Developmental phenotypic-genotypic associations of tyrosinase and melanocortin 1 receptor genes with changing profiles in chicken plumage pigmentation

W. B. Liu,[1] S. R. Chen,[1] J. X. Zheng, L. J. Qu, G. Y. Xu, and N. Yang[2]

National Engineering Laboratory for Animal Breeding and Department of Animal Genetics and Breeding, China Agricultural University, Beijing 100193, China

ABSTRACT The tyrosinase (*TYR*) and melanocortin 1 receptor (*MC1R*) genes have been accepted as major genes involved in the plumage pigmentation of chickens. The co-segregation of plumage coloration and sequence polymorphism in *TYR* and *MC1R* genes were investigated using an intercross between black and white plumage color types of the Dongxiang blue-shelled chicken. Profiles of plumage color changing and genes expression levels of *TYR* and *MC1R* were observed from hatch to 112 d of age using quantitative real-time reverse transcription-PCR. Intercrossed offspring were classified by phenotypes of plumage colors. The phenotypes of black and amber chicks with genotypes of *E_C_* exhibited a black feather pattern, whereas white, gray, and buff chicks with genotypes of *E_cc* and *eecc* belonged to the white feather pattern. Although *TYR* in cooperation with *MC1R* determined the coloration feather patterns, the different phenotypes did not correspond completely with the genotypes. During the period studied, plumage phenotype changed dramatically, and the buff and gray down were gradually replaced by whiteness feathers. Real-time reverse transcription-PCR studies showed that 1) expression levels of *TYR* declined dramatically with age, and expression at hatch was highest ($P < 0.01$) during the ages studied; 2) expression level of *MC1R* was higher at 28 d than at younger and older ages; and 3) expression of *TYR* in chickens carrying *E*/*E* and *E*/*e* alleles on *MC1R* loci were higher than those carrying *e*/*e* alleles from hatch to 28 d.

Key words: chicken, plumage pigmentation, tyrosinase, melanocortin 1 receptor, gene expression

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INTRODUCTION

Color production in chickens takes advantage of pigmentation. Melanins are ubiquitous components of plumage coloration in birds and serve a wide variety of functions, such as sexual selection (Andersson, 1994), geographical differentiation and speciation, and the evolution of sexual dimorphisms (Dunn et al., 2001). The process of melanogenesis includes phases with many loci involved in the complex expression of plumage colors. One of the main loci in plumage coloration is extension (***E***), which controls the relative amounts of eumelanin and pheomelanin in the melanocyte. Molecular studies with several mammalian and avian species have shown that the *E* locus encodes the melanocortin 1 receptor (***MC1R***; Robbins et al., 1993; Jackson et al., 1994; Kerje et al., 2003a,b). The *MC1R* gene encodes the MC1R protein that is a 7-transmembrane domain G protein-coupled receptor expressed primarily in melanocytes (Lu et al., 1994; Cone et al., 1996; Ollmann et al., 1998). In birds, *MC1R* was first cloned from chickens (Takeuchi et al., 1996) and its activation leads to increased synthesis of black or brown eumelanin, whereas those of dysfunction are recessive and associated with a red-yellow phaeomelanin (Robbins et al., 1993).

The biosynthesis and the types of melanin depend on the activity of tyrosinase. Tyrosinase is the key enzyme in melanin biogenesis in pigment cells. The melanin synthesis pathway will be blocked if the enzymatic function of tyrosinase is abnormal, resulting in an albino phenotype. The chicken tyrosinase (***TYR***) gene has been cloned (Mochii et al., 1992), and several mutations at the C locus (Smyth et al., 1986) include the recessive white mutation that has the insertion of a complete avian retroviral sequence in intron 4 of the *TYR* gene, which becomes the diagnostic characteristic of the recessive white mutation (Chang et al., 2006).

In this study, we used an intercross between black and white plumage color types of the Dongxiang blue-shelled chicken. This breed is well known for its varia-

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[1]These authors contributed equally to the work.
[2]Corresponding author: nyang@cau.edu.cn

Table 1. The distribution of plumage color patterns of progeny produced by crossing white males with black females and black males with white females

	Down color pattern[1]					
Mating	White	Buff	Grey	Amber	Black	Total
Parent: white male × black female						
Male	7	28	10	30	30	105
Female	8	15	11	46	32	112
Total	15	43	21	76	62	217
Parent: black male × white female						
Male	2	2	2	20	68	94
Female	3	8	2	7	78	98
Total	5	10	4	27	146	192

[1]Number of chicks.

tion in plumage types and special eggshell pigmentation (Zhao et al., 2006; Wang et al., 2007). It has experienced consistent selection, with skin color ranging from white to gray and heavily pigmented shanks. The research presented here focused on the development of phenotypic-genotypic associations of *TYR* and *MC1R* genes in chicken plumage pigmentation using relative quantitative real-time reverse transcription-PCR (**RT-PCR**) gene expression measurement from hatch to 112 d of age.

MATERIALS AND METHODS

Chickens

An intercross population of 409 individuals was generated by mating 10 Dongxiang white males with 54 black females and 10 Dongxiang black males with 50 white females. Considering that skin pigmentations could affect *TYR* and *MC1R* gene expression independently of feather pigmentations, most parents were individuals with slightly pigmented skin, and so skin coloration effect was limited in this study. Five different phenotypic groups were classified by the types of down colors at hatch, including white, buff, gray, amber, and black (Table 1, Supplemental Figure 1; supplemental figure can be found online at http://ps.fass.org/content/vol89/issue6/). The developmental changing profiles of phenotypes from the 5 different patterns were observed at hatch and 28, 56, and 112 d of age (Supplemental Figure 1). A skin sample with an area of 2 cm^2, including both dermis and epidermis, was collected for RNA extraction from the back region after feathers were removed.

MC1R and TYR Genotyping

Sequence mutations were detected and analyzed by methods of PCR amplification and sequencing on both *TYR* (GenBank: DQ118701/DQ118702) and *MC1R* (GenBank: AY220303, AY220304, and AY220305). The co-segregation of plumage coloration and sequence polymorphism in *TYR* and *MC1R* genes were analyzed for all parents and 131 intercrosses taken at random from the 5 phenotypes. Random samples of 57 birds were used for gene expression studies. One pair of primers was designed for *MC1R* genotyping by using PCR-RFLP based on a restriction site for *Bal*I. Another pair of primers was designed for *TYR* genotyping (Chang et al., 2006; Table 2).

RNA Extraction and cDNA Synthesis

Total RNA was isolated from skin by standard chloroform extraction and dissolved in RNase-free water. Reverse transcription was performed using 250 ng of total RNA from the skin in a total volume of 20 μL, using 10 m*M* oligo T primer and murine leukemia virus reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions.

Relative Quantitative Real-Time RT-PCR

Specific primers used for real-time RT-PCR were designed using Primer Express software (Applied Biosystems, Foster City, CA) and are presented in Table 2. Amplification efficiencies of primers for β-actin, *TYR*, and *MC1R* were calculated, and then primers with similar efficiencies (0.96, 0.95, and 0.98 for primers of

Table 2. Primer sequences for melanocortin 1 receptor (*MC1R*) and tyrosinase (*TYR*) genotyping and real-time reverse transcription-PCR (RT-PCR)

Primer[1]	Sequence (5′ to 3′)
MC1R-g-F[2]	GCCATCCTCAAGAACAGGAA
MC1R-g-R[2]	GCAGATGAGCATGTCGATGA
TYR-CC-F[3]	CAAAACCATAAATAGCACTGGAAATAG
TYR-mL-F[3]	CCTCTGGCTCTATTTGACTACACAGT
TYR-R[3]	TTGAGATACTGGAGGTCTTTAGAAATG
MC1R-q-F[4]	GCCCTTCTTCTTCCACCTCAT
MC1R-q-R[4]	GCTCCGGAAGGCATAGATCA
TYR-q-F[4]	TGGTTTGCATAATGCCCTTCA
TYR-q-R[4]	AACCACCGCTCAAAAATGCT
β-actin-F	GAGAAATTGTGCGTGACATCA
β-actin-R	CCTGAACCTCTCATTGCCA

[1]F = forward; R = reverse.
[2]Primers for genotyping *MC1R*.
[3]Primers for genotyping *TYR*.
[4]Primers for real time RT-PCR.

TYR, *MC1R*, and β-actin, respectively) were chosen for further analysis. Polymerase chain reaction-amplified products of β-actin cDNA were cloned into pMD18-T vectors (Takara Bio Inc., Shiga, Japan) and transfected to DH5α *Escherichia coli* competent cells (Tiangen Biotech, Beijing, China) for amplification. Plasmids containing β-actin segments were extracted as standard samples. Each real-time PCR was run along with a 10-fold serial dilution of the standard sample (10^{-1} to 10^{-7}) that served as the calibrator, and a no-template control was included in each run. Abundances of target genes (*TYR* and *MC1R*) and reference gene (β-actin) were calculated by regressing against the standard curve. Expression levels of target genes are presented as relative abundance values normalized by β-actin.

The thermal cycling was conducted using the ABI Prism 7300 system (Applied Biosystems). The PCR reactions were carried out in a total volume of 15 μL containing 1 μL of cDNA template, 1 m*M* primers, and 1 × PCR mix (Power SYBR Green PCR Master Mix, Applied Biosystems), and amplification cycles were as follows: 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

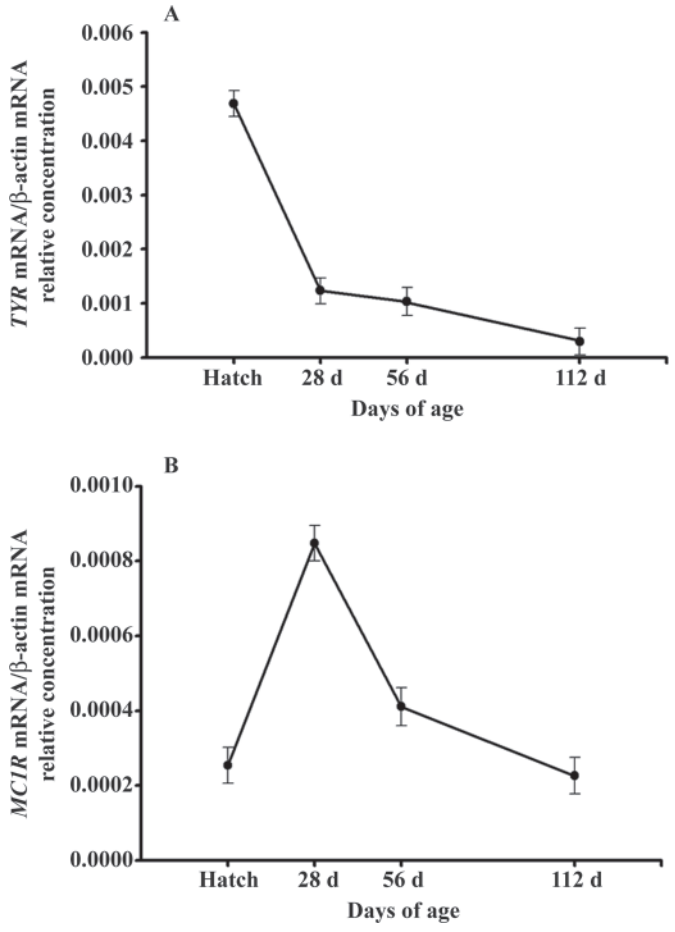

Figure 1. Expression of melanocortin 1 receptor (*MC1R*) and tyrosinase (*TYR*) at several ages. A) The changing profiles in the relative abundance of *TYR* mRNA from hatch to 112 d of age. B) The changing profiles in the relative abundance of *MC1R* mRNA from hatch to 112 d of age.

Statistical Analysis

The effects of age, sex, genotype, and their interactions on the gene expression were analyzed by multifactor ANOVA using the GLM procedure (SAS Institute, 2001). The following model was used for analysis initially:

$$Y = \mu + A + S + G + A \times S + A \times G + S \times G + A \times S \times G + e,$$

where Y was the relative abundance of target gene mRNA; A represented the effect of age; S represented the effect of sex; G represented the effect of genotype; A × S, A × G, S × G, and A × S × G were the effects of interaction between these variables; and e was residual error. It was found that the effects of sex ($P = 0.11$ to 0.17) and all of the interactions ($P = 0.48$ to 0.96) were not significant. Therefore, the final model we used for the following analysis was

$$Y = \mu + F + e,$$

where F served as the effect of age in associated analysis between gene expression and age, or as the effect of genotype in associated analysis between gene expression and genotype, and e was residual error.

RESULTS AND DISCUSSION

Phenotypic-Genotypic Associations of TYR and MC1R Genes

In the parental generation, sequence analysis of *MC1R* indicated that chickens with black plumage were homozygous or heterozygous carriers of *E* allele, whereas those with white plumage were *E*/*E*, *E*/*e*, or *e*/*e*. Parents with white plumage were homozygous carriers with an insertion of 7.7 kb in intron 4 of *TYR*, which was considered as recessive white, whereas those with colored plumage were heterozygous or homozygous carriers without this insertion. Furthermore, white parent chickens were genotyped at the dominant white locus in a previous study (data not shown) to check whether this major pigmentation inhibitor segregated in the population, and it was found that all white ones were recessive homozygous (*i*/*i*) at this locus.

The offspring were classified by phenotypes of down colors at hatch. As expected, there was no marked sexual dimorphism for plumage color. The phenotypes of the population with genotypes of *E*_*C*_ exhibited a black feather pattern, whereas those with genotypes of *E*_*cc* and *eecc* belonged to the white feather pattern (Table 3). The results showed that although *TYR* in cooperation with *MC1R* determined the plumage coloration patterns, different phenotypes did not correspond with different genotypic classes for both *TYR* and *MC1R*

Table 3. Plumage color and melanocortin 1 receptor (*MC1R*)-tyrosinase (*TYR*) genotype distributions in Dongxiang parent and offspring generations

	MC1R-*TYR* genotype[1]						
Phenotypic class	*EECC*	*EECc*	*EeCc*	*EEcc*	*Eecc*	*eecc*	Total
Parent: black male × white female							
White (female)	—	—	—	2	8	40	50
Black (male)	5	1	4	—	—	—	10
Parent: white male × black female							
White (male)	—	—	—	1	3	6	10
Black (female)	21	16	17	—	—	—	54
Offspring (at hatch)							
White	—	—	—	0	4	16	20
White with buff fluff	—	—	—	2	12	14	28
White with gray fluff	—	—	—	12	11	0	23
Amber	0	9	21	—	—	—	30
Black	0	18	12	—	—	—	30
Total number of offspring	0	27	33	14	27	30	131

[1]Number of chickens.

genes, respectively (Table 3). They also provided evidence that the recessive white variation of the *TYR* gene could not completely block melanin synthesis and extension before 28 d. Eda-Fujiwara et al. (2003) reported that estradiol-treated budgerigar females had a darker cere color than controls. Zi et al. (2003) found that coloration strategies in peacock feathers were controlled by the lattice constant and the number of periods in the photonic-crystal structure. Thus, some melanocortin loci or other genetic effects are related to the phenotypes of black, gray, buff, and earthy shades of down coloration. After 28 d, the colored down was gradually replaced by whiteness feather. Apparently these genetic effects functioned partially with age and feather replacement, and genetic variation on the *TYR* gene can inhibit the pathway of the production or expression of the pigments.

Expression Level Changing Profiles of TYR and MC1R Genes

The *TYR* and *MC1R* gene expression profiles at hatch and 28, 56, and 112 d of age were analyzed by the real-time RT-PCR. Expression levels of the *TYR* gene declined from hatch to 112 d, with the expression level at hatch the highest during the period studied ($P < 0.01$, Figure 1A). Tyrosinase is a key rate-limiting enzyme in the metabolic pathway of melanin synthesis and its expression is influenced by hormones and transcription factors, such as placental sphingolipid (Saha et al., 2006) and *Mitf* transcription (Murisier et al., 2007). Therefore, some genetic effects may change the *TYR* expression. Furthermore, the profile of *TYR* expression level was in accordance with the changing of phenotypes per feather replacement with age. That

Table 4. The expression level of melanocortin 1 receptor (*MC1R*) and tyrosinase (*TYR*) according to the genotypes of the *MC1R* gene[1]

MC1R genotype	*MC1R* expression level[2]	*TYR* expression level[2]	Sample size
Hatch			
EE	$(2.19 \pm 0.48) \times 10^{-4}$	$(4.89 \pm 0.64) \times 10^{-3,\ ab}$	5
Ee	$(2.58 \pm 0.39) \times 10^{-4}$	$(5.29 \pm 0.64) \times 10^{-3,\ a}$	5
ee	$(2.78 \pm 0.43) \times 10^{-4}$	$(3.30 \pm 0.64) \times 10^{-3,\ b}$	5
28 d			
EE	$(9.43 \pm 0.79) \times 10^{-4}$	$(1.48 \pm 0.13) \times 10^{-3,\ a}$	5
Ee	$(7.39 \pm 0.79) \times 10^{-4}$	$(1.42 \pm 0.13) \times 10^{-3,\ a}$	5
ee	$(8.61 \pm 0.79) \times 10^{-4}$	$(8.02 \pm 1.34) \times 10^{-4,\ b}$	5
56 d			
EE	$(2.24 \pm 1.16) \times 10^{-4,\ b}$	$(8.78 \pm 1.11) \times 10^{-4,\ b}$	5
Ee	$(4.13 \pm 1.16) \times 10^{-4,\ ab}$	$(1.32 \pm 0.11) \times 10^{-3,\ a}$	5
ee	$(7.21 \pm 1.50) \times 10^{-4,\ a}$	$(8.24 \pm 1.43) \times 10^{-4,\ b}$	3
112 d			
EE	$(2.57 \pm 0.27) \times 10^{-4}$	$(2.91 \pm 0.43) \times 10^{-4}$	5
Ee	$(1.81 \pm 0.27) \times 10^{-4}$	$(3.29 \pm 0.43) \times 10^{-4}$	5
ee	$(2.47 \pm 0.30) \times 10^{-4}$	$(2.46 \pm 0.48) \times 10^{-4}$	4

[a,b]Means in same column without a common superscript differ ($P < 0.05$).

[1]Values are mean ± SE.

[2]Expression level means the abundance of the target gene mRNA normalized by β-actin.

is, after 28 d, the phenotypes did not change dramatically and the gray and buff down faded gradually, being replaced by whiteness (Supplemental Figure 1). Saha et al. (2009) reported that restoring *Mitf* expression and subsequent activating of the *TYR* gene can lead to black hair growth and repigmentation in follicular melonocytes in age-onset gray-haired C57BL/6J mice, demonstrating that the expression of the *TYR* gene can influence hair color. From hatch to 28 d of age, the average relative abundance of *TYR* mRNA in offspring chickens with *E/E* and *E/e* alleles on *MC1R* was higher than those with the *e/e* allele (1.6- to 1.8-fold lower abundance values, Table 4). Ling et al. (2003) and Pape et al. (2008) reported that regardless of the presence of MC1R ligand, the *E* allele results in constitutive activation of MC1R in chicken and murine melanocytes, respectively, resulting in increased cyclic adenosine monophosphate and subsequent tyrosinase activation.

The expression level of *MC1R* at 28 d was higher than at hatch or 56 and 112 d (Figure 1B). Growth is rapid during this period, and skin areas and amounts of melanocytes increase at the same time. Therefore, as a key gene encoding the receptor on the membrane of melanocytes and regulating the synthesis of melanin in the skin and follicle, *MC1R* should be expressed at a high level during such a period of rapid growth and feather replacement.

In summary, the profiles of *TYR* and *MC1R* expression level were in accordance with the changing of phenotypes per feather replacement with age. Specific *MC1R* alleles affected the expression level of *TYR* in early age, but this interaction was diminishing along with declining expressions of these 2 genes after 28 d.

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