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Identification and characterization of a novel splice variant of the *PLCζ1* gene in bull testis tissues

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ABSTRACT. Phospholipase C zeta 1 (PLCζ1), which transcribes a key protein, has an important function in oocyte activation and embryo development because PLCζ1 can trigger a series of intracellular Ca^{2+} oscillations in mammals. In this study, a novel splice variant in the testis tissues of adult and fetal Chinese Holstein bulls was characterized by reverse transcription-polymerase chain reaction (RT-PCR) and sequencing analysis. The novel splice variant *PLCζ1*-sv1 was derived from the *PLCζ1* complete transcript (*PLCζ1*-complete) by alternative splicing; the alternative splicing pattern exhibited alternative 5'-splice sites. The full-length transcript, *PLCζ1*-complete, is the main transcript found in fetal and adult cow testis tissue. Quantitative real-time PCR (qPCR) analysis demonstrated that the expression levels of the *PLCζ1*-complete transcript were significantly higher than those of the *PLCζ1*-sv1 splice variant in bovine testis tissues. PLCζ1 protein sequencing analysis showed that the amino acids at positions 453 to 457 were

deleted in PLCζ1-sv1, thereby terminating transcription prematurely. In summary, this study provided information to elucidate the structure and function of the bovine *PLCζ1* gene.

Key words: *PLCζ1* gene; Chinese Holstein bull; Splice variant; Quantitative real-time polymerase chain reaction; Protein sequencing analysis

INTRODUCTION

Bovine sperm-specific phospholipase C zeta 1 (*PLCζ1*), mapped on SSC5q11-12, comprises 14 exons and encodes a protein with 634 amino acids, which is a key protein during sperm-egg fusion. The *PLCζ1* gene sequence have been cloned in bovine, mouse, human, monkey, chicken, pig, medaka fish, rat, and hamster (Cox et al., 2002; Saunders et al., 2002; Coward et al., 2005; Yoneda et al., 2006; Ito et al., 2008; Young et al., 2009). PLCζ1 contains X and Y catalytic domains that are common to all phosphoinositide-specific PLCs, two pairs of EF hand domains that are possibly involved in Ca^{2+} binding, a C2 domain, which appears to bind to phosphoinositide-containing lipids (Katan, 1998; Rebecchi and Pentyala, 2000), and a putative nuclear localization signal in the linker region between the X and Y catalytic domains. Similar to other PLC family members (Rebecchi and Pentyala, 2000), the PLCζ1 protein catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate and the production of 1,4,5-inositol trisphosphate; diacylglycerol (Kouchi et al., 2005; Nomikos et al., 2005) is necessary to initiate and maintain Ca^{2+} oscillations, which are also observed during fertilization in normal embryo development (Brind et al., 2000; Jellerette et al., 2000; Wu et al., 2001). Ca^{2+} oscillations are observed in experiments in which PLCζ1 is introduced to somatic cells by microinjection (Berrie et al., 1996) and to cell-free systems, such as sea urchin egg homogenates (Jones et al., 1998). Despite the seemingly conserved nature of PLCζ1 among different species, small sequence differences are sufficient to affect structural activity significantly in a species-specific manner (Cox et al., 2002; Ross et al., 2008). Considering these results, we focused on domain regions that determine the specificity of the catalytic activity of bovine PLCζ1.

Alternative splicing has a very important function in protein diversity; therefore, alternative splicing may result in discrepancy between gene expression and individual complexity (Garcia-Blanco et al., 2004; Garcia-Blanco, 2006). This phenomenon is universal to numerous protein-coding genes of multicellular organisms. Several studies have indicated that alternative splicing should be determined (Ast, 2004; Chacko and Ranganathan, 2009). In general, alternatively spliced exons have 5'- and 3'-splice site motifs that differ significantly from the normal motifs. In addition to these splice sites, exons are defined by *cis*-acting regulatory elements, which have been divided into four functional categories: exonic splicing enhancers, exonic splicing silencers, intronic splicing enhancers (also known as intronic activators of splicing), and intronic splicing silencers. These *cis*-acting elements interact directly or indirectly with *trans*-acting activators or repressors of splicing.

In mice, a novel alternative splicing variant of the *PLCζ1* gene that contains a 30-bp insertion following base 159 of the wild-type open reading frame is present in the nucleotide sequence database (accession No. AK006672), which results in the expression

of a different protein (Kouchi et al., 2005; Nomikos et al., 2005). During the cloning of the human and rat *PLCζ1* from testis, Saunders et al. (2002) observed several other incompletely spliced cDNAs that contained internal stop codons. However, the splice variants of bovine *PLCζ1* and *PLCζ1* mRNA expression in bulls have not been characterized yet. In this study, *PLCζ1* splice variants in testis tissues of bulls and the differential expression pattern in adult and fetal cows were identified.

MATERIAL AND METHODS

Tissue sample collection

Testis tissue samples were aseptically collected from eight Chinese Holstein bulls (four adult bulls were three to six years old, and four fetal bulls were newly born). The tissues were snap frozen in liquid nitrogen in a slaughterhouse and transported to our laboratory.

Isolation of total RNA and cDNA synthesis

Total RNA was isolated from 0.1 to 0.2 g testis tissue using TRIzol reagent (Tiangen, China) according to manufacturer instructions. RNA was digested by RNase-free DNase (Takara, Japan) to remove genomic DNA contamination. The RNA concentration was measured using a Biophotometer (Implen, Germany). RNA integrity was determined by electrophoresis on a 1% agarose gel. The RevertAid™ First-Strand cDNA Synthesis kit (Fermentas, Canada) was used to convert approximately 1 μg RNA from each sample to cDNA according to manufacturer specifications.

Identification of *PLCζ1* gene splice variants

Primers P_{A-C} (Table 1) were designed and used to amplify the coding region of the bovine *PLCζ1* gene according to the reference sequence (GenBank accession No. NM_001011680). Polymerase chain reaction (PCR) products were purified and subcloned in a pEASY-T3 cloning vector, which was transformed into *Escherichia coli* DH5α competent cells. The cells were subsequently propagated in Luria broth medium overnight at 37°C. The plasmids were then extracted using a Plasmid Miniprep kit (Biomiga), and the cDNA segment inserts were sequenced using an ABI PRISM 3730 DNA sequencer (Applied Biosystems) and BigDye Terminator v3.1 Sequencing kit (Shanghai Sangon, China). Multiple sequence alignments were performed using the DNAMAN v5.2.2 software (LynonBiosoft) to identify possible splice variants.

Protein structure predictions of different PLCζ1 splice variants

The secondary protein structure of bovine PLCζ1 splice variants was predicted according to http://www.predictprotein.org/; the three-dimensional protein structure was predicted according to http://swissmodel.expasy.org/. Alterations in the binding site of the splicing factor were predicted using ESEfinder 3.0 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi).

Table 1. Primers used in this study.

Primers	Primer sequences (5'→3')	Annealing temperatures (°C)	Fragment size (bp)	Target
P_A	AGTGGCAACAGCGGACGA CCCCAAATGTAGCGAGCAAA	60	943	*PLCζ1* gene cDNA amplification (position 136 to 1078)
P_B	CGATACATTCACCAGCAAG CGAAGAGCCATAGGGACA	58	768	*PLCζ1* gene cDNA amplification (position 925 to 1692)
P_C	GAAACGGGTGGGCGGAAT AGGGAAGCGGGCTCAAGAC	60	810	*PLCζ1* gene cDNA amplification (position 1338 to 2147)
PLCζ1-complete	CAGGAGATTCATTACCAGAG AAGTTCCATAGGGACACC	60	138	*PLCζ1* gene qRT-PCR
PLCζ1-sv1	TGTAGGTTGTCAGATGGTGG CTCGTAGGAAGCGTGGTT	60	122	*PLCζ1* gene qRT-PCR
β-actin	TTAGCTGCGTTACACCCTT TGTCACCTTCACCGTTCC	60	160	β-actin gene qRT-PCR

Nucleotide sequences are numbered relative to the translational start site.

Quantitative real-time PCR analysis of *PLCζ1* splice variants

To determine the relative expression levels of the *PLCζ1*-complete and *PLCζ1*-sv1 transcripts in bovine testis, we performed quantitative real-time PCR (q-PCR) using SYBR Green PCR Master Mix (TaKaRa, Dalian, China). The q-PCR mixture with a final volume of 20 µL consisted of the following components: 10.0 µL 2X SYBR® Pre-mix Ex Taq™, 50 ng cDNA (1.5 µL), 0.5 µL 10 µM sense and antisense primers, and 7.5 µL ddH_2O. After initial denaturation at 95°C for 30 s, the following incubation protocol was used: 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 30 s. PCR was carried out using a LightCycler® 480 II Real-time PCR system. Each sample was run in triplicate. The q-PCR primers of the *PLCζ1* gene and housekeeping internal control gene (β-actin gene; GenBank accession No. BT030480.1) are listed in Table 1. The relative expression was determined as described by Ju et al. (2012).

Statistical analysis

PLCζ1 mRNA expression was quantified relative to a housekeeping gene, and the results are reported as fold-change. Analysis was performed using SPSS v.10.0 (SPSS Inc.). The means of the two groups were compared using a paired-sample Student *t*-test. $P < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Identification of a novel *PLCζ1* gene splice variant in bull testis tissues

mRNA splicing occurs during as post-transcriptional step, in which certain segments of pre-mRNAs are spliced, and the remaining exons are arranged to generate a mature mRNA product. As a result, the same genetic locus can produce various transcripts, thereby producing hundreds, thousands, or even tens of thousands of protein isoforms (Graveley, 2001). The number of proteins expressed by an organism can considerably exceed the number of genes contained in a genome. Among the bovine genes, 21% undergo alternative splicing events. In this study, a novel splice variant of the *PLCζ1* gene was found in bull testis tissues.

The full-length *PLCζ1* transcript (including three fragments) was amplified using cDNA from the testis tissues as a template. To further identify the sequence, we used purified PCR products for subcloning and sequencing. We compared the sequence obtained from each sample with the bovine *PLCζ1* genomic sequence (GenBank accession No. NC_007303.4) and with the *PLCζ1* mRNA sequence (GenBank accession No. NM_001011680). We identified a novel transcript, which we named *PLCζ1*-sv1 (splice variant; Figure 1). The complete transcript was designated as *PLCζ1*-complete. On the basis of isoform frequencies, *PLCζ1*-complete was considered to be the major transcript comprising 90% of 50 sequenced colonies. In contrast, five other transcripts were identified as *PLCζ1*-sv1 with a frequency of 10% (5/50). The *PLCζ1*-sv1 sequence was submitted to the National Center for Biotechnology Information (GenBank accession No. JX442544).

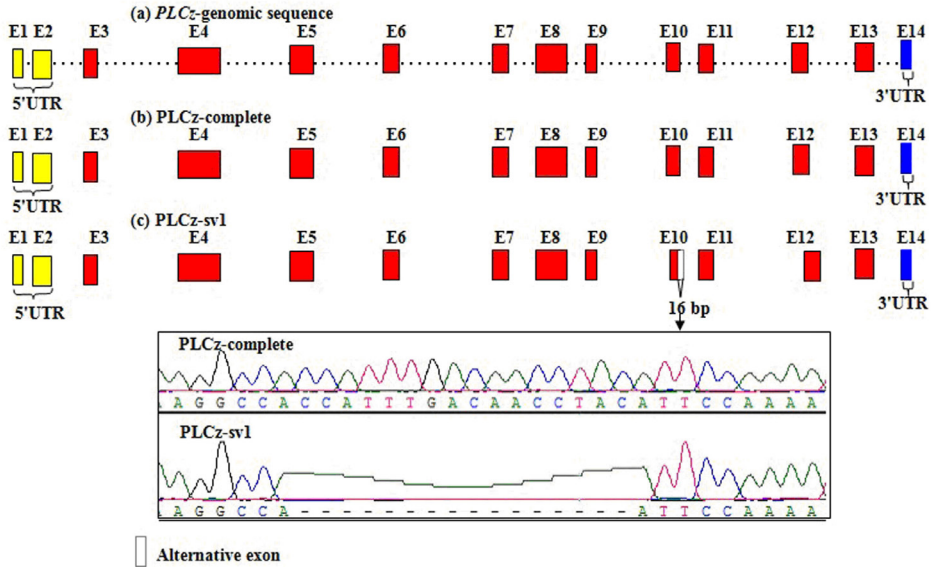


Figure 1. Diagrammatic maps of bovine *PLCζ1* genomic structure and alternative splicing patterns of *PLCζ1* transcripts. Boxes in different colors represent the exons; yellow boxes represent the 5'-untranslated regions (UTRs), including exon 1 and part of exon 2; blue boxes represent the 3'-UTRs containing part of exon 14; and red boxes represent the coding region. The alternatively spliced region is shown as a white box.

Transcription pattern of the novel splice variant

In this study, sequence alignment demonstrated that the splice variant *PLCζ1*-sv1 was truncated at the 3'-end of exon 10 and lacked 16 nucleotides (Figure 1). The alternative splicing pattern of *PLCζ1*-sv1 is found in the alternative 5'-splice site (Keren et al., 2010). Sixteen missense nucleotide deletions correspond to the deleted amino acids located in the primary structure (amino acids at positions 453 to 457); as a result, a transcription termination codon is encountered at an early stage as revealed by bioinformatic analysis. *PLCζ1*-complete encodes a complete protein containing 634 amino acids, whereas *PLCζ1*-sv1 putatively encodes 454 amino acids and lacks 180 amino acids (Figure 2). The deleted section is located in a highly conserved catalytic structure of the Y domain.

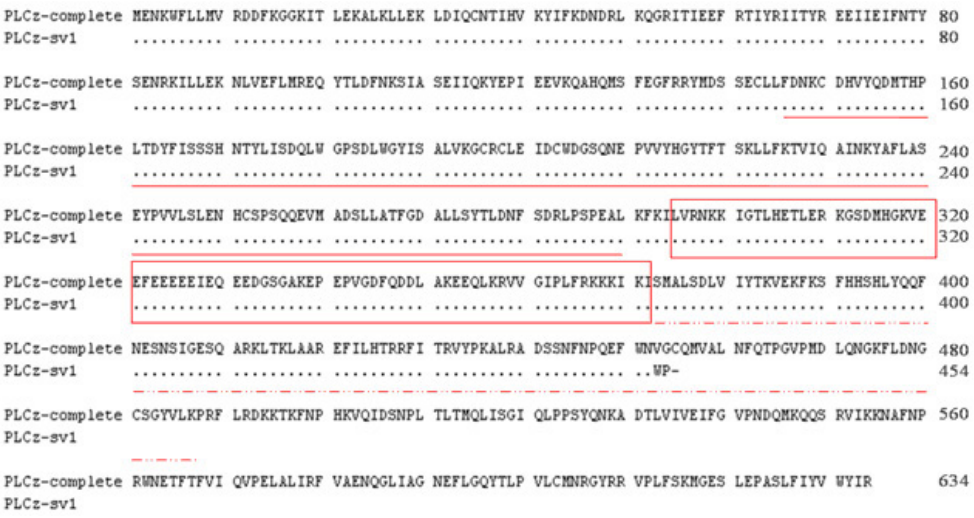


Figure 2. Predicted amino acid sequences of PLCζ1-complete and PLCζ1-sv1. The X catalytic domain is represented by a continuous red underline. The X-Y linker domain is represented by a boxed region in red. The Y catalytic domain is represented by a red broken underline. The EF hand and C2 domains correspond to unmarked N- and C-terminal regions, respectively.

Difference between the predicted protein structures of PLCζ1-complete and PLCζ1-sv1

The results demonstrated that bovine PLCζ1-complete was a mixed-type protein, in which α-helices, β-pleated sheets, and random coils account for approximately 29.3, 16.1, and 54.6% of the protein, respectively. Hydrophilic and hydrophobic regions were also widely distributed in the secondary structure. However, the protein structure of PLCζ1-sv1 was composed of the following: 38.1% α-helices, 8.8% β-pleated sheets, and 53.1% random coils. These results showed that the 16 missense mutations in *PLCζ1*-sv1 altered the secondary protein structure.

The domain, secondary structures, and three-dimensional structures of bovine PLCζ1-complete and PLCζ1-sv1 proteins were predicted by SWISS-MODEL (Figures 3 and 4). PLCζ1-complete contains six domains: calcium-binding EF-hand domain (PS50222: 35-70); phosphatidylinositol-specific phospholipase C, X region domain (PF00388: 156-300; PS50007: 155-299); phosphatidylinositol-specific phospholipase C, Y region domain (PF00387: 375-492; PS50008: 376-492); C2 calcium-dependent membrane-targeting domain (PF00168: 512-598; PS50004: 511-598); C2 calcium/lipid-binding region, CaLB domain (SSF49562: 507-634); and phosphatidylinositol-specific phospholipase C, X and Y box domain (PD001202: 166-504). However, the regions encoding the C2 calcium-dependent membrane-targeting domain and the C2 calcium/lipid-binding region (CaLB domain) were completely spliced out of the *PLCζ1*-sv1 transcript (Figures 3a and 4a). In addition to the differences in the secondary structures of PLCζ1-complete and PLCζ1-sv1 (Figures 3b and 4b), differences in the three-dimensional structures were predicted. The QMEAN-scores of PLCζ1-complete and PLCζ1-sv1 were 0.470 (mean Z-score: -4.98) and 0.520 (mean Z-score: -4.15), respectively (Figures 3c and 4c).

These results demonstrated that the structure of the product encoded by *PLCζ1*-sv transcripts underwent significant changes, thereby forming unusual protein conformations. Two forms of bovine PLCζ1 have been identified; such forms differ from those showing a conservative deletion in the C2 domain. Although the deleted C2 domain exhibits enzymatic activity, this domain fails to cause Ca^{2+} oscillations, possibly because of the loss of a lipid-targeting domain, which is required to direct PLCζ1 to its substrate (Saunders et al., 2007). Deregulated splice variant expression has been identified as the cause of numerous genetic disorders; certain forms of cancer have been linked to an unbalanced isoform expression of genes involved in cell cycle regulation or apoptosis (Faustino and Cooper, 2003; Giesecke et al., 2010).

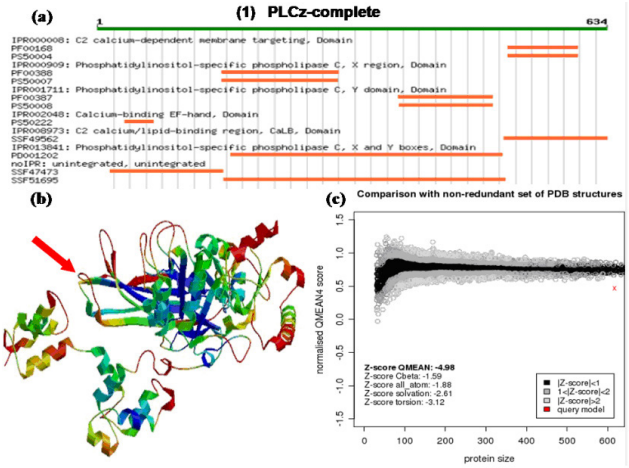


Figure 3. Putative protein structure and functional domains of bovine PLCζ1-complete with the QMEAN results. (**a**) Putative functional domains of bovine PLCζ1-complete. (**b**) Predicted protein structure of bovine PLCζ1-complete. (**c**) The total QMEAN-score of PLCζ1-complete is 0.470 (mean Z-score: -4.98; estimated model reliability between 0 and 1). The red x indicates the differences in the three-dimensional structures of PLCζ1-complete and PLCζ1-sv1.

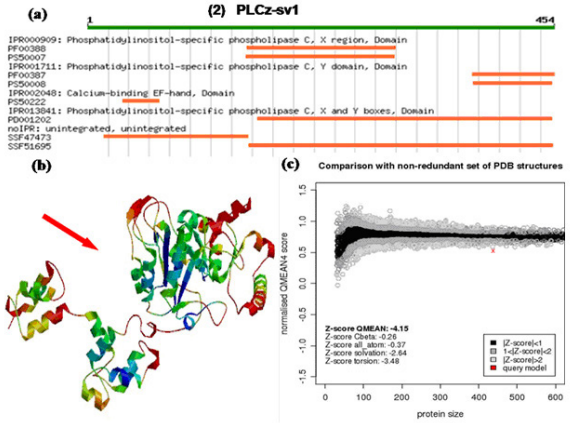


Figure 4. Putative protein structure and functional domains of bovine PLCζ1-sv1 with the QMEAN results. (**a**) Putative functional domains of bovine PLCζ1-sv1. (**b**) Predicted protein structure of bovine PLCζ1-sv1. (**c**) The total QMEAN-score of PLCζ1-sv1 is 0.520 (mean Z-score: -4.15; estimated model reliability between 0 and 1). The red x indicates the differences in the three-dimensional structures of PLCζ1-sv1 and PLCζ1-complete.

Expression of the two transcripts in testis tissues of Chinese Holstein bulls

Northern and Western blot analyses confirmed that PLCζ1 is expressed mainly in the testis (Saunders et al., 2002). In this study, the samples were collected from the testis tissue of fetal and adult bovine to investigate the expression levels of the two *PLCζ1* transcripts. Differential expression of *PLCζ1*-complete and *PLCζ1*-sv1 mRNAs in these adult and fetal cow testis tissues was investigated by q-PCR using the specific primers shown in Table 1. Differential expression of *PLCζ1*-complete transcript between fetal and adult tissues is shown in Figure 5. *PLCζ1*-complete was abundantly expressed in adult testis but was marginally detected in fetal testis ($P < 0.05$). However, no significant differences in *PLCζ1*-sv1 mRNA expression were observed in adult and fetal bulls.

The expression level of the *PLCζ1*-complete transcript was significantly higher than that of the *PLCζ1*-sv1 transcript in adult bull testis ($P < 0.05$). The *PLCζ1*-complete transcript is dominantly expressed by the *PLCζ1* gene. Although the *PLCζ1*-sv1 transcript, which was generated by RNA splicing, was found in bovine testis, defective pre-mRNA splicing may generate non-functional and toxic proteins with formidable effects on cell homeostasis by removing certain parts of exons. Aberrant RNA splicing is associated with many diseases (Wang et al., 2003; Nagao et al., 2005). Therefore, studies should be conducted to investigate the association between a novel *PLCζ1* transcript and bovine reproduction-related diseases.

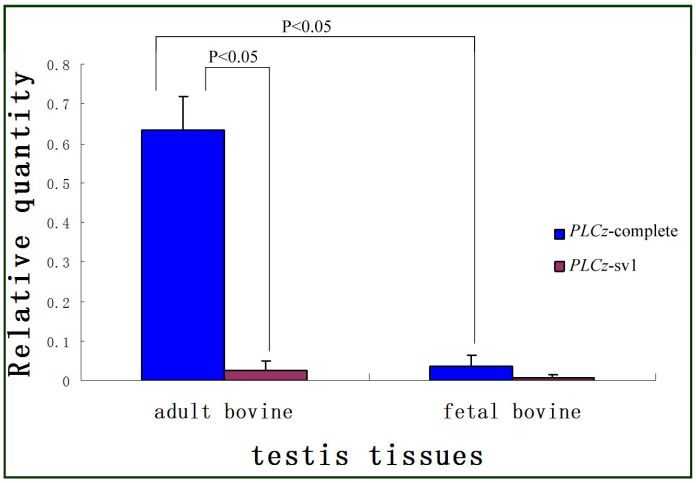


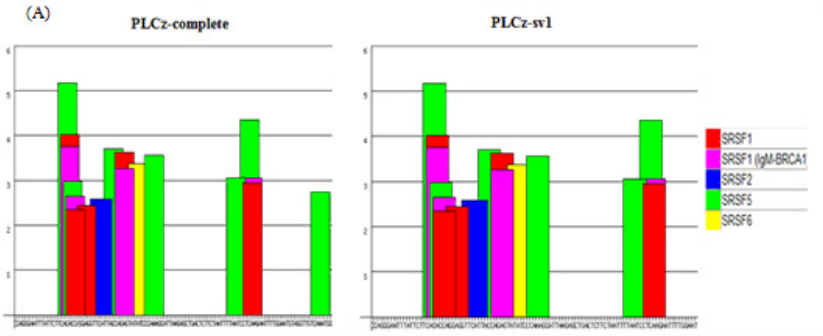
Figure 5. Relative expression of the two transcripts of the *PLCζ1* gene obtained by quantitative real-time polymerase chain reaction (PCR) of bovine testis. Blue represents the relative expression of the bovine *PLCζ1*-complete transcript in adult and fetal bovine testis tissues; purple represents the relative expression of the bovine *PLCζ1*-sv1 transcript in adult and fetal bovine testis tissues. The vertical bars represent standard error.

Exon splicing enhancer prediction of the two transcripts

The mechanism by which the novel splice variant *PLCζ1*-sv1 exists is unknown. Splicing signals, such as the 5'-splice site, 3'-splice site, branch point, and polypyrimidine

tract site, are required for pre-mRNA splicing (Lee et al., 2012). Additional RNA sequence elements, such as exon splicing enhancers, are also important to initiate the pre-mRNA splicing of many genes. Exon splicing enhancers activate exons and promote their inclusion in mature transcripts. Several different exon splicing enhancer families have been recognized; among these families, purine-rich sequences that function by recruiting members of the serine/arginine-rich family of splicing factors (e.g., ASF/SF2) are widely studied. Exon splicing enhancers have been implicated in the regulation of alternative splicing and possibly define many constitutive exons.

Using ESEfinder 3.0, we found that alternatively spliced exons contain RNA sequences that are important for pre-mRNA splicing; these sequences are similar to exons that undergo regular splicing (Figure 6). The *PLCζ1*-sv1 transcript did not change the binding sites of SRSF1, SRSF2 (IgM-BRCA1), SRSF3, SRSF4, and SRSF6. However, the PLCζ1-sv1 transcript did not contain potential binding sites for the splicing factor SRSF5, which differed from the *PLCζ1*-complete transcript in terms of auxiliary splicing proteins (Figure 6). In addition to the essential splicing signals on the pre-mRNA, enhancers or inhibitors/silencers that are found on pre-mRNA are particularly important to regulate alternative splicing.



(B)

Splicing factor	Biding site and score of PLCz-complete	Biding site and score of PLCz-sv1
SRSF1	CACACCA(4.01); CACCAGG(2.35); AGGAGGT(2.43);CAGAGTA(3.62); CTCAAGA(2.94)	CACACCA(4.01); CACCAGG(2.35); AGGAGGT(2.43);CAGAGTA(3.62); CTCAAGA(2.94)
SRSF1(IgM-BRCA1)	CACACCA(3.75); CACCAGG(2.65); CAGAGTA(3.27); CTCAAGA(3.06)	CACACCA(3.75); CACCAGG(2.65); CAGAGTA(3.27); CTCAAGA(3.06)
SRSF2	GTTCATTA(2.58)	GTTCATTA(2.58)
SRSF5	TCACACC(5.18); ACACCAG(2.97); TTACCAG(3.71); CCAAAGC(3.56); TTAATCC(3.05);CCTCAAG(4.35); TCAAATC(2.73)	TCACACC(5.18); ACACCAG(2.97); TTACCAG(3.71); CCAAAGC(3.56); TTAATCC(3.05);CCTCAAG(4.35);
SRSF6	TATATC(3.37)	TATATC(3.37)

Figure 6. Potential exonic splice enhancer motif threshold scores of the two transcripts of the *PLCζ1* gene. The *PLCζ1*-complete sequence: ccagggaatttattcttcacaccaggaggttcattaccagagtatatcccaaagcattaagagctgactcttctaatttt aatcctcaagaattttggaatgtaggttgtcaaatgg. The *PLCζ1*-sv1 sequence: ccagggaatttattcttcacaccaggaggttcattaccagagtatatc ccaaagcattaagagctgactcttctaattttaatcctcaagaattttggaat. (**A**) Red, purple, blue, green, and yellow represent the exonic splice enhancers of *PLCζ1*, including SRSF1, SRSF2, SRSF3, SRSF4, and SPSF5, respectively. (**B**) The novel *PLCζ1*-sv1 transcript contains an additional SRSF5 site (TCAAATC).

A novel splice variant, *PLCζ1*-sv1, was identified in bovine testis tissues. The full-length *PLCζ1*-complete transcript is the main transcript present in fetal and adult bull testis tissues. *PLCζ1*-complete transcript expression was significantly higher than that of the *PLCζ1*-sv1 transcript. The mRNA expression levels of the *PLCζ1*-complete transcript in adult bull testis tissues were higher than those in fetal bull testis tissues. The expression levels of the splice variant *PLCζ1*-sv1 in adult and fetal bull testis tissues did not significantly differ. However, the predicted three-dimensional structures of the products of the two transcripts showed that the PLCζ1-sv1 structure varied from that of PLCζ1-complete. Therefore, *PLCζ1*-complete may be used as a suitable transcript to initiate responses to the environment and bovine reproduction. This study may facilitate a better understanding of the structure and function of the *PLCζ1* gene. Further studies should be conducted to determine the reason why novel splice variants of *PLCζ1* are present in bovine.

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