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Expression of the betaine aldehyde dehydrogenase gene in barley in response to osmotic stress and abscisic acid

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Abstract

When subjected to salt stress or drought, some vascular plants such as barley respond with an increased accumulation of the osmoprotectant glycine betaine (betaine), being the last step of betaine synthesis catalyzed by betaine aldehyde dehydrogenase (BADH). We report here cloning and characterization of BADH cDNA from barley, a monocot, and the expression pattern of a BADH transcript. An open reading frame of 1515 bp encoded a protein which showed high homology to BADH enzymes present in other plants (spinach and sugar-beet) and in *Escherichia coli*. Transgenic tobacco plants harboring the clone expressed high levels of both BADH protein and its enzymatic activity. Northern blot analyses indicated that BADH mRNA levels increased almost 8-fold and 2-fold, respectively, in leaves and roots of barley plants grown in high-salt conditions, and that these levels decreased upon release of the stress, whereas they did not decrease under continuous salt stress. BADH transcripts also accumulate in response to water stress or drought, indicating a common response of the plant to osmotic changes that affect its water status. The addition of abscisic acid (ABA) to plants during growth also increased the levels of BADH transcripts dramatically, although the response was delayed when compared to that found for salt-stressed plants. Removal of plant roots before transferring the plants to high-salt conditions reduced only slightly the accumulation of BADH transcripts in the leaves.

Introduction

Members of several families of vascular plants respond to water or salt stress with the increased synthesis of the osmoprotectant, glycine betaine (betaine) [15, 21, 24, 26, 36]. The synthesis and accumulation of this compound have been best characterized in members of the Gramineae (barley, maize) and the Chenopodiaceae (spinach, sugar-beet). In these plants, betaine biosynthesis occurs by a two-step oxidation of choline via the intermediate betaine aldehyde, which are catalyzed by choline monooxygenase and betaine aldehyde dehydrogenase (BADH, EC 1.2.1.8), re-

The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI Nucleotide Sequence Databases under the accession number D26448.

spectively [8, 26]. Both steps were shown to be localized in chloroplasts in spinach [8, 16, 33, 34], being in this species plastid BADH a dimeric protein of about 60 kDa [3, 35]. The amino acid sequences of BADH from spinach and sugar-beet have been deduced from their respective cDNAs, and found to show 83% identity between them [22, 35]. These proteins show also substantial similarities to other non-specific aldehyde dehydrogenases [7]. The levels of both BADH transcripts and enzymatic activity were found to increase almost 2-fold in leaves of salt-stressed spinach plants, and almost 4- and 3-fold in sugar-beet leaves and taproots, respectively [22, 35]. We have previously reported that betaine levels also increase in leaves and roots of barley in response to salt stress [4], in parallel to the accumulation of BADH [5]. These studies indicate that betaine accumulation in salt-stressed barley plants is regulated (at least in part) via changes in the expression of the genes of the betaine biosynthetic pathway. Nevertheless, little information exists on the nature of the signal(s) that mediate the above-mentioned osmotic response, which could be related to reduced leaf turgor [37], salts taken up by the plants [23], plant hormones or other substances which communicate the roots with the leaves [1, 5, 24, 26], etc. In particular, it is well documented that abscisic acid (ABA) may initiate global physiological reactions in response to environmental stresses [38]. ABA is synthesized in leaves and roots [38], and it has been shown to participate in various developmental and physiological processes including stomatal closure, increased root growth, and inhibition of shoot growth [9, 25]. Moreover, ABA levels increase in tissues subjected to osmotic stress produced by salt, desiccation, or cold [19, 29], suggesting that this compound may constitute a signal that connects roots and leaves [6, 10, 31].

In the present study, we have further characterized the response of barley to osmotic stress, in particular the expression of the BADH gene on plants grown in high-salt conditions, as well as the effects shown by exogenously applied ABA.

Materials and methods

Plant material

Barley (*Hordeum vulgare* L. 116 Jeonju Native Korca) was used in the all experiments reported in this work except for the transgenic expression of BADH in heterologous plants, *Nicotiana tabacum* cv. SR1. Growth conditions were those described previously [4]. To subject the plants to salt stress, the NaCl concentration was raised in 100 mM steps every second day until reaching 300 mM. Water stress and drought were provided by adding 200 g/l of polyethylene glycol 6000 (PEG) to the hydroponic culture medium or withholding water, respectively. For ABA treatments, 7-day old hydroponically grown barley plants were subjected to 100 μM ABA in the conditions and periods indicated in the figures.

Preparation of a cDNA library and screening procedures

Total RNA was extracted from leaves of barley plants grown for 2 days in a culture medium containing 300 mM NaCl, as described above. A cDNA library was prepared from the poly(A)$^+$ RNA using the Predigested Lambda ZAPII/*Eco* RI Cloning Kit (Stratagene), following the procedures recommended by the manufacturers. The library was screened with a 693 bp probe containing the 3′ coding region of spinach BADH [35], which had been obtained by PCR amplification and labeled with [α-^{32}P]dCTP using the random primer labeling system (Amersham). A plasmid clone out of three positives (pBAD), which contained the largest insert, was selected for further characterization after *in vivo* excision of the pBluescript plasmid from the ZAPII vector.

Plasmid construction, plant transformation and regeneration

A 1.9 kb *Bam* HI fragment of pBAD was inserted into the *Bam* HI site downstream of CaMV-35S

promoter in a modified pBI121 from which was removed GUS gene. The resultant plasmid pBBH was electroporated into *Agrobacterium tumefaciens* strain LBA4404 using the method described by Mattanovich *et al.* [20].

Leaf disks were taken from *Nicotiana tabacum* cv. SR1 cultured on Murashige-Skoog (MS) medium containing α-naphthalene acetic acid (0.2 μg/ml), 6-benzylaminopurine (1 μg/ml), kanamycin (200 μg/ml) and carbenicillin (300 μg/ml). Leaf-disk transformation and selection and propagation of kanamycin-resistant plants were as described by An *et al.* [2]. The transformants were grown at 27 °C under fluorescent light (100 $\mu E\ m^{-2}\ s^{-1}$) for 16 h followed by 8 h dark.

Western blot analysis

Total protein (100 μg) from leaf tissue of SR1 and transformants were subjected to sodium dodecyl sulfate polyacryamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the protein was electroblotted onto a PVDF membrane (Millipore). Immunoblot analysis was carried out by epitope-selected antibody raised against BADH protein from spinach leaves according to Arakawa *et al.* [5].

Assay of BADH activity

Crude BADH from tabacco leaves was prepared as described in Arakawa *et al.* [4] with some modifications. BADH was assayed by the fluorometric method described previously [3].

Northern blot analysis

Total RNA was extracted from leaves and roots of barley plants after salt or water stress as described above, and subjected to electrophoresis on a 1.2% agarose gel containing 0.66 M formaldehyde. After transferring and fixing to nylon membranes (Hybond N, Amersham Japan, Tokyo), hybridization was carried out with a ^{32}P-labeled 0.7 kb *Bam* HI/*Apa* I DNA fragment from pBAD in a solution containing 6× SSC, 5× Denhardt's solution, 0.1% SDS and 0.1 mg/ml denatured salmon sperm DNA at 65 °C. The membranes were washed with 6× SSC at 65 °C before exposing to X-ray films. Relative amounts of mRNA were determined by scanning X-ray films (Shimadzu DUAL Wavelength TLC scanner CS-910).

Southern blot analysis

Total DNA was isolated from barley leaves according to Dellaporta *et al.* [11], digested with various restriction enzymes, and separated by electrophoresis on a 0.8% agarose gel. After transferring to nylon membranes (Hybond N^+, Amersham Japan, Tokyo), hybridization was carried out as described above with the ^{32}P-labeled complete insert present in pBAD.

Other procedures

DNA recombinant techniques were used for plasmid constructions [27]. To sequence the insert present in pBAD, a set of exonuclease III deletion mutants were prepared, and the DNA sequence was determined using the *Taq* Dye Primer Cycle Sequencing Kit from Applied Biosystems [28].

Results

BADH cDNA cloning and characterization and its transgenic expression

A cDNA library was constructed in bacteriophage λZAPII using poly(A)$^+$ mRNA from leaves of barley plants grown in high-salt conditions, and screened using a fragment of the 3′ coding region of spinach BADH. From 6×10^5 independent plaques, three clones were isolated, one of which was identified as a nearly full-length BADH cDNA after sequencing. This clone contained a

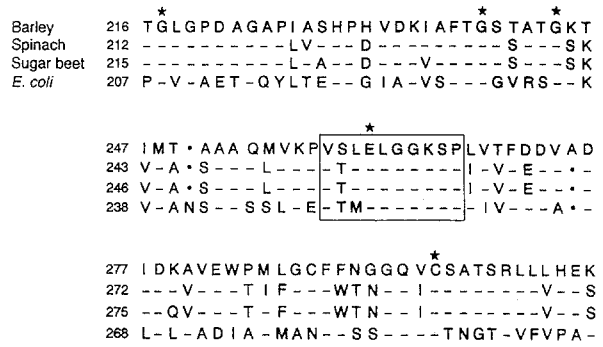

Fig. 1. Comparison of amino acid sequences of BADH from different sources. Amino acid residues found in other BADH identical to those found in the same position in the barley enzyme are indicated by dashes (–). The asterisks indicate the residues presumed to be involved in NAD^+-binding and the box indicates the highly conserved region in catalytic sites of various dehydrogenases.

1826 bp insert with a single open reading frame of 1515 bp, as well as a putative polyadenylation signal within the 3′ non-coding region. The translated polypeptide (505 amino acids) shows 70% and 69% identity, respectively, to the amino acid sequences of BADH from spinach and sugar-beet [22, 35], and 37% identity to the enzyme of *Escherichia coli* [7]. Figure 1 shows some highly conserved amino acid regions, including the decapeptide motif VTLELGGKSP (positions 258–267), and residues probably involved in NAD^+ binding and in catalytic sites, which have been characterized in various dehydrogenases [35].

We have introduced the insert present in pBAD into tobacco plants, which contain negligible endogenous amounts of BADH, and found that the resultant transgenic plants expressed high levels of both BADH protein and enzymatic activity (Fig. 2). The activity was shown to be highly specific for betaine aldehyde except that in wild type of tobacco (data not shown).

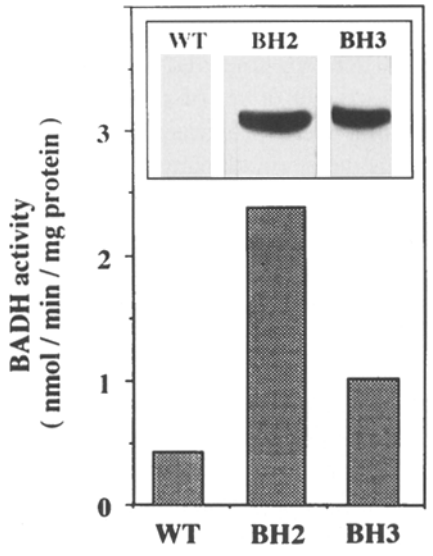

Fig. 2. Activities of BADH enzyme and levels of BADH protein in leaves of wild-type (WT) and transgenic tobacco (BH2 and BH3). Proteins were extracted from mature leaves and used for enzyme assays (*bar graphs*) or SDS-PAGE and immunoblot analysis (inset). Extraction of leaf proteins and $(NH_4)_2SO_4$ precipitation (55–70%) were as previously reported [5].

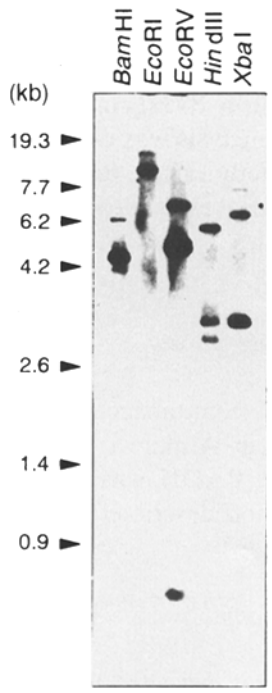

Fig. 3. Southern blot analysis of barley DNA with a BADH gene probe. Total genomic DNA (10 μg) was digested with the following restriction enzymes: lane 1, *Bam* HI; lane 2, *Eco* RI; lane 3, *Eco* RV; lane 4, *Hin*d III; lane 5, *Xba* I. After separation in a 0.8% agarose gel, the fragments were transferred to nylon membranes and hybridized to the complete insert present in pBAD, as described in Materials and methods.

Southern blot analysis

Figure 3 shows the hybridization patterns obtained after hybridization of genomic DNA with the insert contained in plasmid pBAD. The number of bands that were observed upon digestion with different restriction enzymes were two to three, sugesting that BADH is encoded by a small multigene family in barley.

Expression of BADH gene in leaves and roots of barley plants under osmotic stress

A transcript of ca. 1.9 kb was found in barley leaves to hybridize with the BADH DNA probe, whose levels increased about 8-fold after 48 h of salt stress when compared to control plants (Fig. 4a). The levels did not change under continuous salt stress until 96 h, whereas levels of the mRNA in unsalinized plants also increased slightly with time and reached a saturated level at 48 h. Transferring of the salt-stressed plants to a non-salinized medium reduced the levels of BADH transcripts after 36 h to almost one-half of those found in high-salt conditions. Similar results were obtained when the 3′ non-coding region of the insert in pBAD was used as a probe (data not shown).

Drought and water stresses were promoted by withholding water and by addition of polyethylene glycol, respectively, and plants were grown for the time indicated in Fig. 4b before harvesting. In both cases a drastic increase of BADH transcripts was observed after the treatment.

To reveal the organ-specific expression of BADH gene, total RNA was prepared and analyzed from roots of plants treated with 300 mM NaCl for 48 h as described above. The levels of BADH transcripts in the roots increased about 2-fold when compared to those of control plants (Fig. 4c).

Effects of exogenously applied ABA and removal of roots on the expression of the BADH gene in leaves

Treatment of the plants with 100 μM ABA was found to promote a drastic increase in BADH transcripts, an effect observed especially around 96 h after the addition of the plant hormone (Fig. 5a) in culture medium. Nevertheless, the effect of ABA was retarded when compared to that produced by salt stress, which occurred within 12 h (Fig. 4a). Figure 5b shows the expression patterns of BADH gene in the leaves of plants in which the roots had been removed before the salt treatment, as well as the simultaneous addition of

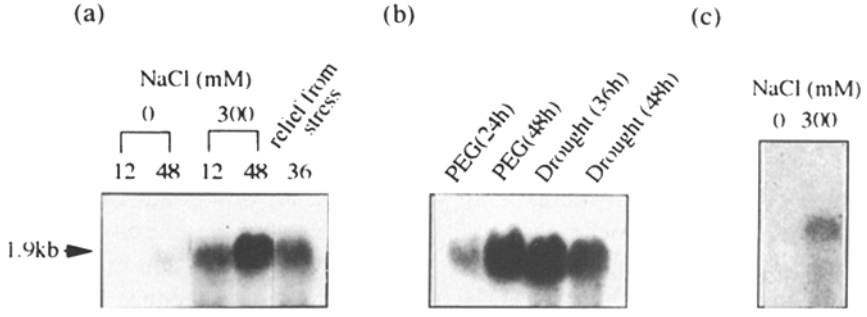

Fig. 4. Levels of BADH transcripts in leaves and roots under osmotic stress. (a) BADH mRNA levels in leaves of barley plants grown in high-salt conditions. RNA extracted from the leaves of control and stressed barley plants was analyzed at the indicated times. The relieving of salt stress was done by transferring the plants after 48 h at high salt to an unsalinized medium for 36 h. The time of treatment (h) is indicated. (b) Effect of water stress and drought on the levels of BADH mRNA in leaves. Total RNA was isolated from stressed plants after the indicated times. The conditions of the treatment are indicated in Materials and methods. (c) Effect of salinity on BADH mRNA accumulation in the roots. After NaCl treatment for 48 h, total RNA was isolated from the roots and analyzed. In all cases 30 μg of RNA were loaded per lane, and the membranes were hybridized to the labeled BADH cDNA fragment.

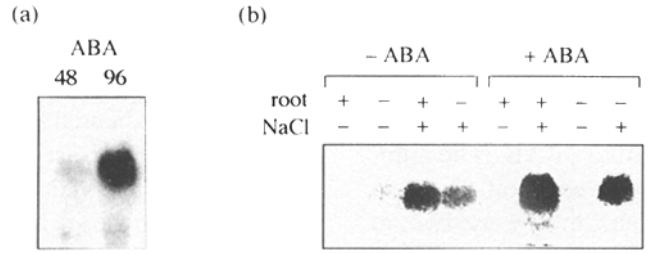

Fig. 5. Effect of removal of roots and exogenously applied ABA on the accumulation of BADH transcripts in leaves. Panel (a) shows the effects of the addition of exogenous ABA on BADH mRNA levels. For hormone treatments, 10 μM ABA were applied to hydroponically grown 7-day old barley plants. Total RNA was prepared and analyzed after 48 and 96 h, respectively, of treatment. Panel (b) shows the effect of the removal of roots on BADH expression. The roots of hydroponically grown 7-day old plants were cut out (root –) before subjecting the rest of the plant to the indicated treatments. Salt stress was induced by adding NaCl to a concentration of 300 mM (NaCl+). ABA was applied exogenously to a final concentration of 100 μM (ABA+). In all cases, total RNA was extracted from the plants after 48 h of treatment, and 30 μg RNA was subjected to analysis as described in Materials and methods.

ABA under these conditions. In the absence of ABA (–ABA), the high-salt treatment induced the accumulation of BADH transcripts in the leaves of plants, regardless of the previous removal of roots. However, in rootless plants (–root, + NaCl) the transcript level was somewhat lower than that observed in plants in which roots were present (+ root, + NaCl). When exogenous ABA was added to these plants (+ABA), the accumulation of BADH transcripts was also detected without salt stress. Although the levels of transcripts after 48 h were low in the absence of salt stress (the differences between Figs. 5a and 5b are due to the different exposure times employed), they were indeed higher when salt stress was combined with ABA in all cases (roots present or absent) than those obtained without ABA treatment when salt stress was present. These results showed that, although the expression of BADH gene in the leaves does not absolutely require the roots, this tissue might modulate to some extent the expression of the BADH gene in the leaves. Moreover, exogenously added ABA enhanced the accumulation of BADH transcripts in leaves during salt stress, suggesting a possibility that there exists an effect of root-sourced ABA on the expression of the BADH gene in the leaves.

Discussion

We have cloned and characterized a cDNA encoding BADH in barley, a plant belonging to the Gramineae family. The identification of the encoded enzyme has been done by comparison of the translated polypeptide with enzymes of spinach, sugar-beet, and *Escherichia coli*. The sequence similarities between the distinct BADH described indicate a high conservation of this gene among monocots and dicots, and probably a common evolutionary origin of the enzymes of plants and bacteria.

Analyzing the transgenic plants revealed that the transformants expressed high levels of both BADH protein and enzymatic activity (Fig. 2). The more interesting aspect of the transgenic plants is its phenotype with respect to osmoprotection. At this point, the transformants did not appear to exhibit increased resistance to osmotic stress, suggesting that introduction of the choline monooxygenase gene is also required for synthesis of enough betaine to act as an osmoprotectant.

We have reported previously that the accumulation of betaine is accompanied with increases in both BADH protein and activity in the leaves of barley plants subjected to salt stress, and that this effect is reversed after relieving stress conditions [4, 5]. In the present work, we demonstrated that

the accumulation of BADH mRNA levels increased up to 8-fold in leaves from salinized barley plants, and that these levels decreased when the stress was attenuated (Fig. 4a). Moreover, the rises on BADH mRNA levels also occurred when barley plants were subjected to drought and water stresses (Fig. 4b), a result consistent with previous findings [4, 5]. Therefore, these observations suggest that betaine accumulation in barley is closely related to osmotic stress, and regulated (at least in part) by changes in the expression of the BADH gene. Moreover, this response was not salt-specific, but rather appeared to be a common reaction to any osmotic stress that affects the plant water status. We cannot exclude at this stage the possibility that the rises observed could result from an increased mRNA stability induced by the stress [12].

Interestingly, we also found that the accumulation of BADH mRNA increased in roots in response to salt stress (Fig. 4c). Our previous studies indicated that BADH is in roots [4, 5]. It is worth mentioning that in sugar-beet the levels of both root BADH activity and mRNA also increased upon salinization [22].

It has been suggested that the signal for the induction of the BADH gene is mediated by some compounds other than the osmotica mentioned above [21]. Many observations have indicated that osmotic stresses such as salt, drought and water stress, etc., lead to the rise of endogenous ABA levels, and consequently to changes in gene expression [29]. Our results indicate that during drought the maximum level of the transcript occurred at 36 h, and decreased to some degree at 48 h (Fig. 4b). Moreover, the exogenous application of ABA induced the accumulation of BADH mRNA, although this response was much slower than that observed with the other treatments (Fig. 5a). This suggests that the BADH gene does not belong to the same category of other ABA-responsive genes (e.g., the *rab* gene family) [13, 16, 29], in which the transcripts increase many fold within hours of ABA application [19]. Recent studies using *Mesembryanthemum crystallinum* (ice plant) indicate that ABA is actually not necessary for the salt stress-induced expression of the PEPCase gene, despite the observation that the level of this hormone in leaves increases in response to the stress [30]. Therefore, our observations in the present work suggest that there exist different signal transduction pathways that regulate the expression of stress-induced genes in barley, and that the expression of BADH gene may require only indirect participation of ABA.

It seems reasonable to assume that the roots sense first the salt stress, and that a signal initiated in these tissues (if any) mediates the response in the leaves [10, 23]. However, salt treatment induced the accumulation of BADH mRNA in the leaves of barley plants in the absence of roots (Fig. 5b), suggesting that the signal for expression did not originate solely from the roots. Recent studies in ice plants suggest that stress-induced and age-related reduction in leaf turgor can trigger PEPCase gene expression [32, 37]. The nature of these signal(s) appears obscure at this stage, although it seems obvious that there exists a mechanism of response that transduces environmental stress into physiological events in the whole plant.

The osmotic stress response of BADH mRNA in barley is more significant than that reported for members of the Chenopodiaceae such as spinach and sugar-beet [22, 35], indicating that this plant can provide an efficient model to study stress promoters and regulatory mechanisms that respond to environmental stress. Further analyses of the regulation of BADH gene expression will allow us to understand the mechanisms that link an environmental stress to a particular response in the different tissues of the plant.

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