Tissue Localization of the Glycine Betaine Biosynthetic Enzymes in Barley Leaves

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Abstract: Barley (Hordeum vulgare L.) plants accumulate glycine betaine (GB), a major compatible solute, in response to salt stress. In barley, GB is produced by a two-step oxidation of choline in a cooperative way in the cytosol and peroxisomes. In this study, we investigated the localization of two GB biosynthetic enzymes, choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH), in the tissues of barley plants (cv. Haruna-nijyo) grown under normal and saline conditions. Three-week-old barley plants grown hydroponically were treated with a hydroponic culture solution containing 200 mM NaCl for 72 h. Salt treatment resulted in increased expression of CMO and BADH proteins mainly in the leaves of barley but not in the roots. The expression of CMO protein was increased by the presence of NaCl in younger leaves but decreased in older leaves. The tissue localization of CMO and BADH proteins was analyzed by immunofluorescent labeling method using their primary antibodies and a fluorescein-conjugated secondary antibody. CMO and BADH proteins were constitutively co-localized in mesophyll and bundle sheath cells under both normal and saline conditions. A possible physiological function of GB in the salt tolerance of barley plants is discussed.

Key words: Barley, Glycine betaine, Immunofluorescent labeling, Salt stress, Tissue localization.

Salt affects 7% of the land on the earth, which corresponds to 930 million ha of land (Szabolcs, 1994). Because plant growth is greatly inhibited by high concentrations of salt, salt stress is one of the main limiting constraints to global agriculture. Therefore, it is important to understand the mechanisms of salt tolerance in plants. Salt-tolerant plants show a variety of salt tolerant mechanisms, one of which is the production/accumulation of compatible solutes.

Glycine betaine (GB) is a compatible solute which is produced widely among flowering plants, bacteria, algae, and animals (Bluden et al., 1992; Rhodes and Hanson, 1993; Kempf and Bremer, 1998). GB accumulation is thought to be important for salt tolerance, because it functions not only as an osmoprotectant but also as a chemical chaperone to protect cellular components such as proteins (Nomura et al., 1998; Chen and Murata, 2011). Since many crop plants, such as rice, do not produce or accumulate GB, genetic engineering of the GB biosynthesis pathway may be a potential way to improve the salt tolerance of crop plants. Therefore it is important to isolate the genes responsible for the production of GB and characterize how GB production is regulated in naturally GB-producing plants.

GB is synthesized in Gramineae, Amaranthaceae, Malvaceae and Compositae (McNeil et al., 1999) by the oxidation of choline via a two-step process: choline → betaine aldehyde → GB (Rathinasabapathi et al., 1997). We have succeeded in isolating choline monooxygenase (CMO), the choline oxidizing enzyme, from gramineous barley (Mitsuya et al., 2011). Barley CMO (HvCMO) requires NADPH for activity and is localized to peroxisomes. In addition to this, it has also been reported that barley has two isoforms of betaine aldehyde dehydrogenase (BADH) (BBD1 and BBD2), the betaine aldehyde oxidizing enzyme (Ishitani et al., 1995; Nakamura et al., 2001). Moreover, within two isoforms, cytosolic BBD2 is mainly involved in the production of GB and expressed in mesophyll and vascular tissues in barley (Fujisawa et al., 2008; Hattori et al., 2009). However, because the tissue localization of CMO has not been determined yet in barley, the production site and the
physiological function of GB in the salt tolerance remain unclear.

In Amaranthaceae such as spinach and sugar beet, GB is produced in the chloroplast stroma (Brouquisse et al., 1989; Rathinasabapathi et al., 1997) and is suggested to protect photosynthesis-related enzymes such as Rubisco and D1 protein in photosystem II (Nomura et al., 1998; Chen and Murata, 2011). Therefore, it is indicated that the physiological role of GB in salt tolerance in gramineous barley is different from that in Amaranthaceae.

In this work, we have investigated the tissue localization of CMO and BADH proteins in barley plants under normal and saline conditions. Also, from the results of the distribution of GB biosynthetic enzymes, the physiological role of GB in barley is discussed.

Materials and Methods

1. Plant materials and growth conditions

A Barley cultivar Haruna-nijyo was used in this study, which is categorized as *Hordeum vulgare* ssp. *vulgare*, with two-rowed spikes and a spring growth habit. The seeds were surface-sterilized and germinated at 25°C for 2 d. Seedlings were hydroponically grown as described previously (Fujiwara et al., 2010) in a growth chamber under a 13-hr light period (25°C, 200 μmol m⁻² s⁻¹, 70% humidity)/11-hr dark period (22°C, 75% humidity). For salt treatment, 3 wk-old barley plants were treated with hydroponic culture solution containing 200 mM NaCl for 72 hr as described previously (Hattori et al., 2009; Fujiwara et al., 2010). Then, the second, third, fifth and sixth leaf blades from the base and whole roots were harvested and stored at −80°C.

2. Protein gel-blot analyses of CMO and BADH proteins

Total soluble protein was extracted from the leaves and roots of barley as reported previously (Burnet et al., 1995). Protein gel-blot analyses were carried out as described previously (Mitsuya et al., 2011). Primary [rabbit anti-HvCMO peptides (Mitsuya et al., 2011) and anti-spinach BADH (Arakawa et al., 1992) IgGs] and secondary [peroxidase-conjugated donkey anti-rabbit IgG (GE Healthcare, Little Chalfont, UK)] antibodies were diluted in Can Get Signal solution (Toyobo, Osaka, Japan) at 1:1,000, 1:5,000, 1:5,000, respectively. Protein concentration was determined with the Bio-Rad Bradford Protein Assay (Bio-Rad, Hercules, CA, USA) using BSA as a standard.

3. Immunofluorescent labeling of CMO and BADH proteins

Immunodetection of CMO and BADH proteins was performed as described previously (Mitsuya et al., 2011). Primary [rabbit anti-HvCMO peptides (Mitsuya et al., 2011) and anti-spinach BADH IgGs] and secondary [Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Wako Pure Chemical Industries, Osaka, Japan)] antibodies were diluted in incubation buffer (20 mM Na phosphate buffer, pH 7.0, 150 mM NaCl, 10 g L⁻¹ BSA, 0.5 mL L⁻¹ Tween-20) at 1:200. The sections were observed using a confocal microscope (Carl Zeiss LSM 5 PASCAL).

Results

1. The expression level of CMO and BADH proteins under salt stress

To determine the accumulation pattern of CMO protein in barley plants grown under normal and saline conditions, we performed Protein gel-blot analysis of CMO protein in individual leaves of barley plants. Plants were grown hydroponically in nutrient solution for 3 wk, and then transferred into salinized nutrient solution containing 200 mM NaCl. The lanes were loaded with 10 μg crude protein extracted from individual leaves (2nd, 3rd, 5th and 6th leaves from the base), which were collected at 0, 24 and 72 hr after the salinization. The expression pattern of CMO protein in 6th leaves and whole roots is shown in Fig. 1A. The expression of BADH under salt stress is shown in Fig. 1B.

![Fig. 1. Effect of salt stress on the expression level of CMO and BADH proteins in the leaves of barley plants. (A) Protein gel-blot analysis of CMO protein in individual leaves of barley plants. Plants were grown hydroponically in nutrient solution for 3 wk, and then transferred into salinized nutrient solution containing 200 mM NaCl. The lanes were loaded with 10 μg crude protein extracted from individual leaves (2nd, 3rd, 5th and 6th leaves from the base), which were collected at 0, 24 and 72 hr after the salinization. (B) Protein gel-blot analysis of CMO and BADH protein in 6th leaves and whole roots. The lanes were loaded with crude protein equivalent to 1 mg FW. Representative results of at least triplicate experiments are shown.](image-url)
CMO protein was increased by treatment with salt for more than 24 hr in the fifth and sixth (younger) leaf blades.

We also determined the expression level of CMO and BADH proteins in the sixth leaf blades and roots of barley grown under normal and saline conditions. For detecting BADH protein in barley, we have used the antibody which was raised in rabbit against SDS-denatured BADH from spinach (Arakawa et al., 1992). The anti-spinach BADH antibody is reactive and specific to the BADH from barley (Arakawa et al., 1992). CMO protein was detected in the sixth leaf blades and increased by the presence of NaCl but little in roots under both normal and saline conditions (Fig. 1B). BADH protein was increased by more than 24 hr treatment with salt in the sixth leaf blades. Although BADH protein was slightly expressed in the roots harvested after 72 hr treatment, the amount of BADH protein in roots was much less than that found in leaf blades.

2. Tissue localization of CMO protein

Immunodetection of CMO protein was performed in 6th leaf blades under normal and saline conditions. The fluorescent signal of CMO protein was detected using an...
Fig. 3. Immunodetection of BADH protein in the leaves of barley plants. Three-week-old plants were grown in fresh nutrient solution (a-f) or salinized nutrient solution containing 200 mM NaCl (g-l) for further 72 hr. The 6th leaves were used for analysis. The red autofluorescence emitted by chloroplasts, the green fluorescence of BADH detected with rabbit anti-spinach BADH antibody and FITC-conjugated goat anti-rabbit antibody and merged image are shown. Scale bars represent 50 μm. Arrowheads in (i) point to BADH signals in stomata cells. BC, bundle sheath cells; MC, mesophyll cells; MS, mestome sheath cells; PPC, phloem parenchyma cells; XPC, xylem parenchyma cells. Representative results of at least triplicate experiments are shown.

anti-HvCMO peptide antibody and a FITC-conjugated secondary antibody as described in Materials and Methods. The signal of CMO protein was detected in the dot-shaped organelles in mesophyll and bundle sheath tissues under both normal and saline conditions (Fig. 2b, e). The fluorescent signals were also detected in the cell wall of xylem and sclerenchyma, which is deduced to be nonspecific signals because they were also seen in the tissues with no primary antibody hybridized (arrows in Fig. 2e, h). The fluorescent signal of CMO protein was not
detected in the vascular parenchyma cells.

3. Tissue localization of BADH protein

Immunodetection of BADH protein was also performed using an anti-spinach BADH antibody. The signal of BADH protein was detected in the cytosol and the dot-shaped organelles in the sixth leaf blades (Fig. 3). This result indicates that the anti-spinach BADH antibody (Arakawa et al., 1992) may react with both isozymes of barley BADH, peroxisomal BBD1 and cytosolic BBD2. The fluorescent signals in the cytosol were detected in mesophyll, bundle sheath, mesostome sheath, xylem parenchyma and phloem parenchyma cells under both normal and saline conditions (Fig. 3b, e, h, k). However, the fluorescent signals in dot-shaped organelles were detected in epidermis and stomata cells (arrowheads) in addition to these tissues (Fig. 3). Although the strong fluorescent signals were detected in the cell wall of xylem and sclerenchyma, we considered them as nonspecific signals because they were also seen in the tissues with no primary antibody hybridized (Fig. 2e, h) and difficult to distinguish from the specific signals.

Discussion

In this study, we used a barley cultivar Haruna-nijyo. This cultivar has been used to generate a large number of ESTs at Okayama University, Japan, and a barley bacterial artificial chromosome library (Saisho et al., 2007). In addition, this cultivar is moderately salt-tolerant (Nakamura et al., 1996) and has been used for the research on the salt tolerance of barley (Arakawa et al., 1992; Ishitani et al., 1995; Fujiwara et al., 2008; Hattori et al., 2009; Fujiwara et al., 2010; Mitsuya et al., 2011).

The expression level of CMO protein was increased by salinization in the younger leaves but decreased in the older leaves under saline conditions (Fig. 1). We previously reported that the accumulation of GB and BADH protein is induced by salt stress preferentially in the younger leaves of barley (Hattori et al., 2009). These results strongly suggest that GB preferentially protects young organs against salt-induced damage by altering the expression of GB biosynthetic proteins at a translational level. Ladyman et al. (1980) reported that GB exogenously applied to mature leaves was translocated to the expanding leaves in barley, which indicates the preferential translocation of GB from older leaves toward younger ones. How GB functions in younger tissues in barley plants remains to be studied.

We have also determined the distribution of CMO and BADH proteins at a tissue level in the sixth leaves grown under normal and saline conditions. CMO and BADH proteins were co-localized in mesophyll and bundle sheath tissues (Figs. 2, 3). The signal of CMO protein was detected in the dot-like organelles, which is in agreement with our previous report that CMO is localized in peroxisomes in barley (Mitsuya et al., 2011). In addition, the signal of BADH protein was detected in the cytosol and the dot-like organelles (Fig. 3). Barley has two BADH isozymes, peroxisomal BBD1 and cytosolic BBD2, which have quite different affinity for betaine aldehyde with an apparent Km of 19.9 mM and 18.9 μM, respectively (Fujiwara et al., 2008). Because cytosolic BBD2 mainly functions in the biosynthesis of GB in barley, GB may be produced by CMO and BBD2 in a cooperative way in the cytosol and peroxisomes in mesophyll and bundle sheath tissues in barley leaves. Although it is not clear how GB functions in the cytosol, its function may be different from that previously mentioned by Chen and Murata (2011) in the direct protection of photosystem II activity in chloroplasts. GB has been reported to be involved in the regulation of the activity of transporter and channel for K+, H+ and Na+ ions under salt stress (Cuin and Shabala, 2005, 2007). Therefore, GB may have a physiological role in the regulation of osmotic potential, the protection of cytosolic enzymes and ion flux under salt stress in the mesophyll and bundle sheath tissues. To determine the physiological function of GB in salt tolerance mechanism, we will study the activity and the localization of GB biosynthesis in salt-tolerant and salt-sensitive barley varieties in a future study.

In this study, the signal of the BADH protein was detected not only in the mesophyll and the bundle sheath, but also in other tissues such as the vascular (xylem parenchyma, phloem parenchyma, and mesostome sheath cells) and epidermal (epidermis and guard cells) tissues under both normal and saline conditions (Fig. 3). Hattori et al. (2009) also reported that BADH protein was detected in parenchyma cells around the xylem of leaves. However, because BADH protein is predicted to be co-localized with CMO protein for the production of GB, it is indicated that BADH may not function in GB synthesis in vascular and epidermal tissues. In barley, two BADHs also catalyze the oxidation of 4-aminobutyraldehyde and 3-aminopropionaldehyde to γ-aminobutyric acid (GABA) and β-alanine, respectively (Fujiwara et al., 2008). Because GABA and β-alanine are accumulated in salt-treated barley plants (Widodo et al., 2009), BADH protein may be involved in the production of GABA and β-alanine in the vascular and epidermal tissues under salt stress. Interestingly, BADH protein was co-localized in mesostome sheath cells with the HvProT2 protein which transports not only GB but also GABA and β-alanine (Fujiwara et al., 2010). Further work is needed to identify the role of BADH protein in vascular and epidermal tissues.

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