An Unusual Posttranscriptional Processing in Two *Betaine Aldehyde Dehydrogenase* Loci of Cereal Crops Directed by Short, Direct Repeats in Response to Stress Conditions[1]

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Various abilities to synthesize and accumulate glycine betaine (GB) are crucial for angiosperms to develop salt and drought tolerances. In higher plants, GB is synthesized by a two-step oxidation of choline via an intermediate form of betaine aldehyde, and catalyzed by choline monooxygenase and betaine aldehyde dehydrogenase (BADH). In this study, numerous truncated and/or recombinant transcripts of two *BADH* homologs resulting from an unusual posttranscriptional processing were detected in rice (*Oryza sativa*) and other cereal crops, including maize (*Zea mays*), wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*). The observed events took place at the 5′ exonic region, and led to the insertion of exogenous gene sequences and a variety of deletions that resulted in the removal of translation initiation codon, loss of functional domain, and frameshifts with premature termination by introducing stop codon. By contrast, the *BADH* transcripts from dicotyledonous species, such as spinach (*Spinacia oleracea*), Arabidopsis (*Arabidopsis thaliana*), and tomato (*Solanum lycopersicum*), had correctly processed mRNA. This suggests the differentiation of posttranscriptional processing in *BADH* genes potentially contributes to the variation of GB-synthesizing capacities among various plant species. In addition, comprehensive sequence analyses demonstrated that extensive sequence similarities (named as short, direct repeats) are of paired presence surrounding the junctions of both the deletion and/or insertion sites in the unusual *BADH* transcripts. The site selection for the deletion/insertion was altered in response to the stress conditions. This indicates that the sequence elements of short, direct repeats are probably required for the recognition of the deletion/insertion sites.

The higher plants, as sessile organisms, are generally characterized by a high degree of homeostatic plasticity in response to environmental fluctuations, thereby optimizing their growth and development in a way that maximizes their opportunities for survival and reproduction. Osmotic stresses, such as salinity and drought, signify the most severe environmental pressures (abiotic stresses) that significantly limit the growth and productivity of plant species (Boyer, 1982). The higher plants, as well as other organisms, have evolved a number of adaptive strategies to overcome such abiotic stresses (Tester and Davenport, 2003; Bartels and Sunkar, 2005). At the cellular level, the most common type of osmotic adaptation involves the accumulation of compatible solutes in cytoplasm, including amino acids, ammonium compounds, and polyols/sugars. Such solutes can lower the osmotic potential for cells without interfering with the metabolic processes or protein structuring and functioning and, consequently, maintain the water content of the cells under stresses (Yancey et al., 1982). Among the few classes of organic compounds that are commonly employed as osmoprotectants, the quaternary ammonium compounds (e.g. Gly betaine [GB]) are widely found in bacteria, cyanobacteria, algae, higher plants, and animals. These compounds are frequently detected being accumulated in those plant species that are exposed to drought or high salinity environments (Rhodes and Hanson, 1993). In principle, GB is known to provide a tolerance to cells under stresses by stabilizing the quaternary structure of the complex proteins and adjusting the osmotic potential in cytoplasm for

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maintaining water content. During photosynthesis, it stabilizes both PSII complexes and Rubisco under salinity and low temperatures (Incharoensakdi et al., 1986; Holmström et al., 2000). Exogenous supply of GB has been demonstrated to protect plants from stresses (Harinasut et al., 1996; Chen et al., 2000) and has also been suggested to have a cryoprotective effect on cell membranes (Sakamoto et al., 2000).

In the known biological systems, GB is synthesized via two distinct pathways from two distinct substrates: choline and Gly, respectively (Sakamoto and Murata, 2002). The conversion of choline into GB has been studied in a number of organisms, and its pathway involves one or two enzymes, depending on the mode of oxidation of choline. The two-enzyme pathway is commonly found across a wide range of plant and animal species and microorganisms, in which GB is formed as the result of a two-step oxidation of choline via the toxic intermediate betaine aldehyde. Different enzymes from plants, animals, and bacteria are involved in the first step of the pathway (Wilken et al., 1970; Ikuta et al., 1977; Lamark et al., 1991; Burnet et al., 1995). In plants, the first step is catalyzed by a novel Rieske-type iron-sulfur enzyme choline monooxygenase (CMO; Burnet et al., 1995; Rathinasabapathi et al., 1997). By contrast, the enzyme involved in the second step of the pathway is supposedly the same in plants, animals, and bacteria: it is NAD-dependent betaine aldehyde dehydrogenase (BADH; Weretilnyk and Hanson, 1989; Falkenberg and Strom, 1990; Chern and Pietruszko, 1995). BADH is a soluble, nonspecific aldehyde dehydrogenase, and its substrates include betaine aldehyde, aminoaldehydes, and dimethylsulfoniopropionaldehyde (Trossat et al., 1997; Livingstone et al., 2003). Plant BADH proteins are dimers (Weretilnyk and Hanson, 1989; Arakawa et al., 1990; Valenzuela-Soto and Munoz-Clares, 1994). The presence of two *BADH* homologs has been widely reported in cereal crops (Wood et al., 1996; Nakamura et al., 2001; Bradbury et al., 2005).

In many plant species under investigation, genes encoding for the two-step enzymatic process have been identified (Weretilnyk and Hanson, 1990; McCue and Hanson, 1992; Burnet et al., 1995; Ishitani et al., 1995; Wood et al., 1996; Nakamura et al., 1997; Russell et al., 1998). However, the abilities to accumulate GB are considerably different. In comparison with Chenopodiaceae species such as spinach (*Spinacia oleracea*) and sugar beet (*Beta vulgaris*) that usually accumulate abundant GB in response to water deficit or salt stress (Pan et al., 1981; Hanson and Wyse, 1982), much less GB (0–20 μmol/g fresh weight) occurs in Poaceous species such as maize (*Zea mays*), wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), and barley (*Hordeum vulgare*; Hanson and Scott, 1980; Lerma et al., 1991; Ishitani et al., 1993; Jagendorf and Takabe, 2001; Yang et al., 2003). Moreover, studies showed that GB accumulation in rice (*Oryza sativa*) plants was undetectable (Flowers and Yeo, 1981; Ishitani et al., 1993; Rathinasabapathi et al., 1993; Shirasawa et al., 2006), although a recent genome sequence of rice revealed that it contains a *CMO* (accession no. AJ578494) and two conserved *BADH* homologs (accession nos. AK103582 and AK071221, named as *OsBADH1* and *OsBADH2*, respectively) that are required for the two-step catalytic reactions. This prompts us to inspect the possible mechanism and molecular processes that underlie the large variation of GB accumulation among various species.

In this study, we extensively examined the expression of the *BADH* genes in rice and their response to stress conditions by including different degrees of ion concentrations and drought. We also examined differential transcriptional products derived from different tissues and treatments that had deletion(s) of 5′ exon material or insertion of exogenous gene sequences, probably resulting from an unusual posttranscriptional processing. Similar experiments were also conducted extending the examination of *BADH* homologs to other cereal crops, including maize, wheat, and barley, as well as dicotyledonous species, including spinach, Arabidopsis (*Arabidopsis thaliana*), and tomato (*Solanum lycopersicum*). In addition, we showed that extensive sequence similarities (named as short, direct repeats [SDRs]) are of paired presence surrounding the junctions at both deletions and insertions sites, and SDRs are probably required for recognition of the deletion/insertion sites in response to stress conditions. These data together with transgenic experiments by other groups (Sakamoto et al., 1998; Takabe et al., 1998; Kishitani et al., 2000) suggest that the lack of precise *BADH* gene products resulting from incorrectly processed *BADH* transcripts may contribute, at least in part, to the large variation in GB accumulation among various species.

RESULTS

Incorrectly Processed Transcripts Derived from Two Rice *BADH* Homologs

To determine the expression pattern of *OsBADH1*, we isolated the total RNAs from various rice tissues grown in different conditions (Table I) and analyzed the transcription by reverse transcription (RT)-PCR. The results showed that drought and salt stresses increased the transcription level of *OsBADH1* (Fig. 1A). Using seedlings of rice *japonica* cv Nipponbare as an example, we found that the expression of *OsBADH1* was very low under conditions without salt stress and detected the increased transcripts when stresses were added. However, a high ion concentration (0.5 M NaCl) inhibited transcription of this gene. Similar results were obtained when different rice varieties were used (Fig. 2, A–D).

To examine whether the expressed products were *OsBADH1* gene, the RT-PCR-amplified fragments were cloned and sequenced. We used primers derived from 5′ and 3′ untranslated regions (UTRs) to isolate

Table I. *The incorrect processing of OsBADH1 transcripts in rice seedlings, callus, and mature leaves*

Rice Variety	Tissue and Growth Condition	No. of Clones	Position and Size of Deletion	SDR	Nearly Identical Sequences
Nipponbare	Seedling, unstressed	1	e1. 16-e2. 72 (172)[a]	CG	GCCGtCGgCG/GCCGCGaCG
Nipponbare	Seedling, drought, 24 h	2	e1. 10-e2. 72 (178)	GGCCGCG	GGCCGCGcCG/GGCCGCGaCG
Nipponbare	Seedling, 0.25 M KCl, 24 h	2	e1. 8-e2. 108 (216)	GGCCG	GGCCGcGC/GGCCGtGC
Nipponbare	Seedling, 0.1 M NaCl, 72 h	3	e1. 1-e2. 100 (215)	GCC	GCCaTGG/GCCTGG
Nipponbare	Seedling, 0.2 M NaCl, 72 h	1	e1. 11-e2. 97 (202)	CGCGC	GcCGCGCcG/GCGCGCG
Nipponbare	Seedling, 0.3 M NaCl, 72 h	4	e1. 16-e2. 72 (172)	CG	GCCGtCGgCG/GCCGCGaCG
Nipponbare	Seedling, 0.3 M NaCl, 72 h	1	e1. 7-e1. 99 (93)	GGCC	GGCCgCG/GGCCaCG
			e2. 27-e2. 82 (56)	GGAG	GGAGgAC/GGAGAC
Nipponbare	Seedling, 0.5 M NaCl, 24 h	1	e1. 10-e2. 72 (178)	GGCCGCG	GGCCGCGcCG/GGCCGCGaCG
93-11	Seedling, unstressed	1	e1. 16-e2. 72 (172)	CG	GCCGtCGgCG/GCCGCGaCG
93-11	Seedling, drought, 24 h	1	e1. 8-e2. 108 (216)	GGCCG	GGCCGcGC/GGCCGtGC
93-11	Seedling, 0.25 M KCl, 24 h	2	e1. 10-e2. 72 (178)	GGCCGCG	GGCCGCGcCG/GGCCGCGaCG
93-11	Seedling, 0.1 M NaCl, 72 h	1	e1. 10-e2. 72 (178)	GGCCGCG	GGCCGCGcCG/GGCCGCGaCG
93-11	Seedling, 0.2 M NaCl, 72 h	1	e1. 16-e2. 72 (172)	CG	GCCGtCGgCG/GCCGCGaCG
93-11	Seedling, 0.3 M NaCl, 72 h	1	e1. 20-e2. 73(169)	GCGA	CgGCGAtC/CcGCGAC
93-11	Seedling, 0.5 M NaCl, 24 h	1	e1. 16-e2. 72 (172)	CG	GCCGtCGgCG/GCCGCGaCG
Suhui 527	Seedling, 0.1 M NaCl, 72 h	1	e1. 7-e1. 34 (28)	GGCC	GGCCG/GGCCtG
			e1. 44-e2. 72 (144)	CG	CGgCGGcGGG/CGaCGGtGGG
Chuanxiang 29B	Seedling, 0.1 M NaCl, 72 h	1	e1. 1-e2. 100 (215)	GCC	GCCaTGG/GCCTGG
Zhonghua 9	Seedling, 0.1 M NaCl, 72 h	1	e1. 16-e2. 72 (172)	CG	GCCGtCGgCG/GCCGCGaCG
Yuanlixiagjing	Seedling, 0.1 M NaCl, 72 h	2	e1. 10-e2. 72 (178)	GGCCGCG	GGCCGCGcCG/GGCCGCGaCG
Newbonnet	Seedling, 0.1 M NaCl, 72 h	2	e1. 10-e2. 72 (178)	GGCCGCG	GGCCGCGcCG/GGCCGCGaCG
Tebonnet	Seedling, 0.1 M NaCl, 72 h	2	e1. 7-e1. 34 (28)	GGCC	GGCCG/GGCCtG
			e1. 44-e2. 72 (144)	CG	CGgCGGcGGG/CGaCGGtGGG
Nipponbare	Callus, unstressed	1	e1. 16-e2. 72 (172)	CG	GCCGtCGgCG/GCCGCGaCG
Nipponbare	Callus, 0.5 M NaCl, 24 h	1	e1. 10-e2. 72 (178)	GGCCGCG	GGCCGCGcCG/GGCCGCGaCG
Nipponbare	Callus, 0.5 M NaCl, 24 h	1	e1. 13-e2. 72 (175)	CG	CGcCGtCGG/CGCGaCGG
93-11	Callus, 0.5 M NaCl, 24 h	1	e1. 13-e2. 72 (175)	CG	CGcCGtCGG/CGCGaCGG
Nipponbare	Mature leaf, field	1	e1. 7-e2. 116 (225)	GGCC	
93-11	Mature leaf, field	1	e1. 8-e2. 72 (180)	CG	GcCGCG/GCGaCG
Basmati	Mature leaf, field	2	e1. 14-e2. 109 (211)	GCCGT	GCCGTCGG/GCCGTgCGG
Della	Mature leaf, field	1	e1. 7-e1. 34 (28)	GGCC	GGCCG/GGCCtG
			e1. 44-e2. 72 (144)	CG	CGgCGGcGGG/CGaCGGtGGG

[a]e1. 16-e2. 72 (172) denotes that exon materials of *OsBADH1* from the 16th base of exon 1 to the 72nd of exon 2 were excised; 172 nucleotides in total were deleted.

the full length of *OsBADH1* cDNA clones (Table II). The resultant sequencing analysis revealed that the *OsBADH1* cDNAs were truncated at the 5′ exonic region. Accordingly, we focused the analysis on a region comprising exons 1 to 6 using primers specific to the 5′ region. As shown in Figures 1A and 2, the observed expressed products were shorter than the expected size of 695 bp of the 5′ exonic region. To examine the structure of *OsBADH1* transcripts, a total of 41 *OsBADH1* cDNA clones were studied in detail. Sequence comparison of the cDNAs revealed a considerable variation in their structural compositions (Table I). All of the cDNAs contained a deletion of the 5′ coding sequence within the *OsBADH1* gene. The deleted exon material ranged from 28 to 225 nucleotides in size. The start-point of the deletions in four cDNAs began with the first nucleotide of the coding sequence, which gave rise to the loss of translation initiation codon. Thirty-two cDNAs encoded derivatives with frame-shifts in the open reading frame (ORF), introducing various stop codons at different positions. Only five cDNA clones showed the potential to encode partial BADH1 proteins with deletions that coded for a part of the putative NAD^+-binding domain. Most of the missing sequences from the truncated transcripts indicated above involved two different exons, and in a few cases the truncation took place within a single exon. In addition, two independent deletions of exon materials within a single cDNA clone were observed in five clones. Therefore, no cDNA was found to have the capacity to encode the full length of the OsBADH1 protein, indicating that correctly processed transcripts represented a very small proportion of the total cytoplasmic mature *OsBADH1* RNA population and consequently that the majority of the *OsBADH1* mRNAs were unlikely to encode functional proteins.

For *OsBADH2*, preliminary experiments based on RT-PCR showed that the mRNA was expressed constitutively and multiple transcription products were detected (Fig. 3A). Primers specific to the 5′ region were used in these experiments (Table II). To analyze the transcripts derived from the *OsBADH2* gene, seedlings from different varieties under different growth conditions were harvested for the total RNA isolation. As a result, all the 59 cDNA clones sequenced also had deletions at the 5′ exonic region (Table III). Similar to

that in the *OsBADH1* gene, various unusual events in the *OsBADH2* locus generated a number of truncated transcripts. The size of the deleted sequences from 5′ UTR and exon(s) ranged from 112 to 523 nucleotides. The start-points of deletions in the 15 cDNA clones varied from −12 to −2 bases of 5′ UTR, resulting in the failure of translation initiation. Twenty-seven cDNA clones encoded derivatives with frame-shifts in the ORF. Eight cDNA clones were able to encode the partial OsBADH2 proteins without frame-shifts. In

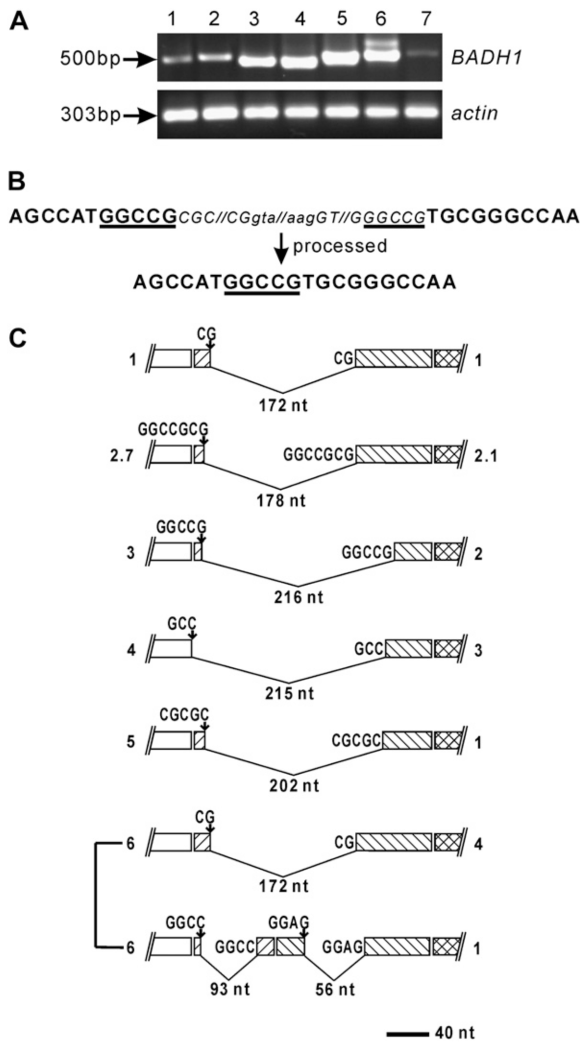

Figure 1. Expression and structure of *OsBADH1* RNAs derived from seedlings of *japonica* cv Nipponbare. A, Agarose gel electrophoresis analysis of RT-PCR amplification products. Comparison of expression abundance under conditions of no stress (lane 1), drought (lane 2), 0.25 M KCl (lane 3), and 0.1, 0.2, 0.3, and 0.5 M NaCl (lanes 4, 5, 6, and 7, respectively) is shown. The position of M_r marker is indicated. *Actin* was amplified as internal positive control. B, Incorrect processing in transcripts from lane 3 of A leading to a deletion comprising intron 1 and partial exons 1 and 2. Introns and exons are marked by lowercase and uppercase letters, respectively. The excised sequence is indicated by the smaller italic letters, while the sequence remaining in the resultant transcript is shown in bold letters. SDRs at deletion junctions are underlined. Notice that one copy of SDRs is excised and the other copy is retained in the resultant transcript. Interrupted lines represent the excised sequence not shown here. C, Structure of incorrectly processed cDNAs derived from *OsBADH1*. Clone numbers are indicated on the right. Numbers on the left correspond to the lane numbers in A. Exons are indicated as boxes, and excised exon materials are shown as folded lines, under which the size is indicated. The positions and sequences of 5′ UTR, exon 1, exon 2, and exon 3 are indicated as white boxes and boxes with slashes, backslashes, and cross-hatching, respectively. Short nucleotide sequences at the 5′ deletion site represent SDRs kept in transcripts, while the nucleotide sequences at the 3′ deletion site are shown as excised SDRs. Scale bar represents 40 nucleotides.

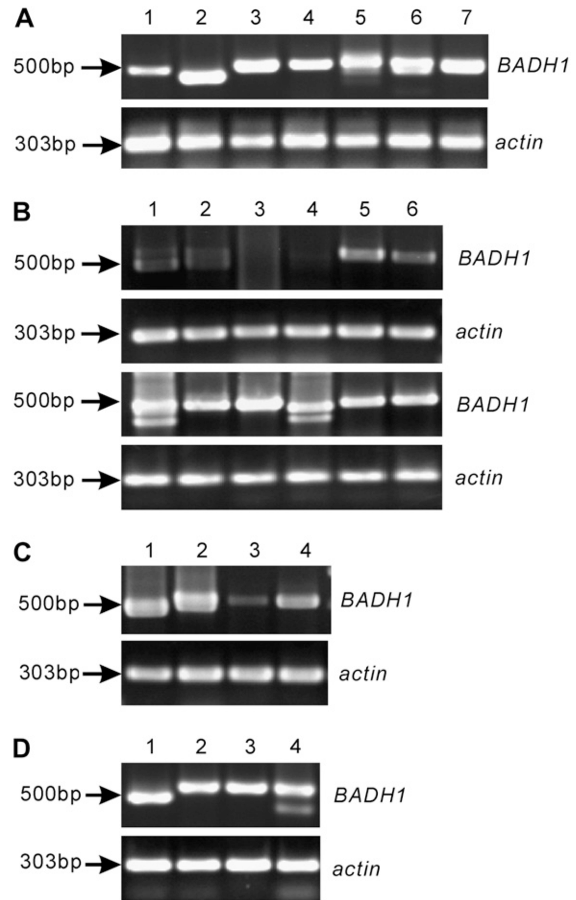

Figure 2. Expression of *OsBADH1* in different rice lines. Agarose gel electrophoresis analysis of RT-PCR amplification products is shown. Positions of M_r markers are indicated. *Actin* was amplified as internal positive control. A, Expressed products derived from seedlings of *indica* cv 93-11 under different growth conditions (lane 1, unstressed; lane 2, drought; lane 3, 0.25 M KCl; lane 4, 0.1 M NaCl; lane 5, 0.2 M NaCl; lane 6, 0.3 M NaCl; lane 7, 0.5 M NaCl). B, Comparison of expression abundance in seedlings between unstressed (0 M NaCl, top) and stressed (0.1 M NaCl, bottom) conditions. Lanes 1 to 6 represent Zhonghua 9, Yuanlixiangjing, Suhui 527, Chuanxiang 29B, Newbonnet, and Tebonnet, respectively. C, Comparison of expression abundance in callus between the unstressed (0 M NaCl) and stressed (0.5 M NaCl) conditions. Lanes 1 and 2 show transcripts derived from the untreated and treated callus of Nipponbare, respectively, and lanes 3 and 4 are indicated as the transcripts derived from untreated and treated callus of 93-11, respectively. D, Expression in mature leaf. Lanes 1 to 4 represent transcripts derived from Nipponbare, 93-11, Della, and Basmati, respectively.

Table II. *Oligonucleotide primers for genes of BADH, ubiquitin, and actin*

Gene (Accession No.)	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Expected Size
			bp
Rice *BADH1* (AK103582)	GCCGCCCCCCAACCGGAAGC	TTCTGTCCGTCCGTTCTG	1,624
	GCCGCCCCCCAACCGGAAGC	GCACCAGCTTCAGTGCCCAG	695
Rice *BADH2* (AK071221)	CAGCTCCAGCTCCTCCTCGA	GGGCGTGTCATGCGTATGCGCA	1,581
	CAGCTCCAGCTCCTCCTCGA	CCAACTACACCGATAGGCTCT	507
Maize *BADH1* (DV031390)	CAGAGTTCAGACCACCGCATC	GTGATAGCAGCAAGCAACTTTACC	1,678
	CAGAGTTCAGACCACCGCATC	AAACAGGCTTAACCATTTGGGC	802
Maize *BADH2* (AY587278)	ACTAGCGTCCGCAACAGCAG	AACTCAACTCTCGCCTTTATCCTGC	1,831
	ACTAGCGTCCGCAACAGCAG	CAAAGCACCCAGCAACATCA	543
Wheat *BADH1* (BJ259181)	CCCATACTCCTACTCACCTCCT	AAGTCCCAGCCAGGATAGAAC	1,670
	CCCATACTCCTACTCACCTCCT	TAGTTCCAGGGAGTGATGAGTCC	563
Wheat *BADH2* (AY050316)	ATGGTCGTCACGGCGAAGATCC	CTCATGCCATTTATTTCGCAAG	1,644
	ATGGTCGTCACGGCGAAGATCC	CCTTTTGTCCAAGGCTTCAG	399
Barley *BADH1* (AB063179)	CGCCCTCATAATACTCCTGC	TGGACGCCTCGTCTCAACTC	1,630
	CGCCCTCATAATACTCCTGC	AGGATAGTTCCAGGGAGTGATG	578
Barley *BADH2* (AB063178)	ACCACCACGCAAGCTCACTC	GAACAGATACAGGTCCTCGAAAC	1,582
	ACCACCACGCAAGCTCACTC	GCTCTAAGCAAGTCACGGAAG	625
Spinach *BADH* (M31480)	ACCAAGAATGGCGTTCCCAA	CTGCATCAACCAACCCTTTATTC	1,634
Arabidopsis *BADH* (AY087395)	CACCACGAATCCACGATCCA	GATTTGAACAGACAGCATTTGGT	1,632
Arabidopsis *BADH* (AF370333)	GAGTATGGCGATTACGGTGC	CTCAGAGCTTGGAAGGAGGT	1,517
Tomato *BADH* (BI935476)	GCATACACTTTGACAAAATA	GCATGTAACGTGCAATTAGT	1,798
Rice *BADH-like* (AK068462)	GGGTTGGCGCCCCAGTCTCCG	CCGATGGGTACATCATAAGAGC	1,879
Rice *actin* (X16280)	AAGATCCTGACGGAGCGTGGTTAC	CTTCCTAATATCCACGTCGCACTTC	303
Maize *ubiquitin* (U29162)	CCACTTGGTGCTGCGTCTTAG	CCTTCTGAATGTTGTAATCCGCA	218
Wheat *actin* (AF326781)	GGTGATGAGGCGCAGTCCAAG	CGACCAGCGAGATCCAAACGA	386
Barley *actin* (AY145451)	CTGGTCGGGATCTCACGGAC	ATCGCTGGGCCAGACTCGTC	565

addition, nine *OsBADH2* cDNAs involved the intermolecular recombination of two different RNA molecules, and the exogenous gene fragments derived either from the putative *acyl-CoA oxidase* or *peptidylprolyl isomerase* (accession nos. XM_476282 and XM_463914, respectively) were incorporated into *OsBADH2* transcripts. These results implicated that the abundance of precisely processed *OsBADH2* transcripts was very low.

Paired Presence of SDRs at Deletion/Insertion Junctions in Rice *BADH* Transcripts

A total of 100 cDNA clones derived from rice *BADH* homologs were sequenced to characterize the sequence structure at deletion or insertion boundaries, and, interestingly, the site selection of deletion/insertion was altered in association with the change of stress conditions (Tables I and III; Figs. 1–3). Moreover, the sequence comparison unraveled extensive sequence similarities between the surrounding sequences at the 5′ and 3′ truncating and/or recombination sites in individual events (Figs. 1, B and C, and 3B). As shown in Figure 1B, deletion occurring in the transcripts in lane 3 of Figure 1A resulted in the excision of the first intron and 216-nucleotide exon materials. In this case, the 5′ and 3′ truncating sites involved exon 1 and exon 2, respectively, and the sequence of GGCCG at the 3′ end was identical to that of the immediate upstream 5′ end of the deleted sequence. We denominated the identical sequences as SDRs. Diagrams described in Figure 1C demonstrated that seven types of different SDRs (CG, GGCCGCG, GGCCG, GCC, CGCGC, GGCC, and GGAG) were identified in the deletion events from the *OsBADH1* gene expressed in *japonica* rice cv Nipponbare. Actually, the length of almost all the SDRs can be extended to larger, nearly identical sequences (Table I). For example, two copies of dinucleotide SDR of CG that are separately located at 5′ and 3′ deletion sites, respectively, can be extended to larger, nearly identical sequences of GCCGtCGgCG and GCCGCGaCG (in which the lowercase letters indicate the differences). Similarly, two copies of trinucleotide SDRs of GCC at 5′ and 3′ deletion sites can be replaced by larger, nearly identical sequences of GCCaTGG and GCCTGG, respectively. It is noteworthy that each type of SDR was somewhat unique depending on the position of the truncating sites and contained GC-rich nucleotides. In many cases, only two copies of individual SDRs were present in a proximal genomic region, and each copy located separately at the 5′ or 3′ truncating boundaries. During posttranscriptional processing, one copy of the SDR was excised and the other one retained in the resultant transcripts. Nevertheless, in the case of multiple copies of the SDRs that occurred in pre-mRNA, only two of the copies were recruited by individual deletion events. For instance, four copies of GGCC were present at the seven, 34, and 99 bases of exon 1 and 116 bases of exon 2, respectively, and arranged three combinations of truncating-site choices, in which the copy GGCC at seven bases of exon 1 was most commonly selected for the 5′ excision site, while one of the other three copies was alternatively used for

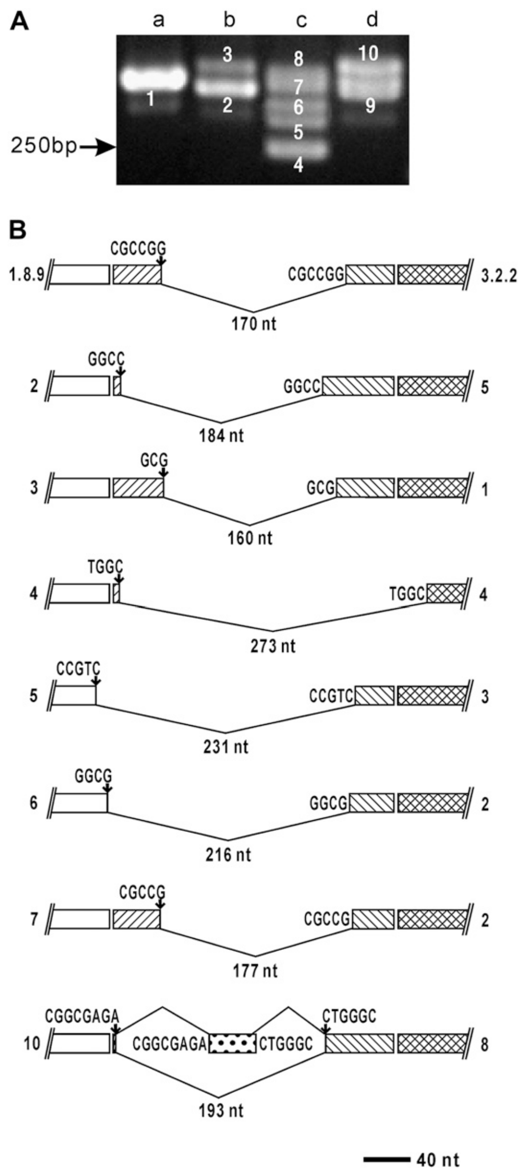

Figure 3. Expression and structure of *OsBADH2* RNAs derived from seedlings of Nipponbare. A, Agarose gel electrophoresis of RT-PCR amplification products. Product fragments are marked by numbers. Position of M_r marker is indicated. Lanes (a–d) are shown as transcripts under four different growth conditions (unstressed, drought, 0.25 M KCl, and 0.5 M NaCl, respectively). B, Structure of incorrectly processed cDNAs derived from *OsBADH2*. Clone numbers are indicated on the right. Numbers on the left correspond to the fragment numbers in A. Exons are indicated as boxes, and excised exon materials are shown as folded lines, under which the size is indicated. The positions and sequences of 5′ UTR, exon 1, exon 2, and exon 3 are indicated as white boxes and boxes with slashes, backslashes, and cross-hatching, respectively. Short nucleotide sequences at the 5′ deletion site represent SDRs kept in transcripts, while the nucleotide sequences at the 3′ deletion site are shown as excised SDRs. Exogenous gene fragment other than *OsBADH2* is indicated as a stippled box. Scale bar represents 40 nucleotides.

the downstream selection of the 3′ excision site (Table I). Moreover, as indicated in the last cDNA clone in Figure 1C, two independent deletion events were found within a single cDNA clone, and one excision event took place within a single exon, suggesting that the intron might not be required during the processing.

Similarly, as shown in Figure 3B, nine types of SDRs (CGCCGG, GGCC, GCG, TGGC, CCGTC, GGCG, CGCCG, CGGCGAGA, and CTGGGC) were found in transcripts of *OsBADH2* induced in *japonica* rice cv Nipponbare under different stress conditions. These types of SDRs can be largely substituted by longer, nearly identical sequences (Table III). Moreover, in the last *OsBADH2* cDNA shown in Figure 3B, the excised sequence was replaced by a 40-bp exogenous gene sequence of *acyl-CoA oxidase* (accession no. XM_476282). More intriguingly, two types of SDRs (CGGCGAGA and CTGGGC, respectively) were present at the junctions, but in this case only one copy of SDR derived from *OsBADH2* and another copy from the *acyl-CoA oxidase*. A similar phenomenon was observed from another exogenous gene fragment (Table III). Based on the comparison of sequence composition in SDRs, we found a commonly used SDR (GGCC) by both of the rice *BADH* homologs for the selection of excision sites. A total of 14 and 23 SDRs were identified in *OsBADH1* and *OsBADH2* for their deletion events, respectively, and their positions in the genomic sequences are shown in Figure 4, A and B. For *OsBADH1*, SDRs at the 5′ deletion site were mainly distributed in a 22-bp region from −3 to 19 bases of exon 1, and SDRs at the 3′ excision site distributed in a relatively large region from 66 to 109 bases of exon 2. For *OsBADH2*, SDRs at the 5′ excision site spanned a region from −17 to 54 bases of exon 1, whereas SDRs at the 3′ excision site primarily covered a 38-bp region from 78 to 115 bases of exon 2.

The Conservation of an Unusual Posttranscriptional Processing Pattern in *BADH* Homologs in Cereal Crop Species

To determine whether the unusual events occurring in the *BADH* transcripts were specific only to the rice genome, we carried out RT-PCR experiments using the total RNA extracted from seedlings of other cereal crop species, i.e. maize, wheat, and barley (Fig. 5, A–C). These experiments used primers either to amplify the full length of mRNA or exclusively the 5′ region of *BADH* homologs corresponding to those in rice (Table II). The sequencing data from a total of 52 cDNA clones (four clones for wheat *BADH1*, 22 for wheat *BADH2*, six for maize *BADH1*, nine for maize *BADH2*, six for barley *BADH1*, and five for barley *BADH2*) demonstrated that all the tested cDNA clones had deletion(s) of the 5′ exonic sequences resulting from the unusual posttranscriptional processing. SDRs and/or their extended, nearly identical sequences at the truncating junctions were identified with no exception for every sequence deletion event (Table IV). Multiple

Table III. *The incorrect processing of OsBADH2 transcripts in rice seedling and mature leaf*

Line	Tissue and Growth Condition	No. of Clones	Position and Size of Deletion	SDR	Nearly Identical Sequences
Nipponbare	Seedling, unstressed	3	e1. 42-e2. 102 (170)[a]	CGCCGG	CGtCGCCGGCG/CGCGCCGGgCG
Nipponbare	Seedling, drought	5	e1. 7-e2. 81 (184)	GGCC	GGCCaCG/GGCCgCG
Nipponbare	Seedling, drought	1	e1. 44-e2. 94 (160 bp)	GCG	GGCGaG/GcGCGcG
Nipponbare	Seedling, 0.25 M KCl, 24 h	4	e1. 6-e3. 26 (273 bp)	TGGC	GAGaTGGC/GAGcTGGC
Nipponbare	Seedling, 0.25 M KCl, 24 h	3	5′ UTR −12-e2. 110 (231)	CCGTC	CCGTCgCG/CCGTCCG
Nipponbare	Seedling, 0.25 M KCl, 24 h	2	5′ UTR −2-e2. 105 (216)	GGCG	CGGCG/CgGGCG
Nipponbare	Seedling, 0.25 M KCl, 24 h	2	e1. 41-e2. 108 (177)	CGCCG	GtCGCCGgC/GCGCCGtC
Nipponbare	Seedling, 0.25 M KCl, 24 h	2	e1. 42-e2. 102 (170)	CGCCGG	CGtCGCCGGCG/CGCGCCGGgCG
Nipponbare	Seedling, 0.5 M NaCl, 24 h	2	e1. 42-e2. 102 (170)	CGCCGG	CGtCGCCGGCG/CGCGCCGGgCG
Nipponbare	Seedling, 0.5 M NaCl, 24 h	8	e1. 2-e2. 85 (193)[b] 40-bp insertion from putative *acyl-CoA oxidase*	CGGCGAGA CTGGGC	CGGCGAGAtGGCCAC/ CGGCGAGAgGGCCAC GaCTGGGCG/GcCTGGGCtG
93-11	Seedling, unstressed	3	e1. 53-e2. 55 (112)	GGCGCG	GtGGCGCG/GcGGCGCG
93-11	Seedling, 0.1 M NaCl, 72 h	1	e1. 55-e2. 123 (178)	CC	CCcCCGCGC/CCtCCGCGC
		2	e2 (143 bp)[c]		
Minghui 63	Seedling, unstressed	1	e1. 42-e2. 102 (170)	CGCCGG	CGtCGCCGGCG/CGCGCCGGgCG
Minghui 63	Seedling, 0.1 M NaCl, 72 h	1	e1. 42-e2. 102 (170)	CGCCGG	CGtCGCCGGCG/CGCGCCGGgCG
Suhui 527	Seedling, unstressed	1	e1. 21-e2. 96 (185)[d] 59-bp insertion from putative *peptidylprolyl isomerase*	GCA C	GCAgCGGCAG/GCAcCGGCgAG CgCCGgG/CaCCGcG
Suhui 527	Seedling, 0.1 M NaCl, 72 h	1	5′ UTR −11-e2. 112 (232)	CG	CCGTCG/CCGTcCG
Chuanxiang 29B	Seedling, unstressed	1	e1. 41-e2. 108 (177)	CGCCG	GtCGCCGgC/GCGCCGtC
Chuanxiang 29B	Seedling, 0.1 M NaCl, 72 h	1	e1. 23-e5. 63 (523)	CAGC	CAGCgG/CAGCtG
Zhonghua 9	Seedling, unstressed	1	e1. 21-e2. 131 (220)	CGCA	TCCCGCA/TCCgCGCA
Zhonghua 9	Seedling, 0.1 M NaCl, 72 h	1	5′ UTR −2-e2. 101 (212)	CG	CGgCGaG/CGcCGgG
Yuanlixiangjing	Seedling, unstressed	1	e1. 52-e2. 106 (164)	GGCGC	GtGGCGCG/GGGCGCcG
Yuanlixiangjing	Seedling, 0.1 M NaCl, 72 h	1	5′ UTR −8-e2. 85 (202)	CGCGA	GtCGCGAtC/GcCGCGAC
Leab	Seedling, unstressed	1	e1. 55-e2. 107 (162)	GCGCC	GGcGCGCC/GGGCGCC
Leab	Seedling, 0.1 M NaCl, 72 h	1	5′ UTR −8-e2. 85 (202)	CGCGA	GtCGCGAtC/GcCGCGAC
Newbonnet	Seedling, unstressed	1	5′ UTR −10-e2. 106 (225)	CGC	GtCGCG/GCGCcG
Newbonnet	Seedling, 0.1 M NaCl, 72 h	1	e1. 18-e2. 140 (232)	CC	
Tebonnet	Seedling, unstressed	1	e1. 24-e2. 98 (184)	GCG	CGCaGCG/CGCGCG
Tebonnet	Seedling, 0.1 M NaCl, 72 h	1	e1. 24-e2. 84 (170)	GCG	GCaGCGgC/GCcGCGaC
Nipponbare	Mature leaf, field	2	5′ UTR −3-e2. 115 (227)	GGC	CGGCgA/CgGGCcA
93-11	Mature leaf, field	1	5′ UTR −10-e2. 130 (249)	CGC	CCGtCGCgATCG/CCGCGCaATCG
Chuanxiang 29B	Mature leaf, field	2	5′ UTR −3-e2. 99 (211)	GC	CGGCG/CGcGCcG

[a]e1. 42-e2. 102 (170) denotes that exon materials of *OsBADH2* from the 42nd base of exon 1 to the 102nd of exon 2 were excised; 170 nucleotides in total were deleted. [b]In these cDNAs, the deletions were replaced by a fragment derived from putative *acyl-CoA oxidase* (accession no. XM_476282). [c]Selective deletion of entire exon 2. [d]In this cDNA, the deletion was replaced by a fragment derived from putative *peptidylprolyl isomerase* (accession no. XM_463914).

deletion events were also observed within a single cDNA clone. These results suggested a highly conserved posttranscriptional processing pattern in *BADH* homologs that distinctly evolved in cereal crop species.

To compare the posttranscriptional processing patterns of the *BADH* homologs between cereal crop species and more distantly related dicotyledonous species, we conducted RT-PCR experiments using total RNA extracted from seedlings of spinach, Arabidopsis, and tomato. Primers designed to amplify the full length of mRNA of *BADH* homologs were used (Table II). As anticipated, the RT-PCR products of *BADH* homologs from spinach (accession no. M31480), Arabidopsis (accession nos. AY087395 and AF370333), and tomato (accession no. BI935476) were of expected size for correctly processed transcripts. Sequencing analysis of 11 cDNA clones (four from spinach, four from Arabidopsis, and three from tomato) confirmed the correct processing. We also sequenced two cDNA clones derived from a distantly related *BADH*-like gene in rice (accession no. AK068462), and no aberrant transcripts were observed.

Based on the sequence comparison of the 5′ exonic region including exons 1 and 2, we did not find, in the distantly related *BADH* homologs, such extensive sequence similarity (SDRs) with paired presence at the deletion/insertion junctions in the tested cereal crop species. This consistently suggests that SDRs may be required for the recognition of deletion/insertion sites.

DISCUSSION

Defective *BADH* Transcripts Resulted from Unusual Posttranscriptional Processing in Cereal Crop Species

Extensive sequence analysis from this study revealed that a number of aberrant *BADH* transcripts

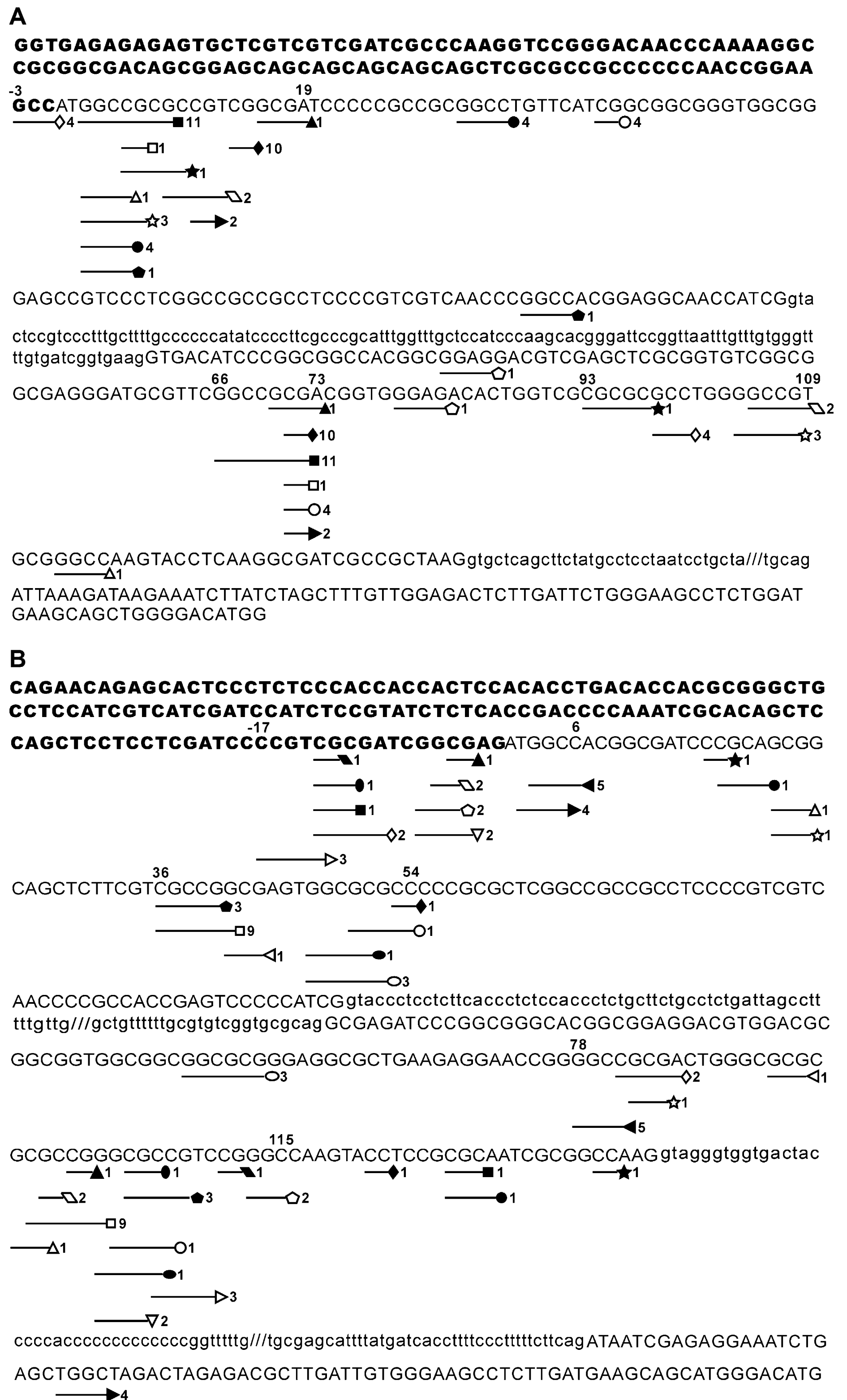

Figure 4. Distribution of SDRs in genomic sequences of rice *BADH* homologs. Exons are indicated as uppercase letters, and intron sequences are indicated as lowercase letters. 5′ UTRs are shown as bold letters. Positions of exon sequences are indicated by numbers. SDRs are underlined. Symbols following underlines indicate specificity and position of SDRs. Clone numbers for individual events are indicated on the right. Notice that those sequences between individual paired SDRs will be excised during the unusual posttranscriptional processing. Distribution of SDRs in *OsBADH1* (A) and *OsBADH2* (B) are shown. Those intron sequences not shown are indicated by interrupted lines.

were derived from an unusual posttranscriptional processing in cereal crop species. The observed events took place at the 5′ exonic region, leading to the removal of translation initiation codon, deletion of functional domain, insertion of exogenous gene fragments, and frame-shifts with premature termination by introducing stop codon. We found that the majority of the incorrectly processed *BADH* transcripts were prematurely terminated by incorporation of stop codon. These transcripts are apparent targets of

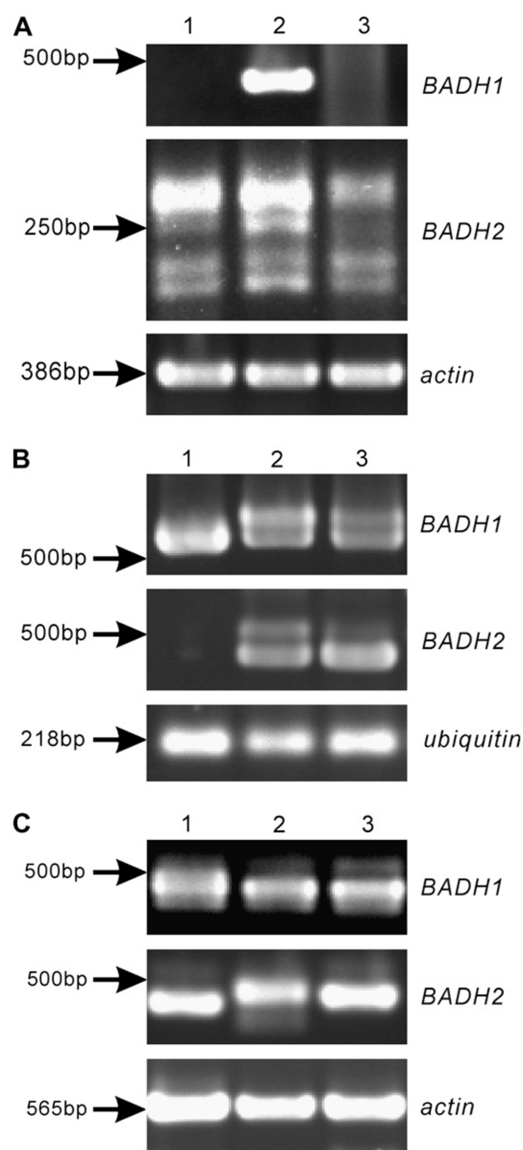

Figure 5. Expression of *BADH* homologs in related cereal crop species. The position of the M_r marker is indicated. *Actin* or *ubiquitin* was amplified as internal positive control. Lanes 1 to 3 represent three different growth conditions (unstressed, 0.1, and 0.5 M NaCl, respectively). Agarose gel electrophoresis analysis of RT-PCR amplification products derived from wheat (A), maize (B), and barley (C) *BADH* homologs are shown.

nonsense-mediated mRNA decay, a surveillance mechanism that selectively degrades nonsense mRNAs (Stamm et al., 2005). This finding suggested that very few functional *BADH* transcripts were made during RNA processing. The phenomenon of abnormally processed transcripts in plant genes has been described previously in maize and wheat (Burr et al., 1996; McKibbin et al., 2002). Most of the transcripts in maize *In1* transcription-factor gene were spliced incorrectly, and missplicing occurred before the helix-loop-helix DNA-binding domain, indicating that truncated protein would not contain specific DNA-binding activity (Burr et al., 1996). Extensive missplicing of transcripts has also been observed for wheat *Vp-1* transcription factor gene. Analysis of *Vp-1* transcript structure showed many combinations of intron insertion/exon deletions, resulting in termination of the ORF before the B3 domain (McKibbin et al., 2002). The highly conserved B3 domain has been shown in maize to bind DNA in a sequence-specific, highly cooperative manner, activating transcription through the Sph cis-element present in the *C1* promoter (Suzuki et al., 1997).

Our analysis of the cDNA sequence structure from various *BADH* genes revealed that numerous deletion events came about within about 200 bp in the 5′ exonic region that encodes a part of the putative NAD^+-binding domain (Incharoensakdi et al., 2000). A variety of partial deletions involving exons 1 and 2 directly led to the termination of ORF before the catalytic domain. A substantial portion of the transcripts had deletions without the original translation initiation codon. These transcripts still contain a shorter ORF with the catalytic and oligomerization domains (Incharoensakdi et al., 2000). Consequently, from all the *BADH* cDNA clones sequenced in this study, no single cDNA clone was found to be processed correctly. However, this type of processing event may not be well represented in the RT-PCR products, as described in the missplicing of *Vp-1* gene (McKibbin et al., 2002). Indeed, correctly spliced *BADH* mRNAs are found in published cDNA sequences in rice (accession nos. AK103582 and AK071221) as well as other cereal crop species (accession nos. AY587278, AY050316, AB063179, and AB063178). As a result, a residual BADH protein activity likely exists in these cereal crops, even though the precise mRNAs were decreased considerably by the unusual posttranscriptional processing.

Nevertheless, GB level accumulated in plants could be altered by any limiting factor in the complex biosynthetic pathway. Either BADH or CMO protein activity is crucial for the ultimate GB-synthesizing ability. Indeed, rice is the only cereal crop that does not accumulate GB (Rathinasabapathi et al., 1993). Although the rice genome contains both *CMO* and *BADH* homologs required for GB synthesis, their activities of coding proteins have not yet been characterized experimentally. In addition to the extensively defective RNA processing of rice *BADH* homologs found in this study, recent transgenic experiments suggest a functional defect in both *CMO* and *BADH* homologs in rice. Kishitani et al. (2000) reported the transgenic rice plants constitutively expressing a precise barley BADH1 converted high levels of exogenously applied betaine aldehyde to GB more efficiently than did wild-type plants. The lower conversion efficiency in the wild-type plants probably results from the limitation of precise native BADH proteins found in this study. More recently, Shirasawa et al. (2006) demonstrated transgenic rice plants harboring a single

Table IV. *The incorrectly processed transcripts of BADH homologs in seedlings of wheat cv Chuanmai 43, maize cv 095, and barley cv Chuannongda 3*

Gene	Growth Condition	No. of Clones	Position and Size of Deletion	SDR	Nearly Identical Sequences
Wheat *BADH1*	0.1 M NaCl, 72 h	4	5′ UTR −13-CDS No. 157 (170)[a]	CTCGCCG	AcCTCGCCG/AgCTCGCCG
Wheat *BADH2*	Unstressed	4	CDS Nos. 44–250 (207)	ATCG	CATCGaCG/CgATCGcCG
Wheat *BADH2*	Unstressed	1	CDS Nos. 55–204 (150)	GCGACTGG	CgGCGACTGGcGC/CGCGACTGGGC
Wheat *BADH2*	Unstressed	2	CDS Nos. 49–176 (128)	CGGC	GaCGGC/GCGGC
Wheat *BADH2*	0.1 M NaCl, 72 h	3	CDS Nos. 44–250 (207)	ATCG	CATCGaCG/CgATCGcCG
Wheat *BADH2*	0.1 M NaCl, 72 h	1	CDS Nos. 30–253 (224)	CG	GCGgCA/GcCGcCA
Wheat *BADH2*	0.1 M NaCl, 72 h	3	CDS Nos. 55–204 (150)	GCGACTGG	CgGCGACTGGcGC/CGCGACTGGGC
Wheat *BADH2*	0.1 M NaCl, 72 h	1	CDS Nos. 49–176 (128)	CGGC	GaCGGC/GCGGC
Wheat *BADH2*	0.5 M NaCl, 24 h	3	CDS Nos. 44–250 (207)	ATCG	CATCGaCG/CgATCGcCG
Wheat *BADH2*	0.5 M NaCl, 24 h	1	CDS Nos. 30–253 (224)	CG	GCGgCA/GcCGcCA
Wheat *BADH2*	0.5 M NaCl, 24 h	1	CDS Nos. 55–204 (150)	GCGACTGG	CgGCGACTGGcGC/CGCGACTGGGC
Wheat *BADH2*	0.5 M NaCl, 24 h	1	CDS Nos. 49–176 (128)	CGGC	GaCGGC/GCGGC
Wheat *BADH2*	0.5 M NaCl, 24 h	1	CDS Nos. 50–174 (125)	GGCG	CGGCGaC/CgGGCGgC
Maize *BADH1*	Unstressed	1	CDS Nos. 22–249 (228)	CGATC	CGATCcCC/CGATCgCC
Maize *BADH1*	0.1 M NaCl, 72 h	1	CDS Nos. 22–249 (228)	CGATC	CGATCcCC/CGATCgCC
Maize *BADH1*	0.1 M NaCl, 72 h	1	CDS Nos. 49–244 (196)	CGGG	CGGGG/CGGGcG
Maize *BADH1*	0.5 M NaCl, 24 h	1	5′ UTR −1-CDS No. 252 (252)	ATCGCC	CGcATCGCC/CGATCGCC
Maize *BADH1*	0.5 M NaCl, 24 h	1	CDS Nos. 47–246 (200)	GGCG	
Maize *BADH1*	0.5 M NaCl, 24 h	1	CDS Nos. 31–75 (45)	GGCGC	GGCGCgGCCT/GGCGCcGCCT
			CDS Nos. 120–247 (128)	GGCGA	CGGCGAC/CgGGCGAtC
Maize *BADH2*	0.1 M NaCl, 72 h	5	CDS Nos. 117–259 (143)	GG	GGcGA/GGtGA
Maize *BADH2*	0.1 M NaCl, 72 h	1	CDS Nos. 50–168 (119)	CGGCG	GtCGaCGGCG/GCGgCGGCG
Maize *BADH2*	0.5 M NaCl, 24 h	3	CDS Nos. 117–259 (143)	GG	GGcGA/GGtGA
Barley *BADH1*	Unstressed	1	5′ UTR −24-CDS No. 210 (234)	GCGC	GGCGCC/GcGCGCgC
Barley *BADH1*	Unstressed	1	5′ UTR −24-CDS No. 208 (232)	GGCGC	GGCGCC/GGCGCgC
Barley *BADH1*	Unstressed	2	5′ UTR −5-CDS No. 197 (202)	GAG	
Barley *BADH1*	0.1 M NaCl, 72 h	1	5′ UTR −24-CDS No. 208 (232)	GGCGC	GGCGCC/GGCGCgC
Barley *BADH1*	0.5 M NaCl, 24 h	1	5′ UTR –24-CDS No. 208 (232)	GGCGC	GGCGCC/GGCGCgC
Barley *BADH2*	Unstressed	2	5′ UTR −7-CDS No. 178 (184)	CCGCCC	CCGCCCgC/CCGCCCtC
Barley *BADH2*	0.1 M NaCl, 72 h	2	CDS Nos. 64–214 (151)	CGCGCCC	CgCGCGCCCgCG/CCGCGCCCcCG
Barley *BADH2*	0.5 M NaCl, 24 h	1	CDS Nos. 58–221 (164)	GGCGC	GGCGCgCG/GGCGCCG

[a]5′ UTR −13-CDS No. 157 (170) means the exon materials from the −13 of 5′ UTR to the 157th base of coding sequence were excised; 170 nucleotides in total were deleted. CDS, Coding sequence.

copy of expressed spinach CMO accumulated detectable GB and had enhanced tolerance to salt stress and temperature stress, strongly suggesting a severe deficit of native CMO protein activity in rice. Because CMO alone only converts choline into betaine aldehyde, these transgenic plants still need native functional BADH proteins for conversion of betaine aldehyde into GB, suggesting an active BADH protein present in rice. In addition, analyses of other transgenic studies have shown that stress tolerance levels as measured by increased GB accumulation could be manipulated via expression of the *BADH* orthologs (Sakamoto et al., 1998; Takabe et al., 1998), implying that the functional transgene is capable of complementing the functional defect in rice *BADH* genes. It is noteworthy that rice may take other defense strategies instead of accumulating GB against osmotic stresses. Ren et al. (2005) reported recently a major quantitative trait locus encoding a sodium transporter confers salt tolerance in rice.

By contrast, other cereal crops have been known to synthesize GB and tolerate various degrees of osmotic stresses. Their precisely spliced *BADH* mRNAs exist in vivo, as evidenced by the published cDNA sequences, and, particularly, the activity of barley BADH protein has been demonstrated in the transgenic experiment (Kishitani et al., 2000). Obviously, barley plants synthesize GB through catalytic reaction of the functional BADH protein, even though a large number of incorrectly processed *BADH* transcripts observed in this study may considerably reduce the precise gene products.

Interestingly, tremendous variation in GB accumulation among dicotyledonous species exists. Spinach has been documented to accumulate far more GB than do cereal crops in response to osmotic stresses (Hanson and Scott, 1980; Pan et al., 1981). We may associate these features with the demonstrated enzyme activities of spinach CMO and BADH proteins that have been well documented in a number of biochemical and genetic engineering experiments (Weretilnyk and Hanson, 1989; Rathinasabapathi et al., 1994; Burnet et al., 1995; Shirasawa et al., 2006). We did not find any abnormal events in splicing of spinach *BADH* homolog after intensive studies. However, the case in Arabidopsis is completely different. Arabidopsis contains one *CMO-like* (accession no. NM_119135) and two *BADH* homologs (accession nos. AY087395 and

AF370333) but does not accumulate this compound of GB (Sakamoto and Murata, 2002). Although our study showed the existence of potential BADH function from correct splicing, another experiment demonstrated that the protein coding for *CMO-like* gene was absent in Arabidopsis plants and there was no enzyme activity in the CMO-like protein expressed in *Escherichia coli* (Hibino et al., 2002). This is sufficient to abrogate the biosynthetic ability of GB in Arabidopsis.

Because no abnormal *BADH* transcripts were detected in the studied dicotyledonous species, it appears that the RNA processing pattern of *BADH* homologs was considerably differentiated between monocotyledons and dicotyledons. But why maintain over such a long evolutionary span a stress-responsive gene and then reduce its activity by incorrect processing in monocotyledons? One possible reason is the differential tolerance to the deleterious effect of the accumulated GB among different plant species. The toxicity was indicated by a destabilizing effect of exogenous supplementation of GB in plant tissues during viability tests of membrane stability (Gibon et al., 1997). Cereal crops may suffer from overaccumulation of GB, and the appropriate level of GB accumulation may be regulated at both the transcriptional and posttranscriptional levels; namely, the transcription is induced abundantly in response to the osmotic stresses, while the proper amount of precise gene products is balanced by posttranscriptional processing. In this view, keeping the appropriate active protein level by the unusual posttranscriptional processing is subject to a favorable evolutionary selection. However, more detailed studies need to be conducted to confirm this hypothesis.

The Selection of Deletion/Insertion Sites Probably Regulated by SDRs

Posttranscriptional splicing accomplishes the excision of introns and the joining of exons into the mature sequences of RNA. Introns are removed from the nuclear pre-mRNA of higher eukaryotes by a system that recognizes only short consensus sequences at exon-intron boundaries and within the intron. In the classic U2-type of spliceosome-mediated splicing, the consensus sequences of the 5′ and 3′ splices sites, AG/GTAAGT and TGCAG/G (the slash denotes the exon-intron boundary), are highly conserved in higher plants and are similar to those in vertebrates (Liu and Filipowicz, 1996; Lorkovic et al., 2000). In this way, intron starts with dinucleotide GT and ends with the dinucleotide AG. Intron border sequences are required for the selection of splice sites by splice machinery. A minor class of nuclear pre-mRNA introns, referred to as U12-type, has recently been described (Tam and Steitz, 1997; Russell et al., 2006). U12-type introns frequently start with AT and terminate with AC. Moreover, primary transcripts of many genes are alternatively spliced by selection of different splice sites, producing different mRNA forms that encode proteins with functional differences (Lorkovic et al., 2000). In plants, the biological relevance of alternative exon inclusion and retention of an unspliced intron regulated by SR (Ser/Arg-rich) proteins has been documented (Isshiki et al., 2006).

In this study, the posttranscriptional processing pattern found in *BADH* homologs in the cereal crops does not conform to either the major GT-AG or the minor AT-AC roles. From a wide variety of unusual deletion/insertion events involving exon materials, we were not able to identify such short consensus at the ends of deleted or inserted sequences. Instead, we did find extensive sequence similarities surrounding the deletion/insertion junctions, at which the putative reactions of excision and joining during the processing were likely directed by the identical sequences called SDRs. SDRs are characterized by a short size (2–7 GC-rich nucleotides), paired recruitment, and direct orientation. More careful inspection of sequence data revealed that di- or trinucleotide SDRs could be extended to relatively larger, nearly identical sequences that might be required for the recognition as signature sequences (Tables I, III, and IV). For each unusual processing event, there are two copies of the same SDRs present in the pre-mRNA, and only one is always excised precisely. There are several sorts of deletion/insertion events that have consistently shown the selection of the deletion/insertion sites relies specifically on the presence and positioning of SDRs in pre-mRNA, although we cannot completely rule out other possibilities. First, the majority of deletion events involve two different exons that are composed of the same SDRs. This type of deletion also occurred in a published maize *BADH1* cDNA (accession no. DW475114) with two copies of the sequence CGCC located separately at exon 1 and exon 2, respectively. Second, deletion detected within exons is probably the result of the presence of two copies of SDRs in the same exon,

Table V. *Plant materials used for RNA isolation*

Species	Variety/Lines
Rice subsp. *japonica*	Nipponbare
Rice subsp. *japonica*	Zhonghua 9
Rice subsp. *japonica*	Yuanlixiangjing
Rice subsp. *japonica*	Newbonnet
Rice subsp. *japonica*	Tebonnet
Rice subsp. *japonica*	Leab
Rice subsp. *japonica*	Della
Rice subsp. *indica*	93-11
Rice subsp. *indica*	Minghui 63
Rice subsp. *indica*	Suhui 527
Rice subsp. *indica*	Chuanxiang 29B
Rice subsp. *indica*	Basmati
Maize	095
Wheat	Chuanmai 43
Barley	Chuannongda 3
Spinach	Dayuanye
Spinach	Chunqiudayebo
Tomato	Ailsa Craig
Arabidopsis	Columbia

suggesting that the presence of an intron is not required. Third, in the case of multiple copies of an SDR present in the proximity of the genomic sequence, only two of them were mutually recruited in a manner that is quite similar to alternative splicing for the selection of splice site (Black, 2003). Fourth, in the form of intermolecular recombination (exogenous sequence insertion) identified in this experiment, two copies of the SDRs are of paired presence between different RNA molecules. Probably due to the sequence differentiation and absence of paired SDRs or nearly identical sequences, we did not observe any abnormal processing event in *BADH* homologs from spinach, tomato, and Arabidopsis, and in a distantly related *BADH*-like gene from rice. This implicates a sequence-dependent manner for the determination of the processing patterns.

However, the special mechanism that enforces the exclusive choice of deletion/insertion sites through recognition of SDRs is elusive. Because the proportion of unusual transcripts derived from *BADH* genes is so great, the event seems unlikely a missplicing that usually fails to recognize exon-intron junction by spliceosome. Instead, an unusual alternative splicing may be responsible for the high incidence of unusual *BADH* transcripts. This investigation indicates that the nature of the putatively SDR-directed deletion/insertion is similar to the intron splicing in conventional RNA processing, both seemingly involving reactions of excision and rejoining of RNA sequences. Similar identical trinucleotide AGG exists at the U2-type spliceosome-mediated splicing junctions (AG/GTAAGT and TGCAG/G at 5′ and 3′ splice sites, respectively) in the same manner of SDRs found in this experiment (Lorkovic et al., 2000). Hence, we argue that the conventional spliceosome or at least some components from it might be involved in the unusual, SDR-mediated posttranscriptional RNA processing. In fact, many components of the splicing machinery appear to be shared by both U2- and U12-type spliceosomes, even though distinct differences (GT-AG versus AT-AC) in signature sequences at U2- and U12-type intron borders exist (Tam and Steitz, 1997; Russell et al., 2006). Possibly, these components from conventional spliceosome could also be shared by the unusual alternative splicing. Additionally, SR proteins and other splicing factors and/or hnRNP proteins found in rice and other organisms play an important role in regulating splice-site selection by binding to sequence elements (enhancers or silencers) present in exons and introns (Lorkovic et al., 2000; Isshiki et al., 2006). Cereal crop genomes probably encode a special type of splicing factors to interact with the sequence elements of SDRs.

Alternatively, the unusual events result from intermolecular and/or intramolecular recombination occurring in the posttranscriptional processing mediated by SDRs without participation of splice machinery. This probably will need additional protein factors other than spliceosome to bring together the sequence elements of SDRs that are not complementary and separately located at putatively 5′ and 3′ recombination sites and to allow the reaction of recombination. Actually, a model of intramolecular recombination mediated by SDRs has been reported in the wheat chloroplast genome (Ogihara et al., 1988). This form of intramolecular recombination mediated by SDRs has also been observed within the ORF of rice chloroplast gene (Kanno et al., 1993). It seems reasonable to propose that numerous unusual *BADH* transcripts were derived from this form of recombination mediated by SDRs.

Interestingly, stressed conditions induced several intermolecular recombinant transcripts derived from different RNA molecules (Table III). Analogous examples of trans-splicing in which separate precursor transcripts contribute sequences to the mature mRNA through intermolecular reactions have been identified in plant chloroplasts and mitochondria (Wissinger et al., 1991). In this type of trans-splicing, discrete pre-mRNA molecules that are independently transcribed have sequences with features characteristic of group II introns. Also, the intermolecular recombination was documented in a rice *calcium-dependent protein kinase* gene encoded in nuclei (Kawasaki et al., 1999). In this case, the splicing process does not involve consensus splicing signals. Obviously, the currently observed unusual intermolecular recombination is different from above trans-splicing events. This process is possibly mediated by SDRs of paired presence with a new kind of mechanism. We should also consider the possibility that intermolecular recombination between two molecules from the same locus may have occurred.

MATERIALS AND METHODS

Plant Materials

Varieties or lines from seven plant species, rice (*Oryza sativa*), maize (*Zea mays*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), spinach (*Spinacia oleracea*), tomato (*Solanum lycopersicum*), and Arabidopsis (*Arabidopsis thaliana*), used in this study are listed in Table V. Seeds of each variety or line were surface sterilized with 1% (v/v) sodium hypochlorite and germinated in a growth chamber (25°C, 14-h/10-h photoperiod at 200 μmol photons m^{-2} s^{-1}). Ten days after germination, seedlings were subjected to different stresses of salt (0–0.5 M NaCl or 0.25 M KCl) or drought (by withdrawing water for 24 h) treatments. Seedling leaves were collected for RNA extraction and analysis. Mature rice leaves were obtained for analysis from plants grown in an experimental field at the Sichuan University. Rice callus was induced in the medium as described (Hiei, et al., 1994).

RNA Isolation and RT-PCR

Total RNAs were extracted by using Trizol reagent following the protocol provided by the manufacturer (Invitrogen). First-strand cDNA was synthesized by using the First Strand cDNA Synthesis kit (Toyobo). The oligonucleotide primers used for amplifying genes of *BADH*, *actin*, *ubiquitin*, and rice *BADH-like* are summarized in Table II. PCR was carried out by using a Taq DNA Polymerase (Takara) in MJ MiniPCR (Bio-Rad) following the instruction given by the manufacturer. *Actin* and *ubiquitin* genes were employed as positive internal controls (Quaggiotti et al., 2003; Liu et al., 2005).

Sequencing and Data Analysis

RT-PCR products containing multiple bands were separated by 2% agarose gel and purified using the QIAquick Gel-Extraction kit (Qiagen). The isolated

fragments were cloned into a pMD18-T vector (Takara). Cycle sequencing was performed with the ABI Prism BigDye Terminators v2.0 cycle sequencing reaction kit (Applied Biosystems). Sequences were determined with an ABI Prism 377 genetic analyzer (Applied Biosystems) and edited with the computer program BioEdit v4.7.8 (Hall, 1999). The sequence alignments were performed using the software ClustalX v1.83 (Thompson et al., 1997), and DNAMEN v5.0 (Lynnon Biosoft) was used to translate DNA sequences into protein sequences to aid the analysis of RNA processing events.

Sequence data from this study can be found in the GenBank data libraries under accession numbers AK103582 (*OsBADH1*), AK071221 (*OsBADH2*), DV031390 (maize *BADH1*), AY587278 (maize *BADH2*), BJ259181 (wheat *BADH1*), AY050316 (wheat *BADH2*), AB063179 (barley *BADH1*), AB063178 (barley *BADH2*), AK068462 (rice *BADH-like*), M31480 (spinach *BADH*), AY087395 (Arabidopsis *BADH*), AF370333 (Arabidopsis *BADH*), BI935476 (tomato *BADH*), X16280 (rice *actin*), U29162 (maize *ubiquitin*), AF326781 (wheat *actin*), and AY145451 (barley *actin*).

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