RAPID COMMUNICATION

Expression of Positional Candidates for Shell Thickness in the Chicken

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ABSTRACT Expression of 12 positional candidates for QTL affecting shell thickness at 53 wk of lay age (ST53) was investigated by real-time PCR in the distal part of chicken oviducts (uterus) with a forming eggshell. In the local chicken breed Green-legged Partridgenous, the complete cDNA CR523443 (ChEST985k21) was downregulated with ratio of means 0.49 ($P \leq 0.01$) in the group with low ST53 (248.6 ± 16.62 μm) relative to the group with the highest ST53 (372.4 ± 2.07 μm). Expression of this gene was highly correlated (0.85, $P \leq 0.01$) with shell thickness. No significant difference in expression between the 2 groups with thick (378.4 ± 3.65 μm) and thin (227.8 ± 8.99 μm) shell and no significant correlation of expression level with ST53 were detected in Rhode Island Red, which could be explained by strict selection to egg quality traits, including optimal shell thickness in this commercial layer breed. These data suggested that CR523443 was a candidate gene for QTL ST53 in the chicken.

Key words: *Gallus gallus*, quantitative trait loci, shell thickness, gene expression profiling, real-time PCR

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INTRODUCTION

Searching for candidate genes affecting quantitative traits could be a tool for MAS. Recently, a large number of genetic markers that facilitate QTL analysis has been generated and mapped in multiple experimental populations. The current availability of highly polymorphic DNA markers in many species renders possible the elaboration of well-saturated genetic maps and, consequently, genetic dissection of complex quantitative traits (Vallejo et al., 1998). Among the genetic markers that are currently used, microsatellites have been found to be abundant, evenly distributed, and highly polymorphic in all resource populations (Cheng et al., 1995).

The results of the whole genome scan for detection and localization of QTL affecting egg quality traits were described by Tuiskula-Haavisto et al. (2002). At 1% genome-wise significance level, 14 chromosomal areas affecting egg quality were found, and at 5% level, only 6 suggestive QTL were found in this study. Another whole genome scan was done in Green-legged Partridgenous (**GLP**), a native Polish breed maintained as a conservative flock, and a highly productive stock of Rhode Island Red (**RIR**; Wardecka et al., 2002, 2003). The significant effect of the genotype (GLP-GLP, RIR-RIR, and GLP-RIR) was found for 16 traits: age at sexual maturity, BW at 20 and 33 wk, feed intake at 33 wk, total individual egg production, egg weight at 53 wk, egg specific gravity at 33 wk, Haugh units at 53 wk, yolk weight at 33 wk, albumen weight at 33 and 53 wk, shell weight at 33 and 53 wk, shell thickness at 33 and 53 wk, and shell color at 33 wk (Wardecka et al., 2003).

The eggshell is a highly ordered structure resulting from the deposition of calcium carbonate concomitantly with an organic matrix upon the eggshell membranes. Mineralization takes place in an acellular uterine fluid, which contains the ionic and matrix precursors of the eggshell (Gautron et al., 2001). It forms in the uterine (shell gland) region of the oviduct in an acellular milieu that is supersaturated with respect to Ca and bicarbonate and which contains a variety of proteins that vary in concentrations during the sequential process of shell formation (Gautron et al., 2001). Formation of eggshell microstructure underlay complex regulations imposed by the resident egg (Lavelin et al., 2000). Significant age and environment effects were found for shell thickness (Edmond et al., 2005).

Shell thickness at 53 wk of lay age (**ST53**) was mapped on Gga4 very close to MCW0114 (Wardecka et al., 2002, 2003). Nine chicken genomic bacterial artificial chromosome clones containing the MCW0114 were fluorescence in situ hybridization-mapped to GGA4q11-12 (Sazanov et al., 2005). Here, expression profiling of 12 positional candidates for QTL affecting ST53 investigated by real-time PCR in the lower part of chicken oviducts with a forming eggshell in RIR and GLP is reported.

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Table 1. Positional candidate genes target sequences used for expression profiling

GenBank accession no.	Position on GGA4 (bp)	Primers[1]	Annealing temperature (°C)	PCR fragment size (bp)
XM_420335	16,137,294 16,158,029	fw 5′-GACAGCAGCCATGCAAAGTA-3′ rv 5′-CCTTCGTGTGATACGTGGTG-3′	60	143
U10548	16,166,813 16,183,769	fw 5′-GTGAACGTTACGCTGCTCAA-3′ rv 5′-GTTTGCTCCTTACGGCACAT-3′	60	119
CR390814	16,351,317 16,353,167	fw 5′-AACAATAGGCCTGACCGTTG-3′ rv 5′-CCATATATCCAGCCCCCTTT-3′	60	120
CR523443 (ChEST985k21)	16,481,729 16,483,417	fw 5′-CATCTCTGGCTGGGTTCTTC-3′ rv 5′-AATTGCTTCGGAACCACAAG-3′	59	115
XM_420348	16,710,015 16,920,778	fw 5′-GAGCTGCCAAAAACTGCTTC-3′ rv 5′-AGATCAGCCTCTTGCACCAT-3′	59	142
NM_001031126	16,941,649 17,036,962	fw 5′-GCAGCAGAAAGAGCAGAGG-3′ rv 5′-TCGTTAGCCTCAGTCCACCT-3′	59	103
XM_420350	17,042,102 17,065,993	fw 5′-AACGACCTGGAGAAGCAGAA-3′ rv 5′-ACATCATGCCTGAACCATCA-3′	59	149
XM_420351	17,078,901 17,105,036	fw 5′-GCTGCTGTGCGTCGTG-3′ rv 5′-CCGTACAGGCCGCTCTC-3′	59	135
XM_420352	17,110,031 17,142,579	fw 5′-CTTCTGTGGAGCTGGGAGTC-3′ rv 5′-GAGCTGCATCCAAGTGACAA-3′	60	101
XM_420353	17,175,913 17,147,736	fw 5′-AGATGCAGTTCCCTGAAAGC-3′ rv 5′-TCTGCTGTCCGAAACATCAC-3′	59	142
XM_420354	17,251,083 17,246,778	fw 5′-TGCTGTCCAAACAAAATGGA-3′ rv 5′-CAGTTTGGGTCAATGCCTTT-3′	60	143
NM_205361	17,374,305 17,347,243	fw 5′-CGATCACAGTGGTGGTTGTC-3′ rv 5′-GTCTCACCCGGTGTTTGACT-3′	60	105
NM_205295	17,388,585 17,387,771	fw 5′-TGGGTGTAATGAGCACCTGA-3′ rv 5′-ACCAACAGCAAAGTGCACAG-3′	60	111
XM_420355	17,417,169 17,437,962	fw 5′-CCGTCTTCCCTTCACCTGTA-3′ rv 5′-ATGCAACCAGATGTCACCAA-3′	60	115
XM_420356	17,477,780 17,443,138	fw 5′-TCACATGTTGAATGGTGCAA-3′ rv 5′-AGTGCCCGATAGTCTCTGGA-3′	59	101
XM_420357	17,520,616 17,479,157	fw 5′-CTCAGCCGACTCTTTCCAG-3′ rv 5′-CTGCCGGGAACTGTAGTCCT-3′	58	106
XM_420358	17,566,453 17,532,690	fw 5′-ACTTTGGGCACCAGCATTAC-3′ rv 5′-CCGCAGTGGGATGTTAAAGT-3′	60	129
XM_420359	17,786,217 17,783,731	fw 5′-GAGGATGAGTCGTGGAGAGC-3′ rv 5′-AGCTGCGATAACTCCTCTGC-3′	59	111
XM_420360	17,795,219 17,787,180	fw 5′-GTTGTTTGCGTTCCATTGTG-3′ rv 5′-TGTTCAGCAATCACGTCCAT-3′	59	110
XM_420361	17,812,226 17,826,844	fw 5′-GGAGAACAGCACGGTGACTT-3′ rv 5′-GGCCATCAGGAAACAGAAGA-3′	60	142

[1]fw = forward; rv = reverse.

MATERIALS AND METHODS

Twenty expressed sequence tags (Table 1) were selected from the Gga4 region 16,137,294 to 17,826,844 (Gga4q11-12, MCW0114 surrounding area) in recently released draft genome sequence of the chicken (http://www.ncbi.nlm.nih.gov/genome//guide/chicken). Design of primers for PCR was done using the Primer Input 3 software (http://frodo.wi.mit.edu). Primer sequences are given in Table 1. Five birds with high ST53 (**STH**) and 5 birds with low ST53 (**STL**) from RIR and GLP were used for expression quantification. The ST53 means and SD were as follows: 378.4 ± 3.65 μm (RIR STH), 227.8 ± 8.99 μm (RIR STL), 372.4 ± 2.07 μm (GLP STH), and 248.6 ± 16.62 μm (GLP STL). The ST53 measurement was done on 3 consecutive eggs from each bird 5 d before isolation of oviducts. The tissue samples were isolated from the distal part of chicken oviducts (uterus) with forming eggshell at 53 wk of age. Fifty to 100 mg of tissue per bird was used to isolate total RNA using Trizol reagent (Sigma-Aldrich Corp., St Louis, MO) according to instructions of the manufacturer. First-strand cDNA for real-time-PCR of candidate genes was synthesized using Enhanced Avian Reverse Transcription PCR Kit (Sigma-Aldrich Corp.) according to the manufacturer's directions. The PCR amplification mix consisted of 2 μL of reverse transcription product, 10 μL of SYBR Green JumpStart Taq ReadyMix Capillary Formulation (Sigma-Aldrich Corp.), and 2 μL of 5 m*M* each primer in a final volume of 20 μL. The PCR reaction conditions consisted of an initial 5-min hold at 95°C, 40 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 15 s in a LightCycler (Roche, Basel, Switzerland). A melting curve analysis was performed from 65 to 95°C. The chicken *GAPDH* (glyceraldehyde-3-phosphate dehy-

Table 2. Expression profiling of candidate EST and correlation between shell thickness (ST) at 53 wk of age and expression level of positional candidates[1]

GenBank accession no.	Gene and homology	RIR		GLP	
		Ratio of means	Correlation of ST and EST expression	Ratio of means	Correlation of ST and EST expression
U10548	Chicken lysosome-associated membrane glycoprotein 2 (*LAMP-2*)	1.12	–0.04	0.80	0.26
CR390814	Chicken finished cDNA, clone ChEST974b18	1.31	–0.14	0.75	0.26
CR523443	Chicken finished cDNA, clone ChEST985k21	1.34	–0.25	0.49**	0.85**
NM_001031126	Chicken mitogen-activated protein kinase 4 (*MAP4K4*)	1.49	–0.18	0.85	0.28
XM_420350	Homology to hypothetical protein FLJ10178	1.10	–0.47	1.20	0.31
XM_420354	Homology to 2610030H06Rik protein	0.74	0.38	0.70	0.41
NM_205361	Chicken *Mel-1c* melatonin receptor	1.10	–0.13	1.42	–0.07
NM_205295	Chicken *HMG2a*	1.35	–0.40	1.39	–0.11
XM_420356	Homology to myotubularin-related protein 1 isoform 1	0.76	0.14	0.96	0.35
XM_420357	Homology to myotubularin	0.70	0.17	0.99	0.06
XM_420359	Homology to hypothetical protein LOC91966	1.34	0.36	1.24	–0.05
XM_420360	Homology to family with sequence similarity 11, member A (*FAM11A*)	1.30	–0.26	0.72	0.28

[1]RIR = Rhode Island Red; GLP = Green-legged Partridgenous; STL = low shell thickness; and STH = high shell thickness.
**$P \leq 0.01$.

drogenase) gene (accession no. NM_204305; GAPDHfw, CCTCTCTGGCAAAGTCCAAG; GAPDHrv, CATCTGCCCATTTGATGTTG) was used as a reference gene (Li et al., 2005). The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) for the calculation of the relative ratio was used. Differences between the mean of candidate expressed sequence tags quantities in oviducts of STH and STL groups were tested for by a 2-tailed *t*-test. All calculations were performed using Excel 2003 (Microsoft Corp., Seattle, WA).

RESULTS AND DISCUSSION

The conventional PCR with specificity for 20 target sequences primers (Table 1) using cDNA prepared from the oviduct tissue samples as templates resulted in getting 12 fragments of expected sizes. The mRNA expression of these 12 target sequences was measured by real-time quantitative reverse transcription PCR (Table 2). No significant difference in expression between STH and STL and no significant correlation of expression level with ST53 were detected in RIR (Table 2). Thus, genetic heterogeneity of factors affecting shell thickness is expected to be less in RIR than in the conserved GLP breed, which has been kept without selection for many generations. In GLP, the CR523443 was downregulated with ratio of means 0.49 ($P \leq 0.01$) in STL relative to STH (Table 2). Expression of this gene was significantly correlated (0.85, $P \leq 0.01$) with shell thickness (Table 2). These data suggested CR523443 is a potential candidate gene for QTL ST53 in chicken.

The 2,102 bp sequence CR523443 (*Gallus gallus* finished cDNA, clone ChEST985k21) primarily was found in mRNA extracted from adult muscle and then was detected in brain, cartilage, female genital, and head (http://www.ncbi.nlm.nih.gov/UniGene). A BLAST search did not show significant homology to other vertebrate sequences.

Relatively little is known about the genes that are involved in the formation of eggshell in birds. In the present study, real-time PCR was used to identify genes affecting this trait based on their position close to the microsatellite loci linked to the QTL. Positional approach was successfully applied for genetic dissection and searching for candidate genes for QTL in different species (Glazier et al., 2002). In livestock, well-approved candidate gene *DGAT1* (diacylglycerol O-acyltransferase 1) was found for milk fat content (Grisart et al., 2002, 2004; Furbass et al., 2006). Optimization of shell thickness has economical importance because it can help to reduce transportation losses. Finding the candidate gene for ST53 provides a tool for searching for QTL, which could be applied for MAS.

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