



# Preferential accumulation of betaine uncoupled to choline monooxygenase in young leaves of *sugar beet* – Importance of long-distance translocation of betaine under normal and salt-stressed conditions

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## Summary

It has been reported that glycinebetaine (betaine) is synthesized in response to abiotic stresses via a two-step oxidation of choline in which choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH) are involved. Here we show that significant amounts of betaine,  $>20 \mu\text{mol/gFW}$ , accumulated in young leaves of *Beta vulgaris* even under normal growth conditions, whereas levels in old leaves, cotyledons, hypocotyls, and roots were low. Under the same conditions, CMO accumulates exclusively in old leaves and is difficult to be detected in young leaves. By contrast, the levels of BADH were high in all tissues. Exogenously supplied choline was converted into betaine in old leaves, but levels were significantly lower in young leaves under the same conditions. When  $d_{11}$ -betaine was applied exogenously to old leaves, it was translocated preferentially into young leaves and roots. In response to salt stress, betaine levels increased in all tissues, but most significantly increased in young leaves. The levels of CMO increased in various tissues, but were low in young leaves. A betaine transporter gene was isolated. Its expression was more strongly

*Abbreviations:* *A. halophytica*, *Aphanothece halophytica*; BADH, betaine aldehyde dehydrogenase; BetT, betaine transporter; C, cotyledons; CBB, Coomassie Brilliant Blue; CDH, choline dehydrogenase; CMO, choline monooxygenase; GFP, green fluorescent protein; H, hypocotyls; L, leaves; MES, 2-(*N*-morpholino)ethanesulfonic acid; ProT, proline transporter; R, roots; TM, transmembrane.

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induced in old leaves than in young leaves. Based on these data, we discussed the role of CMO and betaine transporter under stress and non-stress conditions.  
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## Introduction

Many bacteria, plants, and animals accumulate glycine betaine (betaine) under water or salt-stress conditions. In most organisms, betaine is synthesized by a two-step oxidation of choline: choline → betaine aldehyde → betaine (Rathinasabapathi et al., 1997; Takabe et al., 2006). In plants, the novel Rieske-type iron-sulfur enzyme choline monooxygenase (CMO) catalyzes the first step (Burnet et al., 1995; Rathinasabapathi et al., 1997), whereas membrane-bound choline dehydrogenase (CDH) or soluble choline oxidase (COX) catalyzes the first step in animals and bacteria (Takabe et al., 2006). CMO so far has been found only in Chenopodiaceae and Amaranthaceae, but not in some betaine-accumulating plants such as mangrove (Russell et al., 1998; Hibino et al., 2001). The same enzyme, betaine aldehyde dehydrogenase (BADH), performs the second step in plants, animals, and bacteria. In some bacteria, such as a halotolerant cyanobacterium, betaine is synthesized from glycine by a series of three-step methylation reactions (Waditee et al., 2003).

Although the regulation of betaine synthesis is important, its mechanisms are poorly understood. This is partly due to the difficulty of transforming betaine-accumulating plants such as spinach, barley, and mangrove. Hitherto, it has been shown that the levels of CMO mRNA, protein, and enzyme activity were induced in response to abiotic stresses such as salt and drought in spinach (Rathinasabapathi et al., 1997), sugar beet (Chenopodiaceae), and *Amaranthus caudatus* (Amaranthaceae; Russell et al., 1998). It was also shown that on the removal of drought stress, the levels of CMO mRNA and protein decreased to their original levels (Russell et al., 1998). Very recently, CMO was detected in the seeds of sugar beet (Catusse et al., 2008). Little is known regarding other molecular mechanisms of betaine synthesis.

Few studies have reported on the transporters for betaine in plants. We isolated the betaine transporter genes from betaine-accumulating mangrove *Avicennia marina* (Waditee et al., 2002). It was shown that the expression and activity of betaine transporters were induced by salt stress, and that they transport proline as well as betaine (Waditee et al., 2002). Homologous transporters from tomato and *Arabidopsis*, betaine non-accumulators, have

been shown to transport betaine and proline (Schwacke et al., 1999; Grallath et al., 2005), although the corresponding transporters from rice and barley do not transport proline or betaine (Igarashi et al., 2000; Ueda et al., 2001).

Extensive studies on the accumulation of betaine in betaine-non-accumulating plants by exogenous apply of betaine or genetic manipulation of betaine synthesis gene have been carried out (Chen and Murata, 2002, 2008; Rontein et al., 2001). But, the engineered betaine levels are very low, 0.3–1.0 μmol/gFW and improvements of abiotic stresses have been shown with a limited success. By contrast, accumulation levels of betaine in betaine-accumulating plants are often higher than 30 μmol/gFW. This indicates that some unknown factor(s) limit the accumulation of betaine in betaine-non-accumulating plants. Therefore, it is interesting to study the molecular mechanisms of betaine accumulation in betaine-accumulating plants.

Sugar beet is a betaine-accumulating dicotyledonous plant of the Chenopodiaceae family that has high economic value because it is one of the two main sources of sucrose (Catusse et al., 2008). In addition to sucrose, a large amount of betaine accumulates in the tap roots of sugar beet (Russell et al., 1998). Here, we examined the levels of betaine and CMO in various organs of betaine accumulator. Surprisingly, we found that significant amounts of betaine accumulate in young leaves without the accumulation of CMO, even under control conditions. A betaine transporter gene was isolated. Its expression was more strongly induced in young leaves than in old leaves. In addition to a protective role of betaine, its role on the growth of actively developing cells under normal conditions was discussed.

## Materials and methods

### Plant materials

Sugar beet (*Beta vulgaris* L., cv. NK-219 mm-0) was used throughout this study. The seeds were germinated and grown on soil containing vermiculite with 1/10 MS solutions (130 mL) in a growth chamber with a 16-h light (25 °C, 100 μE m<sup>-2</sup> s<sup>-1</sup>)/8-h dark (20 °C) cycle and 60% relative humidity, unless otherwise stated. One-month-old *B. vulgaris*

plants were used for stress treatment. For salt-stress treatment, 100 mL of 1/10 MS solutions containing 0.3 M NaCl were applied to the culture medium every second day.

### Isolation of *Beta vulgaris* betaine transporter

The cDNA libraries for leaf and root of *B. vulgaris* were constructed using the SMART cDNA library construction kit (Clontech, CA, USA). The betaine transporter gene was isolated using the same mixed oligonucleotide primers which were used to isolate betaine transporter genes from a mangrove *A. marina* (Waditee et al., 2002). One clone containing the full-length gene that encodes putative betaine and/or proline transporter of *B. vulgaris* (BvBet/ProT1) was isolated. The DNA sequence was determined using a DNA Sequencer (ABI PRISM 3100) and analyzed with the DNASIS program (Hitachi Software Engineering Co., Kanagawa, Japan).

### Yeast strain, transformation, and selection

The coding region of BvBet/ProT1 was isolated by PCR. The forward primer *KpnI*-BvBetT-F, 5'-CAGG-TACCATGTCTTCTTCAGACCCAGAA-3', contains the *KpnI* restriction sites. The reverse primer BvBet/ProT-stop-*NotI*-R, 5'-AAGCGGCCGCTCACAAATCAG-CAAA GCAAAGAGATGA-3', contains the *NotI* restriction sites. The amplified fragment was ligated into *KpnI*/*NotI* sites of the pYES2 vector (Invitrogen, CA, USA) and designated as pBvBetT1. The expression plasmid was transferred first to *Escherichia coli* DH5 $\alpha$  cells and then to *Saccharomyces cerevisiae* strain 22574d. *S. cerevisiae* strain 22574d was generously provided by Dr. J.C. Jauniaux, University of Libre, Bruxelles, Belgium (Jauniaux et al., 1987). The 22574d yeast strain carries the following mutations: *mat*  $\alpha$  *ura3-1* (mutated orotidine-5-phosphate decarboxylase), *gap1-1* (mutated general amino acid transporter), *put4-1* (mutated specific proline transporter), and *uga4-1* (mutated GABA transporter). Yeast was transformed using the electroporation technique. Positive BvBet/ProT1 yeast transformants were transferred on selective minimal agar plates containing 0.17% yeast nitrogen base (without amino acids) and 20 mg/mL D-glucose. The yeast strains transformed with the empty pYES2 vector were used as a control.

### Preparation of GFP-BvBet/ProT1 fusion construct

Amplification of the green fluorescent protein (GFP) gene (*SGFP-TYG*) was performed with the

primers 5'-ATGTCGACGGATCCATGGTGAGCAA-3' and 5'-TTCTGCAGGCGCCGCGGTACCCT TGTACAGCTC-GTCCATGC-3' using pTH2 vector as a template (Niwa, 2003) and cloned into pBluescript II SK+. The amplified fragment was digested with *Nco* and *PstI*, and then ligated into *NcoI*/*PstI* site of the pTH2, resulting in the pSGFP plasmid. The BvBet/ProT1 fragment was prepared from pBvBetT1 plasmid by digestion with *KpnI* and *NotI*, and ligated into *KpnI*/*NotI* site of the pSGF. For the expression of the GFP-BvBet/ProT1 fusion protein in onion cells, particle bombardment method was used.

### Transport measurement in yeast

Yeast was grown in minimal liquid medium containing 0.17% yeast nitrogen base (without amino acids) and 20 mg/mL D-galactose to an optical density (OD) of 1.0–1.5 units at 600 nm (mid-log phase) at 30 °C. The cells were collected by brief centrifugation at 2000g at room temperature. The cell pellet was washed twice with water using 500-fold excess over cell volume and finally resuspended with water to OD of 2.0 units at 600 nm. For transport assays, 0.9 mL of cell suspension was added into 3 mL of 25 mM Tris/acetate solution at the desired pH; then 0.75 mL 20% D-galactose was added. Then substrate such as betaine (1 mM) was added; samples were incubated at 22 °C for 1 h. After centrifugation, the pellet was washed twice with 20 mM Tris/acetate buffer containing 3.5% D-galactose; and suspended in 100  $\mu$ L of distilled water. Betaine was extracted and measured as described.

### Analysis of betaine

Betaine was extracted as described previously (Waditee et al., 2005). Briefly, betaine was extracted from the plant tissues (100 mgFW) by extraction buffer (methanol: chloroform: water = 12:5:1) and centrifuged at 15,000g for 5 min. The supernatant was extracted with a mixture containing 25% (v/v) chloroform and 37.5% (v/v) water and centrifuged at 15,000g for 5 min. The supernatant was dried and dissolved in 100  $\mu$ L water. Betaine was measured with time of flight mass spectroscopy (KOMPACT MALDI TOF-MS, Shimadzu/Kratos) using d<sub>11</sub>-betaine as the internal standard.

### *In vivo* betaine synthesis activity

Young and old leaves were removed from 1-month-old *B. vulgaris*. Detached young or old

leaves (35 mg) were cut into 3 mm squares and put on 2 cm square filter paper, to which 200  $\mu\text{L}$  of 5 mM  $\text{d}_9$ -choline and 5 mM MES (pH 5.8) were applied. Leaves were irradiated with  $100 \mu\text{E m}^{-2} \text{s}^{-1}$  at  $25^\circ\text{C}$  for 1 h. Then, betaine was extracted as described. The extracted betaine and  $\text{d}_{11}$ -betaine were precipitated with  $\text{KI-I}_2$  and measured with mass spectroscopy. From the ratio of  $\text{d}_9$ -betaine synthesized from  $\text{d}_9$ -choline to  $\text{d}_{11}$ -betaine and internal standard, the extent of betaine synthesis activity was estimated.

### Betaine translocation

One-month-old *B. vulgaris* plants were used for betaine translocation. A 200- $\mu\text{L}$  aliquot of 10 mM  $\text{d}_{11}$ -betaine was injected into an old leaf via syringe. The leaf was then covered with a wrap. The plant was irradiated with  $100 \mu\text{E m}^{-2} \text{s}^{-1}$  at  $25^\circ\text{C}$ . After 12 h, the plant was dissected into young leaves, root, shoot, and old leaves (which were different from the fed old leaf). The total  $\text{d}_{11}$ -betaine in each part (50 mgFW) was determined as described.

### RT-PCR

Total RNA was extracted from young and old leaves by using RNeasy Plant Mini Kit (QIAGEN Inc., CA, USA). The first-strand cDNA was synthesized from 1  $\mu\text{g}$  of total RNA by using Reverse Transcriptase M-MLV (Takara, Tokyo, Japan) and was used as the PCR template. The amount of template cDNA and the number of PCR cycles were determined for each gene to ensure that amplification occurred in the linear range and allowed good quantification of the amplified products. The first-strand cDNA mixture was used as a template for PCR analysis; the mixture had been normalized with an actin gene (*Act*) of *B. vulgaris*. Amplification of the *Act* was performed with the forward primer 5'-GCCGTTCTTT CTCTGTACCC-3' and the reverse primer 5'-CAGCTTCCATTCCGATCAAT-3'. Primer sets for CMO (forward 5'-GCAACTG GAAGGTTTTCTGTGA-3' and reverse 5'-CCGGAT CCTCACTGCAAAGTTT-CATGCAA-3' and for *BvBetT1*: forward 5'-CAGGA-AATGCATT AAATCCC-3' and reverse 5'-CCTTCTCTC-CAATACAATGC-3' were used for PCR.

### Measurement of sugars, ions, and osmotic concentration

For the quantification of fructose, glucose, and sucrose, leaves were frozen with liquid nitrogen; leaves were extracted with boiling 80% (v/v) ethanol, and centrifuged at 10,000g for 10 min at

$4^\circ\text{C}$ . The alcoholic extracts were dried up and dissolved in sterilized water. The individual sugars were identified using a Shimadzu HPLC system for sugar analysis.

Osmotic concentrations of leaves and root were measured by the vapor pressure method (Miyamoto and Kamisaka, 1988). Frozen leaves were thawed at room temperature and centrifuged at  $4^\circ\text{C}$  for 10 min at 1000g. Aliquots (10  $\mu\text{L}$ ) of the supernatant were subjected to analysis in a vapor pressure osmometer (model 5520; Wescor, Longen, UT, USA).

Cellular ions were determined using Shimadzu Personal Ion Analyzer PIA-1000 (Shimadzu, Japan).

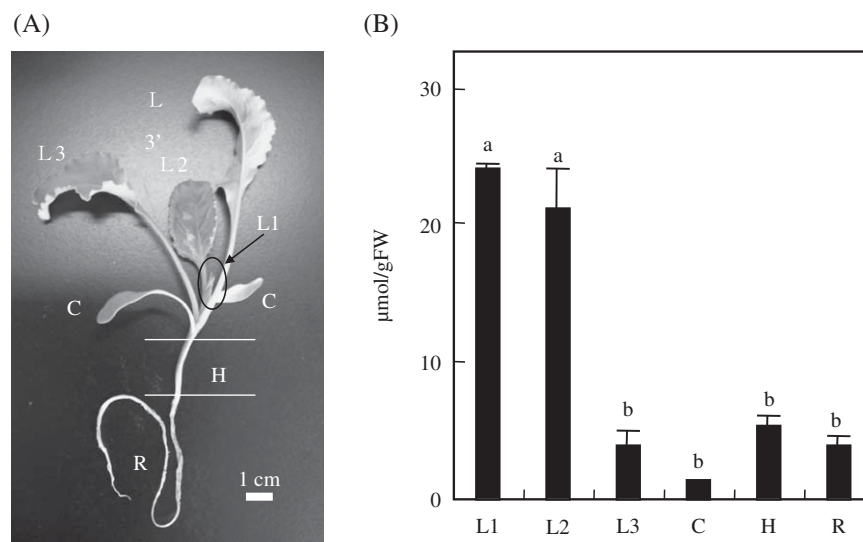
### Computer analysis and other methods

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 the AmT1 sequence were deduced by a computer program TopPredIII (Hofmann and Stoffel, 1992). SDS-PAGE and immuno-blot analysis were carried out according to the standard protocol as described previously (Waditee et al., 2005). Antisera raised against the spinach CMO and BADH were prepared using a white New Zealand rabbit female as previously described (Hibino et al., 2002). Protein contents were determined by Lowry method (Hibino et al., 2002).

## Results

### Differential accumulation of betaine under normal growth conditions

Figure 1A shows a representative photograph of 1-month-old *B. vulgaris* used in this study. Sugar beet was divided into young leaves (L1) ~1.0–2.5 cm, developing leaves (L2) >~3.5 cm, expanded leaves (L3 and L3'), cotyledons (C), hypocotyls (H), and roots (R). Since L3 and L3' leaves appear at the same time, these leaves have the same properties. We measured betaine contents in various tissues of *B. vulgaris*. Figure 1B shows that the levels of betaine are significantly different among tissues. The L1 leaves exhibited the highest value, 24  $\mu\text{mol/gFW}$ . A similar value, 21  $\mu\text{mol/gFW}$ , was also observed in the L2 leaves. By contrast, betaine levels in other tissues such as expanded leaves (L3), cotyledons (C), hypocotyls (H), and root (R) were relatively low, 2–5  $\mu\text{mol/gFW}$ . To our knowledge, these high values of betaine (>20  $\mu\text{mol/gFW}$ ) have not been reported previously under non-stress conditions.



**Figure 1.** Betaine contents under normal growth condition in *B. vulgaris*. (A) Photograph of *Beta vulgaris*. A 1-month-old plant was divided into young and developing leaves (L1 and L2), expanded leaves (L3 and L3'), cotyledons (C), hypocotyls (H), and roots (R). (B) Betaine contents in various tissues. Each value in Figure 1B shows the average of three independent measurements. Different letters denote significant differences ( $P < 0.05$ ) among organs.

### Differential accumulation of CMO, but not BADH, under normal growth conditions

To see whether varying betaine levels were due to the different expression levels of betaine synthesis enzymes, we examined the western blotting of CMO and BADH. Figure 2A shows the protein bands stained with Coomassie Brilliant Blue (CBB). Leaf parts of sugar beet (L1, L2, L3, and C) exhibited a strong band at around 55 kDa, corresponding to the large subunit of ribulose 1,5-bisphosphate (RuBisCO). The intensities of protein bands among these four leaves were similar. By contrast, the protein bands of the large subunit of RuBisCO are weak in the extract of H and R tissues. Figure 2B shows the results of western blotting for CMO. The CMO band was mostly detected in L3 (L3') leaves, although the weak bands could be detected in cotyledon (C) and hypocotyl (H) tissues. Surprisingly, the CMO band in young leaves (L1 and L2) was difficult to detect. The CMO band could not be detected in root (R). In contrast with CMO, BADH protein could be detected in all tissues (Figure 2C). The intensities of BADH bands were similar among all tissues.

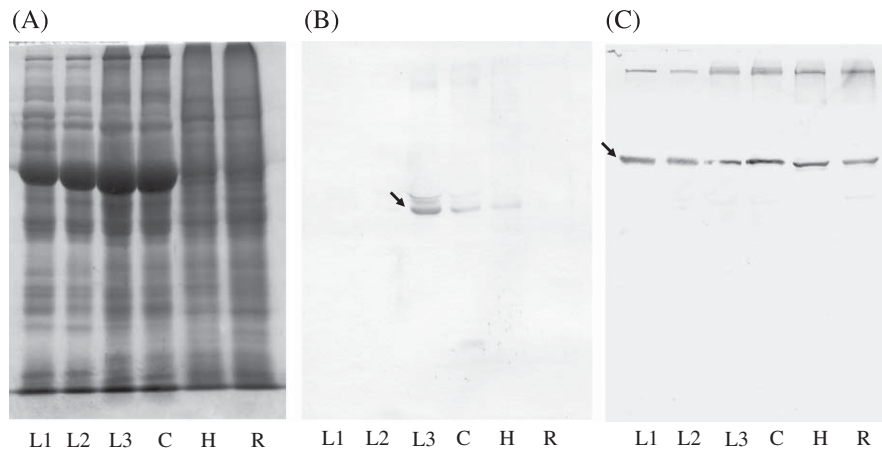
### Young leaves exhibited low betaine synthesis activity *in vivo*

Since Figure 2 shows that the CMO protein accumulates in expanded leaves (L3), but not in

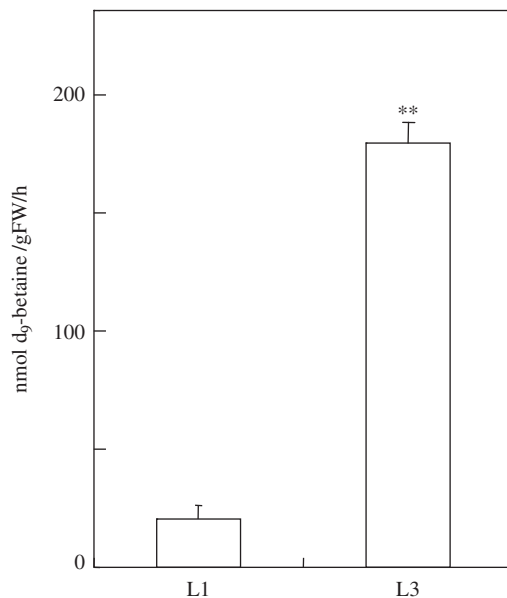
young leaves (L1 and L2), we examined whether the accumulation level of CMO reflects the activity of betaine synthesis *in vivo*. Detached leaf segments (3 mm × 3 mm) of L1 and L3 were put on filter paper, to which  $d_9$ -choline was applied. Samples were then irradiated at  $100 \mu\text{E m}^{-2} \text{s}^{-1}$  for 1 h. After extraction, the  $d_9$ -betaine was detected by TOF-MAS. Figure 3 shows that the rate of  $d_9$ -betaine synthesis in L3 leaves was about 180 nmol/gFW/h, whereas that of L1 leaves was about 20 nmol/gFW/h. L3 leaves exhibited about nine-fold higher betaine synthesis activity, in agreement with the results of Figure 2.

### Preferential translocation of betaine from old leaves to young leaves

The results above caused us to wonder how betaine accumulates in young leaves (L1 and L2) without betaine biosynthesis. To answer this question, we examined the possibility of long-distance betaine transport. Deuterium-substituted betaine ( $d_{11}$ -betaine) was injected into the attached expanded leaf (L3). The plants were irradiated for 12 h. Then, betaine was extracted from various tissues. Figure 4 shows that large amounts of  $d_{11}$ -betaine were detected in L1, L2, and root, but not in C or L3'. These data indicate that betaine synthesis occurs in expanded leaves (L3) and that betaine was transported into young leaves (L1 and L2).



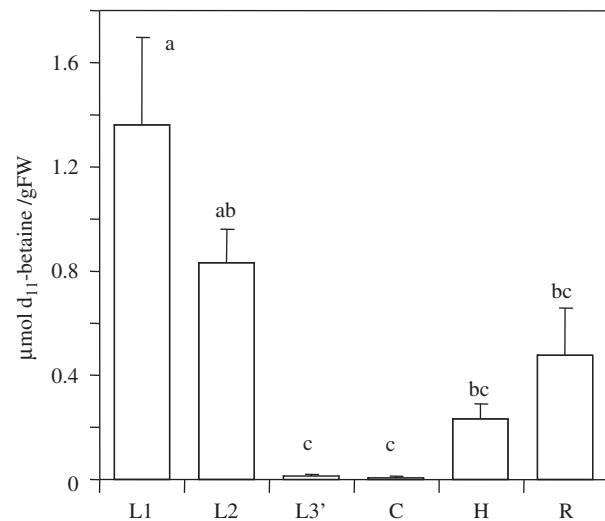
**Figure 2.** Detection of CMO and BADH in various tissues of *B. vulgaris* under normal growth conditions. One-month-old plants were harvested and separated into six tissues. The proteins were applied on SDS-PAGE. (A) Protein bands stained with CBB. (B) Immuno-blotting of CMO. (C) Immuno-blotting of BADH. BvCMO and BvBADH were detected by immuno-blot analysis using the antibodies raised against spinach CMO and BADH.



**Figure 3.** Conversion from choline to betaine in leaves. Detached leaves (L1 and L3) were cut to 3 mm squares and put on 2 cm square filter paper, to which d<sub>9</sub>-choline was applied. Samples were incubated for 1 h under irradiation (100 μE m<sup>-2</sup> s<sup>-1</sup>). Then, d<sub>9</sub>-betaine was detected with a TOF-MAS spectrometer. Each value shows the average of three independent measurements. Double stars (\*\*) represent significant difference ( $P < 0.01$ ) from L1 leaf.

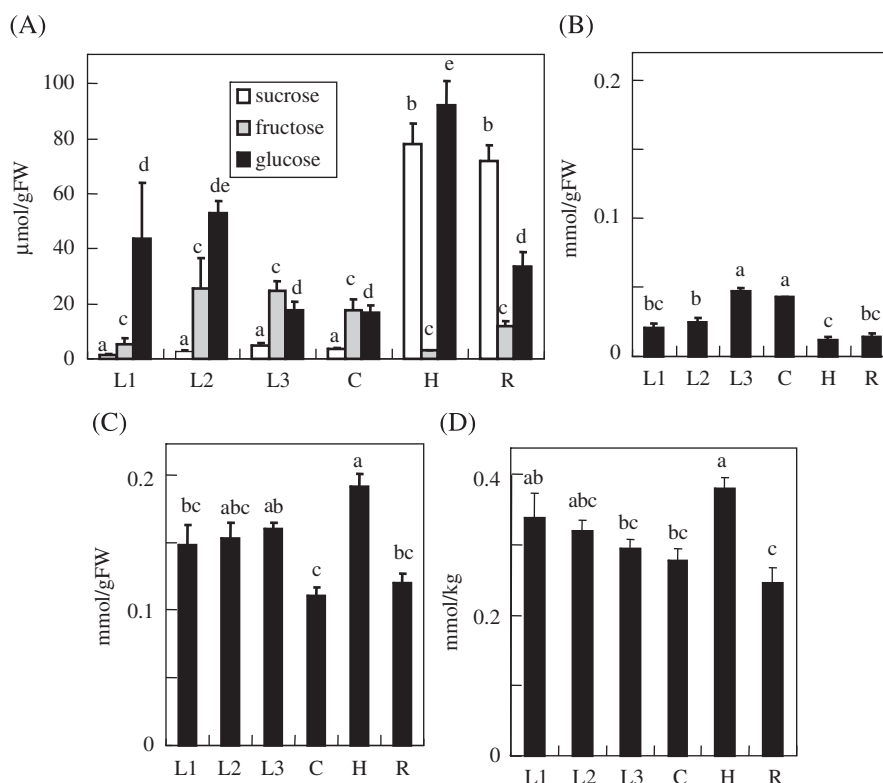
**Sugar, cations, and osmolarity in various tissues under normal growth conditions**

Since betaine is a typical osmoprotectant, we examined whether the levels of sugars, cations, and osmolarity differed among various tissues.



**Figure 4.** Translocation of betaine from old leaves. Deuterium-substituted betaine (d<sub>11</sub>-betaine) was injected into L3 leaf. After irradiation at 100 μE m<sup>-2</sup> s<sup>-1</sup> for 12 h, various parts of the plant were removed and the total d<sub>11</sub>-betaine in each part (50 mgFW) was determined. L3' is the expanded leaf different from the injected one (L3). Each value shows the average of three independent measurements. Different letters denote significant differences ( $P < 0.05$ ) among organs.

Figure 5A shows that young leaves (L1 and L2) preferentially accumulate glucose, whereas expanded leaves (L3) accumulate almost equal amounts of glucose and fructose. The total sugar content was similar among L1 and L3 leaves. In contrast, the sucrose content in H and R was very high, while the fructose content was low. Hypocotyls (H) contained the highest amounts of glucose.

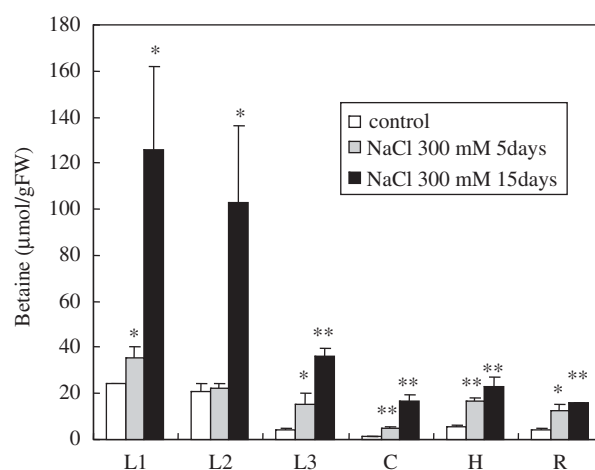


**Figure 5.** Sugars, ions, and osmolarity under normal growth conditions in *B. vulgaris*. One-month-old plants were harvested and separated into six tissues. Then, sugars (A),  $\text{Na}^+$  (B),  $\text{K}^+$  (C), and osmolarity (D) were measured as described in [Materials and methods](#). Each value shows the average of three independent measurements. Different letters denote significant differences ( $P < 0.05$ ) among organs.

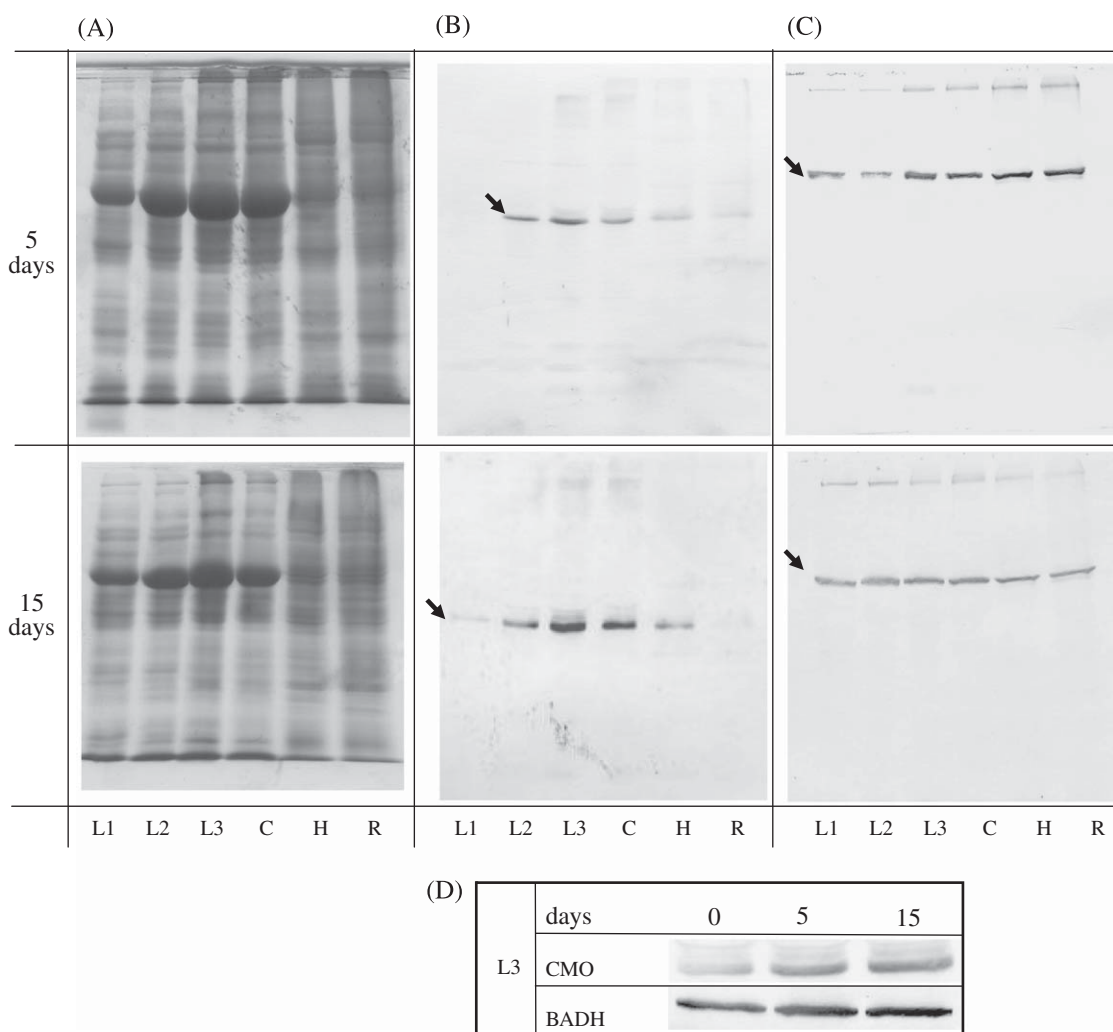
Figures 5B and C show the levels of  $\text{Na}^+$  and  $\text{K}^+$  ions in various tissues. Their values did not vary greatly among tissues. The  $\text{Na}^+$  content in expanded leaves (L3) was slightly higher than that of young leaves (L1 and L2; Figure 5B). The  $\text{K}^+$  contents in C and R were slightly low, whereas that in H was high (Figure 5C). Figure 5D shows the osmolarity of various tissues. The osmolarity was the highest in H and then decreased in the following order;  $\text{L1} > \text{L2} > \text{L3} > \text{C} > \text{R}$ .

### Betaine levels increased with salt stress in all tissues

Figure 6 shows the changes in betaine content following changes in NaCl (300 mM) for 5 and 15 days. The levels of betaine increased in all tissues following salt stress. Betaine content in L1 leaves increased more than  $10 \mu\text{mol/gFW}$  during the first 5 days and reached  $125 \mu\text{mol/gFW}$  after 15 days of 300 mM NaCl stress. Betaine content in L2 leaves also increased significantly after 15 days of salt stress. The betaine levels in L3, C, H, and R



**Figure 6.** Changes in betaine content in various tissues of *B. vulgaris* after salt stress. One-month-old *B. vulgaris* plants were treated with 0.3 M NaCl during the indicated period. Then, betaine was extracted from various tissues and measured as described in [Materials and methods](#). Each value shows the average of three independent measurements. Single star (\*) and double stars (\*\*) represent significant differences,  $P < 0.05$  and  $P < 0.01$ , from the control plants, respectively.



**Figure 7.** Alterations in CMO and BADH in various tissues of *B. vulgaris* after salt stress. One-month-old plants were treated with 0.3M NaCl for the indicated periods. Then plants were harvested and separated into six tissues. The proteins were applied on SDS-PAGE. (A) Protein bands stained with CBB. (B) Immunoblotting of CMO. (C) Immunoblotting of BADH. (D) Changes in L3 leaves. BvCMO and BvBADH were detected by immuno-blot analysis using the antibodies raised against spinach CMO and BADH.

increased at a constant rate, but remained relatively low ( $10\text{--}40\ \mu\text{mol/gFW}$ ) even after 15 days of 300 mM NaCl stress.

#### Differential expression of BvCMO in various tissues under salt-stress conditions

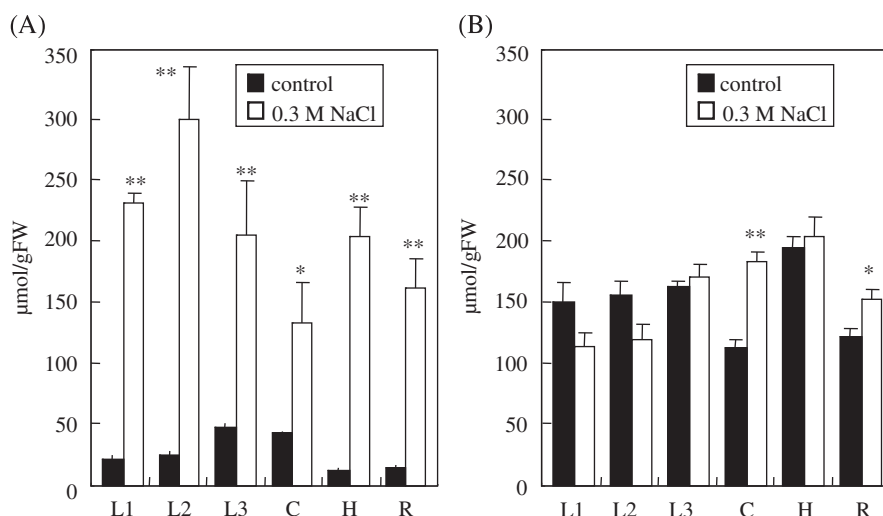
We examined the changes in levels of BvCMO and BvBADH following changes in salinity. One-month-old plants were treated with 300 mM NaCl. Figure 7 shows the results after the 5- and 15-day salt stresses. Left panel (A) shows the protein bands stained with CBB. The pattern of bands was similar to that of non-stressed plants, although the intensity of the band of the large subunit of RuBisCO after 15 days' salt stress was slightly weak compared with those from non-stressed and 5 days' stress plants.

The middle panel (B) shows the results of western blotting for CMO. The CMO band could be detected in all tissues, but was weak in L1 leaves and root (R) even after 15 days' stress. In contrast, the BADH protein could be detected in a similar amount in all tissues. Figure 7D shows the time-course changes of CMO and BADH bands in L3 leaves following salt stress. The results show that the level of CMO in L3 leaves increased significantly on salt stress, whereas the BADH level increased slightly.

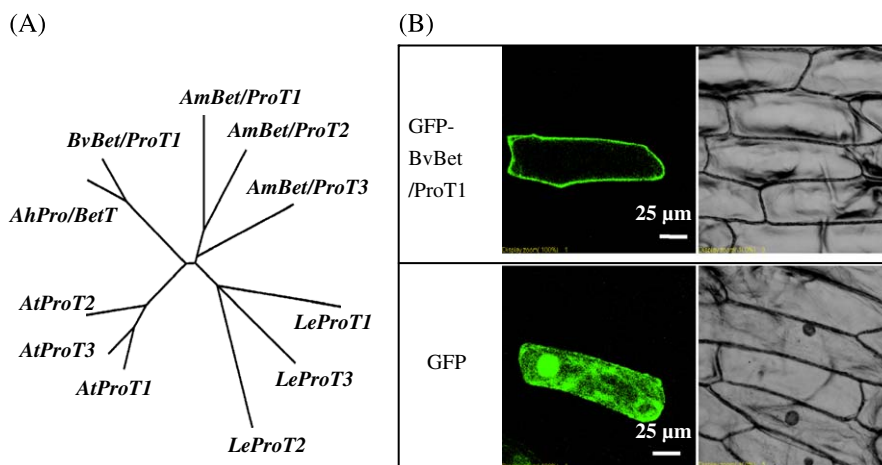
#### Altered $\text{Na}^+$ and $\text{K}^+$ contents in various tissues following salt stress

Next, we examined alterations in  $\text{Na}^+$  and  $\text{K}^+$  content in various tissues. Following salt stress for 5 days, the  $\text{Na}^+$  content increased significantly in all





**Figure 8.** Changes in Na<sup>+</sup> and K<sup>+</sup> contents following salt stress. One-month-old plants were treated with 300 mM NaCl for 5 days. Then samples were collected for analysis. (A) Na<sup>+</sup> and (B) K<sup>+</sup>. Each value shows the average of three independent measurements. Single star (\*) and double stars (\*\*) represent significant differences,  $P < 0.05$  and  $P < 0.01$ , from the control plants, respectively.



**Figure 9.** Phylogenetic trees and localization of BvBet/ProT1. (A) Phylogenetic tree. Phylogenetic analysis of 11 putative proline/betaine transporter genes was constructed as described in [Materials and methods](#). Accession numbers were as follows: BvBet/ProT1 (AB477096), AmBetProT1 (AB075902), AmBet/ProT2 (AB075903), AmBet/ProT3 (AB075904), LeProT1 (AF014808), LeProT2 (genbank:AF0148090), LeProT3 (AF014810), and AhPro/BetT (AF274032). AtProtT1, AtProtT2, and AtProtT3 were TAIR AT2G39890, AT3G55740, and AT2G39890, respectively. (B) Localization. GFP-BvBet/ProT1 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.

tissues, including L1 leaves ([Figure 8A](#)). The levels of K<sup>+</sup> were similar in all tissues and did not exhibit alterations associated with salt stress ([Figure 8B](#)). Under salt-stress conditions, Na<sup>+</sup> content was higher than K<sup>+</sup> content.

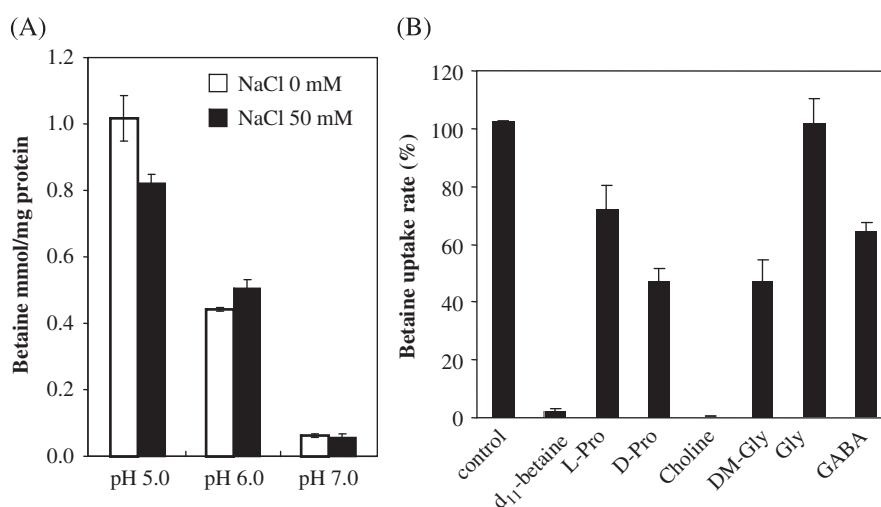
### Isolation of betaine transporter gene and its expression at high salinity

Since the above data suggest that betaine is transported from expanded leaves to young leaves,

we attempted to isolate the cDNA for betaine transporter. To that end, cDNA libraries were constructed from leaves and roots. The betaine transporter gene was isolated using the same mixed oligonucleotide primers that were used to isolate betaine transporter genes from a mangrove *A. marina* ([Waditee et al., 2002](#)). A gene encoding full-length betaine transporter was isolated and designated as BvBet/ProT1. The clone contains 1754 bp DNA, including poly(A) sequences in the 3'-terminal region. The predicted gene product consists of 448 amino acids with a molecular mass

	L1				L3			
	0		0.3		0		0.3	
NaCl (M)	0		0.3		0		0.3	
Cycles	25	27	25	27	25	27	25	27
<i>BvCMO</i>								
<i>BvBet/ProT1</i>								
<i>BvActin</i>								

**Figure 10.** Expression profile of *BvBet/ProT1* and *BvCMO*. Sugar beets were subjected to 0.3 M NaCl for 5 days. Total RNA was extracted from OL and YL as described in [Materials and methods](#). Amplification of *BvCMO*, *BvBet/ProT1*, and *BvActin* were performed for the indicated cycles.



**Figure 11.** Uptake activity of *BvBet/ProT1*. (A) pH dependence of uptake activity of betaine by *BvBet/ProT1*. The transport activity of betaine was carried out as described in [Materials and methods](#). (B) Competition of betaine (1 mM) uptake by *BvBet/ProT1* expressing yeast strain in the presence of the respective competitors at the concentration of 10 mM. DM-Gly, dimethylglycine; GABA, 4-aminobutyrate. Each value shows the average of three independent measurements.

of 49,158. The homology search revealed that *BvBet/ProT1* exhibited the highest homology to the corresponding gene from *Atriplex* (87% identity), followed by mangrove (67%) and *Arabidopsis* (67–68%), and tomato (61–64%; [Figure 9A](#)). By contrast, *BvBet/ProT1* showed low homology to amino acid permease AAP1 (data not shown). These data suggest that the isolated gene encodes a betaine/proline transporter.

Localization of *BvBet/ProT1* was determined by using a fusion protein of *BvBet/ProT1* with green fluorescent protein. Previously, it was shown that the homologous transporters from *Arabidopsis* (*AtProT1-3*) that transport betaine and proline localized at the plasma membrane ([Grallath et al., 2005](#)). The fusion protein GFP-*BvBet/ProT1* was transiently expressed in onion cells under the control of the cauliflower mosaic virus 35S promo-

ter ([Figure 9B](#)). Fluorescent images obtained by confocal laser-scanning microscopy showed that the signal from GFP-*BvBet/ProT1* 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). By contrast, the GFP fluorescence was observed distributing throughout the cytosol ([Figure 9B](#)). These results indicate the localization of *BvBet/ProT* at the plasma membrane.

The expression pattern of *BvBet/ProT1* was examined by RT-PCR. As shown in [Figure 10](#), mRNA for *BvBet/ProT1* was much higher in L3 leaves than that in L1 leaves under normal conditions. Following salt stress, *BvBet/ProT1* transcript levels were slightly increased in comparison with those observed during normal conditions. The CMO transcript level in L1 leaves was weak compared with

that of L3 leaves. Levels increased with salt stress, but the relationship between L1 and L3 leaf levels remained the same as observed under normal conditions.

To determine whether the isolated BvBetT1 really transports betaine, the BvBetT1 gene was expressed in yeast strain 22574d. The expression of BvBet/ProT1 in yeast can be detected by western blotting analysis (data not shown). The kinetic properties of BvBet/ProT1 were examined using  $d_{11}$ -betaine. No measurable uptake of  $d_{11}$ -betaine was observed for the 22574d cells transformed with pYES2, whereas the cells transformed with pBvBetT1 could take up betaine. The uptake of  $d_{11}$ -betaine was faster at lower pH (Figure 11A). The betaine uptake was significantly inhibited by 10-fold excess betaine or choline, but only weakly inhibited by proline (Figure 11B), which is different from those in mangrove betaine/proline transporters.

## Discussion

Hitherto, it has been considered that betaine is synthesized on abiotic stress. However, the present data demonstrated that a significant amount of betaine accumulates even under normal growth conditions (Figure 1). To our knowledge, these high values of betaine,  $>20\ \mu\text{mol/gFW}$ , have not been reported previously to exist under non-stress conditions. It was also found that the levels of accumulated betaine differed significantly among various tissues under non-stress conditions. Young leaves (L1 and L2) accumulated significantly more glycine betaine than did expanded leaves (L3), cotyledons (C), hypocotyls (H), or roots (R) (Figure 1).

One of the most interesting observation in this paper is the uncoupling between the levels of betaine and CMO. Figure 2 shows that the accumulation of CMO in L1 leaves was very low under normal growth conditions. It should be noted that we can detect CMO if we apply more protein. The level of betaine was the highest in L1 leaves as compared with other tissues (Figure 1). A similar pattern was observed under salt-stress conditions (Figures 7 and 8). Uncoupling between the levels of betaine and CMO was surprising because CMO was shown to be induced on salt stress and CMO is the rate-limiting enzyme for betaine synthesis (Russell et al., 1998). The levels of CMO mRNA were higher in L3 leaves than in L1 leaves (Figure 10). Thus, the differential accumulation of CMO was essentially controlled by transcription level, and not by post-

transcriptional events. Moreover, Figures 3 and 4 show that betaine is primarily synthesized in expanded leaves (L3) and translocated into young leaves (L1). Although the translocation of betaine into young leaves has been reported previously (Ladyman et al., 1980; Nakamura et al., 1996), its relation to the CMO and betaine transporter was unknown.

We wondered why sugar beet accumulates betaine under normal condition and sought to determine the role of betaine under non-stress conditions. Although further research is required to clarify these questions, several conclusions are possible. Betaine protects the inactivation of proteins under high salinity and high temperature (Diamant et al., 2001). Betaine functions as a chemical chaperone. In actively developing cells, the levels of protein synthesis and translocation would be high. Betaine might facilitate folding and translocation, serving as a chemical chaperone. Another possibility is that betaine might be utilized to maintain high cellular pressure. It is known that high cellular pressure is required for cell growth. Higher osmolarity in L1 leaves than in L3 leaves is compatible with this idea, although it is difficult to explain why the highest osmolarity was observed in hypocotyls (H). In animal cells, it has been shown that betaine plays a role in cell volume homeostasis (Anas et al., 2008).

We also tried to elucidate how betaine accumulates preferentially in young leaves. In plants, betaine transporter genes are poorly characterized. Previously, we isolated three betaine transporter genes from betaine-accumulating mangrove *A. marina* (Waditee et al., 2002). Here a homologous gene BvBet/ProT1 was isolated from sugar beet. The fusion protein GFP-BvBet/ProT1 showed that BvBet/ProT1 was localized at the plasma membrane (Figure 9B). Levels of mRNA for BvBet/ProT1 were much higher in L3 leaves than in L1 leaves under normal and salt-stress conditions (Figure 10). Since betaine is transported from the expanded leaves to young leaves (Figure 4) and the accumulated levels of BvBet/ProT1 gene transcript were higher in L1 leaves than in L3 leaves (Figure 4), BvBet/ProT1 might play an important role in the efflux of betaine from CMO-expressing cells. In this study, one BvBet/ProT1 gene was isolated from the cDNA libraries constructed from leaves and roots. It will be of interest to identify the betaine transporter(s) that is (are) involved in betaine uptake in young leaves. Further studies are required to understand the mechanisms of betaine transport in plants.

Accumulation levels of betaine in betaine-accumulating plants are often higher than  $30\ \mu\text{mol/gFW}$

(Figures 1 and 2). Introduction of the betaine biosynthetic pathway into plants that do not naturally accumulate betaine resulted in low levels of betaine accumulation. In the latter case, it was shown that the import of choline into chloroplasts and the supply of precursors such as choline limit betaine levels (Nuccio et al., 1998, 2000). However, even if exogenous choline was applied, the betaine level was still low, 0.3–1.0  $\mu\text{mol/gFW}$ . Our recent studies demonstrated that betaine synthesis was not enough under stress conditions even in betaine-accumulating plants (Bhuiyan et al., 2007; Waditee et al., 2007). This indicates that some other factor(s) limits (limit) the accumulation of betaine. Although more extensive studies are required to elucidate the mechanisms responsible for this effect, the present study suggests the importance of betaine transporters in betaine accumulation. Therefore, it might be interesting to introduce the betaine transporter gene as well as betaine synthetic genes in order to improve the stress tolerance of plants.

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