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FUNCTIONAL ENRICHMENT ANALYSIS OF GENES ASSOCIATED WITH NATURALLY OCCURRING HYPERACTIVATED BETA RECOMBINASE RECOGNITION SITES WITH A SIX-BASE SPACER IN THE CAPRA HIRCUS GENOME

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ABSTRACT

Hyperactivated serine recombinases are promising genome engineering tools because they mediate precise DNA recombination without inducing double-strand breaks. However, the genomic distribution of endogenous hyperactivated Beta recombinase recognition sites with six base spacers in goat (Capra hircus) genome has not been systematically investigated. A genome-wide computational search was performed using the Capra hircus ARS1.2 reference genome and a degenerate recognition motif for the hyperactivated Beta recombinase with a 6-bp spacer. Target sites identified on both DNA strands were merged, duplicate loci were removed, and unique sites were annotated using the reference GFF3 file. Functional enrichment of associated genes was assessed using Gene Ontology (GO) and KEGG pathway analyses. A total of 509 unique Beta recombinase recognition sites were identified across the goat genome. Most sites were located in intergenic (52.7%) and intronic (40.7%) regions, while a smaller proportion occurred within CDS (3.1%), promoters (2.2%), and exons (1.4%). Functional enrichment analysis revealed significant GO terms related to cellular regulation, signaling, and small GTPase-mediated signal transduction. GO Molecular Function analysis highlighted protein binding and GTPase regulator activity, whereas GO Cellular Component analysis showed enrichment of cytosolic and cytoplasmic components. KEGG analysis identified axon guidance as the significantly enriched pathway. This study presents the first genome-wide catalogue of naturally occurring hyperactivated Beta recombinase recognition sites with six base spacer in the goat genome. These findings provide a valuable resource for recombinase-mediated genome engineering and future precision genetic improvement in goats.

KEYWORDS: Capra hircus; hyperactivated Beta recombinase; genome-wide identification; site-specific recombinase; genome engineering; Gene Ontology; KEGG.

INTRODUCTION

Since the domestication of livestock, selective breeding has been the primary strategy for improving economically important traits such as productivity, fertility, disease resistance, and adaptability. Initially, breeding programs relied on phenotypic selection, which was subsequently enhanced by marker-assisted selection (MAS). More recently, advances in molecular genetics have enabled the implementation of genome-wide association studies (GWAS) and genomic selection (GS), substantially increasing the accuracy and rate of genetic improvement in livestock populations (Meuwissen et al., 2001; Hayes et al., 2009). Despite these advances, genomic selection depends on naturally occurring genetic variation and may inadvertently increase the frequency of linked unfavorable alleles, thereby limiting long-term genetic gain (Jenko et al., 2015; Gonen et al., 2017).

Genome editing has emerged as a transformative technology that enables precise modification of genomic DNA and offers new opportunities for accelerating livestock improvement. Unlike conventional breeding, genome editing allows targeted insertion, deletion, or substitution of DNA sequences at specific genomic loci, facilitating the direct introduction of desirable alleles into elite breeding populations (Proudfoot et al., 2014; Tan et al., 2013). Successful applications in livestock include the generation of double-muscled animals through MSTN gene editing, production of naturally polled dairy cattle, and development of disease-resistant pigs by modifying genes associated with porcine reproductive and respiratory syndrome (PRRS) and African swine fever susceptibility (Lillico et al., 2013; Proudfoot et al., 2014; Tan et al., 2013; Whitworth et al., 2016).

Current genome-editing technologies are primarily based on programmable nucleases, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR/Cas9 system (Gaj et al., 2013). These technologies introduce DNA double-strand breaks (DSBs), which are subsequently repaired through non-homologous end joining (NHEJ) or homology-directed repair (HDR). Although highly effective, DSB-dependent editing can result in insertion–deletion mutations, chromosomal rearrangements, and off-target modifications, raising concerns regarding editing precision and genomic stability (Wyman and Kanaar, 2006; Cox et al., 2015).

To overcome these limitations, site-specific recombinases (SSRs) have gained attention as an alternative genome engineering platform. SSRs catalyze precise DNA rearrangements by recognizing specific DNA sequences without requiring DNA double-strand breaks (Grindley et al., 2006). Based on their catalytic residues, SSRs are classified into tyrosine recombinases, such as Cre and Flp, and serine recombinases, including φC31 integrase, Gin invertase, Sin recombinase, and Beta recombinase (Grainge and Jayaram, 1999; Smith and Thorpe, 2002). Their ability to mediate accurate DNA integration, excision, and inversion makes them attractive tools for precise genome engineering.

The natural utility of recombinases is restricted by their strict sequence specificity. To expand their genomic targeting range, hyperactivated recombinases with relaxed DNA-binding specificity have been developed through protein engineering and substrate-linked directed evolution (Gaj et al., 2013; Sirk et al., 2014). These engineered enzymes can recognize degenerate endogenous DNA sequences and have been incorporated into programmable genome-editing platforms. A major advance was the development of the RNA-guided recombinase (RGR) system, in which catalytically inactive Cas9 is fused to a hyperactivated recombinase, enabling programmable, site-specific DNA recombination without introducing double-strand breaks (Chaikind et al., 2016; Standage-Beier et al., 2019).

Identification of naturally occurring recombinase recognition sites is a critical prerequisite for developing recombinase-mediated genome engineering strategies. Genome-wide computational analyses provide valuable information on the abundance, chromosomal distribution, and genomic context of endogenous target sites, facilitating the identification of candidate loci for precise DNA integration and functional genome modification (Chaikind et al., 2016). Previous studies have reported genome-wide identification of recombinase recognition sites in livestock genomes, highlighting the potential of engineered recombinases for targeted genome manipulation (Pathak et al., 2019; Pathak et al., 2024).

Goat (Capra hircus) is an economically important livestock species that contributes significantly to global meat, milk, fiber, and skin production. Although genome-editing technologies are increasingly being explored in goats, comprehensive information regarding endogenous recognition sites for hyperactivated Beta recombinase remains unavailable. Therefore, the present study aimed to perform a genome-wide identification and characterization of naturally occurring recognition sites for the hyperactivated Beta recombinase with a 6-bp spacer in the Capra hircus ARS1.2 reference genome. In addition, the chromosomal distribution, genomic features, associated genes, and functional enrichment of these recognition sites were investigated to establish a comprehensive resource for future recombinase-mediated genome engineering and precision livestock breeding.

MATERIALS AND METHODS

Genome sequence retrieval

The reference genome sequence of goat (Capra hircus), assembly ARS1.2 (GCF_001704415.2), was downloaded from the National Center for Biotechnology Information (NCBI) Genome database. The downloaded genome assembly included all assembled autosomes, sex chromosome X, mitochondrial DNA, and unlocalized scaffolds. The genomic FASTA file was used for genome-wide identification of naturally occurring recognition sites for the hyperactivated Beta recombinase, whereas the corresponding GFF3 annotation file was used for genomic feature annotation.

Beta recombinase recognition motif

Sirk et al. (2014) has reported the core sequence for hyperactivated recombinase Beta as: NNNV NNR GT WW AC YNN BNNN. Genome-wide identification of endogenous Beta recombinase target sites was performed using the experimentally validated recognition sequence of the hyperactivated Beta serine recombinase with 6-bp spacer. The degenerate recognition motif used for sequence searching was:

CCNNNNNNNNNNNNNNNNNNNNNNNNNNNNVNNRGTWWACYNNBNNNNNNNNNNNNNNNNNNNNNNNNNNGG

The motif was represented using the International Union of Pure and Applied Chemistry (IUPAC) nucleotide ambiguity codes, where N = A/C/G/T, V = A/C/G, R = A/G, W = A/T, Y = C/T, and B = C/G/T.

Genome-wide identification of Beta recombinase target sites

Genome-wide identification of naturally occurring Beta recombinase recognition sites was carried out using custom Python scripts (Python 3.13). Genomic sequences were parsed using the Biopython package (Bio.SeqIO), and the recognition motif was converted into a regular expression (regex) for efficient genome-wide pattern matching.

Each chromosome and scaffold was scanned independently on both the forward and reverse-complement DNA strands. Reverse-strand matches were converted to genomic coordinates relative to the forward reference genome. Results obtained from both strands were merged to generate a comprehensive list of candidate Beta recombinase recognition sites.

Identification of unique genomic loci

Recognition sites identified on opposite DNA strands frequently represented the same genomic position because the recognition sequence is palindromic. Therefore, duplicate loci were removed by comparing chromosome identifiers and genomic coordinates. Only one representative entry was retained for each genomic position, resulting in 509 unique genomic Beta recombinase target sites, which were used for all downstream analyses.

Genomic annotation of target sites

The unique Beta recombinase target sites were annotated using the corresponding goat genome annotation (GFF3) obtained from the NCBI genome database. A custom Python annotation pipeline was developed to compare the genomic coordinates of each target site with annotated genomic features. Each recognition site was classified into Coding sequence (CDS), Exon, Intron, Promoter and Intergenic region. Promoter regions were defined as the 2 kb upstream of annotated transcription start sites. Recognition sites located outside annotated genes and promoter regions were classified as intergenic. Gene identifiers, gene symbols (when available), chromosome numbers, genomic coordinates, and genomic features were recorded for each annotated locus.

Chromosome-wise distribution analysis and Genomic feature distribution

The chromosomal distribution of Beta recombinase recognition sites was determined by counting the number of unique target sites present on each chromosome. Chromosome accession numbers from the ARS1.2 genome assembly were converted into conventional chromosome names (Chromosomes 1–29, X, mitochondrial genome, and unlocalized scaffolds) to facilitate biological interpretation.

The distribution of Beta recombinase target sites across different genomic features was summarized by calculating the number and percentage of loci located within coding sequences, exons, introns, promoters, and intergenic regions.

Functional enrichment analysis

Genes associated with Beta recombinase recognition sites were extracted from the annotated dataset using custom Python scripts. Duplicate gene entries arising from multiple recognition sites within the same gene were removed to obtain a non-redundant gene list. Intergenic loci lacking annotated genes were excluded from subsequent functional enrichment analyses.

Functional characterization of genes associated with Beta recombinase target sites was performed using the g:Profiler web server (https://biit.cs.ut.ee/gprofiler/) with Capra hircus selected as the reference organism. The non-redundant gene list was queried against multiple functional annotation databases, including Gene Ontology Biological Process (GO:BP),nGene Ontology Molecular Function (GO:MF), Gene Ontology Cellular Component (GO:CC) and Kyoto Encyclopedia of Genes and Genomes (KEGG). Statistical significance of enriched terms was calculated using the built-in g:SCS (Set Counts and Sizes) multiple testing correction method implemented in g:Profiler. Enriched terms with an adjusted P value < 0.05 were considered statistically significant.

RESULTS

Genome-wide identification of hyperactivated Beta recombinase target sites

A genome-wide computational search was performed to identify naturally occurring recognition sites for the hyperactivated Beta recombinase with a 6-bp spacer in the Capra hircus (ARS1.2) reference genome. The initial scan of both forward and reverse DNA strands identified 1,018 candidate target sites. Following the removal of duplicate forward–reverse matches representing identical genomic loci, a total of 509 unique Beta recombinase target sites were retained for subsequent analyses.

Chromosome-wise distribution of Beta recombinase target sites

The 509 unique Beta recombinase target sites were distributed throughout the goat genome, occurring on all assembled autosomes as well as several unlocalized scaffolds. The highest numbers of target sites were identified on chromosomes 2 and 6 (27 sites each), followed by chromosome 8 (26 sites), chromosome 3 (24 sites), and chromosome 19 (23 sites). Chromosomes 4, 7, and 11 each contained 21 target sites, whereas chromosome 5 contained 20 target sites and chromosome 1 contained 19 target sites. The remaining chromosomes contained fewer target sites, and a limited number of recognition sites were identified on unlocalized genomic scaffolds. The widespread chromosomal distribution indicates that naturally occurring Beta recombinase recognition sites are dispersed across the goat genome rather than being restricted to specific chromosomal regions (Figure 1).

Genomic distribution of Beta recombinase target sites

Genomic annotation of the 509 unique target sites revealed that the majority were located within intergenic and intronic regions. Specifically, 268 target sites (52.65%) were located in intergenic regions, whereas 207 sites (40.67%) occurred within introns. Only a small proportion overlapped protein-coding regions, including 16 sites (3.14%) within coding DNA sequences (CDS), 11 sites (2.16%) within promoter regions, and 7 sites (1.38%) within exons. These findings demonstrate that naturally occurring Beta recombinase recognition sites are predominantly associated with non-coding regions of the goat genome (Figure 2).

Gene Ontology enrichment analysis

Following exclusion of intergenic loci, genes associated with Beta recombinase target sites were subjected to Gene Ontology (GO) enrichment analysis (Table 1 and Figure 3).

Biological Process

GO Biological Process analysis identified 25 significantly enriched biological processes. The enriched terms were primarily associated with regulation of cellular signaling, signal transduction, cell communication, developmental processes, and cellular organization. Several signaling-associated genes, including ALK, BMPR1B, NOTCH3, FYN, PLCB1, PRKCB, PRKCE, RASGRF2, and YAP1, contributed to multiple enriched biological processes, indicating that genes containing naturally occurring Beta recombinase recognition sites participate predominantly in regulatory and developmental pathways.

Molecular Function

GO Molecular Function analysis identified significant enrichment of protein binding as the most overrepresented molecular function (adjusted $P = 7.11 \times 10^{-8}$). Additional enriched molecular functions included GTPase regulator activity, nucleoside-triphosphatase regulator activity, enzyme regulator activity, and enzyme activator activity. These results indicate that genes harbouring Beta recombinase recognition sites predominantly encode proteins involved in intracellular signaling, protein interactions, and regulation of enzymatic activity.

Cellular Component

GO Cellular Component enrichment identified nine significantly enriched cellular components. The most significantly enriched components included the cytosol and cytoplasm, followed by cell junction, cell periphery, plasma membrane, synapse, neuron-to-neuron synapse, and the MLL1 and MLL1/2 protein complexes. These results suggest that proteins encoded by Beta target-associated genes are primarily localized to intracellular and membrane-associated compartments involved in signal transduction and intercellular communication.

KEGG pathway enrichment analysis

KEGG pathway enrichment analysis identified a single significantly enriched pathway, Axon guidance, represented by ten genes: ABLIM1, BMPR1B, EFNA1, EFNA5, EPHA4, FYN, NGEF, NTNG2, PLXNA4, and PLXNC1. These genes are involved in neuronal development, axonal navigation, and cell signaling, suggesting that naturally occurring Beta recombinase recognition sites are present within genes participating in conserved developmental signaling pathways.

Overall, the genome-wide survey demonstrates that naturally occurring recognition sites for the hyperactivated Beta recombinase are widely distributed throughout the goat genome, with a predominance in non-coding genomic regions. Functional enrichment analyses further indicate that genes containing these target sites are primarily associated with intracellular signaling, developmental regulation, protein interaction networks, and neuronal communication pathways.

DISCUSSION

The present study provides the first comprehensive genome-wide survey of naturally occurring recognition sites for the hyperactivated Beta recombinase with a 6-bp spacer in the Capra hircus genome. Using a computational motif-search strategy, 509 unique endogenous Beta recombinase recognition sites were identified across the ARS1.2 reference genome. These findings demonstrate that sequences compatible with the engineered Beta recombinase recognition motif are widely distributed throughout the goat genome, highlighting the potential applicability of recombinase-mediated genome engineering in this economically important livestock species.

Engineered serine recombinases have emerged as attractive alternatives to nuclease-based genome-editing systems because they mediate precise DNA recombination without generating DNA double-strand breaks (Sirk et al., 2014; Chaikind et al., 2016). Unlike CRISPR/Cas9 and other programmable nucleases, which depend on cellular DNA repair pathways and may introduce insertion–deletion mutations or chromosomal rearrangements, recombinase-based systems catalyze site-specific DNA integration, deletion, or inversion with predictable outcomes (Gaj et al., 2013; Standage-Beier et al., 2019). Therefore, comprehensive identification of endogenous recombinase recognition sites is an essential step toward evaluating the feasibility of recombinase-mediated genome engineering in livestock genomes.

The identified Beta recombinase recognition sites were distributed across all goat autosomes and the X chromosome, although the number of sites varied among chromosomes. This variation is expected because chromosome length, nucleotide composition, and local sequence organization influence the frequency of degenerate recognition motifs within the genome. Similar chromosome-dependent distributions of engineered recombinase target sites have been reported in previous genome-wide studies of livestock genomes (Pathak et al., 2019; Pathak et al., 2024). The widespread occurrence of endogenous recognition sites suggests that the goat genome contains numerous potential targets for future site-specific recombination strategies.

Genomic annotation revealed that most Beta recombinase target sites were located within intergenic (52.7%) and intronic (40.7%) regions, whereas only a small proportion occurred in coding sequences, exons, and promoter regions. The predominance of intergenic and intronic sites is consistent with the structural organization of mammalian genomes, in which non-coding DNA constitutes the majority of genomic sequence (ENCODE Project Consortium, 2012). Intergenic loci may represent promising candidates for targeted DNA integration because they are less likely to disrupt coding regions or essential regulatory elements. Likewise, intronic regions have frequently been explored for transgene insertion, provided that gene function and RNA splicing are not adversely affected. Nevertheless, experimental validation remains necessary to determine whether individual loci function as genomic safe-harbour sites.

Although relatively few recognition sites were detected within promoters and coding regions, these loci may have particular importance for functional genomics. Site-specific recombination within regulatory or coding sequences could facilitate targeted gene modification, conditional gene activation, or precise replacement of disease-associated alleles. However, because such modifications may directly influence gene expression or protein function, these candidate loci require careful functional evaluation before practical application.

Functional enrichment analysis of genes associated with Beta recombinase target sites demonstrated significant enrichment of Gene Ontology Biological Process terms related to positive regulation of cellular processes, regulation of cell communication, regulation of signaling, and small GTPase-mediated signal transduction. Small GTPases regulate diverse biological processes, including cell proliferation, differentiation, intracellular trafficking, cytoskeletal organization, and developmental signaling pathways (Bos et al., 2007). The enrichment of signaling-related biological processes indicates that genes harboring endogenous Beta recombinase recognition sites participate in fundamental cellular regulatory networks rather than representing random genomic elements.

Gene Ontology Molecular Function analysis further revealed enrichment of protein binding, GTPase regulator activity, and nucleoside-triphosphatase regulator activity, suggesting that many associated genes encode regulatory proteins involved in intracellular signaling and molecular interactions. Similarly, Gene Ontology Cellular Component analysis identified significant enrichment of cytosolic and cytoplasmic components, indicating that these proteins primarily function within intracellular compartments where signaling complexes and regulatory pathways are assembled.

KEGG pathway analysis identified axon guidance as the significantly enriched pathway. Although classically associated with nervous system development, axon guidance signaling also contributes to cell migration, cytoskeletal remodeling, angiogenesis, immune responses, and tissue morphogenesis in multiple organ systems (Kolodkin and Tessier-Lavigne, 2011). Therefore, the enrichment of this pathway likely reflects the involvement of Beta recombinase-associated genes in conserved signaling mechanisms rather than exclusively neuronal functions.

One important observation of this study is that naturally occurring Beta recombinase recognition sites are associated with genes participating in diverse biological processes rather than being concentrated within a single functional category. This broad distribution expands the potential utility of engineered Beta recombinase for applications including targeted transgene integration, gene replacement, conditional genome engineering, and functional genomics in goats. However, the present investigation was entirely computational, and the biological activity of the predicted recognition sites remains

to be experimentally validated. Future studies should evaluate recombination efficiency, genomic accessibility, chromatin context, and off-target recombination using cell-based and in vivo experimental systems.

In conclusion, this study provides the first comprehensive genome-wide catalogue of endogenous recognition sites for the hyperactivated Beta recombinase in the Capra hircus genome. The identified recognition sites, together with their genomic annotations and functional characterization, constitute a valuable resource for the development of recombinase-mediated genome engineering strategies in goats. These findings provide a foundation for future studies aimed at precise genome modification, targeted transgene integration, and functional analysis of economically important genes in livestock.

Table 1. Top enriched Gene Ontology (GO) terms and KEGG pathway associated with genes harboring naturally occurring hyperactivated Beta recombinase recognition sites in the Capra hircus genome

Source	Term ID	Enriched term	No. of genes	Adjusted *P* value
GO:BP	GO:0048522	Positive regulation of cellular process	18	1.38×10^{-4}
GO:BP	GO:0010646	Regulation of cell communication	17	4.34×10^{-4}
GO:BP	GO:0023051	Regulation of signaling	17	4.45×10^{-4}
GO:BP	GO:0007264	Small GTPase-mediated signal transduction	10	6.05×10^{-4}
GO:BP	GO:0048518	Positive regulation of biological process	20	6.29×10^{-4}
GO:MF	GO:0005515	Protein binding	55	7.11×10^{-8}
GO:MF	GO:0030695	GTPase regulator activity	18	1.50×10^{-5}
GO:MF	GO:0060589	Nucleoside-triphosphatase regulator activity	18	1.57×10^{-5}
GO:MF	GO:0030234	Enzyme regulator activity	21	2.65×10^{-4}
GO:MF	GO:0008047	Enzyme activator activity	19	4.18×10^{-4}
GO:CC	GO:0005829	Cytosol	63	3.26×10^{-6}
GO:CC	GO:0005737	Cytoplasm	125	1.89×10^{-5}
GO:CC	GO:0030054	Cell junction	33	1.41×10^{-3}
GO:CC	GO:0071944	Cell periphery	68	3.05×10^{-3}
GO:CC	GO:0045202	Synapse	24	7.36×10^{-3}
KEGG	KEGG:04360	Axon guidance	10	6.24×10^{-3}

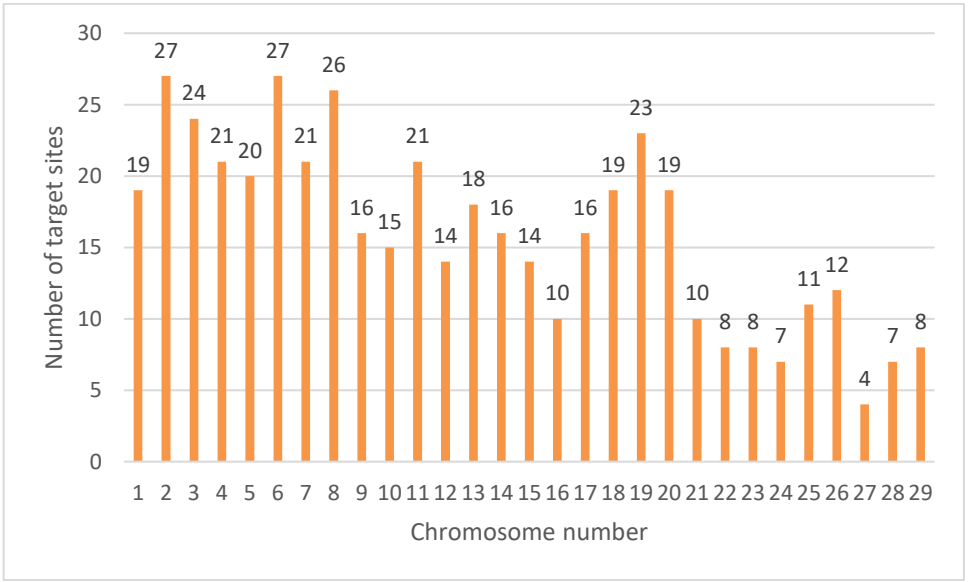

Figure 1. Chromosome-wise distribution of Hyperactivated Beta Recombinase target sites with six base spacers in goat genome.

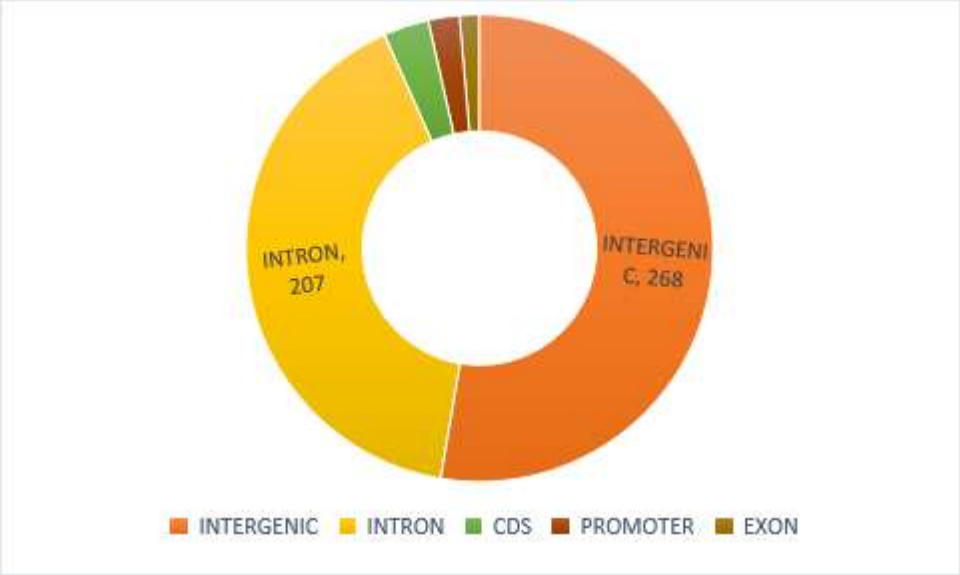

Figure 2: Genomic feature distribution of Hyperactivated Beta Recombinase target sites with six base spacers in Goat genome.

The bar plot shows the most significantly enriched Gene Ontology (GO) terms and KEGG pathway among genes associated with naturally occurring hyperactivated Beta recombinase target sites in the Capra hircus genome. Bar length indicates enrichment significance as -log10(adjusted P value). Adjusted P values were calculated using the g:SCS multiple testing correction method implemented in g:Profiler (organism: Capra hircus). Only terms with adjusted P < 0.05 are displayed.

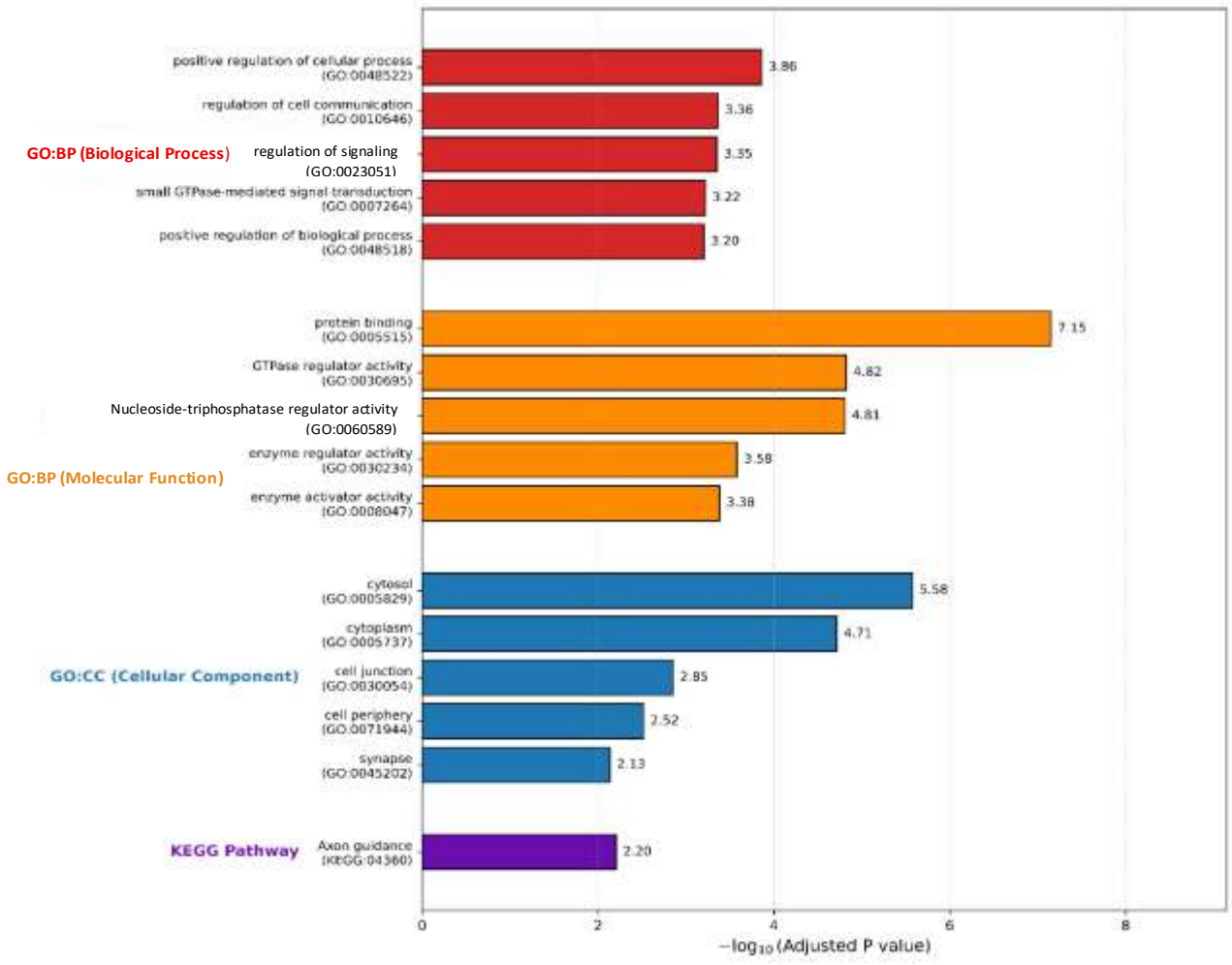

Figure 3. Combined Functional Enrichment Analysis of Genes associated with Naturally occurring yperactivated Beta Recombinase target sites in the *Capra hircus* genome.

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