Cloning and functional analysis of goat SWEET1

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ABSTRACT. SWEETs are a recently discovered class of sugar transporters that mediate glucose uptake in the intestine and mammary glands. Our objectives were to clone goat SWEET1 and conduct a functional analysis of its effect on glucose efflux in goat mammary gland epithelial cells. We cloned and sequenced the goat SWEET1 gene from goat mammary glands, then conducted an analysis of the structure of goat SWEET1, including a prediction of the transmembrane helices and potential N-glycosylation sites. To investigate the biological function of goat SWEET1, we also generated goat SWEET1-transfected goat mammary gland epithelial cells using the eukaryotic expression vector pcDNA3.1-gSWEET1. Goat SWEET1 overexpression can reduce glucose absorption in mammary gland epithelial cells with increasing expression of GLUT1, GLUT4, and GLUT12, which may be attributed to glucose efflux arising from the leading role played by goat SWEET1. This study will improve our understanding of the glucose balance in mammary glands and the level of glucose in milk.

Key words: Goat; SWEET1; Mammary gland epithelial cells; Glucose transporters

INTRODUCTION

Cellular uptake and efflux of sugars across the plasma membrane is one of the most important processes for growth and development, and is always mediated by membrane-associated carrier proteins: Na^+/glucose co-transporters (SGLTs) and facilitative glucose transporters (GLUTs) (Walmsley et al., 1998; Scheepers et al., 2004). Recently, a new class of glucose transporters, named SWEETs, has been identified in *Arabidopsis thaliana* (Chen et al., 2010; Chen et al., 2012). The SWEET family is conserved in bacteria, plants, and humans, and is not structurally related to either the GLUT or SGLT family (Baker et al., 2012; Saudek, 2012; Chardon et al., 2013). SWEET1 participates in glucose efflux activity in *A. thaliana* (Chen et al., 2010). However, the physiological role of SWEETs in mammals remains uncertain (Zhao, 2014).

In this study, we cloned the goat SWEET1 gene (gSWEET1) coding DNA sequence (CDS) region and analyzed the similarities in the SWEET1 gene between different species. Moreover, we also evaluated the biological function of gSWEET1 in glucose efflux activity in goat mammary gland epithelial (GMGE) cells.

MATERIAL AND METHODS

### Cloning gSWEET1 and construction of the expression vector

Total RNA was isolated using a GeneJET™ RNA Purification Kit (Fermentas, Sweden, EU) from mammary gland tissue obtained from Saanen dairy goats (*Capra hircus*). The total RNA was treated with RNase-free DNase I (Fermentas) and used to synthesize the first-strand complementary DNA (cDNA). The sequences of the primer oligonucleotides used in this study are listed in Table 1. The partial gSWEET1 gene was amplified from the first-stand cDNA and cloned into the pJET1.2™ vector (Fermentas) for sequencing. The cDNA was amplified using Phusion™ Hot Start High-Fidelity DNA Polymerase (Fermentas) with primers gSWEET1-F and gSWEET1-R. The 3′ sequence had a frameshift mutation compared with the published sequence (XM_005677408.1). The CDS region of gSWEET1 (681 bp) was amplified from the partial gSWEET1 (Figure 1A). The gSWEET1 gene was cloned into the pcDNA3.1 (+) vector to construct pcDNA3.1-gSWEET1 and verified (Figure 1B and 1C).

Sequence analysis of goat SWEET1

The cDNA sequence was analyzed using CLUSTAL W2.1 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), the National Center for Biotechnology Information BLAST site (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the TMHMM Server v. 2.0 program (http://www.cbs.dtu.dk/services/TMHMM/). The N-glycosylation sites of gSWEET1 were predicted with NetNGlyc 1.0 Server (www.cbs.dtu.dk/services/NetNGlyc). The multiple alignment of the 13 SWEET1 protein sequences was conducted with CLUSTAL W2.1 and the phylogenetic analysis was performed using the neighbor-joining method with the Poisson correction (Tamura et al., 2011).

Table 1. Sequences of oligonucleotide primers used for polymerase chain reaction (PCR) and reverse transcription quantitative PCR (RT-qPCR).

Primer name	Type	Sequence	Product length (bp)
gSWEET1-F1	Forward	CTCGAGATGGAGGCGGGCGGATTGGCCGACTC	681
gSWEET1-R1	Reverse	CTCGAGTCAGGTTTGTAGAAGCTGATAGTTCCTGTC	
gSWEET1-F2	Forward	CTCGAGATGGAGGCGGGCGGATTGGC	693
gSWEET1-R2	Reverse	CTCGAGTCAAGTGAACTCCCTCAGGTTTGTAGAAGC	
GLUT1-F	Forward	CGCTTCCTGCTCATTAAC	134
GLUT1-R	Reverse	GCTCTCCTCCTTCATCTC	
GLUT4-F	Forward	TGCTCATTCTTGGACGGTTCT	111
GLUT4-R	Reverse	CTAGCACCTGGGCGATTAGG	
GLUT12-F	Forward	TCACTGGCCAGCCAAACATA	
GLUT12-R	Reverse	AACGAAGCTGCCATCACAGA	
β-actin-F	Forward	CACCACACCTTCTACAAC	106
β-actin-R	Reverse	TCTGGGTCATCTTCTCAC	
AKT2-F	Forward	TGTCATCAAAGAAGGCTGGC	149
AKT2-R	Reverse	AAGGTATTGGGCCGAGGTCT	
IGF2-F	Forward	GCATCGTGGAAGAGTGTTGC	136
IGF2-R	Reverse	CACGGGGTATGCTGTGAAGT	
EEF1A1-F	Forward	CATCCCAGGCTGACTGTGC	101
EEF1A1-R	Reverse	TGTAAGCCAAAAGGGCATGC	
EEF2-F	Forward	GGGCTGGTGTCTACTGGCC	103
EEF2-R	Reverse	TCAGGATTGTCCTCTGGATC	
gSWEET1-F	Forward	GTGCTCCTTCAGACTACA	144
gSWEET1-R	Reverse	GTGGTGAGAGGTACATACT	
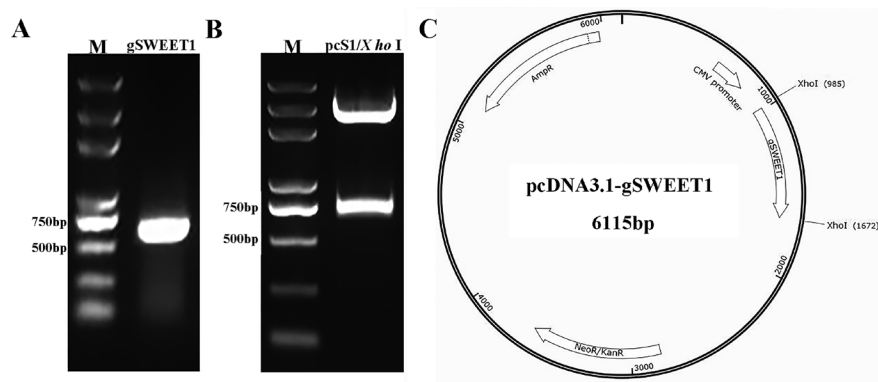
Figure 1. Construction and identification of pcDNA3.1-gSWEET1. **A.** Polymerase chain reaction (PCR) analysis with primers gSWEET1-F/gSWEET1-R. *Lane M* = Trans2KTM plus DNA marker, gSWEET1 681 bp PCR production of goat SWEET1. **B.** Identification of pcDNA3.1-gSWEET1 by digestion with *Xho*I. *Lane M* = Trans2KTM plus DNA marker, pcS1/*Xho*I gSWEET1 inserted into pcDNA3.1(+) and digested with *Xho*I. **C.** pcDNA3.1-gSWEET1 vector construction diagram.

GMGE cell isolation and culture

GMGE cells were isolated from the mammary gland tissue of Saanen dairy goats and cultured with the basal growth medium Dulbecco's modified Eagle's medium (DMEM)/F12 plus 10% fetal bovine serum. Progesterone (5 mg/mL) (ProSpec, ISR, AS), 10^{-7} M hydrocortisone (R&D, CA, USA), and 10 ng/mL ovine epithelial growth factor (ProSpec) were added to the basal growth medium to stimulate the growth of GMGE cells.

Transfection of pcDNA3.1-gSWEET1 into GMGE cells

When the GMGE cells had grown to 80% confluence, we discarded the serum and continued to culture the cells for 48 h. Lipofectamine™ 2000 (60 μL) (Invitrogen, Carlsbad, CA, USA) containing 24 μg pcDNA3.1-gSWEET1 was added to 15 mL DMEM/F12 without fetal bovine serum, then cultured for 6 h. The medium was then discarded and the cells were cultured for 2 weeks with the culture medium mentioned above plus G418 (600 μg/mL) (Invitrogen) until stable gSWEET1-transfected GMGE (ST1-GMGE) cells were ready for selection.

Glucose detection in the cell culture medium

Culture medium was collected from the ST1-GMGE cells and GMGE cells, which were both cultured in 6-well plates at the same density. We isolated cell protein using radioimmuno-precipitation assay lysis buffer and detected the protein concentration using the BCA method. The glucose level in the culture medium was determined using a Glucose Assay Kit II (BioVision, Tucson, AZ, USA).

The effect of the overexpression of gSWEET1

Total RNA was extracted from the ST1-GMGE and GMGE cells using a GeneJET™ RNA Purification Kit (Fermentas). To determine the gene expression profiles, 2 μL cDNA was amplified in a 20 μL reaction mix containing 10 μL of SYBR Premix™ Ex Taq (TaKaRa, Dalian, China), 0.4 μL ROX dye II, and specific primers (Table 1) with an ABI 7500 instrument. The data are reported as values normalized to the housekeeping gene (the gene encoding β-actin) to account for repeated measures. The differences between the means at each stage of development were determined.

Statistical analysis

A statistical analysis was performed using one-way analysis of variance. The comparison of the means was performed, and the differences were considered significant if $P < 0.05$.

RESULTS

Cloning of gSWEET1

A partial 681 bp gSWEET1 gene sequence containing the CDS region was successfully amplified (Figure 1A), and the open reading frame analysis suggested that the gSWEET1 was composed of 226 amino acids. The DNA sequence of gSWEET1 (GenBank: KP997221) is 99% identical to the goat sequence published in NCBI (XM-005677408.1). The phylogenetic analysis of protein sequences showed that gSWEET1 protein was most similar to the sheep and bovine proteins (Figure 2). Moreover, the DNA sequence of gSWEET1 was 97, 85, and 83% identical to the sequences of bovine (XM-003585826.3), human (NM-018845.3), and mouse (NM-009057) SWEET1, respectively. The deduced amino acid sequence of gSWEET1 was 97, 83, and 80% identical to the sequences of bovine (XP-003581980.1), human (NP-061333.2), and mouse (NP-033083.2) SWEET1, respectively (Figure 3).
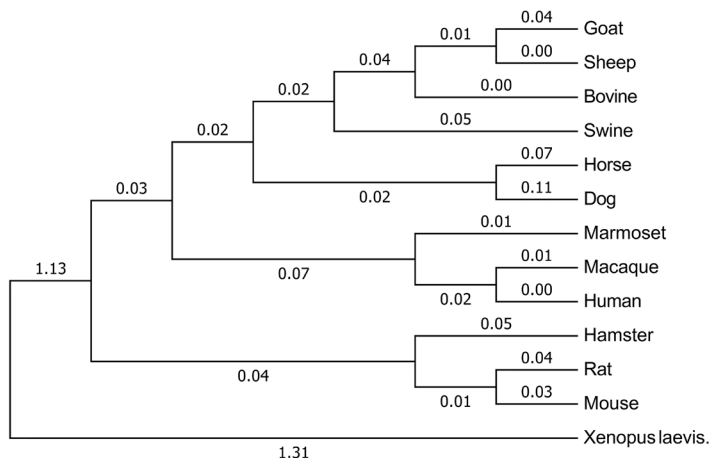
Figure 2. Phylogenetic analysis of goat SWEET1. A phylogenetic tree of protein sequences from goat and other vertebrates. GenBank accession numbers are as follows: SWEET1 sheep (XM_004002573.1), SWEET1 bovine (XM_003585826.3), SWEET1 swine (XM_003125719.2), SWEET1 horse (XM_001498337.2), SWEET1 dog (XM_003434894.2), SWEET1 marmoset (XM_002760075.3), SWEET1 macaque (XM_005541604.1), SWEET1 human (NM_018845.3), SWEET1 hamster (XM_005080168.1), SWEET1 rat (NM_001106445.1), SWEET1 mouse (XM_006501155.1), and SWEET1 *Xenopus laevis* (NM_001091035.1). The scale bar represents the number of substitutions per amino acid site.

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Goat      MEAGGLA-DSLLSG-ACVLFTLGMFSTG-LSDLKHMR-MTRSVDSVQFLP-FLTTDVNN-  54
Bovine    MEAGGLA-DSLLSGXXCVLFTLGMFSTG-LSDLKHMXXMTRSVDSVQFLP-FLTTDVNXX  57
Human     MEAGGFXXDSLIYG-ACVVFTLGMFSAGXXSDLRHMR-MTRSVDNVQFLPXXLTTEVNN-  57
          *****: ***: *  **:*******:*  ***:** ******.*****  ***:**

Goat      LSWLSYGALKGNWTLIVVN-AVGAVLQTLYILVY-LHYCHR-KRAVLLQTTTLLG-VLVL  110
Bovine    LSWLSYGALKGNWTLIIVNXXVGAVLQTLYILVY-LHYCHRXXRAVLLQTTTLLG-VLVL  115
Human     LGWLSYGALKGDXXILIVVNTVGAALQTLYILAXXLHYCPR-KRVVLLQTATLLGXXLLL  116
          *.*********: :::*  ***.*******. **** *  *.*****:**** *:*

Goat      GFAY-FWLLVPDPEMRLQ-HLGLFCS-VFTISMYLSPLAD-LAKVIR-TKSTQRLSFSLT  165
Bovine    GFAXXFWLLVPDPEMRLQ-HLGLFCXXVFTISMYLSPLAD-LAKVIRXXKSTQRLSFSLT  173
Human     GYGY-FWLLVPNPEARLXXQLGLFCS-VFTISMYLSPLAXXLAKVIQ-TKSTQCLSYPLT  173
          *:.  ******:** **  :***** *********** ***** :**** **:.**

Goat      IA-TLLTSAS-WTLYGFRLKDPY-IVVPNLPG-ILTSFIRFWLFWKYSPGVRQELSASTN  221
Bovine    IA-TLLTSAXXWTLYGFRLRDPY-IVVPNLPXXILTSFIRFWLFWKY-PQERDXX--NYQ  228
Human     IXXTLLTSAS-WCLYGFRLRDPYXXMVSNFPG-IVTSFIRFWLFWXXYPQEQDRN---YW  228
          *  ****** * ******:***  :*.*:* *:**********  *  ::

Goat      LREFT  226
Bovine    LLQT-  232
Human     LLQT-  232
          * :
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Figure 3. Multiple sequence alignment of the deduced amino acid sequence of goat SWEET1 with other species. The alignments were performed with the CLUSTAL W2.1 program. An asterisk indicates the positions which have a single, fully conserved residue. A colon indicates conservation between groups of strongly similar properties scoring > 0.5 in the Gonnet PAM 250 matrix. A period indicates conservation between groups of weakly similar properties scoring ≤ 0.5 in the Gonnet PAM 250 matrix.

Sequence analysis of gSWEET1

The prediction of gSWEET1 with TMHMM Server v. 2.0 (Figure 4A) revealed that the gSWEET1 had seven transmembrane (TM) helices, nearly identical to bovine and human SWEET1

(Figure 4B and 4C). The gSWEET1 has two conserved MtN3/saliva motifs embedded in the tandem 3-TM repeat unit, which are connected with a central TM helix. By using the NetNGlyc 1.0 Server (www.cbs.dtu.dk/services/NetNGlyc), four potential N-glycosylation sites were predicted at amino acids 53-56 (NNLS) in the first TM-domain, at 66-69 (NWTL), at 73-76 (NAVG) in the second TM-domain, and at 191-194 (NLPG) in the seventh TM-domain (Figure 5).
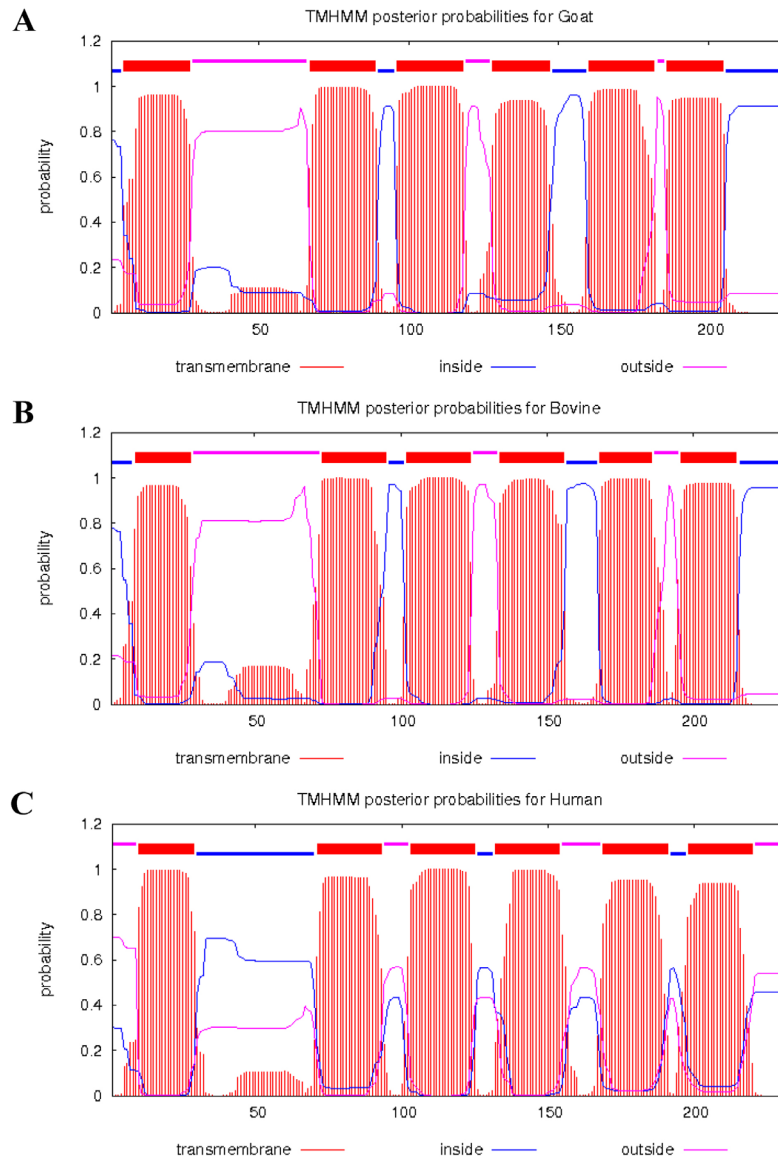
Figure 4. Transmembrane helices prediction of goat SWEET1. Red lines indicate transmembrane regions, blue lines indicate intracellular regions, and pink lines indicate extracellular regions. The vertical axes indicate the probability of transmembrane helices, and the horizontal axes indicate amino acid sequences. **A.** Goat SWEET1 amino acid prediction. **B.** Bovine SWEET1 amino acid prediction. **C.** Human SWEET1 amino acid prediction.

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ATGGAGGCGGGCGGATTGGCCGACTCGCTTCTTTCTGGAGCTTGCGTGCTCTTCACCCTCGGCATGTTC  69
 M   E   A   G   G   L   A   D   S   L   L   S   G   A   C   V   L   F   T   L   G   M   F
TCCACCGGCCTCTCGGACCTCAAGCACATGCGGATGACCCGGAGCGTGGACAGTGTCCAGTTCCTGCCC 138
 S   T   G   L   S   D   L   K   H   M   R   M   T   R   S   V   D   S   V   Q   F   L   P
TTTCTCACCACGGATGTCAACAACCTGAGTTGGCTGAGTTATGGGGCCTTGAAGGGAAACTGGACGCTA 207
 F   L   T   T   D   V   N   N   L   S   W   L   S   Y   G   A   L   K   G   N   W   T   L
ATCGTCGTCAACGCCGTGGGTGCTGTGCTTCAGACTCTGTATATCTTGGTGTATCTGCACTACTGCCAT 276
 I   V   V   N   A   V   G   A   V   L   Q   T   L   Y   I   L   V   Y   L   H   Y   C   H
CGGAAGCGTGCCGTGCTCCTTCAGACTACAACCCTGCTGGGAGTCCTTGTCCTGGGTTTTGCCTACTTT 345
 R   K   R   A   V   L   L   Q   T   T   T   L   L   G   V   L   V   L   G   F   A   Y   F
TGGCTCCTGGTGCCTGACCCTGAGATGCGGCTTCAGCACCTGGGCCTCTTCTGCAGTGTCTTCACCATC 414
 W   L   L   V   P   D   P   E   M   R   L   Q   H   L   G   L   F   C   S   V   F   T   I
AGTATGTACCTCTCACCACTAGCTGACTTGGCCAAGGTCATTCGTACTAAATCAACCCAGCGTCTCTCC 483
 S   M   Y   L   S   P   L   A   D   L   A   K   V   I   R   T   K   S   T   Q   R   L   S
TTTTCACTCACCATTGCCACCCTCCTCACCTCTGCCTCCTGGACACTCTATGGGTTTCGACTAAAAGAT 552
 F   S   L   T   I   A   T   L   L   T   S   A   S   W   T   L   Y   G   F   R   L   K   D
CCCTACATTGTGGTGCCCAACCTTCCAGGAATTCTCACCAGCTTCATTCGCTTCTGGCTTTTTTGGAAG 621
 P   Y   I   V   V   P   N   L   P   G   I   L   T   S   F   I   R   F   W   L   F   W   K
TACTCCCCAGGAGTGAGACAGGAACTATCGGCTTCTACAAACCTGAGGGAGTTCACTTGA            681
 Y   S   P   G   V   R   Q   E   L   S   A   S   T   N   L   R   F   F   T   -
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Figure 5. The coding DNA sequence (CDS) region of cDNA and deduced amino acid sequence of gSWEET1. The odd rows are sequences of cDNA, and the even rows are the sequences of the amino acids. Four potential N-glycosylation sites are at amino acids 53-56 (NNLS), 66-69 (NWTL), 73-76 (NAVG), and 191-194 (NLPG).

## The mRNA expression of gSWEET1 and glucose transporters in ST1-GMGE cells

The mRNA expression of gSWEET1 in the ST1-GMGE cells was significantly increased compared with the GMGE cells ($P < 0.05$) (Figure 6A). With the overexpression of gSWEET1, the mRNA expression levels of GLUT1 and GLUT12 in the ST1-GMGE cells were both increased by over 5-fold compared with the GMGE cells (Figure 6B).
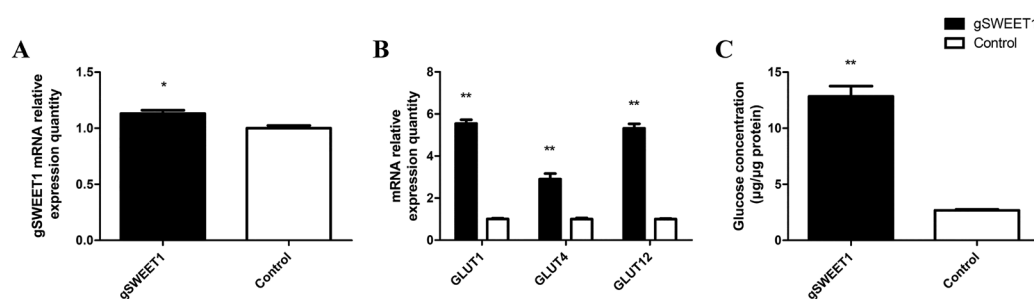
Figure 6. Goat SWEET1 (**A**) and GLUTs (**B**) mRNA levels in ST1-GMGE and GMGE cells (GMGE cells are goat mammary gland epithelial cells). In GMGE cells, the expression levels of SWEET1 and GLUTs were significantly affected by transfection of pcDNA3.1-gSWEET1. (**C**) glucose efflux in GMGE cells. Control means the GMGE cells-transfected pcDNA3.1 (+) vector, gSWEET1 means the stable ST1-GMGE cells. The vertical axes indicate the mean glucose efflux and total protein radio. The horizontal axes indicate the different groups. All data are expressed as mean ± SE (N = 3). *$P < 0.05$, **$P < 0.01$, compared with GMGE cells.

mRNA expression of genes related to the glycometabolism signaling pathway

The overexpression of gSWEET1 significantly reduced the mRNA expression levels of the elongation factors EEF1A1 and EEF2 in ST1-GMGE cells (P < 0.01) (Figure 7B). We also found that the mRNA expression levels of two insulin signaling pathway-related genes, insulin-like growth factor-2 (IGF2) and AKT2, increased significantly (P < 0.01) (Figure 7A).
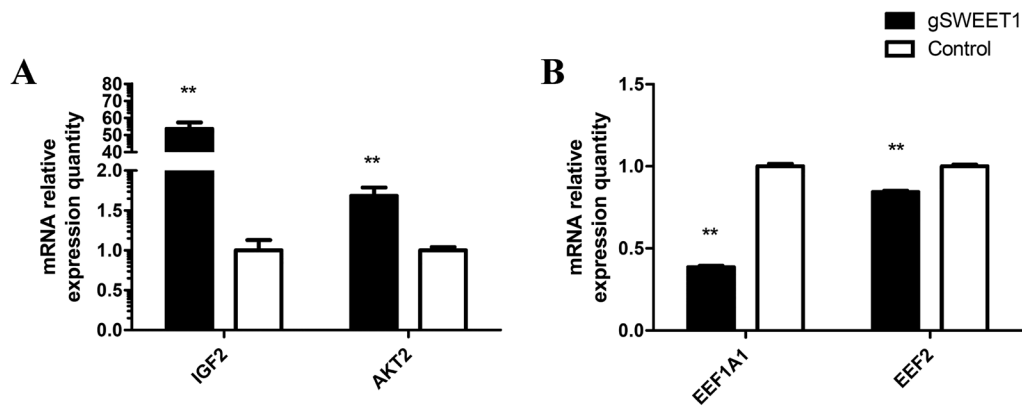
Figure 7. Transcription elongation factor and insulin signaling pathway genes mRNA levels in ST1-GMGE cells and GMGE cells (GMGE cells are goat mammary gland epithelial cells). Expression levels of IGF-2, AKT-2, EEF1A1, and EEF2 were significantly affected by the transfection of pcDNA3.1-gSWEET1 (P < 0.01). Data are expressed as mean ± SE (N = 9). *P < 0.05; **P < 0.01, compared with GMGE cells. Control = GMGE cells; gSWEET1 = ST1-GMGE cells.

Detection of glucose consumption in ST1-GMGE cells

In order to evaluate the biological function of the gSWEET1 gene, glucose consumption was assessed. The glucose concentration in the cell medium of the ST1-GMGE cells was significantly higher (by nearly 60%; P < 0.01) than in that of the GMEM cells, which means that the consumption of glucose in the ST1-GMGE cells was lower than in the GMGE cells (Figure 6C).

DISCUSSION

In this study, we cloned and sequenced partial gSWEET1 cDNA, which closely resembles that of sheep (96%) and cattle (95%). The gSWEET1 had seven transmembrane domains with four potential N-glycosylation sites, which is highly homologous to bovine and human SWEET1. Furthermore, the phylogenetic tree analysis confirmed their genetic relationships. However, this is different from GLUTs and SGLTs, which have 12 and 14 domains, respectively (Zhao and Keating, 2007; De Zutter et al., 2013). The structure of gSWEET1 is consistent with that found in *A. thaliana* (Xuan et al., 2013), indicating high conservation in the different species.

The mRNA expression of gSWEET1 in ST1-GMGE cells was significantly increased, which in turn demonstrated successful transfection. Accompanying the gSWEET1 overexpression, the mRNA expression levels of GLUT1, GLUT4, and GLUT12 were also significantly increased by different degrees. Considering the efflux activity of gSWEET1 in *A. thaliana*, we speculated that gSWEET1 and the GLUTs cooperated in the modulation of glucose balance in mammary gland

cells (Chen et al., 2010). Along with the glucose efflux activity caused by gSWEET1 overexpression, cells need to absorb more glucose to balance the glucose concentration in the cytoplasm, which may be the cause of the high expression of GLUT1, GLUT4, and GLUT12 (Macheda et al., 2003; Thorens and Mueckler, 2010). This is also confirmed by the increase of glucose concentration in the medium of ST1-GMGE cells with overexpression of gSWEET1.

IGF2 and AKT2 can take part in the insulin signaling pathway and promote energy utilization (Nozaki et al., 2013; Bach, 2015). The increasing expression of AKT2 could modulate the insulin signaling pathway and induce the translocation of insulin-sensitive GLUT4 from intracellular pools to the plasma membrane, thereby stimulating the absorption of more glucose from blood to balance the glucose efflux activity caused by gSWEET1 (Stuart et al., 2009; Leto and Saltiel, 2012). Low glucose concentration in the cytoplasm resulting from overexpression of gSWEET1 would then inhibit transcription. This is consistent with the decreasing expression levels of translation elongation factors EEF2 and EEF1A1 (Bionaz and Loor, 2011).

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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