รายงานวิจัยฉบับสมบูรณ์

โครงการ การโคลนยีนบีเทนทรานสปอร์เตอร์และการศึกษาหน้าที่ของโปรตีนบีเทนทรานสปอร์เตอร์จากอะฟาโนทีคี ฮาโลฟิทิคา

Cloning of a betaine transporter gene and functional studies of its product from *Aphanothece halophytica*

โดย

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(ชื่อเดิม) การศึกษากระบวนการและเอนไซม์ในการสร้างโคลีนจากอะฟาโนทีคี ฮาโลฟิทิคา

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

ขอขอบคุณสำนักงานกองทุนสนับสนุนการวิจัยที่ให้เงินทุนวิจัย รศ.ดร.อรัญ อินเจริญศักดิ์ นักวิจัยที่ปรึกษาสำหรับคำแนะนำและการให้คำปรึกษาต่างๆ ที่เป็นประโยชน์กับโครงการ Dr. Teruhiro Takabe สำหรับความร่วมมือและความช่วยเหลือต่างๆ ในการศึกษาวิจัย คุณโสรายา ไทยวาณิช ที่ช่วยเตรียม *Aphanothece halophytica* culture ตลอดการทดลอง คุณสันทนา นาคะพงษ์ สำหรับความช่วยเหลือทั่วไปในห้องปฏิบัติการ อาจารย์และนิสิตภาควิชาชีวเคมีสำหรับคำแนะนำ การอภิปราย และความช่วยเหลือต่างๆ ตลอดโครงการวิจัย

บทคัดย่อ

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ชื่อโครงการ: การโคลนและการศึกษาการแสดงออกของยีนทรานสปอร์เตอร์สำหรับบีเทน/คาร์นิทีน/โคลีน จากอะฟาโนทีคี ฮาโลฟิทิคา

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ระยะเวลาโครงการ: 2 ปี

อะฟาโนทีคี ฮาโลฟิทิคา (*Aphanothece halophytica*) เป็นไซยาโนแบคทีเรียทนเค็มที่สามารถเติบโตได้ในภาวะที่มีเกลือโซเดียมคลอไรด์สูงถึง 3 โมลาร์ เมื่อระดับออสโมลาริทีภายนอกเซลล์เพิ่มขึ้นเซลล์จะสะสมไกลซีนบีเทนซึ่งเป็นโมเลกุลที่มีหมู่ quaternary ammonium ภายในไซโทพลาซึมเพื่อปรับความดันออสโมติกภายในเซลล์ให้เหมาะสม การสะสมไกลซีนบีเทนนอกจากจะเกิดจากการสร้างใหม่ภายในเซลล์แล้วยังมีรายงานว่าเซลล์อะฟาโนทีคี ฮาโลฟิทิคาสามารถดึงไกลซีนบีเทนเข้ามาจากภายนอกเซลล์โดยอาศัยโปรตีนทรานสปอร์เตอร์บางชนิด อย่างไรก็ตามยีนและโปรตีนที่ทำหน้าที่ดังกล่าวยังไม่ถูกค้นพบ การค้นหาและศึกษายีนนี้จึงน่าจะนำไปสู่ความเข้าใจในกลไกรวมทั้งการควบคุมการสะสมไกลซีนบีเทนของเซลล์อะฟาโนทีคี ฮาโลฟิทิคาในสภาวะที่มีเกลือสูงได้ ในงานวิจัยนี้จากการค้นลำดับภายในจีโนมของอะฟาโนทีคี ฮาโลฟิทิคา โดยใช้โปรตีน OpuD ซึ่งเป็นโปรตีนทรานสปอร์เตอร์สำหรับลำเลียงไกลซีนบีเทนจากบาสิลัส สับทิลิส (*Bacillus subtilis*) เป็นคำถาม (query) ผู้วิจัยค้นพบยีนสร้างโปรตีนหนึ่งที่มีความคล้าย (similarity) กับ OpuD มาก และสามารถโคลนยีนดังกล่าวโดยวิธีพีซีอาร์ (PCR) และตั้งชื่อว่า *ApBet* ซึ่งจากการวิเคราะห์โดย DNA gel blot พบว่า *ApBet* เป็นยีนเพียงชุดเดียวและไม่มียีนที่คล้ายคลึงกันภายในจีโนมอีก และในการศึกษาการแสดงออกของยีน *ApBet* โดยวิธีอาร์ทีพีซีอาร์ (RT-PCR) และการวิเคราะห์โดย DNA gel blot พบว่าปริมาณ mRNA ภายในเซลล์อะฟาโนทีคี ฮาโลฟิทิคาลดลงในสภาวะที่มีความเข้มข้นของเกลือโซเดียมคลอไรด์ภายนอกสูงขึ้น ยีน *ApBet* ถูกทำนายได้ว่าสร้างโปรตีนที่มีจำนวนกรดอะมิโน 564 กรดอะมิโน น้ำหนักโมเลกุล 62 kDa ลำดับกรดอะมิโนของ ApBet นี้คล้ายกับโปรตีนทรานสปอร์เตอร์ในกลุ่มที่เรียกว่า Betaine/Carnitine/Choline Transporters (BCCT) จากการทำนายโครงสร้าง ApBet คาดว่าประกอบด้วย transmembrane domain 12 ตำแหน่ง และมีบริเวณที่ลำดับเป็นเอกลักษณ์ตามรูปแบบของโปรตีนในกลุ่ม BCCT คือ [GSDN]-W-T-[LIVM]-X-[FY]-W-X-W-W

คำสำคัญ การแสดงออกของยีน ไกลซีนบีเทน อะฟาโนทีคี ฮาโลฟิทิคา

Abstract

Project Code: TRG4580087

Project Title: Cloning and Expression Studies of a Putative Betaine/Carnitine/Choline Secondary Transporter Gene from *Aphanothece halophytica*

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Project Period: 2 years

Aphanothece halophytica is a halotolerant cyanobacterium that can grow in high external salinity up to 3.0 M NaCl. Under increased extracellular osmolarity, glycine betaine, a quaternary ammonium compound is accumulated within cytoplasm which potentially acts as an osmoprotectant in the *A. halophytica* cell. In addition to accumulation by synthesis, *A. halophytica* has been shown to accumulate glycine betaine in response to salt stress by rapid uptake. However, the gene(s) responsible for this activity has not been found. Identifying and characterizing the gene(s) involved will be a crucial step to better understand how the accumulation occurs in medium of high osmolarity as well as how this process is regulated. In this report, the *A. halophytica* genomic sequence was searched for a glycine betaine transporter gene using the amino acid sequence of OpuD, which is a symporter for glycine betaine from *Bacillus subtilis* as a query. A gene encoding a putative glycine betaine transporter referred as ApBet was identified and isolated by PCR amplification. DNA gel blot analysis indicates a single copy gene encodes ApBet and no closely related gene is present. Expression studies by PCR and DNA blot analysis shows that the *ApBet* mRNA level is decreased under salt stress. The gene was predicted to encode a protein of 564 amino acids and molecular weight of about 62 kDa. The deduced amino acid sequence shares a high level of similarity to several transport proteins belonging to a protein family known as BCCT (Betaine/Carnitine/Choline Transporters). It is predicted to possess 12 transmembrane regions and contains the consensus pattern [GSDN]-W-T-[LIVM]-X-[FY]-W-X-W-W which is a signature of proteins in the BCCT family.

Keywords: *Aphanothece halophytica*, betaine/carnitine/choline transporter, compatible solute, gene expression; glycine betaine

Introduction

Microorganisms have evolutionarily obtained the ability to respond to fluctuating osmolalities in their environments in order to maintain their growth and metabolism. In high osmolarity medium, many microorganisms accumulate organic solutes including sugars and glycosides, polyols, amino acids and quaternary ammonium compounds i.e. betaines (Imhoff, 1993; Kempf & Bremer, 1998). These molecules which accumulate through synthesis or uptake without interfering with the metabolic functions of the cell are referred as compatible solutes. In microbial cells, compatible solutes serve as osmoprotectants to help balance their osmotic strength with that of their surroundings and influence protein structure and stability (Le Rudulier, 1984). Among a few classes of compatible solutes, glycine betaine (N, N, N-trimethylglycine: GlyBet), a quaternary ammonium compound, which intracellularly accumulates at high concentration is a widely used compatible solute in numerous microorganisms. GlyBet accumulation can be accomplished through biosynthesis by choline oxidation or through acquisition of preformed molecules from the extracellular medium by specific transporters (Csonka & Hanson, 1991; Wood et al., 2001)

Aphanothece halophytica, a halotolerant cyanobacteria that can grow in high external salinity up to 3.0 M NaCl, accumulates GlyBet in response to salinity and was reported to possess a two-step oxidation of choline via betaine aldehyde (Incharoensakdi & Wutipraditkul, 1999). Recently, Takabe and colleagues identified in *A.halophytica* an additional and possibly a major biosynthetic pathway of GlyBet mediated by N-methyltransferases that catalyze GlyBet synthesis from glycine (Waditee et al., 2003). In addition to accumulation through biosynthesis, *A.halophytica* was shown able to accumulate GlyBet by rapid uptake of ^{14}C-glycine betaine, coupled with high internal accumulation. In addition, higher ^{14}C-glycine betaine levels occurred in cells grown in medium of elevated osmotic strength (Moore DJ et al., 1987). However, the gene(s) responsible for this transport activity has not been identified. Identifying and characterizing the gene(s) encoding this activity would be a crucial step to better understand how the accumulation of GlyBet, especially in the early stage occurs in medium of high osmolarity. Here, we report the cloning of a gene encoding a putative GlyBet transporter from *A.halophytica* and its regulation under salt stress in the level of gene expression. The study shows that this

putative GlyBet transporter belongs to the Betaine/Carnitine/Choline transporter (BCCT) family of proteins.

Materials and Methods

Materials

Enzymes used for manipulating recombinant DNA were obtained from Fermentas (Hanover, MD, USA) and Life Technologies (Gaithersburg, MD, USA). Kits for purifying and gel extracting plasmid DNA were purchased from Qiagen (Chatsworth, CA, USA). Radioactive chemicals were purchased from Amersham Biosciences (Piscataway, NJ, USA). Synthetic oligonucleotides for reverse transcription-polymerase chain reaction (RT-PCR) were obtained from Bioservice Unit of the National Science and Technology Development Agency (Bangkok, Thailand).

Organisms, grown conditions and salt stress treatment

Aphanothece halophytica originally from Solar Lake in Israel was obtained in axenic culture from T.Takabe (Nagoya Univerisity). *A.halophytica* cells were grown photoautotrophically in BG11 medium supplied with 18 mM $NaNO_3$ as previously described (Incharoensakdi et al., 1986) on a rotary shaker at 30^{o}C without CO_2 supplementation. Continuous illumination was provided by cool-white fluorescence lamps at an irradiance of 60 μmol photon $m^{-2}s^{-1}$. To impose salt stress, cells were transferred from medium containing 0.5 M NaCl to medium containing 2.0 M NaCl and the culture was further grown for 4 hours under the same conditions. Cells were collected by centrifugation, washed with 50 mM HEPES-KOH pH 7.5 and kept at -70^{o}C until use.

DNA gel blot analysis

A.halophytica genomic DNA was isolated as described (Lee et al., 1997). DNA was digested with restriction enzymes, fractionated by agarose gel electrophoresis, transferred to positively charged nylon membranes (Boehringer Mannheim, Indianapolis, IN, USA) and immobilized by UV crosslinking in a Bio-Rad GS Gene Linker^{TM} UV chamber (Hercules, CA, USA). The membranes were hybridized in solution containing 50% (v/v) deionized

formamide, 5XSSPE (20XSSPE is 2.98 M NaCl, 0.2 M $NaH_2PO_4 \cdot H_2O$)/NaOH (pH 7.4), and 20 mM $Na_2 \cdot N,N,N',N'$-ethylenediamine tetraacetic acid (EDTA)$\cdot 2H_2O$), 1X Denhardt's solution (100X Denhardt's solution consists of BSA, Ficoll, and polyvinylpyrrolidone (PVP) at 2% [w/v] each), and 0.2% (w/v) sodium dodecyl sulfate (SDS) at 40°C for 16 hours. The blots were washed in 2X SSPE, 0.1% SDS twice, 15 minutes each at room temperature and hybridizing bands were detected by autoradiography. Hybridization probes were labeled with [α-^{32}P]-dCTP by random priming (Hodgson and Fisk 1987).

Analysis by Reverse transcription polymerase chain reaction (RT-PCR)

A.halophytica cells were collected in 1.5 ml microcentrifuge tubes by centrifugation, immediately frozen in liquid nitrogen, and kept in -70°C until use. Total RNA was isolated according to Verwoerd *et al.* (1989) and used in reverse transcription. Reverse transcription was primed by random hexamer synthetic oligonucleotides, and PCR was carried out using: 5′-CCACCTTTGCTTGGTTTGCG-3′ and 5′-CAATGACGTGACCAGGCCAT-3′ as the sense and antisense primers, respectively. PCR amplification by Taq polymerase consisting of 30 or 16 cycles was conducted using a program of 94°C for 1 minute, 59°C for 1 minute, and 72°C for 1 minute. Products from the 30-cycle PCR were separated by agarose gel electrophoresis and visualized by ethidium bromide staining and UV fluorescencing. Products from the 16-cycle PCR were fractionated by agarose gel electrophoresis, transferred to a positively charged nylon membrane. The membrane was hybridized as described above.

Results

Cloning of a putative Betaine/Carnitine/Choline Transporter gene

Several families of electrochemical potential-driven amino acid transporters were found exclusively in prokaryotes (Saier Jr., 2000). The Betaine/Carnitine/Choline transporter (BCCT) family comprise a family of proteins whose common functional feature is that they all transport molecules with a quaternary ammonium group [$R\text{-}N^{+}(CH_3)_3$]. Transporter proteins in this family possess 12 putative transmembrane segments and vary in length between 481 and 677 amino acid residues. To find similar proteins within the *A.halophytica* database, the amino acid sequence of *OpuD* gene encoding a glycine betaine transporter in *Bacillus subtilis* (Kappes et al., 1996) was used as a query for searching. As a result, a genomic sequence with its encoded amino acid sequence showing a high level of similarity to OpuD transporter was obtained. The gene was isolated by PCR method based on the shot gun sequencing of *A.halophytica*. The PCR product was cloned into pBSII plasmid and its sequence was then confirmed by DNA sequencing (data not shown). Sequence analysis revealed the 1692-bp open reading frame that begins with a GTG start codon and stops with two consecutive stop codons (TAATGA). This gene will be referred as *ApBet*. To determine whether any closely related genes are present in the *A.halophytica* genome, its genomic DNA was digested by restriction enzymes EcoRI, EcoRV, BstE, or XbaI and run on an agarose gel. The gel was blotted onto a positively charged nylon membrane and the membrane was hybridized with a probe made from the cloned *ApBet* gene. Figure 1 shows the autoradiograph of the DNA gel blot analysis. Under relaxed washing conditions, single hybridizing DNA fragments were detected with each of the restriction enzymes tested. Therefore, ApBet is likely to be encoded by a single-copy gene and no closely related gene is present in the *A.halophytica* genome.

Analysis of the ApBet amino acid sequence

The amino acid sequence deduced from the *ApBet* gene gives a 564-residue protein with a calculated molecular mass of 62 kDa. BLAST (Altschul *et al.*, 1997) searching of protein databases using the ApBet amino acid sequence revealed homologous

proteins, which are members of the BCCT family. The known family members exhibiting similarity to ApBet include transporters such as OpuD from *Bacillus subtilis*, BetS from *Sinorhizobium meliloti* (Boscari et al., 2002), CaiT and BetT from *Escherichia coli* (Eichler et al. 1994; Lamark et al, 1991), and BetP and EctP from *Corynebaceterium glutamicum* (Peter et al., 1996; Peter et al., 1998). These proteins are secondary transporters that are coupled to either the proton potential or the electrochemical Na^+ potential. OpuD, BetS, and BetP transport glycine betaine; CaiT and BetT transport carnitine and choline, respectively; and EctP is a low affinity transporter for glycine betaine, proline and ectoine. Transport activity of and expression of genes encoding some of these transporters are enhanced by osmotic upshifts indicating their importance for these bacteria's adaptation to medium of high osmolality. In addition, some transporters exhibit osmosensory properties inherent to their polypeptide chains (Wood el al., 2001).

In Figure 2, the deduced amino acid sequence of ApBet is compared with amino acid sequences from selected BCCT transporters. ApBet amino acid sequence shares about 50, 47, 55, 50, 48, and 44 % amino acid similarity to BetS, BetT, OpuD, BetP, EctP, and Cai respectively. The ApBet amino acid sequence was analyzed by the PredictProtein program available via the WWW (http://cubic.bioc.columbia.edu/predictprotein/) (Rost & Liu, 2003). Like other members of the BCCT family, ApBet was predicted to have twelve membrane helices. Figure 3 shows the predicted model of the secondary structure of ApBet in which both the amino and carboxyl terminal ends of ApBet are located in the cytoplasm. ApBet exhibits a high level of identity in the sequence located in the eighth transmembrane segment and the loop between the eighth and the ninth transmembrane segments. These segments of the BCCT proteins contains a conserved region with four tryptophan residues in the consensus sequence [GSDN]-W-T-[LIVM]-X-[FY]-W-X-W-W, a signature of proteins in this family. ApBet sequence at the corresponding position is SWTVFYWGWW therefore, is likely a homolog of proteins in the BCCT family.

Similar features of ApBet to those of BCCT transporters suggest that ApBet probably possess similar transport functions. To test whether ApBet is a functional transporter, we determined if *ApBet* gene can complement the salt-sensitive phenotype of the *E.coli* mutant MKH13 in the presence of some compatible solutes including glycine betaine and choline. *E.coli* MKH13 cells in which *betT*, *putPA*, *proP*, and *proU* genes are deleted, are unable to grow in high osmolality medium containing betaine due to the lack of

a betaine transport system as well as betaine synthesis genes. The cells transformed with pTrcHis2C expression vector harboring the *ApBeT* gene were grown on agar plates minimal medium A containing 0.2% glucose; 0.7 M NaCl and 1 mM glycine betaine or 1 mM choline as described (Waditee et al., 2002). However, the results have shown that *ApBeT* gene did not complement the salt-sensitive phynotype of MKH13 cells (data not shown).

RT-PCR analyses of ApBet expression

To determine whether the *ApBet* gene is a functional gene, RT-PCR is conducted to detect the presence of its mRNA. *A.halophytica* cells from a 14-day culture grown in BG11 containing 0.5 M NaCl as described in the Materials and Methods section were transferred to media containing either 0.5 M or 2.0 M NaCl and growth was continued for 4 hours under the same conditions. Cells were collected and total RNA was isolated and used to perform RT-PCR. PCR amplification consisting of 30 cycles was conducted using a program of 94°C for 1 minute, 59°C for 1 minute, and 72°C for 1 minute. A control RT-PCR reaction without adding transcriptase was done in parallel with each experimental reaction using total RNA to ensure that the product obtained could be attributed to the product of the reverse transcriptase reaction. Figure 4A shows that a band of the expected size was detected in each condition. These results indicate that the mRNA corresponding to the *ApBet* gene exists, hence verify the functionality of the *ApBet* gene. However, the relative abundance of *ApBet* mRNAs could not be suggested by these results.

To determine the relative abundance of the *ApBet* mRNA in order to examine possible regulation of ApBet at the level of gene expression under salt stress and to infer its involvement in *A.halophytica* adaptation to high osmolality surroundings, similar RT-PCR was carried out except that PCR amplification consisted of only 16 cycles. Then, products from all PCR amplification reactions including the control reactions without adding transcriptase were separated by agarose gel electrophoresis. The gel was blotted onto a positively charged nylon membrane and the membrane was hybridized with a probe made from the cloned *ApBet* gene. Figure 4B shows the DNA gel blot analysis of RT-PCR products of *ApBet* mRNA. Bands of the expected product were detected in the amplification reactions however the salt stress treatment did not increase the level of *ApBet* mRNA as originally anticipated. On the contrary, slightly less amount of the RT-PCR

product for the *ApBet* gene was detected in the cells treated with salt stress (0.5 M to 2.0 M NaCl).

Discussion

In microorganisms, the majority of known amino acid transporters are secondary carriers. Saier has categorized secondary carriers that are known to function in transport of amino acids and their conjugates into 19 families (Saier, 2000). Proteins of the BCCT family transport molecules with a quaternary ammonium group such as glycine betaine, choline, carnitine, and like many transporters in other families, they functions by proton or sodium symport and usually are single polypeptide chains consisting of 12 transmembrane domains. In this study, we report the identification and expression studies of ApBet, a BCCT family member from *Aphanothece halophytica*. ApBet was isolated by PCR and its nucleotide sequence was confirmed by DNA sequencing. The *ApBet* gene encodes a 564-residue protein with a predicted 12 transmembrane domains. DNA gel blot analyses revealed no other closely related genes therefore *ApBet* is probably an only gene encoding a BCCT family member present in the *A.halophytica* genome which confirms the BLAST search result.

The deduced amino acid sequence of ApBet shares a high level of similarity to proteins in the BCCT family especially within the region between the eighth and the ninth transmembrane segments and the region within the eight transmembrane segment that contains the consensus pattern [GSDN]-W-T-[LIVM]-X-[FY]-W-X-W-W, a characteristic of proteins in this family. However, N-terminal domain of approximately 38 amino acids and C-terminal domain of approximately 44 amino acids are diverged from other BCCT family members. Both terminal domains as well as the loop regions of ApBet are highly hydrophilic. The N- and C-terminal domains contain 9 glutamate/aspatate residues and 5 lysine/arginine residues; and 11 glutamate/aspatate residues and 6 lysine/arginine residues, respectively. In the loops, there is a symmetrical distribution of the 16 positively charged residues (14 residues in cytoplasmic loops, 2 residues in extracellular loops) while the 22 negatively charged residues within the loops are more or less evenly distributed. The presence and distribution of these charged residues potentially determine its specificity as well as affinity to the molecule(s) ApBet transports.

Furthermore, we attempted to determine the transport activity of ApBet by functional complementation in *E.coli* MKH13 cells. Our results show that *ApBet* gene did not appear to complement the salt-sensitive phenotype in medium containing glycine betaine or choline. It should be noted that cells expressing the *ApBet* gene grew much slower than cells harboring only the expression vector which may be due to the toxicity of the *ApBet* gene product. To determine regulation of ApBet at the level of gene expression, RT-PCR analyses revealed the presence of *ApBet* mRNA in cells grown in both low and high osmolarity medium however, the levels of *ApBet* mRNA appears lower in the medium containing 2.0 M than the medium containing 0.5 M NaCl. This result suggests the *ApBet* gene is downregulated under increased surrounding osmolarity. This seems to contradict the possible role of compatible solute uptake of this protein that we originally thought. However, expulsion of compatible solute in response to osmotic downshocks, which is equally important in osmotic adaptation can not be ruled out.

Note: Currently, I am under an agreement with my collaborator not to print the genomic DNA and the deduced amino acid sequences of the *ApBet* gene.

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Figures

Figure 1 DNA gel blot analysis of the *ApBet* gene. Genomic DNA (3.75 μg) was digested with EcoRI (lane 1), EcoRV (lane 1), BstE (lane 3), or XbaI (lane4), fractionated by agarose gel electrophoresis, and transferred to a positively charged membrane. The blot was hybridized with a probe made from the the cloned *ApBet* gene.

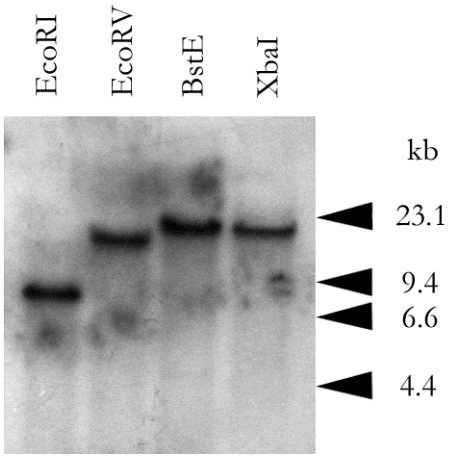


Figure 2 Comparison of the deduced amino acid sequence of ApBet to selected transporters for molecules with a quaternary ammonium group from various microorganisms. Residues identical to those in ApBet are replaced by dashes; ".." indicates a gap inserted for purposes of alignment. The box indicates the location of the consensus pattern of transport proteins in the BCCT family. Horizontal lines indicate the location of the predicted twelve transmembrane regions of ApBet. OpuD_Bs., *Bacillus subtilis* glycine betaine transporter (Accession number P54417); BetS_Sm., *Sinorhizobium meliloti* glycine betaine/praline betaine trasporter (Q8VTN3); CaiT_Ec., *Escherichia coli* carnitine transporter (P31553); BetT_Ec., *Escherichia coli* choline transporter (P17447); BetP_Cg., *Corynebaceterium glutamicum* glycine betaine transporter (CAA63771); and EctP_Cg., *Corynebaceterium glutamicum* low affinity system for glycine betaine, proline and ectoine transport (O86143).

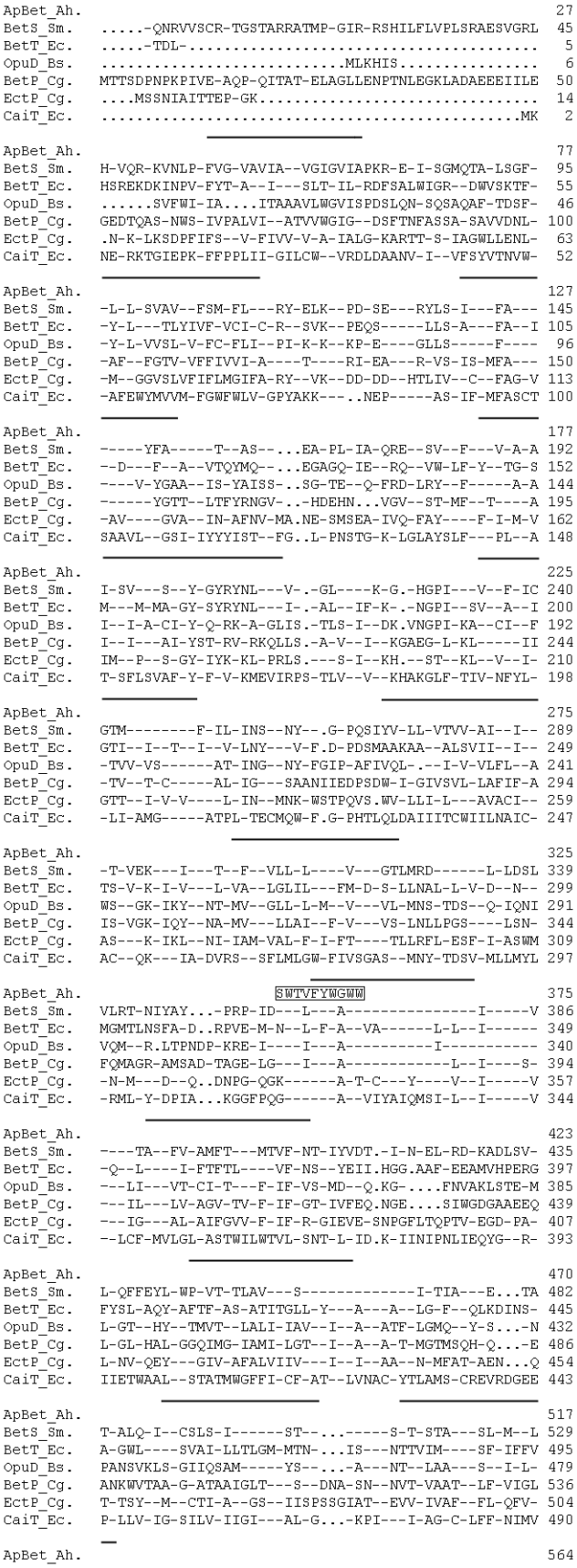


Figure 3 A model of the secondary structure of ApBet with the twelve putative transmembrane segments (I-XII). The grey box located in the eighth transmembrane segment represents the sequence SWTVFYWGWW which is similar to the consensus pattern [GSDN]-W-T-[LIVM]-X-[FY]-W-X-W-W for the proteins in the BCCT family.

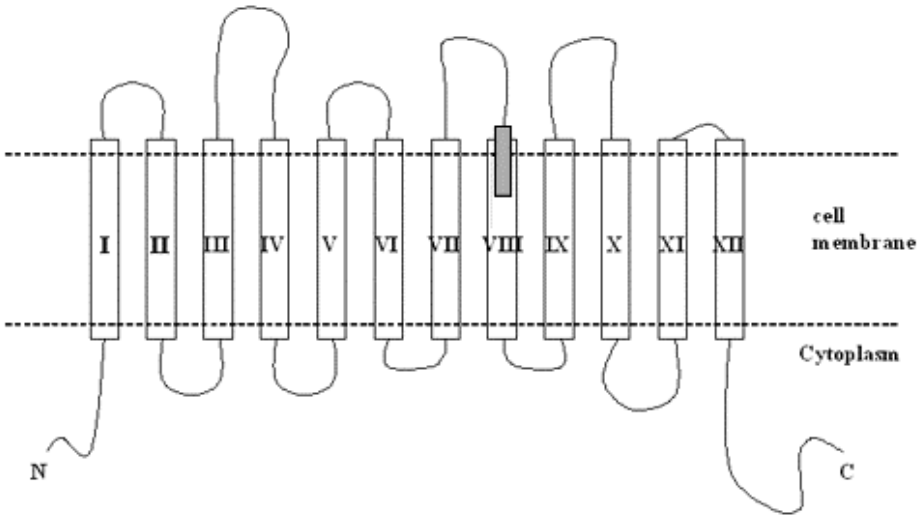
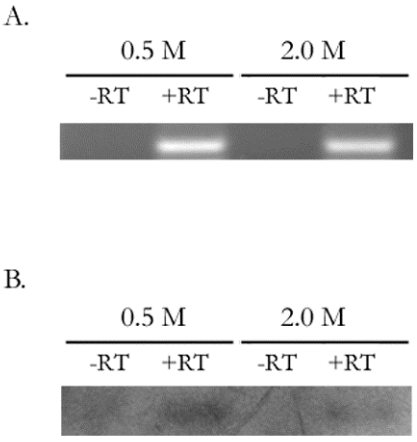


Figure 4 RT-PCR analyses of the *ApBet* mRNAs. A. Total RNA isolated from *A.halophytica* was used in RT-PCR assays either with (+RT) or without (-RT) the addition of M-MLV reverse transcriptase and the cDNAs were amplified by 30-cycle PCR. The products derived from 0.2 μg of total RNA were separated in agarose gels and visualized by ethidium-bromide staining. B. Similar RT-PCR was carried out except that PCR amplification consisted of only 16 cycles. PCR products separated by agarose gel electrophoresis were blotted onto a positively charged membrane. The blot was hybridized with a probe made from the cloned *ApBet* gene and the hybridizing bands were detected by autoradiography.



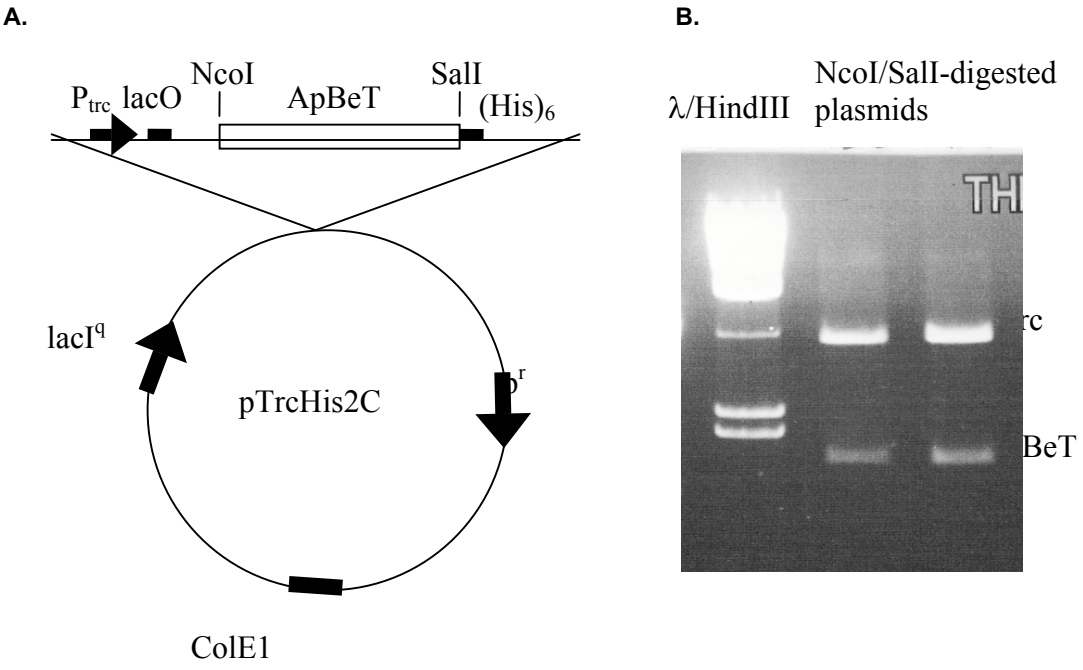
Appendix

Cloning of the *ApBet* gene into pTrcHis2C and functional complemention in MKH13 *E.coli* cells

To investigate the function of the *ApBet* gene, a pair of primers was designed based on the genomic sequence of *A.halophytica* and used to isolate a full length gene with NcoI and SalI restriction sites engineered at the 5′ and 3′ end, respectively. The coding region of the gene then was inserted into the expression vector, pTrc-His2C (Invitrogen) as shown in the schematic diagram in Fig.Ap1A. The recombinant DNA was digested with NcoI and SalI and the products were analyzed by agarose gel electrophoresis (Fig.Ap1B) to confirm the insertion.

To determine whether this gene's product function as a betaine or choline transporter, the first step is to demonstrate whether the product of this gene can complement the salt-sensitive phenotype of the *E.coli* mutant MKH13 in the presence of betaine or choline. *E.coli* MKH13 cells in which *betT*, *putPA*, *proP*, and *proU* genes are deleted, are unable to grow in high osmolality medium containing betaine due to the lack of a betaine transport system as well as betaine synthesis genes. To test if the *ApBeT* gene can complement the salt-sensitive phenotype of the MKH13, the cells were transformed with pTrcHis2C, and pTrcHis2C harboring the *ApBeT* gene. The cells were grown on agar plates minimal medium A containing 0.2% glucose; 0.7 M NaCl and 1 mM glycine betaine or 1 mM choline. Its growth was determined after incubation at 37°C for 2-4 days. So far, the results have shown that *ApBeT* gene did not appear to complement the salt-sensitive phynotype in medium containing either betaine or choline.

Figure Ap1 Cloning of *ApBet* into the expression vector pTrcHis2C. A. A schematic diagram depicts the strategy used to insert the *ApBet* gene into pTrcHis2C expression vector. B. A digested recombinant plasmid was analyzed by agarose gel electrophoresis



Output จากโครงการวิจัยที่ได้รับทุนจากสกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

   อยู่ในขั้นตอนการรวบรวมและเขียนผลงานวิจัยเพื่อลงตีพิมพ์ในวารสารวิชาการนานาชาติ

2. การนำผลงานไปใช้ประโยชน์

   a. เชิงพาณิชย์

      ไม่มี

   b. เชิงนโยบาย

      ไม่มี

   c. เชิงสาธารณะ

      เกิดความร่วมมือทางการวิจัยกับ Dr. Teruhiro Takabe และคณะ, Research Institute, Faculty of Science and Technology, Meijo University, Nagoya, Japan

   d. เชิงวิชาการ

      ยีนที่ได้อาจมีประโยชน์ใช้ในการศึกษากลไกการทนเค็มของ *Aphnothece halophytica* ต่อไป และถ้าได้ทราบกลไกการทำงานของยีนดังกล่าวก็อาจใช้ยีนหรือความรู้ในเรื่องการทำงานและการควบคุมยีนดังกล่าวในเชิงกลไกการตอบสนองต่อความเครียดออสโมติกเพื่อสร้างหรือพัฒนาพืชทนเค็ม ยีนนี้ซึ่งยังไม่มีข้อมูลมากนักรวมทั้งเซลล์ MKH13 ที่ได้รับมาจาก Dr. Takabe น่าจะเป็น material ให้นิสิตศึกษาเพื่อในการศึกษาและงานวิจัยต่อไปได้