Research Paper

Genetic polymorphism within Activating Transcription Factor 4 gene in Sahiwal cattle

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ABSTRACT

Major focus of dairy cattle genomics is to identify genes underlying the genetic variability of economically important traits that could be useful in breeding programmes. The present study on 130 Sahiwal cows by PCR-RFLP and sequencing aimed to explore the polymorphism in the entire coding region of *ATF4* gene. Nucleotide sequences were visualized and edited using BioEdit software. The multiple sequence alignments of the edited sequence with corresponding reference sequences were performed with ClustalW software to identify SNPs. Nucleotide sequences were translated to amino acids by ExPASy Translate tool, edited, and subjected to multiple sequence alignment with the reference ATF4 protein sequences of *Bostaurus*. Amino acid substitutions were analysed using SIFT software (*sift.jcvi.org/* or *sift-dna.org/*). Four set of primers exhibited total 04 SNPs T352C in intron 1, G379A in exon2, A215T and A230C in exon 3 including 02 transitions and 02 transversions. Primer 4 exhibited monomorphic pattern. The identified SNPs can be used as markers for milk traits in Sahiwal cattle. However, association and validation are warranted on larger number of animals and in different herds of Sahiwal cattle.

Key words: *ATF4* gene, Exons, PCR-RFLP, Sahiwal, Single nucleotide polymorphism

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INTRODUCTION

Sahiwal is one of the best dairy breeds of indigenous cattle having its origin in Montgomery district of Pakistan (Mir *et al.,* 2023). National commission on Agriculture (1976) has recommended the improvement in this breed. Sahiwal is one of the few breeds which FAO has undertaken for improvement (FAO, 1983). Sahiwal cattle is famous for its higher milk production, low maintenance cost, the remarkable power of endurance for hot climate of tropics, resistance to tropical diseases and higher feed conversion efficiency (Naha *et al*., 2019). Therefore, it is of paramount importance to improve the production potential of our indigenous livestock to cope up with the continuous increase in demand for milk and milk products and also for providing better economic sustainability to the small-scale farmers for a better livelihood.

Information of the association between polymorphisms in these candidate genes and traits of economic importance is essential for their effective use in marker assisted selection (MAS) (Singh *et al*., 2020). Identification of major genes associated with performance traits could be useful for improvement of milk production for dairy cattle breeding programmes (Ogorevc *et al*., 2009). For dairy traits, genes that are involved in mammary gland development, prolactin signalling and involution pathways are relevant candidates. The genes involved in involution pathway explained the greatest level of variance in milk production traits due to their presence within or close to a previously described QTL with quite large effects on milk production traits (Raven *et al.* 2014). Activating Transcription Factor 4 (*ATF4*) is a member of the ATF/CREB family of bZip transcription factors, characterized by the consensus binding site known as the cAMP responsive element (CRE) (Ameri and Harris, 2007). The basic leucine zipper-containing activating transcription factors (ATFs) regulate mammary gland growth by promoting nuclear translocation of activated *STAT3* and increasing insulin-like growth factor-binding protein-5 expression (Rozita *et al*., 2003); *ATF4*, critical for mammary development, may contain novel mutations influencing milk production traits (Raven *et al*., 2014). Overexpression of the gene decreases proliferation and differentiation of mammary alveolar epithelium, apoptosis and accelerated involution of the mammary gland whereas, downregulation leads to *STAT5A* Tyrosine phosphorylation, resulting in decreased expression of α-lactoalbumin, whey acidic protein and β-casein (Rozita *et al*., 2003). The use of polymorphic markers in breeding programmes could make selection more accurate and efficient. Additionally, selection programmes could start at early ages before traits of interest can be expressed phenotypically. Therefore, present study was aimed to identify polymorphism in the entire coding region of ATF4 gene in Sahiwal cattle.

MATERIALS AND METHODS

Molecular Analysis

The experimental animals for this study were selected from a herd of Sahiwal cattle kept at the ICAR-National

Dairy Research Institute, Karnal's Livestock Research Centre (LRC).To screen for polymorphism in all exons of the ATF4 gene, a total of 130 animals were used for PCR-RFLP and 103 for sequencing.

Genomic DNA Preparation

10 ml of venous blood was collected from the jugular vein of each milch animal under sterile conditions in a 15 ml polypropylene centrifuge tube containing 0.5 ml of 0.5 M EDTA solution and kept in the refrigerator at –20°C until DNA isolation was completed. The phenol-chloroform method, as described by Sambrook and Russell (2001), was used to isolate DNA from Sahiwal cattle with minor modifications.

PCR amplification and RFLP analysis

Primer designing

Detailed sequence of primers used in the present study along withtheir annealing temperatures/ time and amplicon size is presented in Table 1, PCR amplificationwas performed in a final volume of 25 µl containing 100 ng of template DNA, 10 pmol of each primer, 10X PCR buffer (20mM Tris-HCL pH 8.4, 50mM KCl), 1mM $MgCl_2$, 2.0 mM of dNTPs and 1 µl ofTaq DNA polymerase (M/s Genetix Biotech Asia Pvt. Ltd). This solution was initially denatured at 95°C for 5 min, followed by 35 cycles of denaturation (95°C for 1 min), annealing (58°C for 1 min), elongation (72°C for 1 min) and a final extension at 72°C for 5 min. The amplified products were detected in 1.5% Agarose gel electrophoresis (Fig.1). Aliquots of 5 µl of PCR products were applied to the gel. About 10 µl of amplified product was digested with 10 units of *Pfel*and*Acil* enzyme overnight at 37°C in water bath. The amplified product was digested at 37°C for 14 hours. The digested products were detected by electrophoresis in 2% agarose gel in 1X TBE buffer and ethidium bromide (10 mg/µl).

Table 1: Sequence of the primers designed, annealing temperatures and sizeof ampliconsof exons of *ATF4* gene in Sahiwal cattle

Primer set	Sequence (5′-3′)		Annealing temperature (ºC) at 30s	Amplicon size (bp)
Exon1	F	CGGAGAGCTGACAATACGTG	59.6	466
	R	GACACCCAAGCAGGAGTCAC		
Exon2	F	CCCTGTTTTCAACACGGAGT	60	486
	R	AGGAGCCCCTATCACCAGAT		
Exon3,1	F	AGGGGCTCCTACGTTTGATT	60.6	493
	R	GGGCTCATACAGATGCCACT		
Exon3,1	F	GAGCCTGCTATCTCCAGGTG	62.6	487
	R	TACTGACAGCCAATCCCACA		

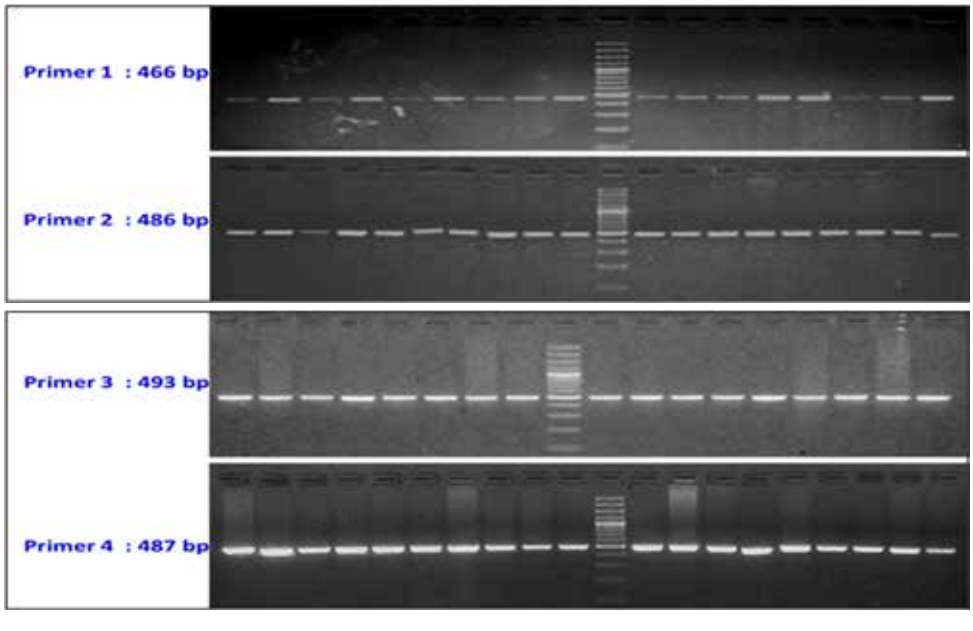

Fig.1: *Resolution of PCR products of ATF4 gene*

DNA sequencing and Protein sequence analysis

Custom sequencing was performed on both ends (5' and 3') of amplified PCR products from two sets of primers. BioEdit software was used to visualise and edit nucleotide sequences. ClustalW software was used to identify SNPs by performing multiple sequence alignments of the edited sequence with corresponding reference sequences. The ExPASy Translate tool (Gasteiger *et al*., 2003) was used to convert nucleotide sequences to amino acids, which were then edited and aligned with the reference OLR1 protein sequence of *Bostaurus*. SIFT software (sift.jcvi.org/ or sift-dna.org/) was used to examine amino acid substitutions (Sim *et al*., 2012).

RESULTS AND DISCUSSION

In the present study one hundred and thirty samples for PCR-RFLP and one hundred and three samples for sequencing were included for analysis with an aim to identify DNA polymorphism in complete CDS region of *ATF4* gene.

For determining the change in all exons of *ATF4* gene of Sahiwal cattle, the reference sequences in ENSEMBL ENSBTAG00000017462 for *Bostaurus*were compared and aligned with the edited sequences of Sahiwal cattle by ClustalW2 software.Comparison of all exon of Sahiwal cattle of *ATF4* gene with that of reference sequence of *Bostaurus* by ClustalW2 multiple alignments revealed a total of 4 mutations including 2 transitions and 2 transversions. The 4 SNPs were detected at positions C352T (Primer 4), A379G (Primer 5), T215A and C230A (Primer 6) as compared to *Bostaurus* (Ref. Seq. ENSBTAG00000017462) (Table 2). The chromatogramsreflecting thechange in nucleotide as compared to *Bostaurus* are given in Fig. 2, 3, 4, 5. The coding sequences were translated into amino acid sequence using ExPAsy translate tools and multiple sequence alignment with corresponding *Bostaurus* sequence revealedno amino acid change. All loci under study except C352T werein Hardy-Weinberg equilibrium.

Table 2: Summary of nucleotide changes in *ATF4* gene in Sahiwal cattle as compared to *Bostaurus*(Ensembl Gene ID ENSBTAG00000017462)

Primer	Locus	Position	Region	*Bos Taurus*	Sahiwal	Type of Variation
Primer 1	352	111463075	Intron	C	T	Transition
Primer 2	379	111463888	Exon 2	A	G	Transition
Primer 3	215	111464200	Exon-3	T	A	Transversion
	230	111464215		C	A	Transversion

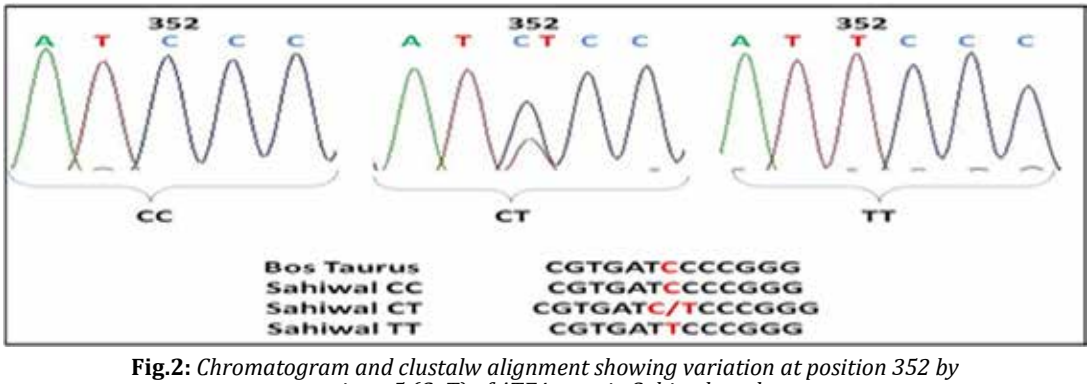

Fig.2: *Chromatogram and clustalw alignment showing variation at position 352 by primer 5 (C>T) of ATF4 gene in Sahiwal cattle*

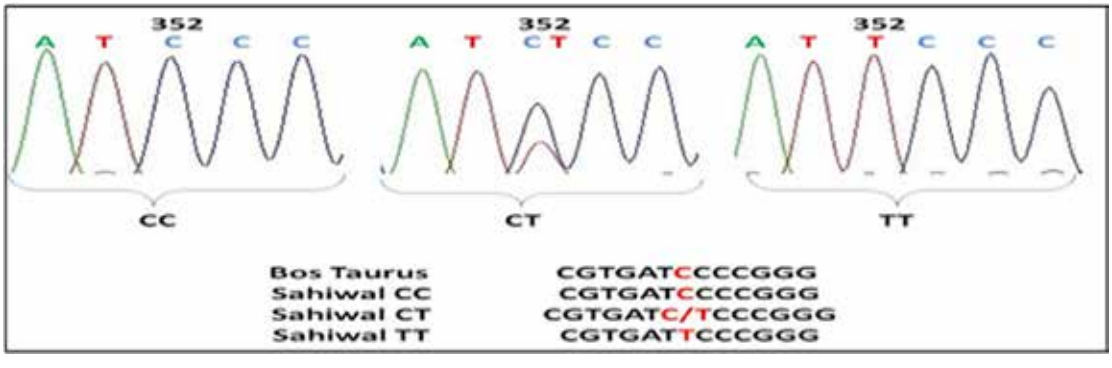

Fig.3: *Chromatogram and clustalw alignment showing variation at position 379 by primer 4 (A>G) of ATF4 gene in Sahiwal cattle*

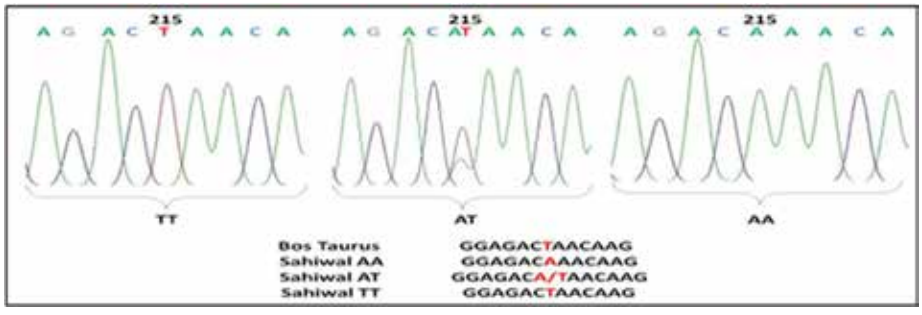

Fig.4: *Chromatogram and clustalw alignment showing variation at position 215 by primer 6 (T>A) of ATF4 gene in Sahiwal cattle*

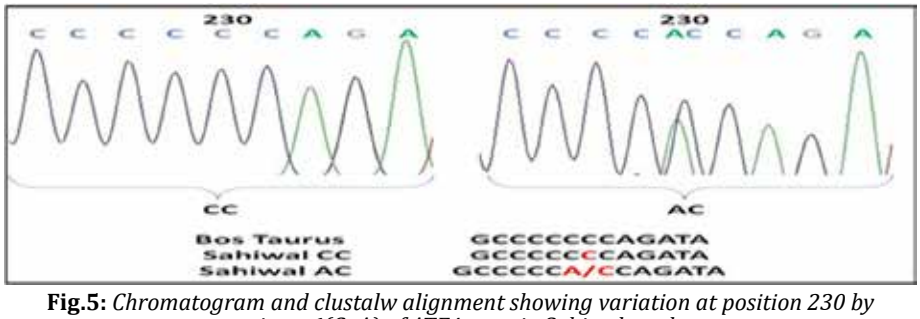

Fig.5: *Chromatogram and clustalw alignment showing variation at position 230 by primer 6(C>A) of ATF4 gene in Sahiwal cattle*

PCR-RFLP analysis of intron 1 was carried out using *Pfel*restriction enzyme.*Pfel*-RFLP for intron 1 revealed polymorphic pattern showing three genotypes CC (466bp), CT (466bp, 349bp and 117bp) and TT (349bp and 117bp). Genotypic frequencies of CC, CT and TT were 0.72, 0.26 and 0.02 respectively. The allelic frequencies for T and C were 0.15 and 0.85 respectively (Fig. 6). PCR-RFLP analysis of exon 2 was carried out using *Acil*. *Acil*-RFLP for exon 2 revealed polymorphic pattern showing three genotypes, AA (486bp), AG (486bp, 377bp and 109bp) and GG (377bp and 109bp). Genotypic frequencies of AA, AG and GG were 0.61, 0.37 and 0.02 respectively. The allelic frequencies for A and G were 0.795 and 0.205 respectively (Fig. 7). The identification of SNPs was done by sequencing using primer 6 for *ATF4* gene. The genotype and allele frequency are presented in the Table 3.

Fig.6: *PCR-RFLP patterns of ATF4 gene using Pfel restriction enzyme*

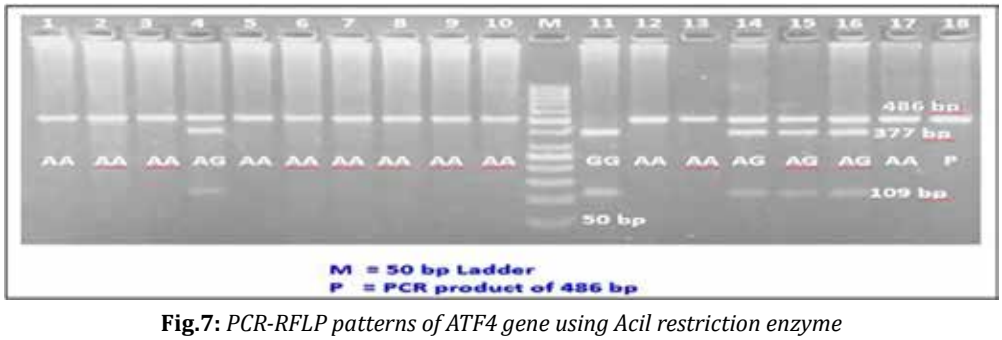

Fig.7: *PCR-RFLP patterns of ATF4 gene using Acil restriction enzyme*

Table 3: Genotypic and allelic frequencies of *ATF4*gene using sequencing and PCR–RFLP in Sahiwal cattle

Gene	ATF4 gene						Ne*	I*	Chi-Square (p-value)
Locus	Gene region	Frequency							
		Genotype			Allele				
P1 352 C>T	Intron 1	CC	CT	TT	T	C	1.3301	0.4142	0.008
		0.72(71)	0.26(17)	0.02(5)	0.15	0.85			
P2 379 A>G	Exon 2	AA	AG	GG	G	A	1.3376	0.4195	0.13
		0.64(57)	0.32(24)	0.04(2)	0.20	0.80			
P3 215 T>A	Exon 3	AA	AT	TT	A	T	1.6220	0.5716	0.59
		0.07(3)	0.38(24)	0.55(31)	0.26	0.74			
P3 230 C>A		CC	AC		A	C	1.0351	0.0871	0.93
		0.96(56)	0.04(2)		0.02	0.98			

*__Ne__=Effective No. of alleles; __I__=Shannon's Information Index

CONCLUSION

The present study was aimed to explore the polymorphism among *ATF4* gene in Sahiwal cattle. A total of 4 SNPs viz. T352C in intron 1, G379A in exon2, A215T and A230C in exon 3 including 2 transitions and 2 transversions were discovered. The identified SNPs can be used as potential markers for milk yield and composition traits in Sahiwal cattle. However, validation and association of these SNPs is warranted on a larger number of animals and in different herds of Sahiwal cattle.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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