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Molecular cloning, polymorphisms, and association analysis of the promoter region of the *STAM2* gene in Wuchuan Black cattle

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ABSTRACT. The STAM protein plays an important role in the cytokine-related JAK/STAT pathway. We selected the *STAM2* gene as a candidate gene that could be linked to growth performance in analysis of a Chinese cattle breed (Wuchuan Black cattle). We examined genetic variants in the promoter region of the *STAM2* gene and their associations with eight growth traits in 159 individuals. Seven SNPs, which included six new SNPs for the SNP database, were found. The core promoter region was identified with a bioinformatic software. This analysis also showed that the SNPs have a significant influence on the function and structure of the *STAM2* promoter in terms of RNA secondary structure, CpG island, and transcription factor binding sites. Association analysis demonstrated that G-102A is significantly associated with withers height, heart girth, cannon circumference, chest width, and hip height in this population, which leads us to suggest that G-102A is a useful SNP marker for cattle growth performance. Animals with the genotype

AA had higher mean values for withers height, cannon circumference, chest width, and hip height than those with GG and AG genotypes. This SNP of the *STAM2* gene could be applied in marker-assisted selection for improving growth performance in cattle.

Key words: *STAM2* gene; Promoter; Growth performance; Wuchuan Black cattle; Association analysis

INTRODUCTION

Janus kinase/signal transducer and activator of transcription (JAK/STAT) signal pathway is one of the major signaling pathways involved in regulation of many cytokines and growth factors that play key roles in cell proliferation, differentiation, cell migration, and apoptosis (Rawlings et al., 2004; Stark and Darnell, 2012). Signal transducing adaptor molecules (STAM), which include STAM1 (Takeshita et al., 1997) and STAM2 (Endo et al., 2000), are the key regulatory proteins of JAK/STAT pathways. Both of them contain an Src homology 3 domain, STAM-specific motif, Ub-interacting motif, Vps27-Hrs-STAM homology, and immunoreceptor tyrosine-based activation motif (ITAM) (Lim et al., 2011b). STAM proteins can interact with Jak2 and Jak3 tyrosine kinases via its ITAM domain, which results in the induction of cytokine signal transduction pathways in growth and other biological responses (Rismanchi et al., 2009), such as epidermal cell growth factor, interleukin 2-4, and platelet-derived growth factor (Takeshita et al., 1996). However, it has also been shown that STAM proteins can inhibit receptor tyrosine kinase and cytokine receptors through ubiquitination by binding to ubiquitin (Kong et al., 2007). The specific function and mechanism of STAM proteins in growth-related signal transduction pathways are still unknown, although the interaction mechanism between STAM proteins and other members in JAK/STAT pathway has been studied so far (Lim et al., 2011a; Lange et al., 2012; Malik et al., 2012).

Wuchuan Black cattle (WB) are a native breed in Guizhou Province, China, which is famous for superior meat quality, resistant coarse feed, and disease resistance (Xu et al., 2010). However, their poor growth performance has significantly limited their utilization in the production system. The characterization of candidate genes for growth traits may provide a foundation to select for improved growth performance of WB. Our preliminary research has successfully detected SNPs in the bovine *STAM* gene among different cattle breeds (Yang et al., 2012a,b). Therefore, the objective of this study was to investigate the association of STAM2 promoter SNPs with growth traits for WB. The effect of SNPs on the functional elements of *STAM2* promoter was also studied by using various bioinformatic tools.

MATERIAL AND METHODS

Population and DNA sample collection

WB (N = 60) and Guizhou Holstein cows (GH, N = 60) with a consistent concentration of 100 ng/μL were used to construct two DNA pools. A total of 159 individuals of WB were used to conduct PCR-restriction fragment length polymorphism (RFLP) and association

analysis with growth traits, which included the following traits: withers height (WH), body length, hip height (HH), heart girth (HG), cannon circumference (CC), chest width (CW), chest depth, and body weight. For each of the body measurements, the same traits were measured three times by the same person to minimize error. Blood samples were collected and genomic DNA was extracted by using the genomic DNA kit (Sangon Biotech, Shanghai, China). The results were detected by 1.5% agarose gel electrophoresis.

Primer design and PCR amplification

According to cattle *STAM2* gene sequences (GenBank accession No. AC_000159.1), one primer pair (Table 1) was designed by the Primer-Blast software to amplify the cattle *STAM2* gene 5'-flanking region and to detect the SNP of the promoter region by constructing two DNA pools with two breeds. PCR was conducted in 25-µL reaction mixtures containing 12.5 µL 2X *Taq* PCR Master Mix, 1.5 µL 10 pmol/µL of each primer, 2.5 µL DNA template, and 7 µL ddH_2O. The PCR procedure was 94°C for 4 min followed by 35 cycles at 94°C for 40 s, 61.3°C annealing for 45 s, and 72°C for 50 s, and a final extension at 72°C for 10 min. PCR products were purified using a DNA gel extraction kit and subsequently subjected to two-way direct sequencing, three times independently. DNAstar and BLAST combined with the Cattle dbSNP database were used to screen the SNPs in the *STAM2* gene.

Table 1. Sequences of primer, annealing temperature, and predicted length of amplified DNA.

Primer	Annealing temperature (°C)	Product size (bp)	Forward primer (5'→3')	Reverse primer (5'→3')
STAM2	61.3	1066	GGAGAAAGGGACGACAGAGCA	CTTTCCAGTTCCCAGAGTCGC

Genotyping and association analysis

The appropriate SNP was genotyped by using the RFLP technique, which was performed by mixing 5 µL PCR product with 10 U *Sma*I, and 1 µL 10X reaction buffer and incubating at 25°C for 12 h following the supplier instructions. The digestion products were separated by electrophoresis on a 2.0% agarose gel stained with Goldview (Fermentas, Shenzhen, China). The genotypic frequencies were calculated using the SPSS software and the Hardy-Weinberg equilibrium was analyzed using the χ^2 test. Association analysis between genotypes of the *STAM2* gene and body measurement traits was carried out on our resource population by the least-square method with the SPSS software.

Data analysis and bioinformatic tools

Different softwares may lead to distinguishing results by using various mathematical models and algorithms, and comparative analysis between different softwares can improve accuracy for prediction. The result prediction for various indexes can be obtained online by submitting diverse sequences that changed by the mutation of SNPs.

Core promoter prediction: Promoter SCAN: http://www-bimas.cit.nih.gov/molbio/proscan/; Neural Network Promoter Prediction: http://www.fruitfly.org/seq_tools/promoter.html; Promoter 2.0: http://www.cbs.dtu.dk/services/Promoter/; Softberry: http://linux1.softberry.com/.

Prediction of transcription factor binding sites (TFBS): Tfsitescan: http://www.ifti.org/; TESS: http://www.cbil.upenn.edu/cgi-bin/tess/tess; TFSEARCH: http://www.cbrc.jp/research/db/TFSEARCH.html; Softberry: http://linux1.softberry.com/berry.phtml; Prediction of RNA secondary structure: Genebee: http://www.genebee.msu.su/services/rna2_reduced.html; Prediction of CpG island: Bio-soft: http://www.bio-soft.net/sms/cpg_island.html; MethPrimer: http://www.urogene.org/methprimer/index1.html; Webgene: http://zeus2.itb.cnr.it/cgi-bin/wwwcpg.pl; Softberry: http://linux1.softberry.com/berry.phtml?topic=cpgfinder&group=programs&subgroup=promoter; CpG Island Searcher: http://www.uscnorris.com/cpgislands2/cpg.aspx.

RESULTS

Characterization of the bovine *STAM2* gene promoter

The 1066-bp fragment sequence that contained the 853-bp fragment upstream from the start codon was amplified (Figure 1). Almost all softwares indicated that there was a core promoter region in the target sequence that had been amplified except for Softberry, and two promoter regions were determined by using Neural Network Promoter Prediction (Table 2). Canonical TATA boxes, GC box, and CCACT box, which play a central role in eukaryotic transcription initiation and improve transcription frequency, were found in the promoter region. Potential transcription factor binding sites in the region were predicted using online software. Promoter prediction analysis indicated that they shared several important confirmed binding sites for transcriptional factor, which included SP1, STAT, c-Myb, CCAAT/enhancer-binding proteins (C/EBPs), AP-1/2/4, NF-Y, IRF-2, γ-IRE, MyoD, and ROR-α.

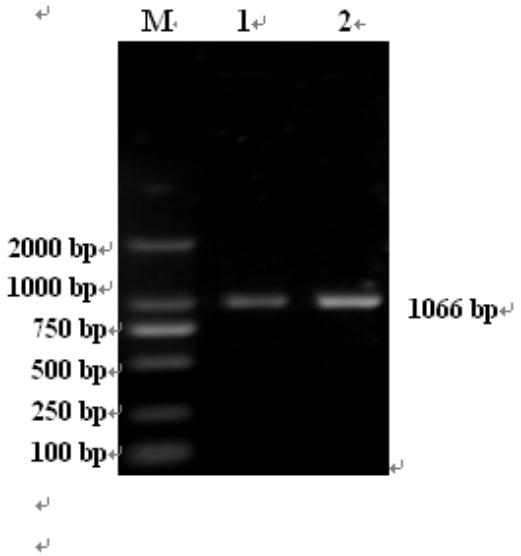

Figure 1. Results of DNA pooling of *STAM2* in two breeds. *Lane M* = DL2000 marker; *lane 1* = Wuchuan Black cattle; *lane 2* = Guizhou Holstein cow.

Table 2. Results of promoter prediction.

Software	Start site	Termination site	Score
Neural Network Promoter Prediction	-60 bp	-10 bp	0.72
	-5 bp	+45 bp	0.83
Promoter 2.0	-453	-	0.546
Softberry	-	-	-
Promoter SCAN	-173	+77	0.63

Polymorphism of the *STAM2* gene

A 1066-bp fragment in the 5'-flanking region of the cattle *STAM2* gene was amplified in two DNA pools representing two breeds (WB and GH). Sequence analysis by DNAStar and BLAST softwares showed that there were 7 SNPs between the two cattle breeds, which included A-523C, A-390C, A-324T, G-102A, A-46G, G-4T, and T+37C (Figure 2). Furthermore, the T+37C mutation conformed to rs109680819 in SNP BLAST databases and the others were first found in cattle. The SNP information was then submitted to the SNP database in NCBI, and the respective accession numbers ss538994483, ss538994485, ss538994487, ss538994489, ss538994491, and ss538994493 were obtained.

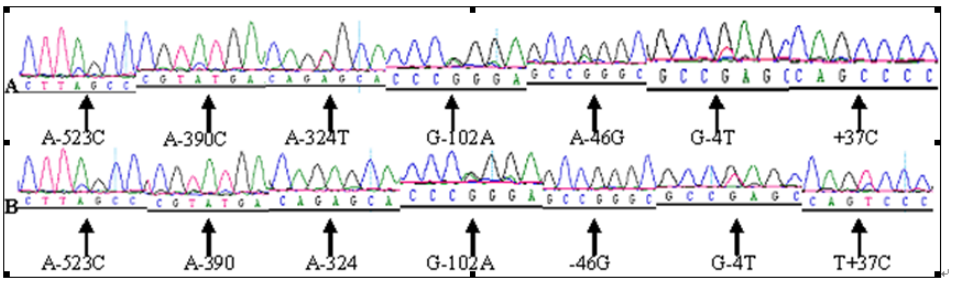

Figure 2. Sequencing and BLAST of *STAM2* PCR products in two breeds. **A.** Wuchuan Black cattle. **B.** Guizhou Holstein cow.

Effect of SNP on functional elements of *STAM2* promoter

RNA secondary structure and CpG island

The results showed that the free energy of the RNA secondary structure of the *STAM2* promoter changed from -1.38 x 10^6 to -1.332 x 10^6 J/mol, resulting from the SNPs found in this study and further affecting the stability of RNA secondary structure. Moreover, the pattern of RNA secondary structure also changed dramatically after SNP mutation, almost because of the change in the stem-loop structure at different positions (Figure 3).

In general, the CpG island in the promoter region consists of non-methylated sequences, which could bind with specific transcription factors; nevertheless, abnormal methylation of the CpG island will reduce the binding activity between CpG island and trans-acting factors. The expression level of the gene could be inhibited as a result of the effect of DNA methylation on the interaction between transcription factors and promoter-specific sequence (Tamura et al.,

2000). The bioinformatic softwares of Webgene, MethPrimer, Bio-soft, CpG Island Searcher, and Softberry, based on some evaluation standards in terms of GC content greater than 50%, Obs/Exp >0.6, and range of CpG island greater than 200 bp, were used in this study to predict the range of the CpG island of the *STAM2* gene and to investigate the effect of different SNPs on the profile of the CpG island; the results are shown in Table 3 and Figure 4. All of them demonstrated that there was a CpG island with similar range, which indicated that the CpG island plays an important role on the expression of the *STAM2* gene. Comparative analysis showed that there was no significant effect of SNP on the range of the CpG island and GC content; however, it was amazing that the SNPs led to the vanishing of CpG island by Softberry only.

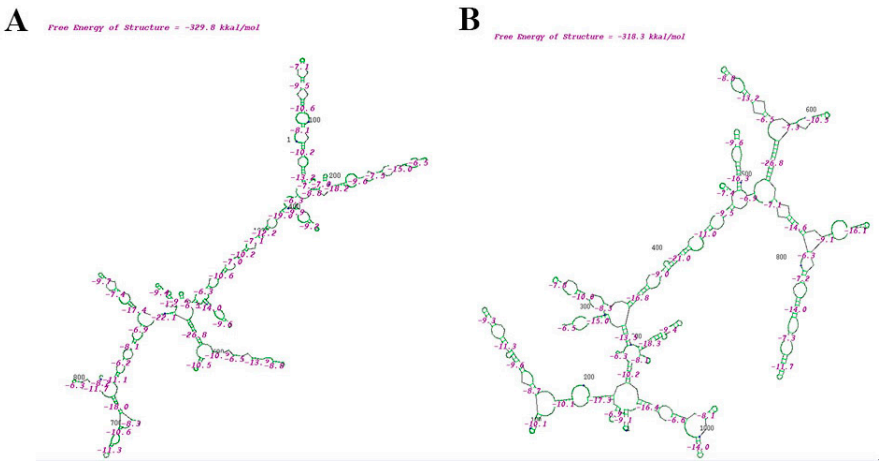

Figure 3. RNA secondary structure prediction of the *STAM2* gene. **A.** Pre-mutation. **B.** Post-mutation.

Table 3. Prediction of the CpG island of *STAM2* gene by various softwares.

Software	Number of CpG island		Size (bp)		Range of CpG island		Content of GC (%)	
	Pr-M	Po-M	Pr-M	Po-M	Pr-M	Po-M	Pr-M	Po-M
MethPrimer	1	1	930	930	-774 to +155 bp	-774 to +155 bp	64.2	64.2
Bio-soft	1	1	1066	1066	-853 to +213 bp	-853 to +213 bp	63.0	62.9
CpG Island Searcher	1	1	1066	1066	-853 to +213 bp	-853 to +213 bp	63.0	62.9
Webgene	1	1	1066	1066	-853 to +213 bp	-853 to +213 bp	63.0	62.9
Softberry	1	0	255	0	-160 to +94 bp	0	74.1	0

Pr-M = pre-mutation; Po-M = post-mutation.

TFBS

The effect of the SNPs on TFBS of the *STAM2* gene promoter was researched by submitting different sequences changed by polymorphism sites to several software programs. A number of TFBS were found in the target sequence for any program (Table 4). We found that the SNPs could lead to the disappearance of some important TFBS that existed previously with some new TFBS emerging (Figure 5). The SNP A-523C led to the vanishing of the binding site for IS1-1 at -527 bp but produced the TFBS for heat shock factor and the disappearance of c-Myb at -322 bp for the consequence of A-324T regulation. Beginning with

-101 bp, the TFBS for JCV, MAZ, CS, and SP1 disappeared as a result of the G-102A mutation. Furthermore, the site of -11 bp, close to the transcriptional start site lost one TFBS, but TFBS for SP1, AP2- α/γ, emerged near the +33 bp position as a result of the SNP T+37C. It demonstrated that the SNPs could lead to a remarkable change in TFBS near the mutation sites and regulate the interaction between transcription factors and promoter sequence directly, and that the expression level of the *STAM2* gene may be subsequently regulated as a result of the possible change in promoter activity.

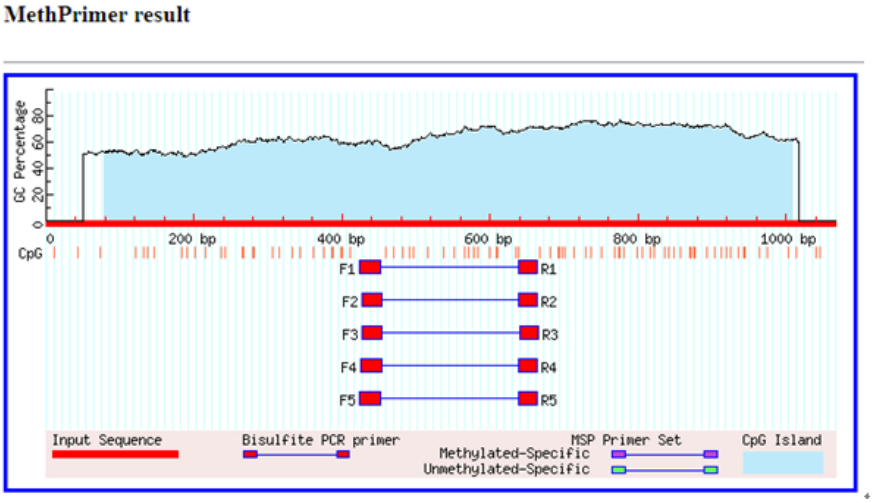

Figure 4. Prediction of CpG island by the MethPrimer software.

Table 4. Prediction of transcription factor binding sites of the *STAM2* gene.

Software	Number of TFBS		Number of TFBS with score 10	
	Pr-M	Po-M	Pr-M	Po-M
Tfsitescan	390	386	20	20
TESS	374	377	-	-
GeneBuilder	213	214	0	0
Softberry	35	34	-	-
TFSEARCH	186	188	7	7

TFBS = transcription factor binding sites. For other abbreviations, see legend to Table 3.

Allele and genotype distribution

We found that the G-102A mutation existed in the restriction site (CCC^GGG) and changed the recognition site of restriction endonucleases *Sma*I. The PCR-RFLP for the SNP G-102A genotyping in all 159 WB populations in our study was subsequently analyzed. Our data showed that genotype AA represents the occurrence of one band of 1066 bp, genotype AG represents three restriction fragment bands of 1066, 750, and 316 bp, and genotype GG represents two bands of 750 and 316 bp (Figure 6). The results (Table 5) indicated that at 159 loci, the A allele was slightly preponderant in WB. The data shown here demonstrate that the frequencies of *STAM2*-G and *STAM2*-A were 0.4717 and 0.5283 in the total population, respectively. Furthermore, the frequency of the GG genotype (0.2453) was lower compared

with the AG and AA genotypes in all populations. The SNP locus in the population was in agreement with Hardy-Weinberg equilibrium (P > 0.05).

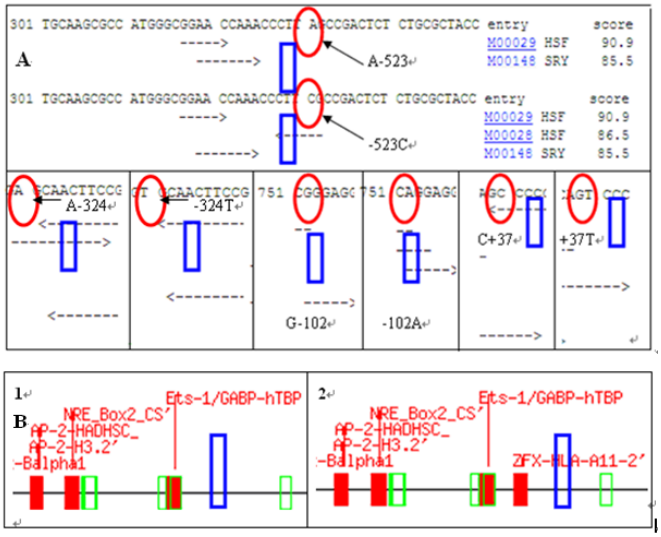

Figure 5. Change of transcription factor binding sites by SNP mutation. **A.** Prediction by TFSEARCH: red circles show the SNP site and blue rectangles show the difference of TFBS result from the SNPs. **B.** Prediciton by Tfsitescan: 1 represents profile of TFBS pre-mutation and 2 represents TFBS post-mutation of the SNPs. The difference of TFBS results from the SNPs.

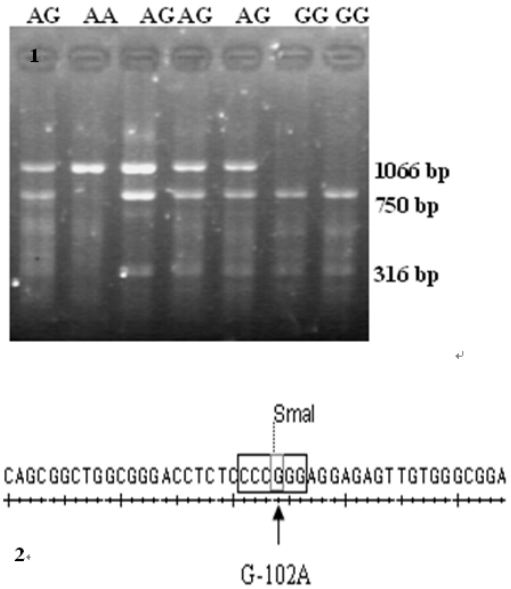

Figure 6. PCR-RFLP analysis of G-102A in the *STAM2* gene. **1.** Detection result of PCR-RFLP. Strands with 1066 bp for the AA genotype, 750 and 316 bp for the GG genotype, 1066, 750, and 316 bp for the AG genotype appeared at this locus. The 316 bp was unclear in the figure. **2.** G-102A mutation site located in the recognition of restriction site for *Sma*I.

Table 5. Allele and genotype frequencies at G-102A locus of the *STAM2* gene in Wuchuan Black cattle.

Population	Number	Genotype frequencies			Allele frequencies		χ^2 (HWE)
		GG	AG	AA	G	A	
Wuchuan Black cattle	159	0.2453 (39)	0.4528 (72)	0.3019 (48)	0.4717	0.5283	1.4376

χ^2 (HWE) = Hardy-Weinberg equilibrium value.

Association analysis

Subsequently, 8 body measurement traits were analyzed by comparing the genotypes of 159 individuals and their phenotypic data. The results of the association analysis of the G-102A are given in Table 6. At the G-102A SNP site, there were significant effects on WH, HG, CC, CW, and HH in the total population. Animals with the genotype AA had higher mean values for WH, HG, CC, CW, and HH than those with GG and AG genotypes, which indicated that the AA genotype may be the superior genotype for growth performance of WB. There were significant differences between genotype GG and AA or AG for WH, HG, CC, CW, and HH. Furthermore, heterozygotes (AG) had lower HG, CC, and CW than those of homozygotes (AA or GG). The results showed that G-102A had an important influence on the body measurement traits of WB.

Table 6. Association analysis of G-102A SNP genotypes with body measurement traits at the Wuchuan Black cattle *STAM2* gene.

SNP	Genotypes	Traits (cm/kg)							
		WH	BL	HG	CC	CW	CD	HH	BW
G-102A	GG	108.51 ± 4.56^a	135.74 ± 10.43	157.46 ± 14.75^a	18.02 ± 0.90^a	30.87 ± 6.63	54.59 ± 2.12	107.37 ± 3.68^a	298.66 ± 33.80
	AG	109.28 ± 4.12	134.08 ± 8.17	152.56 ± 11.19^b	17.88 ± 1.35^a	28.67 ± 6.51^a	54.03 ± 2.69	108.72 ± 4.10^a	291.18 ± 25.14
	AA	110.78 ± 4.15^b	136.29 ± 7.48	156.72 ± 9.37	18.56 ± 1.32^b	31.48 ± 6.29^b	54.64 ± 2.48	110.68 ± 3.21^b	300.64 ± 21.05
P		0.038	0.344	0.055	0.013	0.048	0.341	0.0009	0.123

Data are reported as means ± SE. Different superscript letters (a, b) represent significant differences in genotypes GG, AA, and AG. WH = withers height; BL = body length; HG = heart girth; CC = cannon circumference; CW = chest width; CD = chest depth; HH = hip height; BW = body weight.

DISCUSSION

The STAM family is highly conserved with the evolutionary process and exists in the cytoplasm and early endosomes, indicating that the STAM protein plays a consistent important role in organisms with the long evolutionary process (Bache et al., 2003). The STAM protein can promote the cytokine signal transduction pathways by activating the expression of downstream genes when its 198 tyrosine residues are phosphorylated (Steen et al., 2002). However, it also demonstrated that the STAM protein is involved in the process of endocytosis to downregulate the expression level of cytokine receptor by interacting with the Hrs protein (Takata et al., 2000). However, its intricate role in the JAK/STAT pathway network is still unknown.

A previous study showed that mice lacking the *STAM* gene were smaller than the wild-type, and that the mutation led to the disappearance of hippocampal CA3 pyramidal neurons, which caused the death of the mice 6 months later (Yamada et al., 2001). Moreover, mice with double mutations for the *STAM1* and *STAM2* genes had no normal morphogenesis and were

embryonically lethal as a consequence of significant reduction in thymocytes and peripheral mature T cells (Yamada et al., 2002). The candidate gene approach is a powerful method to investigate associations of gene polymorphisms with economically important traits in farm animals. It is important to choose *STAM* genes as candidate genes for livestock breeding because of the almost blank background for exploration of the *STAM* gene polymorphism in livestock.

In the present study, we found for the first time several novel variants in the promoter region of the *STAM2* gene in cattle by DNA pooling and PCR-RFLP methods, which are main located in the core promoter region with typical promoter characteristics and functional elements predicted by various softwares excepted for A-523C locus. We also found that the SNPs screened in this study had a significant influence on the functional elements and structure of the *STAM2* gene promoter, such as RNA secondary structure, TFBS, and CpG island. The remarkable change in RNA secondary structure caused by SNPs mutation may lead to a change in the stability of the *STAM2* gene structure. Almost all SNPs caused a change in TFBS near the SNP position even for some important transcription factors. Furthermore, a large range of CpG island was found in the target sequence, and the SNP led to the disappearance of the CpG island shown by the result of the Softberry software. The SNPs may regulate the expression level of the *STAM2* gene by affecting promoter function.

We also reported for the first time the *Sma*I polymorphism in the cattle *STAM2* gene, and the SNP of G-102A was studied further for association analysis by PCR-RFLP. It demonstrated that there was no significant difference in frequencies of A/B, although the A allele was slightly preponderant in populations. However, genotype frequencies of AG heterozygotes were higher compared to homozygotes (AA or GG). Association analysis showed that G-102A had significant effects on WH, HG, CC, CW, and HH in the total population, which suggested that G-102A is an important SNP marker that affects cattle growth performance. Moreover, animals with the genotype AA had higher mean values for WH, CC, CW, and HH than those with the GG and AG genotypes, which indicated that the AA genotype may be the superior genotype for growth performance of WB.

In conclusion, the SNPs found in this study could regulate *STAM2* gene expression by affecting the promoter activity. The SNP of G-102A may be treated as an important marker for cattle breeding and the animals with the AA genotype may have more outstanding growth performance than those with the AG and GG genotypes. More importantly, the polymorphisms of the *STAM2* gene had a significant effect on growth performance of WB. It seemed that the *STAM2* gene can be used as a candidate gene for cattle breeding in further studies. However, more cattle breeds and data on growth traits need to be collected for further research to confirm its function.

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