*PP2A*) can be regulated by glutathionylation, hydrogen peroxide, and Ca^{2+} (Rao and Clayton 2002). Interestingly the gene whose expression in the gene network was directly correlated ($r \geq 0.9$) with a local gene (*PRDX4*) was protein phosphatase 2A regulatory subunit B *PPP2R2B* (Figure 4). It may suggest that regulation of *PPP2R2B* can be a downstream effect of the local-eQTG (*PRDX4*). Interestingly the hub molecules of the gene network (with 32 and 30 connections with other nodes in the network), namely phospholipase A2 and catalase, are also directly involved in oxidative stress. An enhanced activity of phospholipase A2 has been shown to induce excessive release of Ca^{2+} leading to meat drip loss and PSE meat (Soares *et al.* 2003). Interestingly, catalase mimics activity of peroxidase and protects cells against ROS (Day 2009). Together, all these genes appear to be regulated to protect muscle cells against oxidative stress. *PRDX4* is a potential eQTG and variation in the expression of the other genes mentioned (*PPP2R2B*, *CAT*, and *PLA2G7*) can be interpreted as downstream effects of the variation in the *PRDX4* expression.

The *PRDX4* had two up- and two down regulated probes. The two upregulated probes, in the qq genotypes, showed much higher abundance (average expression of 6 and 4) compared to the two other probes (average expression of -0.4 and -0.5). These results would suggest the existence

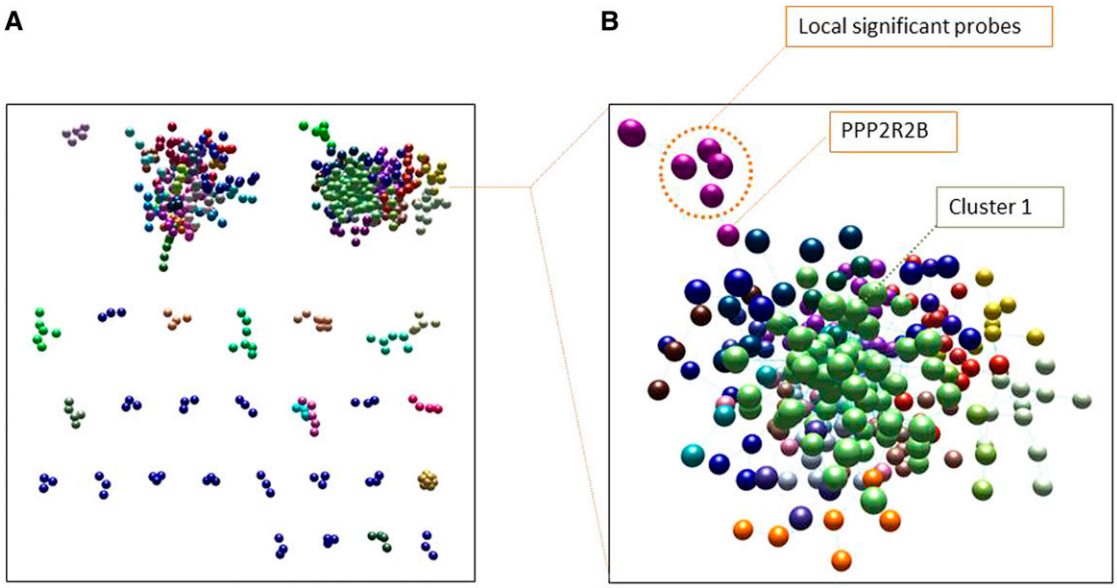

Figure 4 Co-expressed groups. (A) All 28 co-expressed networks. (B) A higher resolution of part of the network including a cluster of local significant probes, which are connected to other clusters by *PPP2R2B*. This network includes several clusters of similar expression patterns in which cluster 1 (the green cluster) is the biggest and a more interconnected cluster, whose hub molecules are phospholipase A2 and catalase. For more details see the text.

of at least two splice variants of the gene in the chicken muscle cells. *PRDX4* is one of the strong functional and positional candidates for the targeted QTL. However, other differentially expressed genes in the region of 204 kb are involved in energy metabolism (*ACOT9* and *APOO*) and could potentially play roles in the variation due to the QTL.

Targeted region and more hypotheses

Characterization of genetic architecture of complex traits is very challenging, and so far it has been done for a few QTL of major effect and more specifically in model animals. Little information is available about genetic control of gene expression affecting moderate-effect QTL. In this study we targeted a QTL moderately affecting pH. The results provided us with valuable information and significant improvement in characterization of the QTL. The confidence interval of the QTL was about 50 cM; however, using the targeted genetical genomic approach, significant eQTL targets were mainly localized in a few hundred kilobases.

Whatever the biological processes underlying the QTL, and potential functional effects of the local eQTG on pH and other traits, one question remains: What caused such remarkable intensively local signals at the target region? From a statistical point of view, improved power at the targeted region was expected for this approach. Sensitive microarray technology, efficient design, and good information content at the QTL can also play important roles. However, such intensively local signals for several genes spanning a small region around a moderate QTL (~204 kb) is quite remarkable. It must be noted that this region is much smaller than the shared chromosomal segments within the QQ and qq birds. On the other hand, it is clear that some genomic regions may affect one or several traits in varying degrees, which may be due to several linked QTL or a pleiotropic effect of one (or a few) QTL. For example, in this population a region on chromosome 4 was identified with major effects on several traits (Nadaf *et al.* 2009). It is possible that the targeted region also has effects on several traits not yet studied in this population. In addition, it is also possible that more than one QTL (or gene) with major but antagonistic effects in this region are affecting pH 15, leading to a QTL with moderate effect.

Finally, a similar approach was used for characterization of a QTL on GGA4 for body weight in a broiler × layer cross (Cabrera *et al.* 2012). They found a spread of differentially expressed genes across the genome with no particular enrichment in the QTL region and only one reasonably significant candidate gene in the QTL region. This shows that similar approaches can have very different results depending on the underlying biology of the targeted QTL. Here we have a few highly significant known genes as well as highly significant unknown probes, which all are restricted to the QTL region. These results are remarkable and worthy of further investigation.

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GENETICS

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An Expression QTL of Closely Linked Candidate Genes Affects pH of Meat in Chickens

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DOI: 10.1534/genetics.113.160440

Individual Sample Comparison			Pooled Sample Comparison		
QQ		**qq**	**QQ**		**qq**
1	⟷	13	1		13
2		14	2	⤫	14
3		15	3		15
4		16	4	⤫	16
5		17	5		17
6		18	6		18
7		19	7		19
8		20	8	⤫	20
9		21	9		21
10		22	10	⤫	22
11		23	11		23
12	⟷	24	12		24

Figure S1 Design of the 2 experiments comparing individuals or pooled samples of 3 individuals

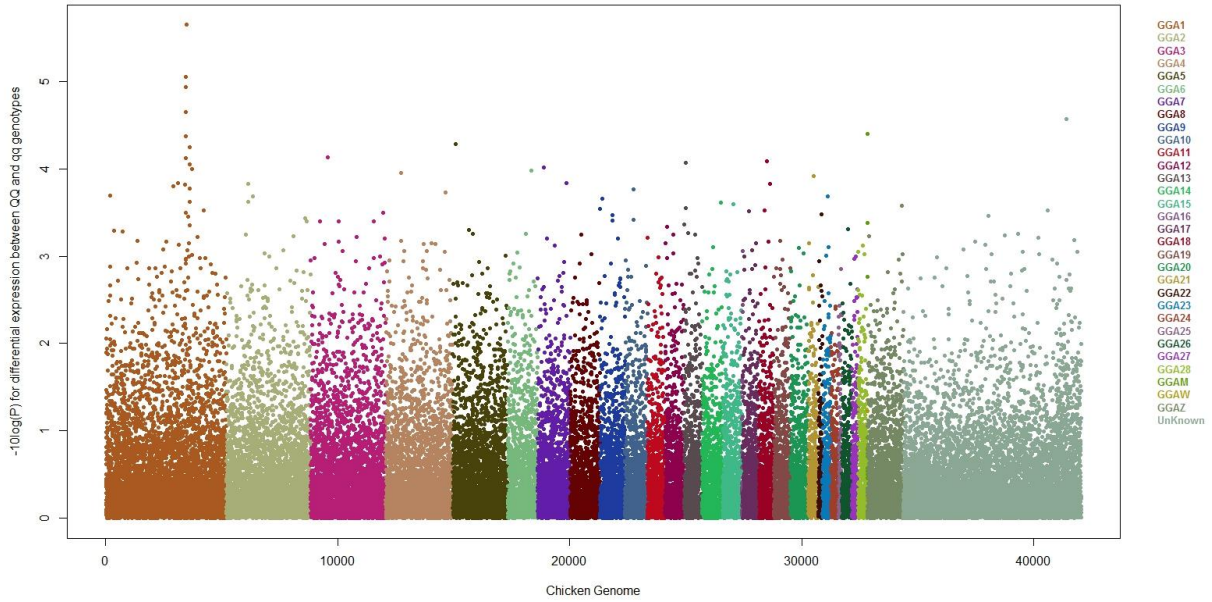

Figure S2 Pooled samples: Manhattan plot of differential expression statistic –*log*10 (*P*-value) against the chicken genome. The signals are highly enriched at the QTL position on GGA1 (Chromosome 1). Different colors indicate different chromosomes.

Files S1-S3

Available for download at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.160440/-/DC1

File S1 Genotype, phenotype and marker information in GridQTL input file format

File S2 R script describing different steps of the gene expression analysis

File S3 List of all probes used for gene network analysis, DAVID annotation, and MCL clustering information

Table S1 Annotation for the top probes from differential expression analysis (see also Table 1)

Probe Name	Genbank Accession	Ensembl ID	Genomic Coordinates[1]	Seq
A_87_P016951	CR385747	NA	chr1:121675397-121675456	TGTAGGGGAGGTCAAACATCAGGGCTGCGCGGAAGTTGCACATAATGCAACAAAATAAAA
A_87_P014348	NM_001012823	ENSGALT00000026377	chr1:121797704-121797763	GCACCACACAACAAACATCTTCCATTTCACATTTATATCAGAGAACGAGGTCCCACGTGT
A_87_P030344	NA	NA	chr1:121805614-121805657	ATATACTTAATGGGAGTAGCGAAAACTAGTTATTAATTGGGGAAAGAGAGAAGTGCACGC
A_87_P034725	NA	NA	chr1:121537871-121537850	TTAGAGACTTGCTTGGGTAGGATGGGGTGTCGTGAATAAATTCTATCATTGTCAAACTCT
A_87_P014256	CR390282	NA	chr1:121718672-121720141	AAGTGAAGGATAACATTCAGCAGGAGGTGATTTTCCCTAGCTGTGGGTACAGTATGCTCA
A_87_P011383	CR523763	NA	chr1:121705248-121705307	TCTGTATTTTTCACCTCTCAGTGAATTAATAGGATTTAATGCCCTGTGTATTTTGTTGGG
A_87_P032384	BU230994	NA	chr1:121879616-121879557	TGTGAGGTTCCCATTTCCACTATCCAATTTTCAAAAGATTCTTCATACTCTGTTCCTATG
A_87_P006189	NA	NA	chr1:127451013-127450954	AGGCATTTCCATCAAAGTAGTAGTGTATTTCTCGTGCCTCTGATCTCTGTGGATGTTTTT
A_87_P025536	XM_416798	NA	chr1:121759707-121759764	GACAAGCTTTAATTTTATCCCTTGTGTTGTGCTGGGTGAACTTTATGGAGGCGACAAGAG
A_87_P034683	NA	NA	chr1:121804107-121804079	TGGGGCTGATTTCGTTTATTACTTAGGTCACGTTCAACTTGATTTTGGCATTAAATTCGT
A_87_P028458	NA	NA	chr1:121804677-121804735	ATCATCCACGTAACCTCCTCTGGACTTGCTTTTGATACAGCCCAGGATATGTTGGCTTTT
A_87_P036477	NM_001030802	ENSGALT00000026363	chr1:121703895-121703954	CAGCTACAGGCTTCAGTCATGTCCTCCAATTCATGTATTATGGAACTATTGAACTGAGTA
.	.	.	.	.
A_87_P021966	BX935739	ENSGALT00000026387	chr1:121804122-121804063	GCTGACTTCCGTTTAATTACTTAGGTCACGTTCAACTTGATTTTTGCCAATAAAATTTCG

[1]Genome assembly galGal3, May 2006.

Table S2 Primers used for real-time PCR analysis.

Probe Name	Genbank Accession	Gene Symbol	Forward	Reverse
A_87_P036477	NM_001030802	*KLHL15*	ATGCGAGAACGAGATCAGGAC	CGCACAGTTTTCTAGGCAGATT
A_87_P025536	XM_416798	*APOO*	CCGTTCCAAAAAAAACCAGC	TCTTGTCGCCTCCATAAAGTTC
A_87_P014348	NM_001012823	*ACOT9*	ATGCAGATACCAGAGAGCACCA	CAAAGTGTCGCTTCCCATCTAA
A_87_P021966	BX935739	*PRDX4*	TCGTTTTCTTTCTTGTGCAGAT	CGTGAAAGTTAAGAAAATTAACGAAA
-	AF173612	18S rRNA	TCCAGCTAGGAATAATGGAATAGGA	CCG GCCGTCCCTCTTAAT