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Research article

Expression and functional characterization of sugar beet phosphoethanolamine/phosphocholine phosphatase under salt stress

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ABSTRACT

Choline is a vital metabolite in plant and synthesized from phosphocholine by phosphocholine phosphatase. The *Arabidopsis* At1g17710 was identified as the first plant gene encoding the phosphatase for both phosphoethanolamine and phosphocholine (PECP) with much higher catalytic efficiency (> 10-fold) for former. In betaine accumulating plants, choline is further required for betaine synthesis. In this report, we found three putative PECP genes in sugar beet, betaine accumulating plants. Two genes encode the proteins of 274 amino acid residues and designated as BvPECP1S and BvPECP2S. Another gene encodes the 331 amino acid protein (BvPECP2L) consisted of BvPECP2S with extra C-terminal amino acid. Enzymatic assays of BvPECP1S revealed that BvPECP1S exhibited the phosphatase activity for both phosphoethanolamine and phosphocholine with higher affinity (> 1.8-fold) and catalytic efficiency (> 2.64-fold) for phosphocholine. BvPECP2L exhibited low activity. RT-PCR experiments for BvPECP1S showed the increased expression in young leaf and root tip under salt-stress whereas the increased expression in all organs under phosphate deficiency. The expression level of BvPECP2L in salt stressed young leaf and root tip was induced by phosphate deficient. Physiological roles of BvPECP1S and BvPECP2L for the betaine synthesis were discussed.

1. Introduction

Choline (Cho) is a vital metabolite in plants, eukaryotes and bacteria. Cho is required for the synthesis of phospholipid phosphatidylcholine (Ptd-Cho) (Lin et al., 2015). Ptd-Cho is sythesized from Cho *via* P-Cho and cytidine diphospho-Cho (CDP–Cho). Ptd-Cho is also synthesized from ethanolamine (EA) *via* three step methylation reactions of phosphoethanolamine (P-EA) and phosphatidylethanolamine (Ptd-EA) (Fig. 1) (McNeil et al., 2001). In addition, certain plants such as spinach have chloroplast enzymes that catalyze the two-step oxidation of Cho to glycinebetaine (GB) (Rathinasabapathi et al., 1997). GB is crucial because it has strong osmoprotectant properties and confers the tolerance to salinity, drought, and other stresses (Rhodes and Hanson, 1993; Takabe et al., 2015). The extensive research has been conducted to introduce the GB synthetic genes into the betaine non-accumulating plants. Genetically engineered plants so far have faced with the limitation of being unable to produce sufficient amounts of GB (McNeil et al., 2001; Takabe, 2012). One of the reason was Cho deficiency in betaine non-accumulating plants. Therefore, it is essential to understand the limiting factor for Cho production in plants (Nuccio et al., 2000; Rontein et al., 2002).

Among Cho metabolic pathways, Cho production via P-Cho which is catalyzed by P-Cho phosphatase is largely unknown. This would be due to the broad substrate specificity of phosphatase. P-Cho phosphatase produces phosphate and Cho. Oppositely directed reaction is catalyzed by the Cho kinase (CK) (Tasseva et al., 2004). Both are irreversible reactions.

Recently, phosphoethanolamine/phosphocholine phosphatase (PECP) gene was identified in *Arabidopsis* (betaine non-accumulating plants) (May et al., 2012). Based on the transcriptional analysis of

Abbreviations: BADH, betaine aldehyde dehydrogenase; Cho, choline; CDP, cytidine diphosphate; CK, choline kinase; CMO, choline monooxygenase; EA, ethanolamine; GB, glycine betaine; NPC, nonspecific phospholipase C; PECP, phosphoethanolamine/phosphocholine phosphatase; PLD, phospholipase D; Ptd, phosphatidyl; SDC, serine decarboxylase

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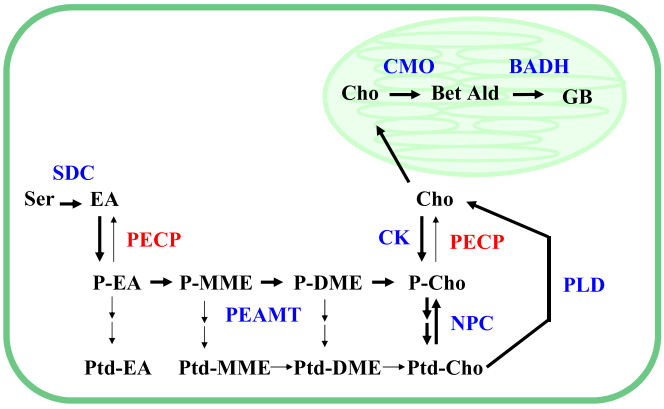

Fig. 1. Simplified scheme of Cho and Ptd-Cho biosynthesis pathways in sugar beet. Arrows indicate de novo biosynthesis. Compounds are indicated in black letters and enzymes in blue. BAD-ald, betainealdehyde; BADH, betainealdehyde dehydrogenase; Cho, choline; CK, choline kinase; CMO, cholinemonooxygenase; EA, ethanolamine; NPC, nonspecific phospholipase C; PEAMT, phosphoethanolamine methyltransferase; PLD, phospholipase D; SDC, serine decarboxylase. The conversion of phospho-bases (P-EA; P-MMEA, phosphomonomethylethanolamine; P-DMEA, phosphodimethylethanolamine; P-Cho) to phosphatidyl-bases (Ptd-EA; Ptd-MMEA; Ptd-DME; Ptd-Cho) implicates cytidine-diphosphate (CDP)-containing intermediates which were omitted for simplicity. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

phosphate starvation-induced genes (Morcuende et al., 2007), two genes, At1g73010 and At1g17710, were mentioned as the candidate. May et al. (2011) isolated the gene At1g73010 and functionally characterized. It turned out that At1g73010 catalyzed the cleavage of pyrophosphatase and designated as AtPPsPase1. Subsequently, they found that the recombinant protein of At1g17710 catalyzes phosphatase activity for both P-EA and P-Cho, and designated as AtPECP. The PECP gene is unknown in plants except *Arabidopsis*.

In this study, based on gene similarity search, we found three putative PECP genes (BvPECP1S, BvPECP2S, BvPECP2L) in sugar beet. Biochemical studies showed that BvPECP1S and BvPECP2L exhibited the phosphatase activity for both P-EA and P-Cho, but much higher affinity and catalytic efficiency for P-Cho than for P-EA. RT-PCR experiments suggest the importance of BvPECP1S and BvPECP2L for production of choline from P-Cho under salt stress and phosphate deficient conditions.

2. Materials and methods

2.1. Plant materials

Sugar beet (*Beta vulgaris* L., cv. Abend) was used throughout this study (Yamada et al., 2015). The seeds were germinated on paper towels moistened with distilled water in darkness at 25 °C. After germination, seedlings were transplanted into plastic pots (100 mL) containing sterile vermiculite. Plants were grown on 1/10 Murashige and Skoog solutions prepared to pH 6.5 in a growth chamber with a 16 h light (25 °C, 100 μE m^{-2} s^{-1})/8 h dark (20 °C) cycle and 60% relative humidity, unless otherwise states (Yamada et al., 2009). Four-week-old seedlings of *B. vulgaris* plants were used for stress treatment. For salt-stress treatment (Na), 100 mL of the nutrient solution containing 0.3 M NaCl were applied to the culture medium for 1 week every second day. For phosphate deficient treatment (-P), the nutrient solution without phosphate was applied for 1 week. For the simultaneous treatment of phosphate deficiency and salt stress (-P + Na), the nutrient solution containing 0.3 M NaCl without phosphate was applied for 1 week. After harvesting, the plants were divided into mature leaf, young leaf, hypocotyl and root tip, and were immediately frozen at −80 °C.

2.2. Isolation of putative PECP genes from Beta vulgaris

Total RNA was extracted from leaves using the RNeasy Plant Mini Kit (Qiagen Inc., CA, USA). The first-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA using reverse transcriptase Prime Script RTase (Takara, Tokyo, Japan) and was used as the PCR template. The coding regions of BvPECP1S and BvPECP2L were isolated from cDNA by PCR. The same forward primer was used for BvPECP1S and BvPECP2L. It was *Nco*I_BvPECP1S_F 5′-CCATGGAA GGAATAGTTGTTGTTT-3′. The reverse primers are *Sal*I_BvPECP1S_R 5′-GTCGACAAAAGGTACTCCGAGAGCTT-3′ and *Sal*I_BvPECP2L_R 5′-GTCGACGAAACC GTGCGAAATTC-3′, respectively. The PCR products were cloned into the pMD20-T vector (Takara, Japan) and transformed into *E. coli* strain DH5α. The *E. coli* cells were grown in LB media containing ampicillin. The DNA sequence was determined using a DNA Sequencer (ABI PRISM 3100) and analyzed with the DNASIS program (Hitachi Software Engineering Co., Kanagawa, Japan).

2.3. Construction and expression of recombinant putative BvPECP proteins

The expression vectors for the recombinant BvPECP1S and BvPECP2L proteins were constructed by the insertion of the coding region of BvPECP1S into *Nco*I/*Sal*I sites of the pTrcHis2C vector (Invitrogen) and designated as BvPECP1S/pTrcHis2C and BvPECP2L/pTrcHis2C, respectively. The *E. coli* BL21 (DE3) cells were transformed with BvPECP1S/pTrcHis2C and BvPECP2L/pTrcHis2C. The transformed *E. coli* BL21 (DE3) cells were grown in LB broth media at 37 °C. When the optical density at 620 nm reached to 0.5, the isopropyl-1thio-β-D-galactopyranoside (IPTG) was added to 0.1 mM. After 2 h incubation, the cells were harvested by centrifugation and used for protein purification.

2.4. Purification of recombinant proteins

Pellets were resuspended in buffer A containing 50 mM HEPES/NaOH (pH 7.0), 300 mM NaCl and 0.042% (v/v) Triton-100. Subsequently, cells were lysed by sonication. Crude extracts were clarified by centrifugation at 15,000 g, 4 °C for 15 min. The His-tagged fusion protein was purified by affinity chromatography on HisTrap HP columns (GE Healthcare, Piscataway, NJ, USA) equilibrated with 20 mM imidazole containing 20 mM sodium phosphate and 0.5 M NaCl at pH 7.4. The recombinant protein was eluted with 500 mM imidazole containing 20 mM sodium phosphate and 0.5 M NaCl at pH 7.4. Then, BvPECP proteins were applied to a column of HiTrap Desalting (GE Healthcare) and eluted by buffer A. The protein content was determined by Bradford (1976) method using BSA as standard.

2.5. Enzyme assays

The phosphatase activity of BvPECP enzymes was assayed by measuring the production of inorganic phosphate (Baykov et al., 1988). Recombinant BvPECP proteins were incubated with various substrates. The reaction mixture contained 50 mM HEPES/NaOH (pH 7.0), 25 ng mL^{-1} BSA, 1 mM substrate, and 18 mM $MgCl_2$. The reaction was started by addition of BvPECP proteins and incubated for 30 min at 37 °C. The inorganic phosphate released in the reaction mixture was estimated by the colorimetric malachite green assay by measuring absorbance at 630 nm (Baykov et al., 1988). A KH_2PO_4 solution was used for the calibration.

2.6. RT-PCR

Total RNA was extracted from leaves (old L1 and young L2), hypocotyls and root tips by using RNeasy Plant Mini Kit (QIAGEN Inc., CA, USA) (Yamada et al., 2011). The first-strand cDNA was synthesized from 1 μg of total RNA by using Prime Script RT-PCR kit (TaKaRa) 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 proper quantification of the amplified products. The actin gene (Act) of *B. vulgaris* was used as a control. The primers used for PCR amplification are shown in Table S1.

2.7. Other methods

The nucleotide sequences were determined by using an ABI310 genetic analyzer (Applied Biosystems, Foster City, CA). The protein concentration was measured by using the Bradford method. SDS-PAGE and Western blot analysis were carried out as described previously (Yamada et al., 2009). For the phylogenetic analysis, CLUSTAL W (Thompson et al., 1994) was used to generate a phylogenetic tree of amino acid sequences of putative PECPs.

3. Results

3.1. Identification of three putative PECP genes in sugar beet genome

Blast search was performed in sugar beet genome using the AtPECP1 sequence as query. Three putative PECP genes (accession number; XP010690045, KMT01671, XP010690046) which exhibited high similarity to AtPECP1 were found in sugar beet. Among them, two genes (XP010690045, KMT01671) encode the proteins with 274 amino acid residues and third gene (XP010690046) encodes a protein containing 331 amino acid residues (Fig. 2). KMT01671 is a truncated version of XP010690046. We tentatively designated XP010690045, KMT01671 and XP010690046 as BvPECP1S, BvPECP2S and BvPECP2L, respectively (Fig. 2). The amino acid sequences of BvPECP1S and BvPECP2S were 91% identical. Fig. 2 shows that BvPECP1S, BvPECP2S and BvPECP2L sequences possessed the characteristic structural features of the HAD (Haloacid Dehalogenase) superfamily comprising of the N-terminal motif I DxDxT, the conserved Ser residue in motif II, and the C-terminally located motif III, K-(x)$_{18–22}$ (GDxxxD) (Fig. 2). These motif sequences were exactly matched to the AtPECP1 sequences (May et al., 2012), suggesting that BvPECP1S, BvPECP2S and BvPECP2L belongs to the HAD subfamily IB, phosphoserine phosphatase-like (PSP-like) proteins (Burroughs et al., 2006).

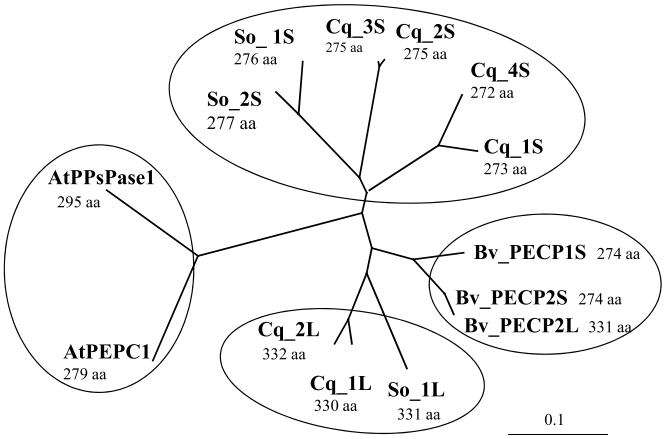

Fig. 3. Phylogenetic tree of putative PECPs in *Arabidopsis*, *Chenopodium quinoa*, *Spinacia oleracea* and sugar beet. The following sequences were used (accession numbers): AtPECP1 (At1g17710); AtPPsPase1 (At1g73010); Bv_PECP1S (XP010690045); Bv_PECP2S (KMT01671); Bv_PECP2L (XP010690046); So_ 1S (XP021856236); So_2S (XP021856307); So_1L (XP021858900); Cq_1S (XP021717918); Cq_2S (XP021757819); Cq_3S (XP021757820); Cq_4S (XP021728869); Cq_1L (XP021732785); Cq_2L (XP021774194). The numbers of amino acid were shown.

Blast search was performed both in betaine non-accumulator (*Arabidopsis*) and accumulator (*Chenopodium quinoa* and *Spinacia oleracea*) using the BvPECP1S sequence as query. Two, three and six genes were found in *Arabidopsis*, *Spinacia oleracea* and *Chenopodium quinoa*, respectively. Multiple alignment of their encoded proteins together with three sugar beet proteins were carried out and the phylogenetic

```
                                I
Bv_PECP1S    1 MEGIVVVFDFDKTIIDCDSDNWVVDELGFTDLFNQLLNTMPWNSMMDVLMKRMHEKGITI
Bv_PECP2S    1 MEGIVVVFDFDKTIIDCDSDNWVVDELGFTDLFNQLLNTMPWNSMMDVLMKEMHEEGITI
Bv_PECP2L    1 MEGIVVVFDFDKTIIDCDSDNWVVDELGFTDLFNQLLNTMPWNSMMDVLMKEMHEEGITI
               **************************************************. ***:****
                                                   II
Bv_PECP1S   61 NEIAEVLKRIPIHPRIVPAIRDAHAAGCDLRIVSDANLFFIETILEHLGLSDCFSEINTN
Bv_PECP2S   61 DDIADVLKRIPIHPRIVPAIRAAHAAGCDLRIVSDANLFFIETILEHLGLSDYFSEINTN
Bv_PECP2L   61 DDIADVLKRIPIHPRIVPAIRAAHAAGCDLRIVSDANLFFIETILEHLGLSDYFSEINTN
               ::**:**************** ******************************** *******
                                                          III
Bv_PECP1S  121 PGYVDEEGRLRILPHHDFTKSLHGCTNPCPPNMCKGSVIKRLLCEHGNKKFIYLGDGLGD
Bv_PECP2S  121 PGYVDEEGRLRILPHHDFTKSSHGCTNPCPPNMCKGMVIKRLLCKHGNKKFIYLGDGLGD
Bv_PECP2L  121 PGYVDEEGRLRILPHHDFTKSSHGCTNPCPPNMCKGMVIKRLLCKHGNKKFIYLGDGLGD
               ********************* ************** ********:****************

Bv_PECP1S  181 YCPSLKLREGDHVMPRKNFPVWDLISNNPERMTSKIHEWSDGEDFERILLSLIQAIISNA
Bv_PECP2S  181 YCPSLRLREGDHVMPRKNFPVWDLISNNPELITSKIHEWTDGEDFERVLLSLIQAIISNA
Bv_PECP2L  181 YCPSLRLREGDHVMPRKNFPVWDLISNNPELITSKIHEWTDGEDFERVLLSLIQAIISNA
               *****:************************ :********:********:************

Bv_PECP1S  241 DDNNAAQLLNLDWKFKSFPIASHKTLPQALGVPF 274
Bv_PECP2S  241 DDNDAAHFFNRDCKFESLPISSPDTLPQALGVPF 274
Bv_PECP2L  241 DDNDAAHFFNRDCKFESLPISSPDTLPQALGVGSQKCCIVHPHISRSASFGISYKNKILK
               ***:**:::* * **:*:**:* .********

Bv_PECP2L  301 GSRGGKTSGPNQLKNLLTARLQSNSRISHGF 331
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Fig. 2. Multiple alignment of sugar beet Bv_PECP1S, Bv_PECP2S and BV_PECP2L.The multiple sequence alignment was carried out using the ClustalW program. Amino acids important for catalysis are highlighted in underline. The conserved active site motifs of the HAD superfamily were shown. The accession numbers of Bv_PECP1S, Bv_PECP2S and Bv_PECP2L are XP010690045, KMT01671 and XP010690046, respectively.

tree was generated by the ClustalW program (Fig. 3). It is interesting to note that betaine accumulating plants could be separated into two forms, short (S) and long (L) forms with the molar ratio 2:1. Spinach has two short (276, 277 aa) and one long (331 aa) sequences, whereas chenopod has four short (272, 273, 275, 275 aa) and two long (330, 332 aa) sequences. Short sequences of spinach and chenopod form the same group whereas long sequences of these plants form another group.

Arabidopsis has two sequences, AtPEPC1S (279 amino acid)) and AtPPsPase1 (295 amino acid). Although the biochemical study demonstrated that AtPPsPase1 catalyzes the cleavage of pyrophosphatase (May et al., 2011), *in vivo* study suggested that AtPPsPase1 functions as the PECP activity (Angkawijaya and Nakamura, 2017).

3.2. Expression, purification and functional characterization of BvPECP1S and BvPECP2L

To investigate the functional properties of BvPECP1S and BvPECP2L, we cloned the ORFs from total RNA of sugar beet and expressed in *E. coli* BL21 (DE3) as described in Materials and Methods. BvPECP1S activity was assayed by measuring the production of inorganic phosphate. First, the substrate specificity of BvPECP1S was examined. Fig. 4 showed that P-Cho phosphatase activity was the highest among seven substrates, then P-EA was followed. The activity for P-EA was about 70% of that for P-Cho. Pyrophosphate was a poor substrate for BvPECP1S. It was about 24% of P-Cho phosphatase activity. No activity was observed for P-Ser and nitrophenyl phosphate. These results indicate that BvPECP1S displayed essentially P-Cho and P-EA phosphatase activities.

To examine the metal specificity, the enzyme BvPECP1S was assayed in the presence of 1 mM P-Cho and 20 mM of respective metal ion (Mg^{2+}, Ca^{2+}, Fe^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+}). Fig. 5 shows that the highest activity was observed with Mg^{2+}. Then, a comparable activity was displayed in the presence of Ca^{2+}, Zn^{2+} and Fe^{2+}. These results are similar to that in AtPECP1 (May et al., 2012). Next, the effect of Mg^{2+} concentration was investigated. The observed rate depended in a hyperbolic manner with an apparent K_m value of 1.0 mM (Fig. 5B), which is also similar to that of AtPECP1 (May et al., 2012). Finally, pH profile was examined. BvPECP1S exhibited a bell-shaped curve (Fig. 5C). A relatively broad pH optimum was observed in the range of pH 6.0–9.0.

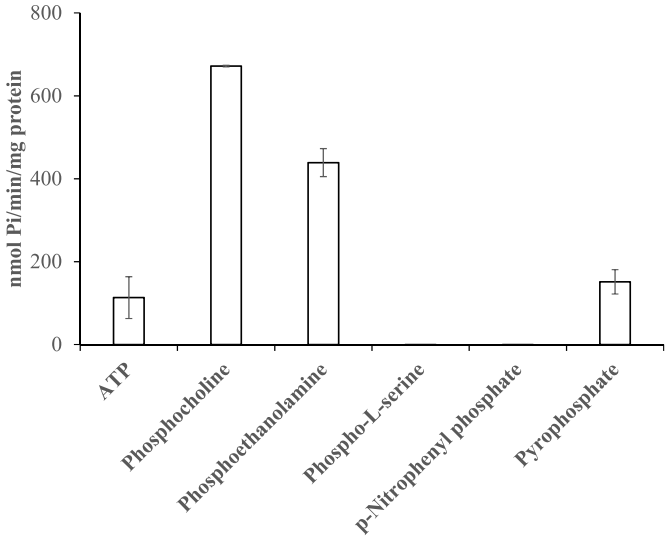

Fig. 4. Substrate specificity of BvPECP1S. The hydrolysis reaction was measured as describing Materials and Methods using the colorimetric phosphate assay according. The concentrations of substrate and $MgCl_2$.were 1 mM and 18 mM, respectively. Means ± the SEM are shown. Each value shows the average of three independent measurements.

Next, the effects of substrate concentration were examined. The kinetics were hyperbolic for both substrates, P-Cho and P-EA. Analysis by Michaelis–Menten equation yielded K_m and V_{max} values for P-Cho as 0.22 mM and 820 nmol Pi min^{-1} mg^{-1} protein, respectively (Fig. 6B). K_m and V_{max} values for P-EA were 0.40 mM and 501 nmol Pi min^{-1} mg^{-1} protein, respectively (Fig. 6A). The affinity for P-Cho was 1.8-fold smaller than that for P-EA. Therefore, the catalytic efficiency V_{max}/K_m for P-Cho phosphatase was 2.98-fold higher than that for P-EA phosphatase. The enzyme activities of BvPECP2L were very small compared with that of BvPECP1S. The V_{max} and K_m for P-Cho in BvPECP2L were about 8.4 Pi min^{-1} mg^{-1} protein and 0.10 mM, respectively, and the V_{max} and K_m for P-EA in BvPECP2L were 35.6 Pi min^{-1} mg^{-1} protein and 4.14 mM, respectively. Due to the lower affinity for P-EA (4.14 mM) compared with that for P-Cho (0.10 mM), the catalytic efficiency for P-Cho phosphatase was 9.77-fold higher than that for P-EA phosphatase.

3.3. Expression of BvPECP1S and BvPECP2L genes in sugar beet

Under the control growth condition, the level of *BvPECP1S* mRNA was low in young leaves, hypocotyl and root tips compared with that in mature leaves (Fig. 7). The levels of *BvPECP1S* mRNA in young leaves and root tips was increased after treatment with phosphate deficient (-P) and salt stress (Na) conditions (Fig. 7B and D) but were almost the same or slightly induced in mature leaves (Fig. 7A). Significant induction of *BvPECP1S* under phosphate deficient conditions was observed (Fig. 7C). The simultaneous addition or successive addition of phosphate and salt did not change the effect of phosphate deficiency.

The levels of *BvPECP2L* mRNA were increased in mature and young leaves under phosphate deficient conditions (Fig. 7A and B). Salt stress significantly increased the levels of *BvPECP2L* mRNA in root tips (Fig. 7D), whereas phosphate deficiency increased the level of *BvPECP2L* mRNA in young leaves (Fig. 7B).

4. Discussion

Data presented in Figs. 4–6 showed that BvPECP1S is a P-Cho/P-EA phosphatase. However, the present results showed that P-Cho is a preferred substrate for BvPECP1S compared with the P-EA considering substrate specificity (1.8-fold smaller K_m for P-Cho) and catalytic efficiency (3.0-fold higher V_{max}/K_m for P-Cho phosphatase). This might be worthwhile to note. In AtPECP1, the catalytic efficiency for P-Cho phosphatase was lower (> 10-fold) than that for P-EA phosphatase (May et al., 2012). suggesting that the main function of AtPECP1 would be the production of inorganic Pi by the P-EA phosphatase activity under phosphate deficient condition. By contrast, Cho is required under salt stress conditions in betaine accumulating plants such as sugar beet. The kinetic data support the viewpoint that the main function of BvPECP1S is the production of Cho and Pi from P-Cho under abiotic stress such as salt stress and phosphate deficient conditions.

Compared with the BvPECP1S, the enzyme activities of BvPECP2L were very low, and it would difficult to discuss the *in vivo* function of BvPECP2L at the present stage. However, BvPECP2L showed high affinity for P-Cho (0.10 mM) than that for P-EA (4.14 mM) and higher catalytic efficiency (9.77-fold) for P-Cho phosphatase than P-EA phosphatase as described in text, and suggested that BvPECP2L functions as P-Cho phosphatase *in vivo*. Our data presented the first example on PECP genes from betaine accumulating plant and demonstrated that the existence of at least two PCEP genes in the same plant.

In *Spinacia oleracea* and *Chenopodium quinoa*, three and six putative PECP genes could be found (Fig. 3). The phylogenic distance between long form (So_1L, Cq_1L, Cq2L) and short form (So_1L, Cq_1S, Cq_2S) is far compared to that of long form (Bv_2L) and short form (Bv_1S, Bv_2S). from sugar beet. In this study, we did not examine the substrate specificity for putative BvPECP2S. Further studies are warranted to clarify the function properties of PECPs from betaine accumulating

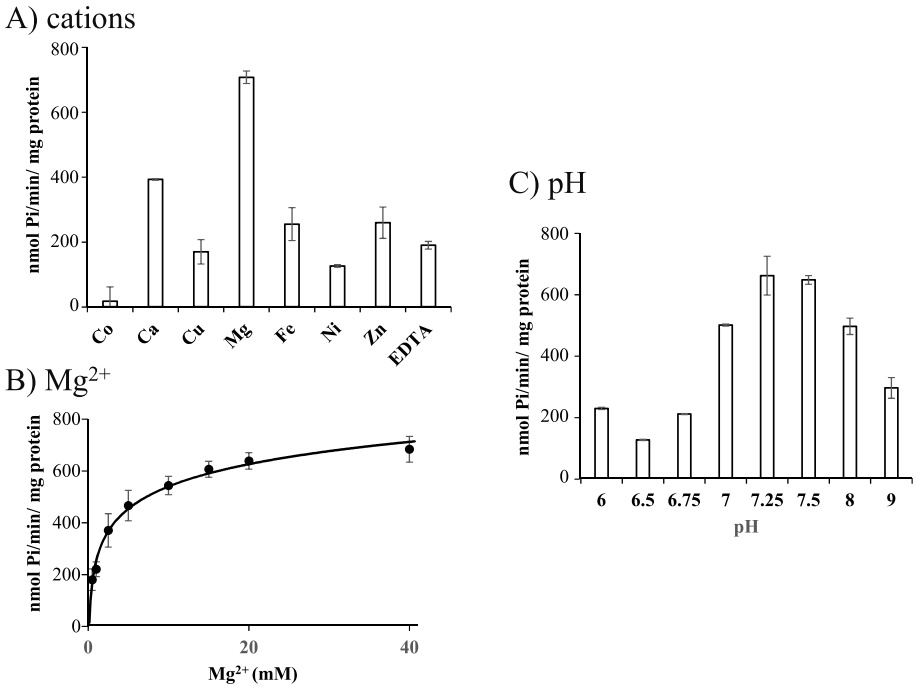

Fig. 5. Effects of cations and pH for the activity of BvPECP1S. A) Effects of divalent cations; B) Effects of Mg^{2+} concentrations; C) Effect of pH. Means ± the SEM are shown. Each value shows the average of three independent measurements.

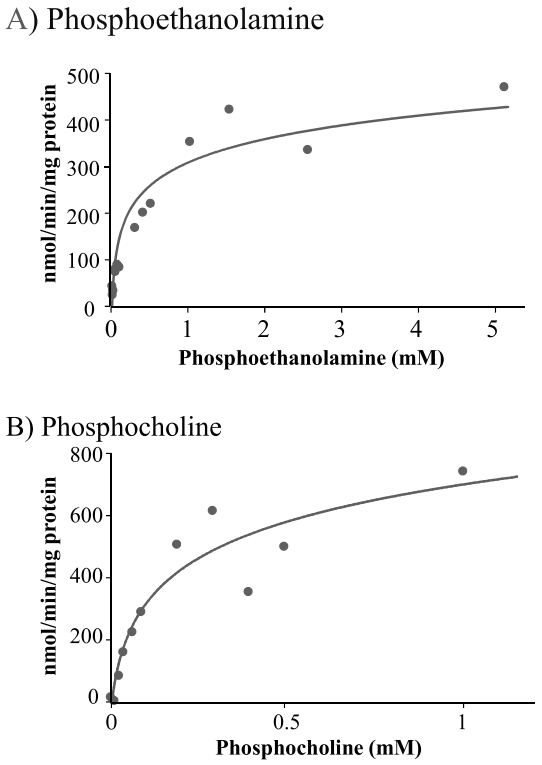

Fig. 6. Enzyme activity toward phosphocholine and phosphoethanol amine. The enzyme assay contained 1 mM P-Cho and 10 mM Mg^{2+}. The K_m and V_{max} values were calculated from triplicate measurements using nonlinear regression. Means ± the SEM are shown. Each value shows the average of three independent measurements.

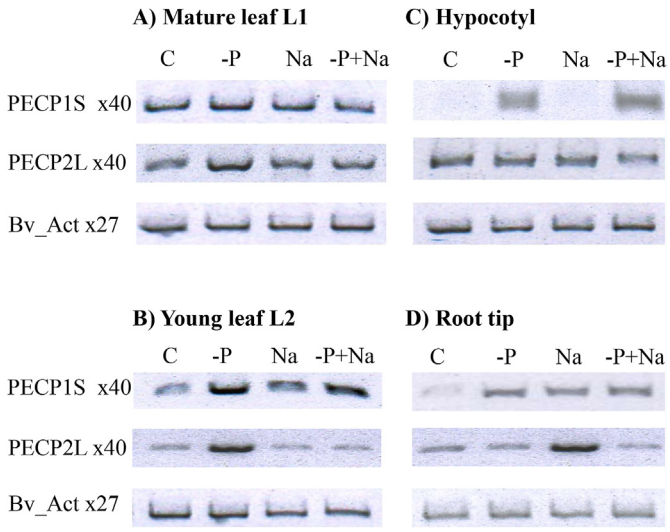

Fig. 7. Semiquantitative RT-PCR for BvPECP1S and BvPECP2L. 4. Total RNA was extracted from mature leaf L1, young leaf L2, hypocotyl and root tip. Semi-quantitative RT-PCR was carried as described in Material and Methods. The products were electrophoresed and detected.

plants including sugar beet.

In the case of *Arabidopsis*, the substrates for AtPPsPase1 and AtPECP1 were strongly differ (May et al., 2011; May et al., 2012). Biochemical studies showed that pyrophosphate is the preferred substrate for AtPPsPase1, whereas P-EA and P-Cho are the preferred substrates for AtPECP1. Recently, Angkawijaya and Nakamura (2017) showed that only the double knockout mutant, but not the single knockout mutant, of AtPPsPase1 and AtPECP1 in *Arabidopsis* decreased choline content, suggesting that *in vivo* AtPPsPase1 and AtPECP1 function as P-Cho phosphatase. However, the unchanged P-Cho content and no significant phenotype under phosphate starvation in the double knockout mutant were observed (Angkawijaya and Nakamura, 2017). P-Cho could be produced by the three step methylations of P-EA (BeGora et al., 2010) or by the hydrolysis of phosphatidylcholine with non-specific phospholipases C (Nakamura et al., 2005). The metabolic fate of P-Cho remains elusive.

The mRNA for BvPECP1S and BvPECP2L in the mature leaves did

not change so much under salt stress and phosphate deficient conditions (Fig. 7A). The data in Fig. 7 are consistent with the previous reports that P-Cho is an abundant component of the xylem sap in plants and is easily available after onset of Pi deprivation (Martin and Tolbert, 1983; Gout et al., 1990). It has also been reported that only moderate increase of P-Cho phosphatase activity is observed in salt stressed spinach leaves, betaine accumulating plant (Summers and Weretilnyk, 1993). Summers and Weretilnyk (1993) interpreted their data that P-Cho phosphatase activity in spinach leaves is high even without salt stress, and therefore P-Cho phosphatase activity did not increase so much under salt stress. Our Fig. 7A results are consistent with these observations. However, we observed an interesting result that the mRNA for BvPECP1S was significantly induced under phosphate deficient conditions (Fig. 7B) which is consistent with the previous reports showing that P-Cho is an abundant component of the xylem sap in plants and is easily available after onset of Pi deprivation (Martin and Tolbert, 1983; Gout et al., 1990).

Different from the mature leaves, the significant induction of BvPECP1S under salt stress and phosphate deficient conditions in young leaf and root tip was observed (Fig. 7C and D) which depicted the pivotal role of BvPECP1S in young leaf and root tip.

By contrast, the expression of BvPECP2L was not enhanced under phosphate deficient and salt stress conditions (Fig. 7A and C). The expression of BvPECP2L in root tip increased under salt stress (Fig. 7D). It should be noted that root tips are the main site for P-Cho biosynthesis in *Arabidopsis* (Alatorre-Cobos et al., 2012). Therefore, the production of Cho and inorganic Pi in the root tip by BvPECP2L would play important role for salt stress in sugar beet, which needs to be tested. Taken together, the presented data suggest the importance of BvPECP1S and BvPECP2L in choline and Pi production in young leaf and root tip of sugar beet.

Conflicts of interest

The authors declare that they have no conflict of interest.

Author contribution

Study conception and design: Takabe.

Acquisition of data: Sahashi, Yamada-Kato, Maeda, Kito, Tanaka.

Analysis and interpretation of data: Sahashi, Yamada-Kato, Maeda, Kito, Tanaka, Takabe.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.07.011.

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