



## Research paper

# Isolation and characterization of proline/betaine transporter gene from oil palm

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Oil production from oil palm is adversely affected by drought and salt. Under drought and salt stress, proline content increases in oil palm; the mechanism for this is unknown. Here, an 8319-nucleotide sequence including cDNA, genomic DNA and the promoter region of proline transporter gene from oil palm *Elaeis guineensis* was determined. The transporter gene exhibited high similarity to *Bet/ProT* genes from several plants, but the highest homology was found with rice *ProT1*. The exon–intron structure of genomic DNA was unique, and numerous stress-response *cis*-elements were found in the promoter region. Expression of cDNA *EgProT1* in *Escherichia coli* mutant exhibited uptake activities for glycinebetaine and choline as well as proline. Under salt-stressed conditions, exogenously applied glycinebetaine was taken up into the root more rapidly than the control. These data indicate that oil palm has a unique *ProT1* gene. Nucleotide sequence data for the cDNA and genomic DNA of proline transporter gene from *Elaeis guineensis* are available in the DDJB database under accession numbers AB597035 and AB597036, respectively.

**Keywords:** betaine transport, *Elaeis guineensis*, oil palm, proline transporter, salt stress.

## Introduction

Oil palm is an economically important monocotyledonous plant that produces the greatest amount of edible oil in the world and is widely cultivated in the tropical zone (Yusof and Chen 2003). Oil yield and growth rate are the focus of oil palm genetic improvement (Jalani et al. 1997, Cochard et al. 2005). Oil yield and growth rate are dependent not only on genetic background, but also on environmental factors such as salt, drought and high temperature (Henson and Dolmat 2003, Kallarackal et al. 2004, Henson and Harun 2005). However, physiological and molecular biological studies on abiotic stresses of oil palm are scarce. Regulation of cell volume by adjusting the osmotic balance between inside and outside cells is one of the important mechanisms to cope with salt and drought stresses (Chen and Polle 2010). To maintain osmotic balance, plants synthesize ‘organic osmolytes’ such as glycinebetaine (betaine) and proline

(Rhodes and Hanson 1993, Takabe et al. 2006). It seems clear now that all plants have a proline biosynthetic pathway whereas the betaine biosynthetic pathway is restricted to plants such as *Amaranthaceae*. Accumulation levels of osmolytes would be determined by the rates of biosynthesis, metabolism and transport. Compared with biosynthesis, the transport properties of osmolytes such as proline and betaine are largely unknown.

Proline transporters (ProTs) were first isolated from *Arabidopsis* as highly selective transporters for proline (Rentsch et al. 1996). Although tomato is a betaine non-accumulating plant, it was shown that LeProTs also transport betaine (Schwacke et al. 1999, Grallath et al. 2005). We also isolated homologous transporters from betaine-accumulating mangrove *Avicennia marina* (AmBet/ProT1–2) and showed that they transport betaine and proline (Waditee et al. 2002). Hitherto, functional properties of Bet/ProTs have been reported for

betaine non-accumulating plants—*Arabidopsis* (*Arabidopsis thaliana*, AtProT1–3: Rentsch et al. 1996, Grallath et al. 2005), tomato (*Lycopersicon esculentum*, LeProT1–3: Schwacke et al. 1999) and rice (*Oryza sativa*, OsProT1: Igarashi et al. 2000)—and on betaine-accumulating plants—mangrove (*A. marina*, AmBet/ProT1–2: Waditee et al. 2002), sugar beet (*Beta vulgaris*, BvBet/ProT1: Yamada et al. 2009) and barley (*Hordeum vulgare*, HvProT1: Ueda et al. 2001; HvProT2: Fujiwara et al. 2010). Among them, the selectivity of rice ProT1 for betaine has not been investigated (Igarashi et al. 2000), and barley HvProT1 is the only known transporter that recognized proline, but not betaine (Ueda et al. 2001). These facts suggest that Bet/ProTs from dicot plants transport betaine as well as proline. In contrast, the substrate specificity of Bet/ProTs from monocot plants seems to be unclear.

In previous papers (Cha-um et al. 2010a, 2010b), we showed that proline content of oil palm increased upon abiotic stresses such as salt and drought. Here, we isolated cDNA, genomic DNA and the promoter region of ProT from oil palm. It will be shown that oil palm has a unique EgProT1 that could uptake betaine, choline and proline.

## Materials and methods

### Plant materials, culture conditions and stress treatment

Oil palm fruits were obtained from Suksomboon Palm Co. Ltd (Chonburi, Thailand). The kernel of the fruit was removed. The seeds, with the seed coat, were dried in a hot air oven at 45 °C for 12 h and then the seed coat was broken. The embryos, along with the endosperm, were surface-disinfected once in 15% Clorox® for 20 min and once in 5% Clorox for 30 min. The embryos were then excised to germinate in Murashige and Skoog (MS) medium (Waditee et al. 2005). The media were adjusted to pH 5.7 before autoclaving.

Oil palm seedlings were cultured under conditions of 25 °C ambient temperature, 60% relative humidity and 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density provided by fluorescent lamps with a 16 h light per day photoperiod. After 2 months, the seedlings were transferred to soil containing vermiculite with 1/10 MS solution (130 ml) in a growth chamber with a 16 h light (28 °C, 70  $\mu\text{E m}^{-2} \text{s}^{-1}$ )/8 h dark (25 °C) cycle and 60% relative humidity, unless otherwise stated. After 2 weeks, the seedlings were used for stress treatment. For salt-stress treatment, 100 ml of 1/10 MS solution containing the indicated concentrations of NaCl was applied to the culture medium every second day.

### Bacterial strains and culture conditions

*Escherichia coli* DH5 $\alpha$  cells were grown at 37 °C in Luria–Bertani (LB) medium. *Escherichia coli* MKH13 cells deficient in *betT*, *putPA*, *proP* and *proU* genes were grown at 37 °C in minimal medium A (MMA) containing 0.2% glucose and

ampicillin (50  $\mu\text{g ml}^{-1}$ ) (Waditee et al. 2002). Radiolabeled 1- $^{14}\text{C}$ betaine (55 mCi mmol $^{-1}$ ), choline and 1- $^{14}\text{C}$ proline (50 mCi mmol $^{-1}$ ) were purchased from American Radiolabeled Chemicals, Inc. (St Louis, MO, USA).

### Isolation of *E. guineensis* ProT gene (EgProT1)

First-strand cDNA was synthesized from the total RNA of *E. guineensis* leaves using oligo(dT) $_{18}$  primer. Then, the partial gene that encodes putative ProT of *E. guineensis* (EgProT1) was amplified using degenerate primers (ProT-F/ProT-R) that were designed from the conserved regions of ProTs (Waditee et al. 2002). To obtain the upstream and downstream sequences of *EgProT1*, genome walking was performed using LongAmp (LA) PCR in vitro Cloning Kit (TaKaRa, Shiga, Japan). The exons and introns of *EgProT1* were identified by polymerase chain reaction (PCR) amplification and sequence analysis. Nucleotide sequence data for the cDNA and genomic DNA of ProT gene from *E. guineensis* are available in the DDJB database under accession numbers AB597035 and AB597036, respectively.

### Construction of expression vector for EgProT1 in *E. coli*

The coding region of *EgProT1* was amplified from the cDNA of *E. guineensis* with primer pairs EgproTF-Bam (5'-GGATC CAATGGAGGTCGAGGAGGAGAAG-3') and EgproTstopR-Xho (5'-AACTCGAGTTACAGATCAGCAAAAAAATG-3'). Polymerase chain reaction products were subcloned into pCR2.1 and sequenced. Then, the DNA fragments covering the coding region of *EgProT1* were prepared by digestion with BamHI and XhoI. The resulting fragments were ligated into the corresponding site of pTrcHis2C. The generated plasmid pTrc-EgproT was transferred to *E. coli* MKH13 cells. Transformants were selected on LB agar containing 50  $\mu\text{g ml}^{-1}$  ampicillin.

### Transport assay

*Escherichia coli* MKH13 cells transformed with pTrcHis2C empty vector and pTrc-EgproT were grown overnight at 37 °C in LB medium containing ampicillin (50  $\mu\text{g ml}^{-1}$ ) and were then inoculated into the same fresh medium to an absorbance at 620 nm (OD $_{620}$ ) of 0.05. Isopropyl- $\beta$ -D-thiogalactopyranoside (0.1 mM) was added to mid log-phase cells (OD $_{620}$  between 0.6 and 0.8). After a 1 h incubation (OD $_{620}$  of 2.0), cells were harvested and resuspended in 300  $\mu\text{l}$  of MMA and pH was adjusted by solid 2-(*N*-morpholino)ethanesulfonic acid (MES).  $^{14}\text{C}$ Choline,  $^{14}\text{C}$ betaine or 1- $^{14}\text{C}$ proline was added and then incubated at 30 °C for 5 min. The incubated cells were washed with 400  $\mu\text{l}$  of water twice. Cells were disrupted by the addition of 20  $\mu\text{l}$  of 10% H $_2$ O $_2$  and heated at 95 °C for 5 min. Then, 1 ml of liquid scintillation fluid was added and mixed vigorously with a vortex. The resulting mixtures were subjected to liquid scintillation analysis.

### Preparation of expression vector for green fluorescent protein-fused *EgProT1* in onion cells

To investigate the localization of *EgProT1* in onion cells, green fluorescent protein (GFP) gene was used as a reporter gene (Yamada et al. 2009). Green fluorescent protein gene was tagged to either the N- or C-terminal end of the *EgProT1* gene. The coding region of *EgProT1* was amplified using pTrc-EgproT (described above) with specific primer sets: GFP-EgproT-up (5'-GGT CCATGGAGGTCGAGGAGGAGAAG-3') and GFP-EgproT-low (5'-GCGGCCGCTT ACAGATCAGCAAAAAAATG-3') for the N-terminal end and EgproT-GFP-up (5'-TCTA GAATGGAGGTCGAGGAGGAGAA-3') and EgproT-GFP-low (5'-GGATCCCAGATC AGCAAAAAAATGGAT-3') for the C-terminal end. Polymerase chain reaction products were subcloned into pCR2.1 and sequenced. For the construction of *EgProT1* tagging GFP at the C-terminal, DNA fragments covering the coding region of *EgProT1* were prepared by digestion with XbaI and BamHI, and then ligated into the XbaI/BamHI site of pEL2 $\Omega$ -MCS (Ohtsubo et al. 1999). This plasmid was digested with HindIII and BamHI, and then ligated into the HindIII/BamHI site of pTH2 (Niwa 2003), resulting in the pEgproT-GFP plasmid. The pEgproT-GFP plasmid was digested with NcoI to remove the *EgProT1* region, and self-ligated, resulting in the pGFP plasmid. For the construction of *EgProT1* tagging GFP at the N-terminal, the GFP gene (SGFP-TYG) was amplified with the primers 5'-ATGTCGACGGATCCATGGTGAGCAA-3' and 5'-TTCTGCAGGCGCCGCGGTACCCTTGACAGCTCGTCCATG C-3' using pTH2 vector as a template and cloned into pBlue-script II SK+. The amplified fragment was digested with NcoI and PstI, and then ligated into the NcoI/PstI site of pGFP, resulting in the pGFP1 plasmid, which did not contain the stop codon. EgproT was prepared by digestion with KpnI/NotI and then ligated into the KpnI/NotI site of pGFP1, resulting in the pGFP-EgproT plasmid. The generated plasmids pGFP, pGFP-EgproT and pEgproT-GFP were introduced into onion cells by the particle bombardment method.

### Computer analysis and other methods

The hydropathy profile of the deduced amino acid sequence was predicted according to the method of Kyte and Doolittle (1982). The possible transmembrane (TM) segments of *EgProT1* were deduced by the computer program TopPredII (Hofmann and Stoffel 1992). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immuno-blot analysis were carried out according to the standard protocol as described previously (Waditee et al. 2005). Protein content was determined by the Lowry method (Hibino et al. 2002).

## Results and discussion

### Proline content and betaine uptake under salt-stress conditions in oil palm *E. guineensis*

In a previous paper (Cha-um et al. 2010a), we showed that proline content of oil palm leaves increased ~2.9-fold after 14 days of salt stress (0.2 M NaCl). Here, we examined the uptake of betaine in various tissues using 2-month-old oil palm. It was found that oil palm could uptake betaine from the growth medium. The levels of betaine in the middle leaf and base were increased upon salt stress, although a slight decrease was observed in the root (data not shown). These data indicate that under salt-stress conditions, oil palm accumulates more proline or betaine regardless of whether they are synthesized in vivo in the case of proline or exogenously applied via growth medium in the case of betaine.

It was shown that the activity of photosystem II decreased gradually upon salt stress. However, its decrease was protected by exogenously applied betaine, indicating that exogenously applied betaine was taken up via the root and transported to leaves where it protects photosystem II.

### *EgProT1* has large genomic DNA with a unique intron-exon pattern

Genomic sequences of ProTs are available for many plants. For example, *Arabidopsis* has three genes with seven exons and similar intron-exon structures (Figure 1). Poplar has two *ProT*

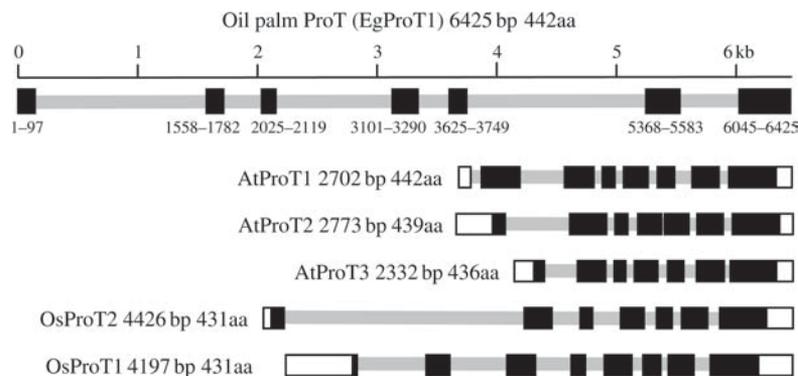


Figure 1. Exon-intron structure of genomic DNA of ProTs. Lines represent introns and closed boxes represent exons.

genes and their intron–exon structures were also very similar to those of the *Arabidopsis* ones (data not shown). In rice, the OsProT2 gene has seven exons and OsProT1 has eight exons. The first intron of OsProT2 is much longer than that of OsProT1. The size of genomic DNA and the intron–exon structure of oil palm are quite different from those of rice, indicating that the EgProT1 gene is less well conserved in structure.

### Cloning of ProT genes from oil palm

We constructed a cDNA library from the leaves of *E. guineensis*. A cDNA encoding ProT homologous to mangrove BetT was isolated as described in Materials and methods. But it turned out that the 5′-terminal region was missing. After genome walking, it was found that the *EgProT1* gene contains a large first intron (1460 bp). A putative cDNA encoding *EgProT1* was obtained. EgProT1 consists of 442 amino acid residues. Alignments of amino acid sequences from several amino acid transporters showed that EgProT1 exhibits high homology to BetT/ProTs from several plants such as tomato ProTs (LeProT1–3) and sugar beet (BvBet/ProT1) (see Figure 3).

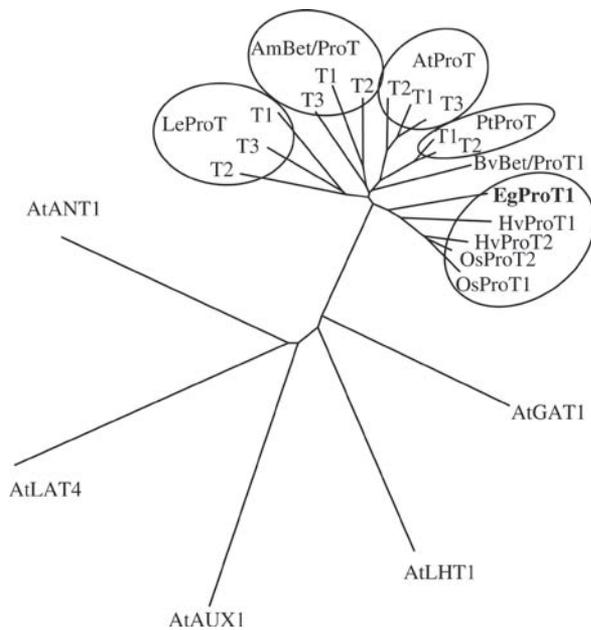


Figure 2. Phylogenetic analysis of plant Bet/ProTs. Amino acid sequences of Bet/ProTs from barley (HvProT1: Ueda et al. 2001; HvProT2: Fujiwara et al. 2010), rice (OsProT1: Igarashi et al. 2000; OsProT2: NP\_001058673), poplar (*Populus trichocarpa*; PtProT1: accession number XP\_002316242; PtProT1: accession number XP\_002311196), *A. thaliana* (AtProT1–3; Rentsch et al. 1996, Grallath et al. 2005), tomato (LeProT1–3; Schwacke et al. 1999), sugar beet (BvBet/ProT1; Yamada et al. 2009) and mangrove (AmBet/ProT1–3; Waditee et al. 2002) were aligned with ClustalW using default settings (<http://clustalw.ddbj.nig.ac.jp/top-j.html>). Amino acid sequences of amino acid permeases (AtAAP, AT1G31830; AtLHT1, At5g40780; ANT1, At3g11900), gamma aminobutyric acid transporter (AtGAT1, AT1G08230) and auxin transporter (AUX1, NP\_565882) were also included. The phylogenetic tree using the neighbor-joining algorithm was constructed with TreeView software.

EgProT1 exhibits the highest homology to rice ProT2 (NP\_001058673) (74% identity at the amino acid level), followed by rice ProT1 (Igarashi et al. 2000) (72%) and barley ProT1 (Fujiwara et al. 2010) (71%). Since oil palm, rice and barley are monocots, the above data suggest that Bet/ProTs evolved after the separation between monocot and dicot plants. The homology of EgProT1 to the gamma aminobutyric acid transporter (Meyer et al. 2006) and amino acid permeases was low, as shown in Figure 3. As mentioned by Grallath et al. (2005), Figure 2 shows that ProT members from a single plant species always cluster together, indicating that duplication of the genes was a relatively recent event.

### Many aromatic amino acid residues are located in TM domains

Analysis of the hydropathy plot (Kyte and Doolittle 1982) and the TM prediction program (Hofmann and Stoffel 1992) predicted 11 putative TM segments, as shown in Figure 3. Putative TM helical segments are boxed, and the numbers of the first and last amino acids in each segment are indicated. Many conserved aromatic amino acid residues were found in TMs III, IV and VII. Among them, TM VII has an extremely large number of aromatic residues. Among 17 Bet/ProTs shown in Figure 2, the conserved amino acids in the TM segments of Figure 3 are underlined. There are several amino acid residues, including Phe169, which are only conserved among ProTs from monocot plants. Charged amino acid residues in TM X (Asp) and TM XI (Arg) were also found (Figure 3). In the highly conserved loop region connecting TMs II and III, many basic amino acid residues were found.

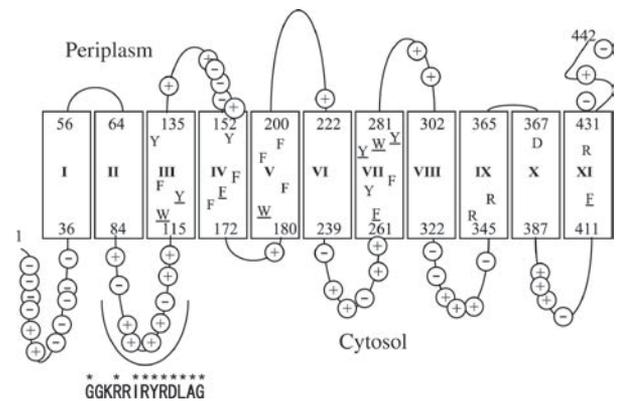


Figure 3. Topological model of EgProT1. Hypothetical secondary structure model of the EgProT1 protein. The possible TM segments of the EgProT1 sequence were deduced by the computer program TopPredII. Putative TM helical segments are boxed, and the first and last amino acids of each segment are indicated. Unique aromatic and charged amino acids in TM segments are shown. Conserved aromatic amino acids within 17 Bet/ProTs in Figure 3 are shown by underlines. Charged amino acids in loops are indicated by pluses (Arg and Lys) and minuses (Asp and Glu). Highly conserved amino acids in the loop connecting TM2 and TM3 are shown by asterisks.

Recently, the three-dimensional structure of the betaine transporter (BetP) from *Corynebacterium glutamicum* has been resolved (Ressler et al. 2009). It was shown that betaine is bound in a tryptophan box occluded from both sides of the membrane with aromatic side chains lining the transport pathway. We could also find aromatic amino acid residues in the betaine transporter from a halotolerant cyanobacterium *Aphanothece halophytica* (ApBetT) (data not shown) (Laloknam et al. 2006). Although the homology of amino acid residues between EgProT1 and BetP or ApBetT is low and the numbers of TMs are different between EgProT1 (11 TMs) and BetP or ApBetT (12 TMs), the importance of aromatic residues seems to be the same.

### Promoter analysis

To characterize the promoter of EgProT1, the 1895 bp 5' flanking region of *EgProT1* gene was sequenced. Numerous potential *cis*-acting elements were predicted by database analysis (PLACE; <http://www.dna.affrc.go.jp/PLACE/>). Apart from the TATA-box and the CAAT-box, several stress-related transcription-binding motifs are found. The core motif of dehydration-responsive element/C-repeat (DRE/CRT) *cis*-acting element (Simpson et al. 2003) was found at -55 and -1474 (data not shown). The MYB binding sites for rd22 (Abe et al. 1997) were found at -137, -337 and -416. Low-temperature-responsive elements (Baker et al. 1994) at -54, -835, -1132 and -1536, an abscisic acid-responsive-like element (Nakashima et al. 2006) at -460, and heat shock elements (Rieping and Schoffl 1992) at -319, -490, -804 and -1182 were also found. More *cis*-regulatory elements could be found, although we did not test the roles of these putative elements in transcriptional regulation of *EgProT1*. These facts suggest that expression of *EgProT1* is induced by abiotic stress.

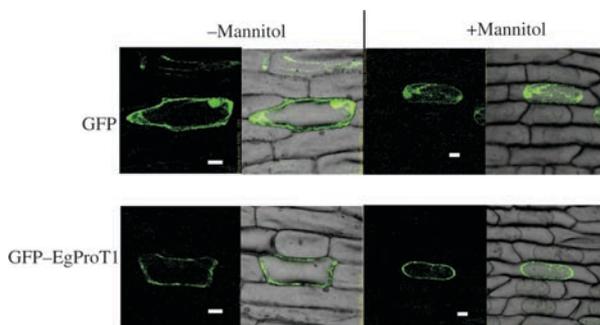


Figure 4. Localization of EgProT1. GFP-EgProT1 and GFP were transiently expressed in onion cells under the control of the cauliflower mosaic virus 35S promoter. Fluorescent images were obtained by confocal laser-scanning microscopy. The onion epidermal cells expressing GFP and GFP:EgProT1 were plasmolyzed with 0.8 M mannitol for 10 min. Green fluorescence of GFP was observed by confocal laser-scanning microscopy. Bright-field images were overlaid with GFP fluorescence. Scale bars represent 50  $\mu$ m.

### EgProT1 is a plasma membrane localized transporter

Localization of EgProT1 was determined by using a fusion protein of EgProT1 with GFP. Previously, it was shown that the homologous transporters from *Arabidopsis* AtProT1-3 (Grallath et al. 2005), sugar beet BvBet/ProT1 (Yamada et al. 2009) and barley HvProT2 (Fujiwara et al. 2010) localized at the plasma membrane. The fusion protein GFP-EgProT1 was transiently expressed in onion cells under the control of the cauliflower mosaic virus 35S promoter (Figure 4). Fluorescent images obtained by confocal laser-scanning microscopy showed that the signal from GFP-EgProT1 was present as a single fluorescent ring at the periphery of the cell. A similar fluorescence image was observed for GFP-AtProT1 (data not shown). In contrast, GFP fluorescence was observed distributed throughout the cytosol (Figure 4). These results indicate localization of EgProT1 at the plasma membrane.

### EgProT1 uptakes betaine, choline and proline

To determine whether the isolated EgProT1 really transports proline, the *EgProT1* gene was expressed in yeast strain 22574d (Jauniaux et al. 1987, Yamada et al. 2009). However, we could not detect uptake activity for proline and betaine (data not shown). Since we could not detect the EgProT1 protein by western blotting analysis (data not shown), the reason for failure to detect uptake activity was due to the absence of expression. Deletion of 4 or 58 amino acid residues in the N-terminal and start from the second and third Met, and

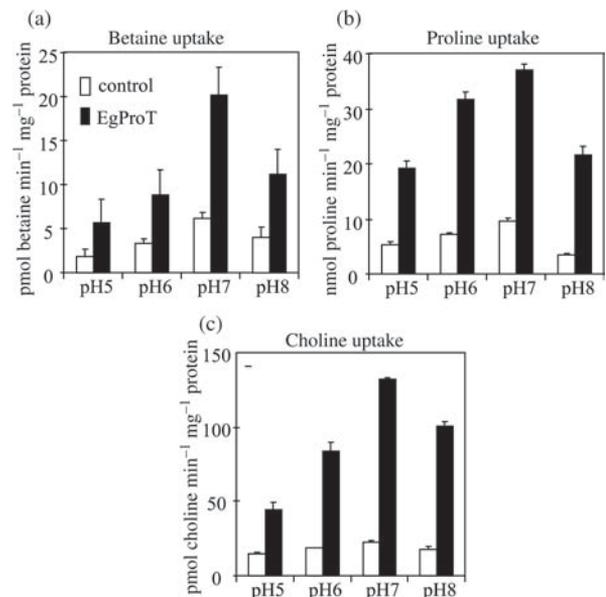


Figure 5. Uptake activity of EgProT1. (a–c) pH dependence of uptake activity of betaine, proline and choline by EgProT1, respectively. Transport activity was carried out as described in Materials and methods. Control and EgProt represent MKH13 cells transformed with vector-alone pTrcHis2C and pTrc-EgProT1, respectively. Each value shows the average of three independent measurements.

removal of the His-tag in the C-terminal were also negative. Then, we examined expression in *E. coli* mutant MKH13 (Haardt et al. 1995). After optimizing the expression conditions, we could detect uptake activity although it was low. No measurable uptake of [<sup>14</sup>C]betaine was observed for MKH13 cells transformed with vector-alone pTrcHis2C, whereas cells transformed with pTrc-EgProT1 could take up betaine (Figure 5a). Similar results were observed for proline uptake (Figure 5b). Interestingly, we observed the highest uptake activity for choline, as shown in Figure 5c.

Figure 5 shows that the rates of betaine uptake by EgProT1 increased with increasing pH, reaching a maximum at pH 7, and then decreased, which is very similar to the pH dependence of mangrove BetT1 and BetT2 (Waditee et al. 2002). Similar pH-dependent uptake patterns were obtained for proline (Figure 5b) and choline (Figure 5c). These data indicate that EgProT1 is a transporter for betaine, choline and proline.

## Conclusion

In previous papers (Cha-um et al. 2010a, 2010b), we showed that proline content in oil palm increased upon abiotic stresses such as salt and drought. In this study, we isolated cDNA, genomic DNA and the promoter region of ProT from oil palm. The transporter gene exhibited high similarity to those of rice ProTs. The fusion protein GFP-EgProT1 revealed the localization of EgProT1 at the plasma membrane. The exon-intron structure of genomic DNA was unique, and stress-response elements were found in the promoter region. Promoter analysis of EgProT1 in tobacco and oil palm, and in vivo functional analysis using a transgenic approach are in progress.

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