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Study on the pathway of glycinebetaine biosynthesis in a monocotyledonous grass, *Aneurolepidium chinense*, grown in semiarid area

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Introduction

Glycinebetaine (GB) is regarded as one of the most effective osmoprotectants, and known to protect activity of PSI, PSII and ribulose 1,5-bisphosphate carboxylase/oxygenase from various abiotic-stresses (Nomura et al., 1998). In higher plants, it has been reported that GB is synthesized in a two-step oxidation of choline, via betaine aldehyde as an intermediate, by a ferredoxin-dependent choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH), although CMO has not been characterized yet in many families except chenopods. High levels of GB are present in leaves of some families of dicotyledons (Weretilnyk et al., 1989) and monocotyledons (Ishitani et al., 1993). Existence of BADH isozymes was suggested in sugar beet (Weretilnyk et al., 1988), mangrove (Hibino et al., 2001), sorghum (Wood et al., 1996) and barley (Nakamura et al., 1997, 2001). We reported one type of those monocotyledonous BADHs is localized in microbodies (Nakamura et al., 1997). Although rice is one of the most important crops, it is a non-GB-accumulating plant sensitive to various stresses. Therefore to confer the ability to synthesize GB into rice may enhance stress tolerance and improve its productivity under stresses. Towards this goal in mind, we chose to exploit the GB-producing enzyme system of another poaceous plant, sheep grass (*Aneurolepidium chinense*), which forms the dominant variation in circumference of semiarid area and is always exposed to high temperature, water deficit and strong light. Sheep grass accumulates GB in cells at high level in response to drought and salt. So it is important to elucidate how can the plants accumulate GB at such an enormous level up to 100 μmoles/gFW, since so far GB accumulation in rice was reported to be only 1-5 μmoles/gFW by genetic engineering using a bacterial gene (Sakamoto et al., 1998). In the present work, we cloned sheep grass *BADH* genes to characterize BADHs from sheep grass.

Materials and Methods

Sheep grass (*Aneurolepidium chinense*) was grown in a greenhouse hydroponically in a modified Hoagland solution. To subject the plants to salt stress, NaCl concentration was raised in 100 mM steps every second day until 400 mM or NaCl was directly added at the concentration of either 300 mM or 500 mM. Plants were exposed to 42 ˚C for heat stress (humidity 70 % under illumination, 100 μmol m^{-2} s^{-1}).

Total RNA was extracted from leaves of salt-stressed sheep grass plants. A cDNA library was prepared from poly $(A)^+$ RNA using the cDNA Synthesis Kit (Amersham Pharmacia

Biotech) and the Predigested Lamda ZAP II/ *Eco*RI Cloning Kit (Stratagene), following the procedures recommended by the manufacturers. For screening the cDNA library, the *BADH* cDNA of sheep grass was used as a probe as described previously (Nakamura et al. 1997). We confirmed to 5'- noncoding region and coding region of *BADH* cDNA by using the 5'-rapid amplification of cDNA end (RACE) kit (Clontech). DNA sequences were determined by the dye-primer sequencing method on a DNA sequencer (model 373A: Perkin-Elmer).

For Southern blot analysis, genomic DNA was digested with various restriction enzymes, and separated by electrophoresis on a 0.8 % agarose gel. For Northern blot analysis, total RNA was extracted from sheep grass leaves. RNA (20 μg) was denatured by treatment with formaldehyde, and fractionated by electrophoresis on 1.2% agarose gel containing formaldehyde. Hybridization was conducted by using specific probes from 3'-UTR of *BADH* cDNAs generated by PCR amplification of about 300 bp fragments. The probes were labeled with [α-^{32}P] dCTP using random primer labeling system (Amersham), and hybridization signals were detected by means of a Bio-image analyzer (Fuji BAS 2000).

Results

By screening the cDNA library, we isolated two types of *BADH* cDNA (*AcBADH1* and *AcBADH2*) from leaves of salt-stressed sheep grass. Predicted amino acid sequence shares 57 % identity between AcBADH1 and AcBADH2. However, AcBADH1 had the signal sequence SKL at its C-terminus, which is known to target proteins to microbodies, while AcBADH2 did not have the SKL signal. Moreover, the *AcBADH2* gene had high homology to *BADH* gene of dicotyledons (spinach, sugar beet) than those of monocotyledons (barley, sorghum, rice) (Fig. 1).

To examine the expression pattern of both *BADH* genes, we carried out Northern blot analysis (Fig. 2). Under normal growth conditions, *AcBADH1* gene was detected at only very low level, but it remained at low levels under heat stress. Transcriptional levels of both *AcBADH1* and *AcBADH2* genes decreased after 6 h of heat stress. However, *AcBADH1* mRNA was clearly induced under salt stress. *AcBADH2* was constitutively expressed under salt stress conditions. Expression of *AcBADH2* was always higher than that of *AcBADH1*.

Discussion

Up to date, it is reported that spinach BADH is localized in chloroplasts (Weigel et al., 1986) and monocotyledonous BADH with SKL at C-terminus is localized in microbodies (Nakamura et al., 1997). In this study, we confirmed the existence of BADH isozyme (AcBADH2) in sheep grass, which lacks the SKL signal at the C-terminus of its deduced amino acid sequence. Although overall amino acid sequence of AcBADH2 was more similar to those of dicotyledonous BADHs, its N-terminal amino acid sequence was quite different from those of dicotyledonous BADHs. Recently, a *BADH* gene (*BBDH2*) similar to *AcBADH2* was also isolated from barley (Nakamura et al., 2001). Its expression pattern was different from barley *BBDH1* (microbody type). *AcBADH1* was more inducible than *AcBADH2* in leaves of sheep grass under salt stress. AcBADH2 may be primarily important in adaptation of the plants to continued salt stress, whereas AcBADH1 may support secondarily the function of AcBADH2 for GB synthesis under more stressed conditions. However, both the two genes were sensitive to heat stress at 42 ˚C in the present study. Although we have to examine protein expression of the two BADHs, we suggest that use of *AcBADH2* gene into rice plants might be more effective for synthesis of GB to protect photosynthesis and improve their growth under salt stress conditions.

Fig. 1 A, Comparison of the deduced amino acid sequences around the C-terminus end of BADHs**;** B, Phylogenic tree of BADHs in higher plants.

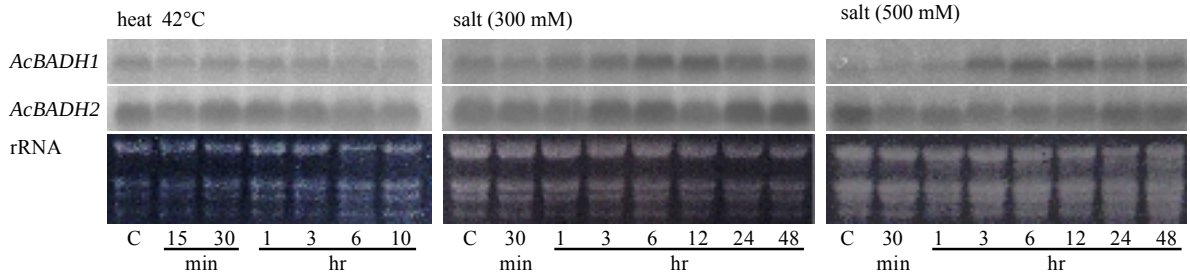

Fig. 2 Northern blot analysis of sheep grass leaves (time course)

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