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Associations of *SLC45A2* variation and MC1R/cAMP-related molecular characteristics with the "cloudy feather" phenotype in Qiangshan Cloud chickens

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Abstract: Qiangshan Cloud chicken is a local chicken breed unique to Aba Prefecture, Sichuan Province, characterised by its distinctive "cloudy feather" phenotype, where black feathers form the base with attached cloud-like white feathers. This study aimed to evaluate genetic associations with this phenotype and to explore whether *MC1R* expression, cAMP concentration, melanogenesis-related transcript patterns, and qualitative histology were consistent with a multi-gene working model. The research was conducted in two phases. Phase I involved 220 adult chickens (110 Qiangshan Cloud chickens and 110 Fengwei chickens) for *SLC45A2* and *TYR* genotyping and reciprocal crossing experiments. Phase II comprised the same 220 birds plus 30 recessive white-feathered chickens for *MC1R* genotyping. For qPCR and cAMP ELISA, 24 biologically independent birds were selected from each breed; qPCR was performed in three technical wells per bird and ELISA in two technical wells per bird, with the technical-well mean used as one biological observation. Histology included three independent birds per breed and three microscopic fields per bird and was evaluated qualitatively. In Phase I, genotyping and crossbreeding segregation results supported an association between the *SLC45A2* c.1039C>A (Leu347Met) variant and the cloudy feather phenotype; 87 of 110 Qiangshan Cloud chickens (79.1%) carried an A-containing genotype, and the phenotypic segregation patterns in the crossbred offspring were consistent with sex-linked inheritance. The 7.7 kb insertion in *TYR* intron 4 was present in 20% of Qiangshan Cloud chickens, but no obvious feather-colour difference was detected between sampled cc and CC birds. The *MC1R* E allele frequency was 98.2% in Qiangshan Cloud chickens. *MC1R* expression and cAMP concentration were higher in Qiangshan Cloud chickens than in the comparison breeds ($P < 0.01$). *MC1R* and *TYR* expression were positively correlated in the Qiangshan Cloud wild-type subgroup ($n = 18$, $r = 0.656$, $P = 0.003\,1$), whereas the mutant subgroup showed a nonsignificant inverse trend ($n = 6$, $r = -0.710$, $P = 0.114$). Positive correlations were also observed in Fengwei chickens ($n = 24$, $r = 0.813$, $P = 1.35 \times 10^{-6}$) and recessive white-feathered chickens ($n = 24$, $r = 0.616$, $P = 0.001\,34$). Representative histological sections qualitatively showed more extensive brown-black staining in Qiangshan Cloud chickens; melanin content was not quantified. The data support *SLC45A2* c.1039C>A as a sex-linked candidate variant associated with cloud-like white-feather distribution. The high frequency of the *MC1R* E allele, higher *MC1R* expression and cAMP concentration, and the observed TYRP1 and DCT expression patterns are consistent with enhanced melanogenic signalling in Qiangshan Cloud chickens. However, direct negative regulation of *TYR*, functional compensation for the *TYR* insertion, and causal pathway relationships were not established and require functional validation. Because the comparisons involved genetically distinct breeds and *MC1R* was nearly fixed within Qiangshan Cloud chickens, the present design does not isolate an *MC1R* single-locus effect.

Keywords: cAMP signalling pathway; *MC1R*; multi-gene regulation; Qiangshan Cloud Chicken; *SLC45A2*; *TYR*

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Feather colour is an important breed identifier and economic trait in poultry, governed by precise regulation of multiple loci and their interaction networks (Huang et al. 2020; Mastrangelo et al. 2023). Qiangshan Cloud Chicken, a local breed developed through years of selection in Aba Prefecture, Sichuan Province, was approved as a national livestock genetic resource in 2023. As shown in Figure 1, this breed exhibits a black feather base with characteristic white feathers on the neck, chest, wings, and other areas, resembling floating clouds from a distance, hence the name "cloudy feather" (Meng et al. 2024; Li et al. 2025). Deciphering the genetic basis of this unique feather colour is crucial for breed conservation, purification, and marker-assisted breeding.

Known major genes influence chicken feather colour. *SLC45A2* encodes a membrane transporter, and variants such as Leu347Met have been associated with the silver feather allele (S) and lighter coloration (Xu et al. 2024). Tyrosinase (TYR) is required for melanin synthesis, and a 7.7 kb endogenous viral insertion in intron 4 has been associated with reduced TYR function and recessive white plumage (Zheng et al. 2020). The coexistence of the insertion genotype and a non-albino phenotype in some Qiangshan Cloud chickens raises the possibility of genetic-background or epistatic modification, but does not by itself demonstrate a compensatory mechanism (Cho et al. 2021).

Melanocortin 1 receptor (MC1R) is an important regulator of eumelanin synthesis. In established melanocyte systems, activation of MC1R can increase cAMP-PKA-MITF signaling and influence the transcription of melanogenesis-related genes, including *TYR*, *TYRP1*, and *DCT* (Liu-Smith and Meyskens 2016; Ghosh Roy et al. 2025). We therefore hypothesised that the *MC1R* genetic background and associated signaling measurements might contribute to the stable black-feather base of Qiangshan Cloud chickens despite the presence of the *TYR* insertion in some individuals. This hypothesis was evaluated as an exploratory model rather than a demonstrated regulatory pathway.

This study had two objectives: first, to evaluate associations of *SLC45A2* and *TYR* variants with the cloudy feather phenotype; and second, to characterise *MC1R* genotype, melanogenesis-related gene expression, cAMP concentration, and qualitative melanin staining to develop a testable multi-gene model. The study did not directly manipulate these genes or signaling pathways and therefore was not designed to establish gene-gene regulatory causality.

MATERIAL AND METHODS

Experimental animals and sampling

Samples were provided by Maoxian Jiuding Original Ecological Livestock and Poultry

Illustration of the Qiangshan Cloud chicken

(A) Qiangshan Cloud chicken (rooster) (B) Qiangshan Cloud chicken (hen)

Figure 1. Qiangshan Cloud chicken
(A) Rooster; (B) Hen

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Breeding Co., Ltd. A total of 250 adult chickens were used in stages:

- Phase I (gene association analysis): 220 chickens, including 110 Qiangshan Cloud chickens (10 males and 100 females) and 110 Fengwei chickens (10 males and 100 females), for *SLC45A2* and *TYR* genotyping and crossbreeding trials.
- Phase II (expression and pathway-associated measurements): the full cohort comprised 250 chickens, including the same 110 Qiangshan Cloud chickens and 110 Heishui Fengwei chickens described in Phase I and 30 recessive white-feathered chickens (10 males and 20 females; *TYR* cc genotype), for *MC1R* genotyping. For qPCR and cAMP ELISA, 24 biologically independent birds were selected per breed (Qiangshan Cloud: 6 cc and 18 CC; Fengwei: 2 cc and 22 CC; recessive white-feathered: 24 cc). For histology, three biologically independent birds were selected per breed.

Wing-vein blood was collected for DNA extraction. For the qPCR and ELISA subsets, feather follicle tissues from neonatal feather regions on the neck were collected, flash-frozen in liquid nitrogen, and stored at −80 °C until RNA or protein extraction. Separate feather follicle specimens from the histology subset were fixed in formalin and processed for paraffin embedding.

Primer design and PCR amplification

Detection of SLC45A2 gene variant sites. Based on the chicken whole-genome sequence published by NCBI and reference (He et al. 2019), primers for the fourth exon of this gene (primer information see Table 1) were designed using Premier v5.0 to amplify the fourth exon. The primers were synthesised by the Chengdu Qingke Experimental Technology Department. The PCR reaction system was 30 µl : 27 µl of 1× TSE101 Mix, 0.5 µl of DNA template, 0.4 µl each of the forward and reverse primers, and ddH_2O to a final volume of 30 µl. The amplification program was as follows: initial denaturation at 98 °C for 3 min; followed by 37 cycles of denaturation at 98 °C for 10 s, annealing at the specific primer Tm for 15 s, and extension at 72 °C for 15 s; with a final extension at 72 °C for 10 minutes. The PCR amplification products were detected by 1.0% agarose gel electrophoresis, and the imaging results were photographed and saved. Sequencing results were read using SnapGene to screen for variant sites in the exons of the *SLC45A2* gene, and genotyping for the c.1039C>A (Leu347Met) mutation site in the *SLC45A2* gene of the male and female Qiangshan Cloud chicken population was performed.

Detection of TYR gene viral insertion/deletion mutation. Based on the chicken whole-genome sequence published by NCBI and reference (Chang

Table 1. Sequences of PCR primers used in the study

Gene	Primer name	Primer sequence (5'→3')	Product size (bp)	Annealing temperature
SLC45A2	SLC45A2-F	ATGTTTAGCACCCAACCAAC	331	52 °C
	SLC45A2-R	TATGAGGAAAGAAAGGCAAG		
TYR	TYR-F	CCTCTGGCTCTATTTGACTACACAGT	500/350	58 °C
	TYR-R1	CAAAACCATAAATAGCACTGGAAATAG		
	TYR-R2	TAGATACTGAGGTCTTTAGAAATG		
MC1R	MC1R-F	CATCCCCTCTGCCTCGTGAC	550	59 °C
	MC1R-R	GCGTTGTTGCGGTAGTAGGT		
TYRP1	TYRP1-F	AAGTTCATAGGCAGGTCAG	160	58 °C
	TYRP1-R	TCAGTGAGAGAGGCTGGTT		
DCT	DCT-F	AGCAGAGGAGACAGTGGACT	180	58 °C
	DCT-R	GCATCTGTGGAGGGTTGTT		
β-actin	β-actin-F	GAGAAATTGTGCGTGACATCA	138	60 °C
	β-actin-R	CCTGAACCTCTCATTGCCA		

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et al. 2006), primers for this gene were designed and used for amplification (primer information see Table 1) using Premier v5.0. The primers were synthesised by the Chengdu Qingke Experimental Technology Department. DNA samples from Qiangshan Cloud chickens and Fengwei chickens were used as templates, and the viral insertion in intron 4 of the *TYR* gene was detected using PCR technology. The retroviral insertion in intron 4 of the *TYR* gene was detected using a three-primer PCR genotyping assay adapted from Cho et al. (2021).

The PCR reaction system was 30 µl : 27 µl of 1× TSE101 Mix, 0.5 µl of DNA template, 0.4 µl each of the forward and reverse primers, and ddH_2O to a final volume of 30 µl. The amplification program was as follows: initial denaturation at 98 °C for 3 min; followed by 37 cycles of denaturation at 98 °C for 10 s, annealing at the specific primer Tm for 15 s, and extension at 72 °C for 15 s; with a final extension at 72 °C for 10 minutes. The PCR amplification products were detected by 1.0% agarose gel electrophoresis, and the imaging results were photographed and saved.

Detection of MC1R gene variant sites. Based on the chicken whole-genome sequence published by NCBI and reference (Xu et al. 2022), primers for the fourth exon of this gene (primer information see Table 1) were designed using Premier v5.0 to amplify the fourth exon. The primers were synthesised by the Chengdu Qingke Experimental Technology Department.

The PCR reaction system was 30 µl : 27 µl of 1× TSE101 Mix, 0.5 µl of DNA template, 0.4 µl each of the forward and reverse primers, and ddH_2O to a final volume of 30 µl. The amplification program was as follows: initial denaturation at 98 °C for 3 min; followed by 37 cycles of denaturation at 98 °C for 12 s, annealing at the specific primer Tm for 15 s, and extension at 72 °C for 15 s; with a final extension at 72 °C for 10 minutes.

The PCR amplification products were detected by 1.0% agarose gel electrophoresis, and the imaging results were photographed and saved. Sequencing results were read using SnapGene to screen for variant sites in the exons of the *MC1R* gene, and genotyping for the A>C mutation at position 926 (resulting in the amino acid substitution Glu926Ala) in the *MC1R* gene of the male and female Qiangshan Cloud chicken population was performed.

Reciprocal crossbreeding experiments

Based on the genotyping results of the *SLC45A2* gene, three crossbreeding schemes were designed and implemented (for specifics, refer to the Results and Analysis section). The segregation ratio of feather colour in the F1 generation chicks was statistically analysed to validate the genetic relationship between the *SLC45A2* mutation and the cloudy feather phenotype.

The crossbreeding populations were housed separately in standardised poultry houses and provided with consistent feed and water. Natural mating was employed, with one rooster and ten hens constituting a single mating group. All fertilised eggs were collected and incubated under identical conditions (temperature 37.8 °C, relative humidity 55–60%). After hatching, the primary feather colour phenotypes (cloudy feather, speckled feather, or solid black feather) of the F1 generation chicks were independently recorded by two experienced researchers.

Quantitative Real-Time PCR (qPCR)

RNA extraction and quality assessment. Total RNA was extracted from approximately 30 mg of liquid nitrogen-ground feather follicle tissue using TRIzol™ Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), with an A260/A280 ratio between 1.8 and 2.0 considered acceptable. RNA integrity was verified by 1.2% agarose gel electrophoresis, observing clear 28S and 18S rRNA bands.

cDNA synthesis. cDNA was synthesised following the removal of genomic DNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan). The reaction system had a total volume of 10 µl, containing 500 ng of total RNA. The reaction procedure was as follows: 42 °C for 2 min (genomic DNA removal), 37 °C for 15 min (reverse transcription), and 85 °C for 5 s (enzyme inactivation). The synthesised cDNA was diluted with sterile nuclease-free water and stored at –20 °C.

qPCR reaction. The qPCR reactions were performed on a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific). The 20 µl reaction mixture consisted of: 10 µl of 2× SYBR Premix Ex Taq™ II (Takara), 0.8 µl each of the forward and reverse primers (10 µM each; primer sequences

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listed in Table 1), 2 µl of diluted cDNA template (equivalent to 50 ng of total RNA), and 6.4 µl of nuclease-free water. Twenty-four biologically independent birds were analysed per breed. The Qiangshan Cloud group included 6 *TYR* insertion-mutant birds (cc) and 18 wild-type birds (CC), the Fengwei group included 2 cc and 22 CC birds, and the recessive white-feathered group included 24 cc birds. Each bird was analysed in three technical wells, and the mean of the three wells was used as one biological observation. A no-template control was included in each run. The amplification program was as follows: initial denaturation at 95 °C for 30 s; followed by 40 cycles of denaturation at 95 °C for 5 s, and combined annealing/extension at 60 °C for 30 s, with fluorescence signal acquisition at this stage. Following the amplification cycles, a melt curve analysis was performed (95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s) to confirm the specificity of the amplification products.

Data analysis. The relative expression levels of the target genes (*MC1R*, *TYR*, *TYRP1*, *DCT*) were calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$) (Kumari et al. 2023). The *β-actin* gene was used as the internal reference gene for normalisation.

cAMP concentration detection

Tissue sample preparation. Approximately 20 mg of fresh or –80 °C stored feather follicle tissue was accurately weighed and placed in a pre-cooled glass homogeniser. Then, 200 µl of pre-cooled 0.1 M HCl tissue lysis buffer (provided with the kit) was added, and the tissue was thoroughly ground on ice until completely homogenised.

Sample processing. The homogenate was transferred to a 1.5 ml centrifuge tube and centrifuged at 4 °C and 12 000 × *g* for 15 minutes. The supernatant was carefully aspirated for subsequent detection. Simultaneously, an aliquot of the supernatant was taken to determine the total protein concentration using a BCA protein assay kit (Pierce, USA) for standardising the cAMP concentration to unit protein content.

cAMP ELISA detection. Feather follicle samples from 24 biologically independent birds per breed were analysed for cyclic adenosine monophosphate (cAMP). Each biological sample was assayed in two technical ELISA wells, and the mean of the duplicate wells was used as one biological observation. Detection was performed according to the manufacturer's instructions for the chicken cAMP Enzyme-Linked Immunosorbent Assay Kit (Jiangsu Jingmei, P.R. China), as follows:

- Standard solutions, prepared samples, and biotinylated cAMP were sequentially added to the antibody-coated microplate wells. The plate was then incubated at room temperature for 2 hours. Following incubation, the plate was washed five times thoroughly with the provided wash buffer to remove unbound substances.
- Subsequently, horseradish peroxidase (HRP)-conjugated streptavidin was added to each well, and the plate was incubated at room temperature for 30 minutes. After this incubation, another series of five washes was performed.
- The chromogenic substrate, 3,3',5,5'-Tetramethylbenzidine (TMB), was then added to the wells, and the plate was incubated at room temperature for 15 min, protected from light to allow for colour development. The enzymatic reaction was stopped by adding the stop solution. The optical density (OD value) of each well was immediately measured at a wavelength of 450 nm using a microplate reader (BioTek, USA).

Concentration calculation. A standard curve was constructed by plotting the known concentrations of the cAMP standards against their corresponding mean OD values, typically using a four-parameter logistic (4PL) or linear regression curve fit. The OD value obtained for each sample was then interpolated from this standard curve equation to calculate the raw cAMP concentration in the sample. Finally, the raw cAMP concentration was normalised to the total protein content of the sample, and the results are expressed as picomoles of cAMP per milligram of protein (pmol/mg prot).

Histological analysis – Masson-Fontana melanin staining

Feather follicle tissues were fixed in 10% formalin, subsequently embedded in paraffin, and sectioned to a thickness of 5 µm. Following deparaffinisation, the sections were stained according to the protocol provided with the Masson-Fontana ammoniacal silver staining kit (Solarbio, P.R. China). After staining, the sections were mounted with neutral balsam and observed under an optical microscope for imaging.

Tissue fixation. Feather follicle tissue blocks were fixed in 10% neutral buffered formalin at 4 °C for 24–48 hours. Subsequently, the tissues were

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dehydrated through a graded series of ethanol solutions (70%, 80%, 95%, and 100%), cleared in xylene, and finally infiltrated and embedded in molten paraffin wax.

Sectioning and mounting. The paraffin-embedded tissue blocks were continuously sectioned at a thickness of 5 μm using a Leica RM2235 rotary microtome. The resulting ribbons were floated in a 40 °C water bath to flatten the sections, which were then mounted onto poly-lysine-coated glass slides. The mounted sections were dried in a 60 °C oven for 2 hours.

Masson-Fontana staining. Staining was performed strictly following the instructions of the Masson-Fontana Melanin Staining Kit (Solarbio, P.R. China). The detailed procedure was as follows:

- Deparaffinisation and hydration: Sections were deparaffinised in xylene I and II, 10 min each, and then rehydrated through a descending graded ethanol series (100%, 95%, 80%, 70%) to distilled water.
- Ammoniacal silver solution impregnation: The sections were immersed in a pre-warmed (56 °C) Fontana ammoniacal silver working solution and stained in the dark for 30–60 min, until the tissue sections turned brownish-black.
- Rinsing: The sections were thoroughly rinsed with distilled water.
- Toning and fixation: Sections were immersed in a 0.2% gold chloride aqueous solution for 1 min for toning, followed by a water rinse. Subsequently, they were immersed in a 5% sodium thiosulfate solution for 2 min for fixation, followed by another water rinse.
- Counterstaining: Cell nuclei were counterstained with Nuclear Fast Red or Neutral Red for 1–2 min, followed by a water rinse.
- Dehydration, clearing, and mounting: The sections were progressively dehydrated through a graded ethanol series, cleared in xylene, and finally mounted with a coverslip using neutral balsam.

Image acquisition and analysis. Stained sections were observed and imaged using an optical microscope (Nikon Eclipse E100) at 200× and 400× magnification. Three biologically independent birds were examined per breed, and three non-overlapping microscopic fields were observed per bird. Brown-black to black deposits were recorded as melanin-positive staining. The fields were used only for qualitative description of the apparent distribution and staining intensity and were not treated as independent biological replicates. No histomorphometric measurement or biochemical quantification of melanin content was performed.

Statistical analysis

Statistical analyses were performed using SPSS v26.0, and figures were prepared using BioRender and Origin. Genotype frequencies were compared using the chi-square (χ^2) test. Gene-expression and cAMP data are presented as mean ± standard deviation.

For qPCR and ELISA, technical-well values were averaged within each bird before analysis, and the bird was treated as the biological unit. Multiple-group comparisons used one-way analysis of variance followed by the Least Significant Difference post-hoc test when the overall test was significant. Associations between *MC1R* and *TYR* expression were assessed at the bird level using Pearson correlation coefficients with two-sided *P*-values. $P < 0.05$ was considered statistically significant.

Histological fields were evaluated qualitatively and were not included in inferential tests of melanin quantity. Because the study compared established breeds rather than an isogenic or segregating population, breed-group differences were interpreted as composite background-associated differences and not as isolated *MC1R* locus effects.

RESULTS AND ANALYSIS

Polymorphism of the *SLC45A2* gene and its association with feather colour. The mutation site in the fourth exon of the *SLC45A2* gene was detected in the experimental populations of Cloud chickens and Fengwei chickens. This mutation was present only in the Cloud chickens, as illustrated in Figure 2.

As shown clearly in Figure 3, the *SLC45A2* c.1039C>A variant was not detected in the Fengwei chickens, which had the wild-type CC (males) or CW (females) genotypes and characteristic speckled plumage. Among Qiangshan Cloud chickens, all 10 roosters had the AA genotype. Of the 100 hens examined, 77 had the AW genotype and 23 had the CW genotype. Thus, 87 of 110 Qiangshan Cloud chickens (79.1%) carried an A-containing genotype

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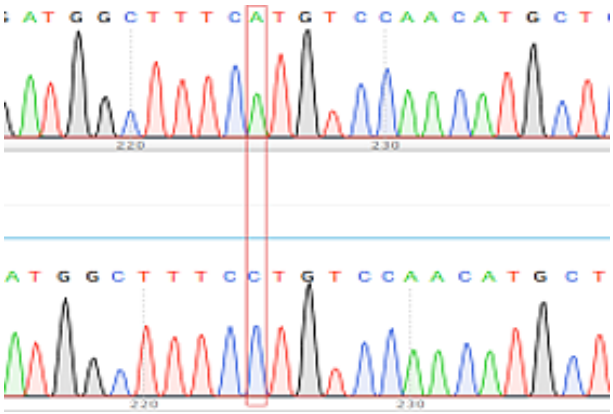

Figure 2. C→A mutation at the fourth locus of the *SLC45A2* gene

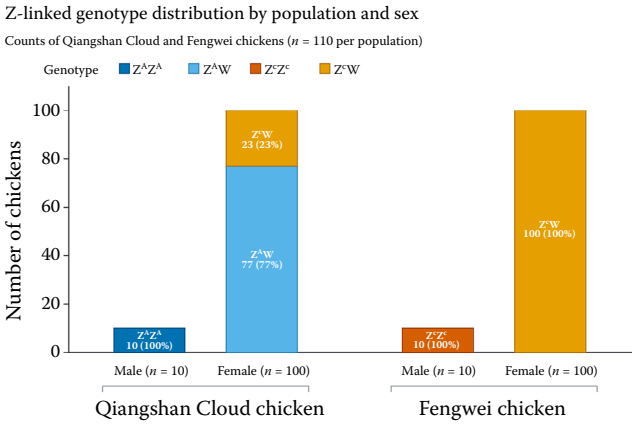

Figure 3. Distribution of the mutation at the fourth locus of the *SLC45A2* gene in Cloud chickens and Fengwei chickens

Bars show observed counts; percentages are calculated within each sex-by-population group

associated with the cloudy feather phenotype in this population.

Based on the genotyping results of the *SLC45A2* gene, three crossbreeding schemes were designed and implemented as follows:

- Scheme 1: 5 Qiangshan Cloud roosters (genotype Z^AZ^A) were crossed with 50 Fengwei hens (genotype Z^cW).
- Scheme 2: 5 Fengwei roosters (genotype Z^cZ^c) were crossed with 50 Qiangshan Cloud hens (genotype Z^AW).
- Scheme 3: 2 Fengwei roosters (genotype Z^cZ^c) were crossed with 20 Qiangshan Cloud hens (genotype Z^cW).

For each scheme, 50 F1 offspring were randomly selected. It was predicted that for Scheme 1, male offspring would exhibit the mottled feather phenotype, and female offspring would exhibit the cloud feather phenotype; for Scheme 2, all offspring would be mottled feather; and for Scheme 3, all offspring would be mottled feather, with no cloud feather individuals. The observed F1 phenotypes were compared to the predicted genotypes to check for consistency, as illustrated in Figure 4.

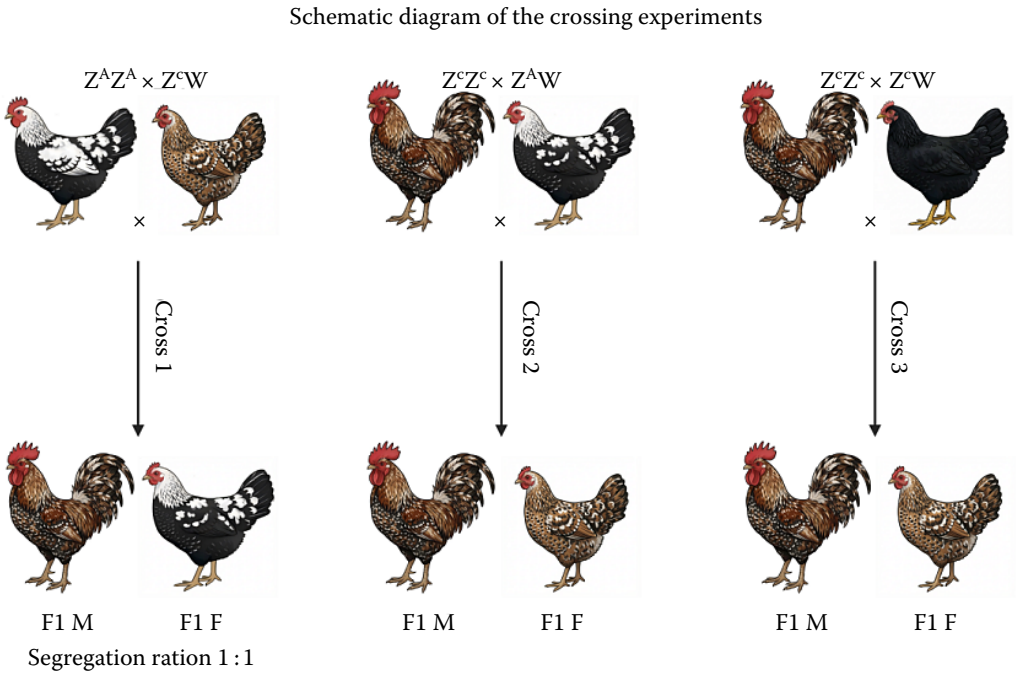

Figure 4. Schematic diagram of phenotype predictions for the three experimental schemes

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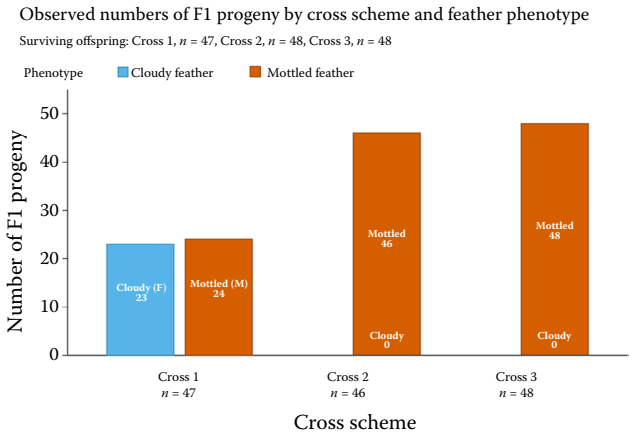

Figure 5. Statistics of phenotypic traits and genotypes in the F1 generation from crossbreeding verification
Totals below 50 reflect mortality during the experiment
F = female; M = male

As shown in Figure 5, the observed phenotypes were consistent with the crossing predictions: in Scheme 1, the observed counts of cloudy-feathered and speckled-feathered offspring were 23 and 24, respectively, and did not differ significantly from those expected under a 1 : 1 segregation ratio (χ^2 = 0.021 3, df = 1, P = 0.884); all offspring from Schemes 2 and 3 exhibited speckled feathering. These segregation results support an association between *SLC45A2* c.1039C>A and the cloudy feather phenotype and are consistent with sex-linked inheritance, but they do not by themselves establish that this single variant is sufficient to cause the complete phenotype.

Detection and distribution of the *TYR* recessive white locus insertion mutation. The results of PCR amplification using primer pairs Tu1 with Td and Tu2 with Td for samples 1 to 12 and samples 13 to 24, respectively, are shown in Figure 6.

Analysis of the electrophoretograms revealed a distinct pattern of band sizes corresponding to different genotypes. In the lanes containing Qiangshan Cloud chicken samples, only samples 2, 3, 8, 9, 19 and 22 exhibited a 350 bp band, while all other samples showed a 500 bp band. Furthermore, all individuals tested were homozygous for their respective alleles. Similarly, in the Fengwei chicken lanes, only samples 9 and 20 displayed the 350 bp band, with the remaining samples showing the 500 bp band, and all individuals were also homozygous.

As shown in Figure 7, the overall mutation rate in the Qiangshan Cloud chicken population was 20%. The mutant individuals exhibited the cc genotype, while the wild-type individuals accounted for 80% of the population, all of which were homozygous for the CC wild-type genotype. In the Fengwei chicken population, the overall mutation rate was 6.4%. Due to the small number of mutant individuals overall, it was not possible to determine whether the mutation exhibited any sex-specific differences in distribution.

***MC1R* genotype distribution.** The *MC1R* A>C variant at position 926 (Glu926Ala) was used to characterise the dominant black-feather E locus. In Qiangshan Cloud chickens, 106 of 110 birds had the EE genotype and four had the Ee genotype; the E allele frequency was 0.982. This frequency was higher than that in Fengwei chickens (E allele frequency, 0.250) and recessive white-feathered chickens (E allele frequency, 0) (χ^2 = 239.16, P < 0.01) (Table 2), consistent with near fixation of the E allele in the sampled Qiangshan Cloud population.

Expression of melanogenesis-related genes and cAMP pathway activity. Bird-level qPCR analysis included 24 chickens per breed, with the three technical wells averaged for each bird (Figure 8).

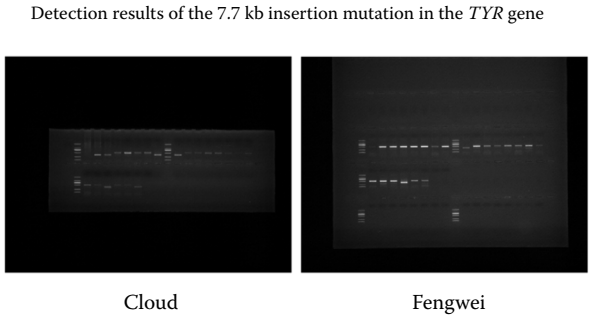

Figure 6. Detection results of the 7.7 kb insertion mutation in the *TYR* gene

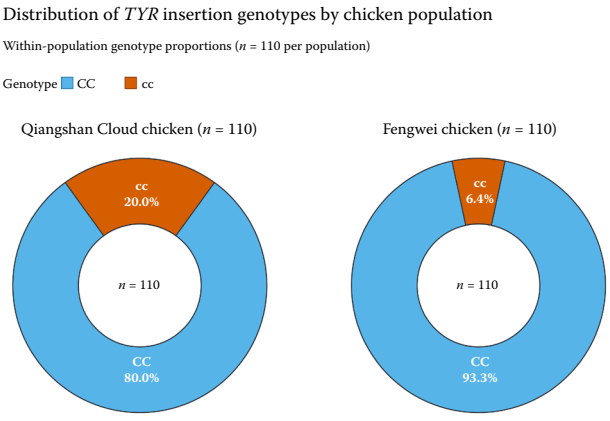

Figure 7. Distribution of *TYR* gene insertion mutations in Cloud chickens and Fengwei chickens

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Table 2. Genotype and allele frequencies of the *MC1R* gene (E locus) in different chicken populations

Population	*n*	EE	Ee	ee	E allele frequency
Qiangshan Cloud chicken	110	106	4	0	0.982
Fengwei chicken	110	7	41	62	0.250
Recessive white-feathered chicken	30	0	0	30	0.000

The Qiangshan Cloud group was analysed as 6 *TYR* insertion-mutant birds (cc) and 18 wild-type birds (CC). *MC1R* expression did not differ significantly between the two Qiangshan Cloud genotype subgroups, but was higher than in Fengwei and recessive white-feathered chickens. In the mutant Qiangshan Cloud subgroup, *TYR* expression was 0.42 ± 0.11, whereas *TYRP1* (1.98 ± 0.35) and *DCT* (2.11 ± 0.40) expression was higher than in the two comparison breeds ($P < 0.05$). Pearson analysis at the bird level showed a positive association between *MC1R* and *TYR* expression in wild-type Qiangshan Cloud chickens ($n = 18$, $r = 0.656$, $P = 0.003\ 1$) and a non-significant inverse trend in the mutant subgroup ($n = 6$, $r = -0.710$, $P = 0.114$). Positive associations were observed in the pooled Fengwei group ($n = 24$, $r = 0.813$, $P = 1.35 \times 10^{-6}$) and the recessive white-feathered group ($n = 24$, $r = 0.616$, $P = 0.001\ 34$) (Figure 9). The inverse trend in the small mutant subgroup was exploratory and does not provide evidence of direct negative regulation.

The ELISA analysis included 24 biologically independent birds per breed, with two technical wells averaged for each bird. The cAMP concentration in Qiangshan Cloud feather follicles (15.23 ± 2.11 pmol/mg protein) was higher than that in Fengwei chickens (8.45 ± 1.67 pmol/mg protein) and recessive white-feathered chickens (7.98 ± 1.54 pmol/mg protein) ($P < 0.01$) (Figure 10). This pattern is consistent with greater cAMP-associated signalling in Qiangshan Cloud chickens, but cAMP concentration alone does not demonstrate consti-

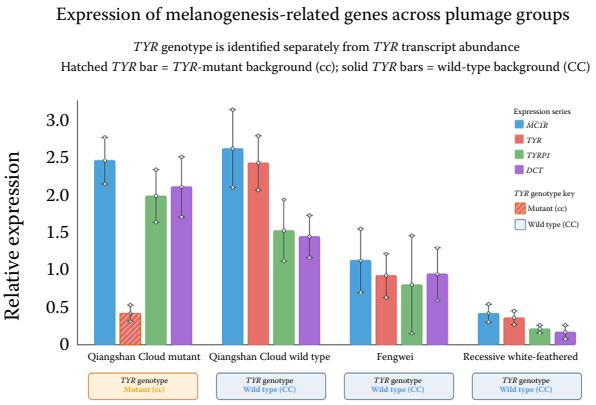

Figure 8. Bar chart of relative expression levels of melanogenesis-related genes in different chicken breeds

Twenty-four biologically independent birds were examined per breed. The Qiangshan Cloud group comprised 6 birds with the homozygous *TYR* insertion genotype (cc) and 18 wild-type birds (CC), which are displayed separately according to *TYR* genotype. The Fengwei and recessive white-feathered groups each comprised 24 independent birds. For each bird, qPCR was performed in three technical wells, and the mean of the technical replicates was used as one biological observation. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Bars represent the mean ± SD. Bar colours indicate the measured transcript, whereas the hatched *TYR* bar identifies the *TYR* insertion-mutant background

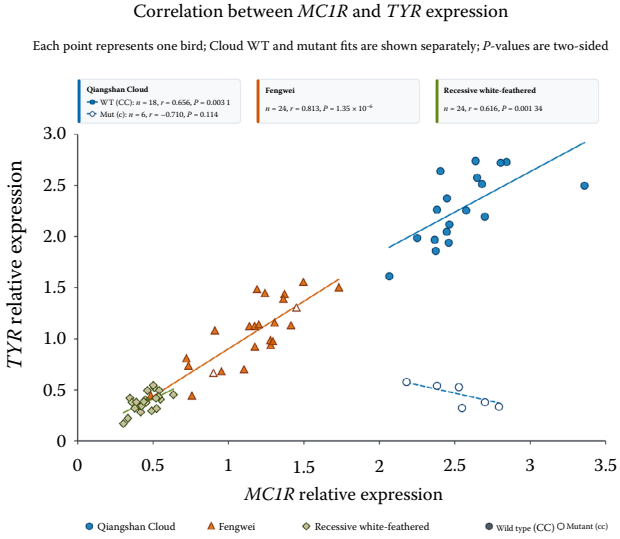

Figure 9. Correlation analysis between *MC1R* and *TYR* expression

Each point represents one independent bird. Qiangshan Cloud chickens are indicated by blue circles, Fengwei chickens by orange triangles, and recessive white-feathered chickens by olive diamonds. For Qiangshan Cloud and Fengwei chickens, filled and open symbols represent the wild-type (CC) and mutant (cc) *TYR* genotypes, respectively. Solid lines indicate least-squares regression fits, whereas the dashed blue line represents the fit for the Qiangshan Cloud mutant subgroup. Pearson's *r* was calculated using bird-level values, and all *P*-values are two-sided

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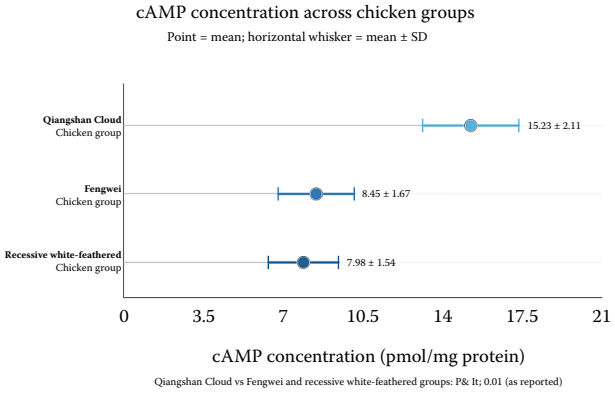

Figure 10. Comparison of cAMP concentrations

Twenty-four biologically independent birds were examined per breed. Each biological sample was measured in two technical ELISA wells, and the mean of the duplicate wells was used as one biological observation. Points represent group means, and horizontal whiskers indicate SD. cAMP concentrations are expressed as pmol/mg protein. Differences among breeds were evaluated using one-way ANOVA followed by the specified post-hoc comparisons

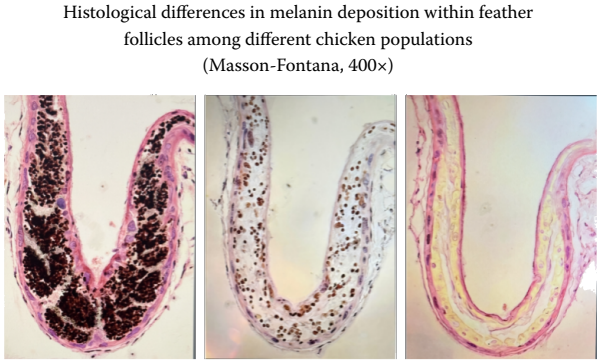

Figure 11. Histological differences in melanin deposition within feather follicles among different chicken populations

Representative sections are shown for (A) Qiangshan Cloud chickens, (B) Fengwei chickens, and (C) recessive white-feathered chickens. Three biologically independent birds were examined per breed, and three microscopic fields were observed per bird. The microscopic fields were used for qualitative assessment and were not treated as independent biological replicates. Brown-black staining indicates melanin deposition. Images were acquired at 400× magnification. No histomorphometric or biochemical quantification of melanin content was performed

tutive MC1R pathway activation or a causal effect on downstream genes.

Histological observation of melanin deposition. Representative Masson-Fontana-stained sections are shown in Figure 11. Within the examined fields, brown-black staining appeared more extensive in feather follicles from both wild-type and mutant Qiangshan Cloud chickens, intermediate in Fengwei chickens, and sparse in recessive white-feathered chickens. This comparison was descriptive: three biologically independent birds were examined per breed and three fields were observed per bird, without histomorphometric or biochemical quantification. Therefore, the images illustrate staining patterns but do not support a statistical ranking of melanin quantity among breeds.

DISCUSSION

Association of the missense mutation site in exon 4 of the *SLC45A2* gene with the cloudy feather phenotype. Feather colour, as an important phenotypic trait, can serve as a genetic marker in genetic breeding research (Mastrangelo et al. 2020; Yuan et al. 2023). Different breeds exhibit corresponding morphological characteristics, and the naming of some breeds is directly related to their feather colour, such as the Qiangshan Cloud chicken, whose name is derived from its unique appearance. Furthermore, by utilising molecular markers for feather colour genes, the separation of feather colour traits can be conducted more efficiently during selective breeding, which is beneficial for the genetic purification of local chicken breeds, thereby achieving better economic benefits. Sex identification in poultry based on the principle of sex-linked inheritance through feather colour separation can enhance production efficiency (Yang et al. 2025).

SNP genotyping of *SLC45A2* exon 4 showed that all 10 Qiangshan Cloud roosters had the AA genotype, whereas 77 of 100 hens had the AW genotype and 23 had the CW genotype. Reciprocal crosses with Fengwei chickens produced segregation patterns that support an association of c.1039C>A with the cloudy feather phenotype and are consistent with a sex-linked locus. Because the study did not include functional manipulation or fine mapping that excluded linked variants, the present data do not establish c.1039C>A as the sole causal determinant of the complete plumage pattern.

Li et al. (2024) also found the silver feather mutation site c.1039C>A in black-white feathered indi-

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viduals. Furthermore, by crossing Taihang chickens (speckled feather) with white-feathered chickens and analysing the feather colour segregation in the F1 generation, they observed differences in the segregation ratios from reciprocal crosses between black-white and speckled feather types, which also confirmed the association of the black-white feather trait with the sex chromosome. Liu et al. (2023) investigated the correlation between mutations at different sites of the *SLC45A2* gene and the silver feather trait in White Leghorn chickens. They found that both the AG point mutation (Tyr277Cys) in the third exon and the CA point mutation (Leu347Met) in the fourth exon coexisted in the White Leghorn strain. Their study concluded that the third exon mutation was not associated with the silver feather trait, whereas the fourth exon mutation was associated with it. He et al. (2019) discovered that a single nucleotide polymorphism in the coding region (site C.1154) of the *SLC45A2* gene was the causal mutation for the white-speckled feather trait in Wenchang chickens. Genetic analysis showed complete linkage between this site and the phenotype, but the CA point mutation (Leu347Met) was absent in Wenchang chickens.

Synthesising existing research, mutations in the *SLC45A2* gene primarily affect pheomelanin synthesis and do not impact eumelanin synthesis. This variation is sex-linked. Both male and female Qiangshan Cloud chickens exhibit the cloudy feather trait with consistent distribution. The absence of heterozygous Z^AZ^C variants in the roosters is analysed to be due to the incomplete dominant inheritance pattern of the cloudy feather trait in Qiangshan Cloud chickens. The mutant allele A is incompletely dominant over the wild-type allele C. In heterozygous Z^AZ^C individuals, the cloudy feather trait appears but is mixed with other feather colour characteristics, or the areas exhibiting white feathers are randomly reduced, failing to meet the breed standard. Consequently, such individuals were actively eliminated during the selection process, and only homozygous individuals were retained. During the breeding process, a very small number of Z^CW individuals, which do not exhibit the cloudy feather phenotype but instead show a solid black feather colour, were also observed. In subsequent conservation and breeding efforts, these phenotypic individuals should be culled as much as possible to purify the target trait and breed homozygous cloudy feather traits, laying a solid foundation for future commercial production.

Impact of *TYR* gene mutation on the feather colour phenotype of Qiangshan Cloud Chickens. The 7.7 kb endogenous viral mutation in intron 4 of the *TYR* gene functions by inhibiting the gene's transcription and expression, leading to a decrease in tyrosinase enzyme activity. The loss of this enzymatic activity mediates the occurrence of albinism, resulting in a lightening of individual feather colour (Liu et al. 2010; Liu et al. 2019). Studies by Li et al. (2024) and Guo et al. (2021) both observed that individuals carrying the gold feather gene still exhibited a white feather phenotype under the action of the homozygous recessive white (cc) genotype. Furthermore, homozygous cc variant individuals exist in local chicken breeds and are consistently associated with the recessive white feather trait. Based on previous research summaries, the insertion mutation in intron 4 of the *TYR* gene causes individual albinism, with the feather colour phenotype being white.

In the population survey, the *TYR* insertion genotype (cc) occurred in 20% of Qiangshan Cloud chickens, whereas 80% had the CC genotype. No obvious difference in the cloudy feather phenotype was detected between sampled cc and CC individuals. These observations indicate that the *TYR* insertion alone was insufficient to produce an albino phenotype in this genetic background under the conditions examined; they do not exclude a conditional effect of *TYR* or modification by other loci.

Several non-mutually exclusive explanations may be considered for the absence of an obvious albino phenotype, but the present observational data do not distinguish among them:

- The detected *TYR* variant is a 7.7 kb intronic insertion rather than a coding single-nucleotide substitution; therefore, synonymous-versus-missense explanations do not apply. Because TYR protein abundance and tyrosinase enzymatic activity were not measured, the extent of residual TYR function in insertion carriers remains unresolved.
- The higher *TYRP1* and *DCT* transcript levels in Qiangshan Cloud chickens may reflect broader activation of a melanogenic program and could contribute to downstream pigment processing. However, TYRP1 and DCT catalyse steps distinct

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from the TYR-initiated reactions, and transcript abundance alone cannot demonstrate that these enzymes substitute for TYR or functionally compensate for a TYR deficiency.

- MITF is a transcriptional regulator of melanocyte differentiation and melanogenesis-related genes, including *TYR*, *TYRP1*, and *DCT* (Yu et al. 2021). MITF expression, activation, and DNA binding were not measured in the present study; consequently, its involvement in the observed expression pattern remains an inference from established pathways rather than a result demonstrated here.

Previous studies have shown that MITF can regulate melanocyte-lineage genes, including *TYR*, *TYRP1*, *TYRP2*/*DCT*, *SILV*/*PMEL17*, *AIM1*, and *TRPM1* (Zu 2020). An association between *MITF* expression and melanin deposition has also been reported across tissues in Silky Fowl (Zhen et al. 2015). These findings provide biological context, but they do not establish the same regulatory relationship in Qiangshan Cloud feather follicles without direct measurement of MITF activity.

Associations among *MC1R*, *TYR*, cAMP, and melanogenesis-related expression. *MC1R* E-locus alleles have been associated with eumelanic plumage and may contribute to the black-feather background; however, the present study did not test whether MC1R activity masks a functional TYR defect (Ribani et al. 2023). In their study on the association between feather colour traits and the *MC1R* gene in small black-feathered chickens, Xu et al. (2022) found that the black feather trait in these chickens is controlled by an autosomal dominant gene. The molecular mechanism involves a gain-of-function mutation in the coding sequence (CDS) of the *MC1R* gene, forming a CTG haplotype highly associated with the black feather phenotype.

Research by Ran (2017) indicated that chicken feather colour traits are strongly related to polymorphisms in the *MC1R* and *TYR* genes. Specifically, the T398A mutation in the *MC1R* gene can serve as an important genetic marker to distinguish black and speckled feathers from other feather groups; the G920C mutation site can be used as an SNP marker for breeding black-feathered chickens. On the other hand, the C47G and T172C mutations in the *TYR* gene are associated with the barred feather phenotype and can be used as genetic markers for trait selection; furthermore, the T120C mutation in this gene can also serve as an SNP marker for the breeding of black-feathered chickens.

The Qiangshan Cloud population had a high *MC1R* E allele frequency (0.982), and *MC1R* expression and cAMP concentration were higher than in the comparison breeds. These results are compatible with greater melanogenic signalling. However, the association between *MC1R* and *TYR* expression depended on *TYR* genotype: it was positive in wild-type Qiangshan Cloud chickens (n = 18, r = 0.656, P = 0.003 1) but negative and not statistically significant in the mutant subgroup (n = 6, r = –0.710, P = 0.114). The small, nonsignificant mutant-subgroup result does not support the conclusion that MC1R negatively regulates mutant *TYR*.

Between-breed contrasts should be interpreted with caution because the Qiangshan Cloud, Fengwei, and recessive white-feathered populations differ in overall genetic background and at multiple pigmentation-related loci, including *SLC45A2* and *TYR*. Consequently, differences in *MC1R* expression, cAMP concentration, and downstream transcripts cannot be attributed specifically to *MC1R*. Furthermore, with 106 EE and only 4 Ee birds among 110 Qiangshan Cloud chickens, the available within-breed *MC1R* variation was insufficient to test whether *MC1R* genotype explains individual variation in *TYR* expression. Isogenic, segregating, or functional-intervention designs would be required to isolate an *MC1R*-specific effect.

Figure 12 therefore presents a working conceptual model rather than a demonstrated mechanism. Higher *MC1R* expression and cAMP concentration, together with elevated *TYRP1* and *DCT* transcripts, are consistent with increased melanogenic signalling in Qiangshan Cloud chickens. Nevertheless, this study did not measure MITF abundance or binding, TYR/TYRP1/DCT enzyme activity, or the effects of experimentally altering MC1R-cAMP signalling. It therefore cannot determine whether TYRP1 or DCT functionally compensates for the *TYR* insertion.

A feedback effect on *TYR* transcription is one possible explanation for the subgroup-specific expression pattern, but no direct evidence for such feedback was obtained. Alternative explanations include genotype-linked background differences, biological variation, and limited statistical power in the six-bird mutant subgroup. SLC45A2 has established roles in melanosome pH regulation and

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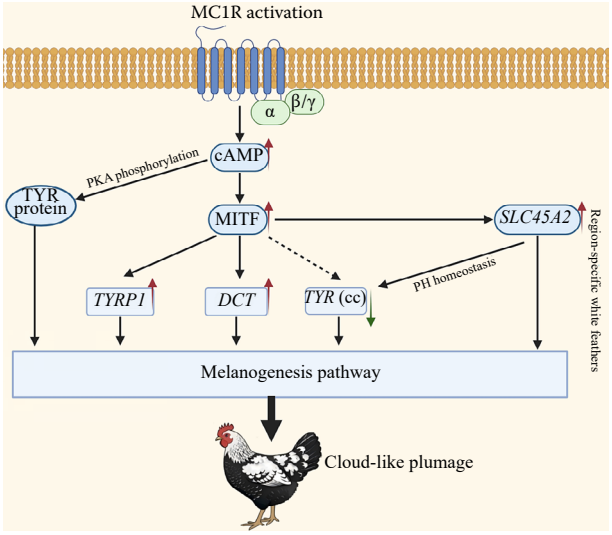

Figure 12. Proposed conceptual model of genetic and signalling associations with plumage colour in Qiangshan Cloud chickens

In this figure, *TYR* represents the insertion-mutant *TYR* background. Red upward arrows indicate upregulation, whereas green downward arrows indicate downregulation. The cross-breed design does not isolate an *MC1R* single-locus effect

melanocyte pigmentation (Le et al. 2020), but the specific functional effect of c.1039C>A on these processes was not directly tested in this study.

Post-translational regulation of melanogenic enzymes through cAMP-PKA-associated processes is also biologically plausible (Zhai et al. 2023). However, phosphorylation status and enzyme activity were not measured; thus, post-translational compensation for low *TYR* transcript abundance remains speculative.

The representative Masson-Fontana sections qualitatively showed more extensive brown-black staining in Qiangshan Cloud feather follicles despite lower *TYR* transcript abundance in the mutant subgroup. Because the histological assessment involved three birds per breed, three fields per bird, and no quantitative measurement of melanin, it cannot independently validate a negative-regulation or compensation mechanism. The staining pattern should instead be viewed as descriptive evidence that is compatible with continued pigment deposition in the sampled Qiangshan Cloud follicles.

Previous studies have emphasised associations between *MC1R* variation and black plumage (Ran 2017; Xu et al. 2022). The present study extends this context by identifying genotype, expression, cAMP, and qualitative histological patterns in Qiangshan Cloud chickens, but the specific regulatory and compensatory pathways proposed here require experimental validation.

Several limitations should be considered. The Qiangshan Cloud *TYR*-insertion subgroup contained only six independent birds, and its inverse correlation was not statistically significant. The study measured transcript abundance and cAMP concentration but did not manipulate MC1R-cAMP signalling, quantify MITF binding, or measure TYR, TYRP1, and DCT enzyme activities. Masson-Fontana staining was assessed qualitatively in three birds per breed and three fields per bird, without histomorphometry or biochemical melanin quantification. Accordingly, the pathway shown in Figure 12 should be interpreted as a testable working model rather than a demonstrated mechanism. Cross-breed genetic-background confounding and near fixation of the MC1R E allele also prevented estimation of an isolated *MC1R* single-locus effect.

CONCLUSION

This study identified several genetic and expression associations relevant to the formation of the cloudy feather phenotype in Qiangshan Cloud chickens:

- The *SLC45A2* c.1039C>A (Leu347Met) variant was strongly associated with cloud-like white-feather distribution, and the segregation pattern was consistent with a sex-linked major locus; direct causality remains to be functionally validated.
- The 7.7 kb insertion mutation in the *TYR* gene is present in some individuals but does not, by itself, produce an albino phenotype.
- The *MC1R* E allele was nearly fixed in the sampled Qiangshan Cloud population, and these chickens showed higher *MC1R* expression and cAMP concentration together with higher *TYRP1* and *DCT* expression than the comparison breeds. These observations are consistent with enhanced melanogenic signalling but do not establish a unique negative-feedback suppression of *TYR* or complete functional compensation for the *TYR* insertion. The nonsignificant inverse trend in the

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six-bird mutant subgroup requires confirmation in a larger independent sample and functional assays. Therefore, the between-breed differences should be regarded as breed-associated patterns rather than effects attributable solely to *MC1R*.

In summary, the findings support a testable model in which *SLC45A2*-associated variation, the *MC1R* genetic background, the *TYR* insertion, and melanogenesis-related expression patterns jointly contribute to the observed plumage phenotype. This model provides a basis for subsequent functional studies and marker validation, but causal regulatory interactions should be confirmed before application in molecular breeding.

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Conflict of interest

The authors declare no conflict of interest.

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