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Expression of *OsBADH1* gene in Indica rice (*Oryza sativa* L.) in correlation with salt, plasmolysis, temperature and light stresses

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Abstract

The relationship between environmental factors, salt tolerant and the expression of betaine aldehyde dehydrogenase (*BADH*) gene, salt stress related gene, was investigated in Indica rice. The expression was observed in various rice cultivars as well as under different environmental conditions. Northern blot analysis revealed that salt-tolerant in each rice cultivar is correlated to the expression level of *OsBADH1* mRNA. The expression studies showed that *OsBADH1* can be induced by a variety of environmental factors such as salinity, drought, cold, heat, light intensity and CO_2 concentration. The results demonstrated that the *OsBADH1* mRNA expression was up-regulated by salinity, drought, cold and high light intensity but down-regulated by CO_2 enrichment and heat stress. The primary response of *OsBADH1* gene expression was induced within 24 h after salinity, cold or drought stress treatment. Moreover, these results suggest that the expression of *OsBADH1* gene in response to salt stress could be magnified under high light conditions. Interestingly, the effect of salt stress on the expression of *OsBADH1* gene was alleviated by CO_2 enrichment. This report showed that *BADH1* gene not only plays role in the response of indica rice to salt stress but also to plasmolysis, temperature and light stresses. Therefore, *BADH1* gene might involve in multifunctional mechanisms in response to environmental stresses of indica rice.

Keywords: Betaine aldehyde dehydrogenase (BADH); CO_2 enrichment; Environmental stress; Light stress; Rice (*Oryza sativa*); Salinity stress.
Abbreviations: RuBisCO- Ribulose-1,5-Bisphosphate Carboxylase Oxygenase; PSII-Photosystem II; ORF- Open Reading Frame; CMO-Choline Monooxygenase; KDML105- Khao Dowk Mali 105; PPFD-Photosynthetic Photon Flux Density.

Introduction

Environmental conditions are the main elements that directly affect the growth and production of crop plants (Boyer, 1982). Environmental conditions such as salinity, drought, cold, heat, light intensity and CO_2 level are currently the main focus in many molecular researches (Xiong and Zhu, 2001; Kotak et al., 2007; Xie and Gong, 2008; Javid et al., 2011). These stress conditions have caused enormous problems in most agricultural areas. Salinity stress produce toxic salt ions that directly damage the plant cells and further inhibit the water use efficiency in the plant, resulting in poor growth rate and low productivity. Moreover, high salinity level causes both hyperionic and hyperosmotic stress effects, and the consequence of these can be plant cell death (Hasegawa et al., 2000). Drought and cold conditions are other environmental factors that directly interfere with the water-use system in plants. All of these conditions lead to the reduction of plant water potentials and osmotic pressure of the soil (Beck et al., 2007; Cha-um et al., 2011). Likewise, high light intensity above saturation point of photosynthesis initiates over-heating (heat stress) and high-light stress, inducing various responses including protein degradation, photoinhibition of the photosynthetic pigment apparatus and increase in heat emission (Jiao et al., 2004). On the other hand, CO_2 produces positive effect on plants growing under salinity, osmotic and high light stresses. Elevated CO_2 level has been reported to increase osmoregulation solutes by reducing the transpirational intake of salts or by improving RubisCO activity (Azam and Farooq, 2003). Osmotic adjustment of the cell is the main response of plants under salt, drought (water deficit), heat and cold stress conditions (Delauney and Verma, 1993; Rhodes and Hanson, 1993; Bohnert et al., 1995; Stoop et al., 1996; Holmström et al., 2000). Previous reports have shown that, an osmoprotective substance, glycine betaine, plays an important role in cell stabilization by balancing both the quaternary structures of proteins and the highly ordered structures of membrane against the adverse effects of salinity (Sakamoto and Murata, 2000). Furthermore, it facilitates osmotic adjustment by lowering the internal osmotic potential that contributes to the water stress tolerance ability. In addition, it stabilizes both PSII complex and RuBisCO during photosynthesis under stress conditions (Holmström et al., 2000). The positive effect of exogenous glycine betaine application in plant growing under salinity stress has been proven. Plant cell could be protected from the adverse effect of salinity induced oxidative stress by the exogenous application of glycine betaine (Demiral and Türkan, 2004).

Therefore, Glycine betaine may be an effective substance that protects plant cell and photosynthetic machinery from the effect of osmotic and high light stress. In higher plants, glycine betaine is synthesized *via* a two-step oxidation of choline. First, the enzyme choline monooxygenase (CMO) oxidizes choline to an intermediate compound, betaine aldehyde. Second, betaine aldehyde is catalyzed by BADH enzyme to produce the end product "glycine betaine" (Rhodes and Hanson, 1993). The betaine aldehyde dehydrogenase (BADH) enzyme is known as the key enzyme for glycine betaine biosynthesis. Recently, many researchers have reported the accumulation of glycine betaine and the expression of *BADH1* gene in response to salinity, drought and cold (Rhodes and Hanson, 1993). Nakamura et al. (1997) reported the expression of *BADH* gene and glycine betaine accumulation in Japonica rice. However, the expression of BADH gene was found to be very low level and did not induce accumulation of glycine betaine. Therefore, Japonica rice was considered to be a glycine betaine-nonaccumulator (Rathinasabapathi et al., 1993). Recent information on rice genome sequence revealed that rice contains two *BADH* homologs, *OsBADH1* on chromosome 4 and *OsBADH2* on chromosome 8 (International Rice Genome Sequencing Project, 2005). Both OsBADH1 and OsBADH2 contain SKL signal peptide at the C-terminus, which targets the peroxisome (Chen et al., 2008; Fitzgerald et al., 2008). Studies on the transcription of *OsBADH1* and *OsBADH2* in response to salt and drought stresses revealed that the constitutive expression of *OsBADH2* was demonstrated under normal and stress conditions, but that *OsBADH1* expression level was up-regulated by salt and drought stresses (Niu et al., 2007; Fitzgerald et al., 2008). Recently, Indica rice cv KDML105 was reported as glycine betaine accumulator under salt stress. The accumulation of glycine betaine and the activity of BADH enzyme in rice seedlings were gradually increased when exposed to high salt stress (342 mM NaCl) and reached the highest peak after 4 days of treatment, which were about 8 times higher than those under 0 mM NaCl (Cha-um et al., 2004). Glycine betaine may, therefore, play an important role in salt-stress responses and may be involved in the response of rice to other environmental factors. In this study, the expression of *OsBADH1* was investigated in KDML105 rice, which is well-known as Thai jasmine rice that has high impact for rice export. The expression of this gene was compared with other indica cultivars that have different salt-resistant capability, which are Pokkali (salt tolerance), IR29 (salt sensitive), and Pathumthani 1 (salt sensitive). Up to date, there are currently no reports on the expression pattern of *BADH1* gene in response to light intensity and CO_2 concentration in rice. Therefore, our study aims to investigate the expression of *OsBADH1* gene under various environmental conditions including high and low light intensity or CO_2 concentration. Results obtained, not only confirmed the positive effect of *OsBADH1* gene expression in response to osmotic and cold stress, but also highlighted its effect of *OsBADH1* expression in response to high light stress and CO_2 level, which have not been report yet.

Results and discussion

Cloning and analysis of OsBADH1 gene

In the present study, open reading frame (ORF) of *OsBADH1* cDNA was cloned from indica rice and used as a probe to study the expression of *OsBADH1* gene in glycine betaine-accumulating rice, KDML105. Sequencing analysis revealed that *OsBADH1* ORF fragment consists of about 1,515 bp nucleotide sequences (Accession number: DQ234303) and encoded 505 amino acid residue. The deduced amino acid sequence was 97% identical to Japonica rice *BADH1* (Accession number: AK103582), 81 % with *Hordeum brevisubulatum* (Accession number: AAS66641), 85 % with *Zoysia tenuifolia* (Accession number: BAD34957), 74 % with *Sorghum bicolor* (Accession number: AAC49268), 72 % with *Zea mays* (Accession number: AAT70230) (Fig. 1). SKL tripeptide was found at its carboxyl terminus which was normally observed in *BADH* gene from Poaceae species (Nakamura et al., 1997). This result appears to be consistent with previous report that showed the similarity of *BADH* gene polymorphism between Indica and Japonica rice by southern blotting analysis (Nakamura et al., 1997). Even the similarity of *BADH1* gene between Indica and Japonica was previously reported but there is currently no report on the accumulation of glycine betaine in Japonica rice. On the contrary, high BADH1 enzyme activity and accumulation of glycine betaine under salt stress condition was recently reported in Indica rice, KDML105 (Cha-um et al., 2007). This implies that *OsBADH1* mRNA in Japonica rice is probably degraded by some phenomena such as micro RNA interference (Kusaba, 2004), that may not occur in Indica rice. Moreover, increased salt tolerance in transgenic tobacco over-expressing *OsBADH1* coding sequence cloned form Indica rice has been reported (Hasthanasombut et al., 2010). Therefore, besides the sequence of *OsBADH1* gene, an investigation on the sequence of promoter, 3' and 5' untranslate region (3'-UTR and 5'-UTR), which regulates translation and mRNA stability of *OsBADH* gene and compare between Indica rice and Japonica rice is necessary.

Expression of OsBADH1 gene in different rice cultivars

The expression of *OsBADH1* gene among the different cultivars of Indica rice having a different response to salt stress was also investigated in this study. The result showed the correlation between salt tolerant capacity of each cultivar and *OsBADH1* gene expression level. Under control condition (0% NaCl), the expression of *OsBADH1* gene was highest in salt tolerance rice (Pokkali) and lowest in salt-sensitive rice (Pathumthani 1) (Fig. 2). When rice seedlings were exposed to moderate salinity (137 mM NaCl) for 3 days, the expression levels of *OsBADH1* gene increased in all rice cultivars. *OsBADH1* gene expression of each rice cultivar under salt stress coincided with the observed expression under normal condition, the expression was highest in Pokkaki and lowest in Pathumthani1 (Fig. 2). A noteworthy observation in this study was that the increasing level of *OsBADH1* gene induced by the effect of salt treatment was high in salt sensitive line, Pathumthani1and IR29, and low in salt tolerant line, Pokkali and KDML105. The increasing level of *OsBADH1* gene expression after salt treatment in Pathumthani1 was 6-folds higher than the increasing level observed in Pokkali. However, the total expression level of *OsBADH1* mRNA in salt-tolerant lines (Pokkali and KDML105) was higher than those of salt-sensitive lines (IR29 and Pathumthani 1) in both normal and salt-stress conditions. These results show that *OsBADH1* gene in salt tolerant rice is expressed at high level even under normal condition. In agreement with these results, Moghaieb et al. (2004) demonstrated high expression level of *BADH* gene in two halophytic plants (*S. europaea* and *S. maritime*) under normal condition (0 mM NaCl). In the case of rice, Lee et al. (2003) reported that, Indica rice has higher salt tolerance level than Japonica rice. Therefore, the low salt tolerant level in Japonica rice might be partly due to lower expression of

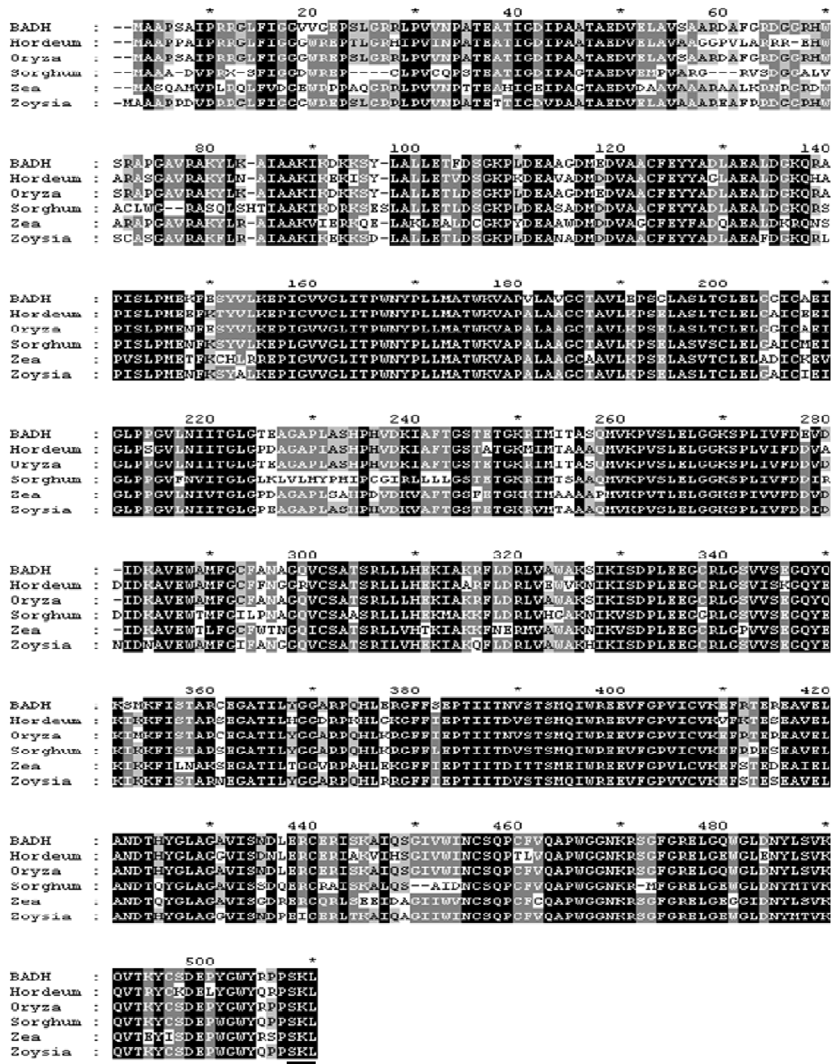

Fig 1. Alignment of the deduced amino acid sequences of rice *BADH1* (*OsBADH1*) with *BADH1* from *Hordeum* (*Hordeum brevisubulatum*), *Oryza* (*Oryza sativa* cv. Japonica), *Sorghum* (*Sorghum bicolor*), *Zea* (*Zea mays*), and *Zoysia* (*Zoysia tenuifolia*).

BADH mRNA in Japonica rice under normal condition (Nakamura et al., 1997).

Expression of OsBADH1 gene under different environmental stresses

In our study, the expression of *OsBADH1* gene was observed under a variety of environmental stresses such as salinity, temperature, light intensity and CO_2 enrichment. From the previous reports, the expression of *BADH1* mRNA in plants such as barley (Ishitani et al., 1995), sorghum (Wood et al., 1996) and rice (Nakamura et al., 1997) normally increases after stress treatment. In this study, we investigated the expression of *OsBADH1* gene at the initial period (6, 12 and 24 h) after stress treatment. At 6 h, the expression of *OsBADH1* gene was dramatically increased by drought (2-fold) and salinity (1-fold) stresses when compared with the control condition. Surprisingly, the expression of *OsBADH1* gene did not increase continuously after stress treatment. The expression significantly declined at 12 h of drought (2-fold) or salt (0.6-fold) treatment and then slightly increased again at 24 h. (Fig. 3). However, the expression of *OsBADH1* gene at 24 h was still lower than that at 6 h after salt or drought stress was applied. Mccue and Hanson (1992) reported similar expression pattern of *BADH* gene in sugar beet. After salt stress, *BADH* mRNA level decreased for several hours, and then increased. Ye et al. (2005) also reported similar expression pattern of *BADH* gene in oilseed rape (*Brassica napus*), the expression was first induced at 6 h after salinity treatment and then declined at 18 h. This result suggested that the defense response of rice against stresses trough the osmoregulation mechanism was induced within a few hour after stress application. Moreover, the trend of *OsBADH1* gene expression in rice under drought stress was similar to expression under salinity stress. Ibraheem et al. (2011) also showed similar expression pattern of sucrose transporter gene under salinity and drought stress in rice plant. It has been explained previously that the early response of

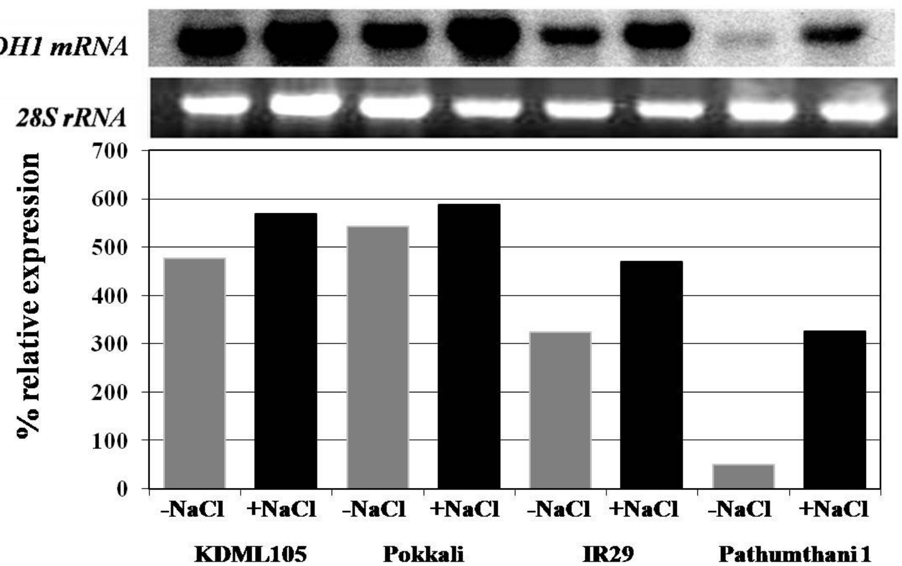

Fig 2. Upregulation of *OsBADH1* transcript levels in KDML105 rice (moderate salt tolerant) under salt stress compared with Pokkali (salt tolerant), IR29 (salt sensitive), and Pathumthani1 (salt sensitive) rice. The level of *OsBADH1* expression in each rice cultivar was investigated under normal (-NaCl) and salt stress (+NaCl) conditions. Total RNA was extracted from fifteen-day-old *in vitro* plants after 24 h of salt treatment (137 mM NaCl). Northern blot analysis was performed using 20 µg of total RNA per lane. A radio labeled full length cDNA of *OsBADH1* was used for hybridization. Relative expression percentage was calculated from the intensity of the autoradiograph signals compared with intensity of 28s rRNA on agarose gel. The relative expression was obtained with three independent plants showing a similar trend of *OsBADH* mRNA expression.

plant to water and salt stress were mostly identical because drought and salinity share a physiological water deficit. Under salinity, in addition to osmotic stress, plants are also responding to ion toxicity (Munns, 2002). Nevertheless, the expression level of *OsBADH1* gene in rice under salt stress was almost 1 times lower than drought stress. This result seems to be in conflict with the study of *BADH* gene expression in *Jatropha curcas*, a high salt and drought resistant plant, in which the level of *BADH* mRNA induced by salt stress was higher than that induced by drought stress (Zhang et al., 2008). These finding suggested that KDML105 rice, a moderate salt tolerant cultivar, was greatly injured by high salt concentration (342 mM NaCl) which led to lower *BADH* mRNA expression under high salt stress condition. Kumar et al. (2004) also indirectly showed the decrease of *BADH* mRNA by the decreasing of enzyme activity resulting from elevated salt concentration in culture of transgenic carrot. This phenomenon could be explained by the accumulation level of glycine betaine, an osmoprotective substance produced from *BADH* gene expression. In *Lactuca sativa*, the accumulation level of glycine betaine was dramatically enhanced when increase salinity level from low to moderate level. However, the rising of accumulation level was slightly increased under high salinity stress (Younis et al., 2009). Expression of *OsBADH1* gene was also induced by cold and heat stresses. When compared with normal condition (25°C), the expression of *OsBADH1* gene was up-regulated by cold treatment (4 °C) and down-regulated by heat treatment (40 °C) (Fig. 4). The expression of *OsBADH1* was first induced at 12 h after cold treatment. Consequently, the expression was dramatically increased at 24 h (2 fold) after cold stress was initiated. In contrast, the expression of *OsBADH1* gene under heat stress was slightly down-regulated (0.2 fold) in first 12 h of stress period, and gradually decreased again (0.6 fold) at 24 h (Fig.4). The interesting point revealed from this result is that the pattern of *OsBADH1* mRNA expression was differently induced by temperature stress and osmotic stress. The regulation of *OsBADH1* mRNA expression was induced by osmotic stresses (drought and salinity) faster than temperature stresses (cold and heat). In general, temperature stress has some similarities to osmotic stress, because it creates concentrated solutions of solutes, thereby subjecting plants to a shortage of liquid water. It was proven that glycine betaine maintains plasma membrane integrity of transgenic *BADH* plants under salinity and cold stresses (Zhang et al., 2010; Rhodes and Hanson, 1993). However, drought and salinity do not only cause hyperosmotic stress but also ionic stress which generates reactive oxygen species (Bohnert and Jensen, 1996). Perhaps, *OsBADH1* mRNA expression in rice plant was greatly induced in a few hours by the synergistic effect of hyperosmotic and ionic stresses. Therefore, this may suggested that drought and salinity have influence on *OsBADH1* mRNA expression in rice than cold stress. It could also be possible that plants grown under salt stress condition got adapted to these adverse effects within a few hours thereby decreasing the level of *OsBADH1* gene expression. In contrast, rice plants which were cultured under cold condition could not adapt within 24 h, hence, the expression level of *OsBADH1* gene was continuously increased.

Yang et al. (2007) reported heat resistance and high accumulation of glycine betaine in transgenic tobacco by over-expression of *BADH* gene. However, a decrease in *OsBADH1* mRNA by the effect of heat stress was detected in this study. The decreased of *OsBADH1* mRNA in rice under high temperature might be caused by heat-induced degradation of mRNA which has been reported previously in carrot cell lines cultured under high temperature (Milioni et al., 2001). Moreover, Alia et al. (1998) also demonstrated heat-inducted degradation of choline oxidase (codA) protein, an enzyme for glycine betaine production in bacteria. Surprisingly, the degradation of this protein did not decrease the accumulation of glycine betaine and heat tolerance of transgenic plants. Light intensity and CO_2 concentration were found to be the main environmental factors affecting photosynthesis (Brun

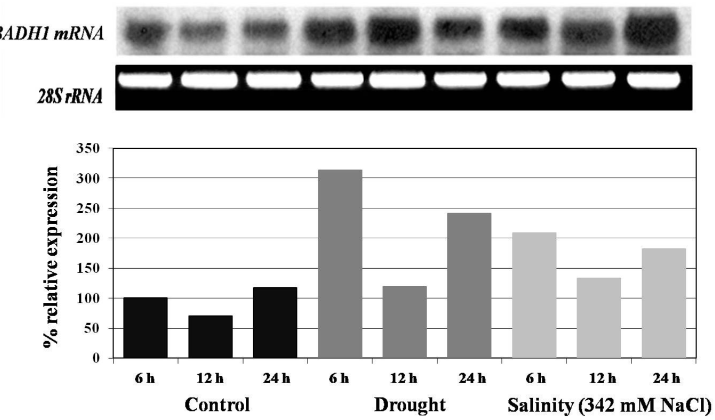

Fig 3. Upregulation of *OsBADH1* transcript levels in KDML105 rice (moderate salt tolerant) caused from drought and high salinity (342 mM NaCl) stresses. Total RNA was extracted from fifteen-day-old *in vitro* plants after 6, 12 and 24 h of drought or high salinity treatment.

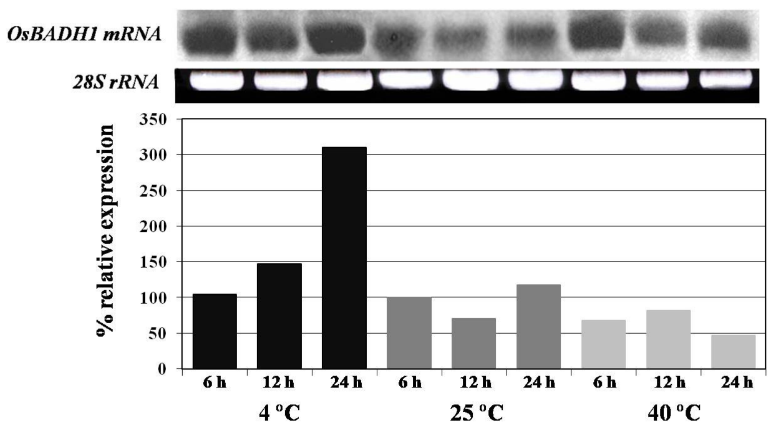

Fig 4. Regulation of *OsBADH1* transcription in KDML105 rice plant by cold or heat stress. Northern blot analysis showed the expression pattern of *OsBADH1* mRNA expression under cold (4℃) or heat (40 ℃) compared with normal temperature (25℃). Total RNA was extracted from fifteen-day-old *in vitro* plants after 6, 12 and 24 h of cold or heat treatment.

and Cooper, 1967). High light intensity caused photoinhibition and protein degradation of photosystem I in plants (Jiao et al., 2004). Murata et al. (1992) reported that glycine betaine prevents the selective dissociation of the extrinsic polypeptides from the PSII complex in the presence of high concentrations of salts. Furthermore, glycine betaine also protects the oxygen-evolving PSII complex against heat-induced inactivation which is caused by high light stress (Allakhverdiev et al., 1996). Therefore, the effect of salinity combined with high light intensity on *OsBADH1* gene expression was investigated in this study. The result showed the significant increase of *OsBADH1* mRNA expression when applied to high light intensity (150 µmol m^{-2} s^{-1} PPFD) was applied under normal condition (0 mM NaCl) (Fig. 5). When moderate salinity (137 mM NaCl) was applied in combination with high light intensity, the expression was drastically increased (2-folds) when compared with the expression induced by high light stress under normal condition. This remarkable enhanced expression of *OsBADH1* mRNA suggests that expression of *OsBADH1* gene in rice could be accelerated by the combined effect of salinity stress and high light intensity. Elevated CO_2 level has been reported as a major factor that enriches the overall growth and reproductive of plants (Jones et al., 1984). In addition, enrichment of CO_2 could enhance tolerance of plants to several environmental stresses such as light, temperature, salinity and nutrients (Azam and Farooq, 2003). In our study, we investigated the positive effect of CO_2 enrichment on the expression of *OsBADH1* gene under salinity stress. The result showed that the expression of *OsBADH1* mRNA was dramatically down regulated (7.5 fold) under moderate salt stress (137 mM NaCl) by the application of high CO_2 concentration (1500 µmol mol^{-1}) (Fig. 6). Interestingly, there was no significant difference between salt stress and normal conditions in the expression of *OsBADH1* mRNA when enriched CO_2 was applied. This may suggests that the enrichment of CO_2 causes strong and healthy growth in plants with an apparent decrease in *OsBADH1* gene expression and stress resistibility.

Materials and methods

Plant Materials

Rice plants, KDML105 (moderate salt tolerance), Pokkali (salt tolerance), IR29 (salt sensitive), and Pathumthani 1 (salt sensitive) were multiplied using tissue culture technique to minimize error that may arise from different seedlings. In order to reduce the effect of environmental condition such as relative humidity and photo period, rice plants were transferred and grown in close system under photoautotrophic

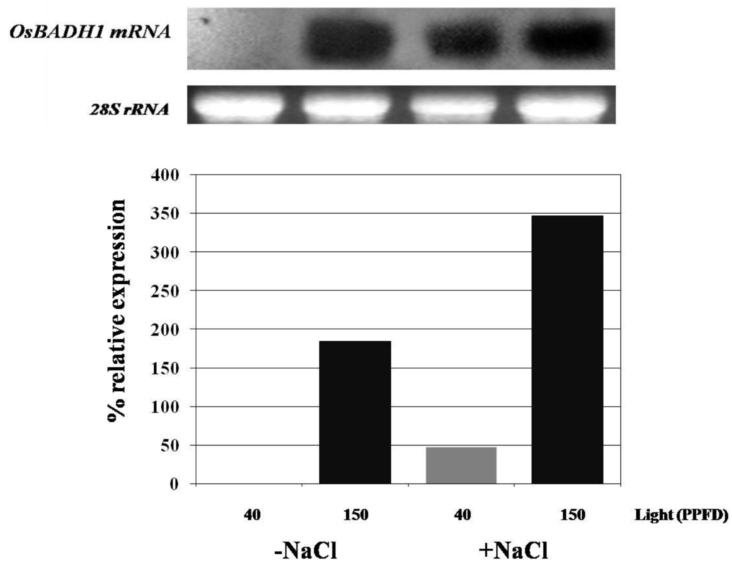

Fig 5. Northern blot analysis of *OsBADH1* mRNA expression under low or high light intensity signal (40 and 150 µmol m^{-2} s^{-1} PPFD) with or without moderate salinity stress (137 mM NaCl, 24 h). The relative expression was observed in 15 day-old *in vitro* plants of KDML105.

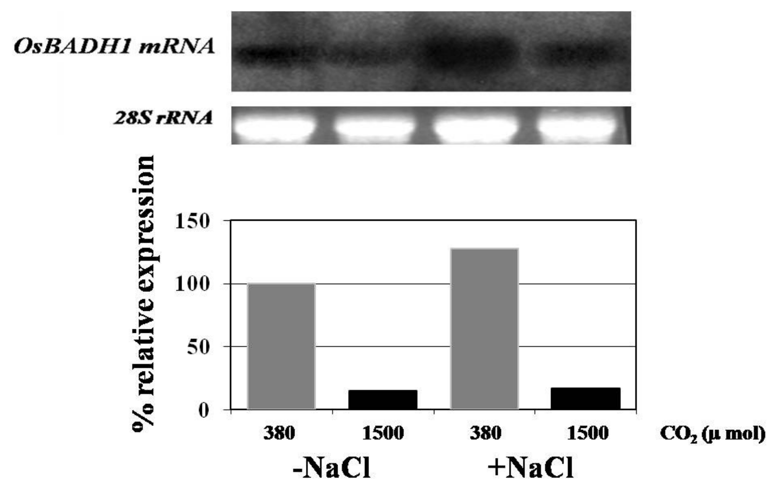

Fig 6. Northern blot analysis of *OsBADH1* mRNA expression under low or enriched CO_2 concentration (380 and 1,500 µmol mol^{-1}) with or without moderate salinity (137 mM NaCl, 24 h). The relative expression was observed in 15 day-old *in vitro* plants of KDML105.

conditions fixed at 25 ± 2 ºC, 60 ± 5% RH and 16 h d^{-1} photoperiod of 60±5 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). After stress treatment, leaf tissues were collected for RNA extraction and northern blot analysis.

Stress treatments

Salinity stress

In order to investigate the relationship between expression of *OsBADH1* and salt tolerance in different rice cultivars including salt sensitive, moderate salinity stress (137 mM NaCl) was used. Fifteen-day-old *in vitro* plants of each cultivar (KDML 105, Pokkali, IR29, and Pathumthani 1) were incubated for 1 week in close system as described previously. Then, salt stress was applied by replacing the sugar-free-liquid MS medium in the chamber box with sugar-free-liquid MS medium containing 137 mM NaCl. The expression of *OsBADH1* gene was investigated 3 days after salt treatment. The primary response of rice to salinity and drought stress was also examined in glycine betaine-accumulating rice cultivar (KDML105) which is a moderate salt tolerant type. In this treatment, high salt stress (342 mM NaCl) was applied as described previously. For drought stress, rice plants were transfer to new close chamber boxes without liquid medium and incubated under normal condition as explained previously. Primary response of gene expression to high salinity and drought stress were observed at 6, 12 and 24 h after stress treatments.

Temperature stress

The effect of temperature stress (heat and cold) on *OsBADH1* gene expression was also investigated in this study. Plant materials were prepared as described for salt stress treatment. The culture chamber boxes were incubated at 4 ºC or 40 ºC, and gene expression was recorded at 6, 12 and 24 h. The pattern of gene expression was compared with gene expression under normal condition (25ºC).

Light stress

The effect of light intensity on *OsBADH1* gene expression was also studied by applying two light intensities (40 and 150 µmol m^{-2} s^{-1}PPFD) at 16 h d^{-1} photoperiod.

CO_2 enrichment

Also, the effect of CO_2 concentration was investigated by applying CO_2 at 380 and 1500 µmol mol^{-1}.
The combined effect of moderate salinity (137 mM) and other environmental factors such as CO_2 and light intensity was investigated as well in this study.

Study of the betaine aldehyde dehydrogenase (BADH) gene expression

All the investigations were done by detection of mRNA level using Northern blot analysis and cloned *OsBADH1* ORF fragment as specific probe. *OsBADH1* ORF fragment was cloned from rice RNA using RT-PCR technique. The first cDNA strand was synthesized using Superscript III RNase H^- RT reverse transcription system (Invitrogen, Auckland, New Zealand). The second cDNA strand was synthesized using *OsBADH1* gene specific primer, which is *OsBADH1* forward primer (5'-ATTCCATATGGCCGCGCGTCGGCGATCC-3') that contains the start codon ATG and *Nde*I site and *OsBADH1* reverse primer (5'-AAACGGATCCCAGCTTGGATGGAGGCCGGTAC-') that contains stop codon TAG and *Bam*H I site. These primers were designed with regards to *BADH1* cDNA sequence of *Oryza sativa* subtype japonica cv. Nipponbare, AK103582 (Kikuchi et al., 2003)*, Suaeda liaotungensis*, GenBank accession number AF359282, *Brasica napus,* GenBank accession number AY351634*, Gossypium hirsutum*, GenBank accession number AY461804 and *Spinach oleracea*, X69771 (Xiao et at., 1995). The PCR reaction was performed according to the following conditions: 95 ºC for 4 min and 35 cycles of 95 ºC for 1 min, 60 ºC for 2.30 min, 72 ºC for 2 min, and finally with, 72 ºC for 5 min, the expected size was 1,500 bp. The fragment of *OsBADH1* cDNA was ligated to TA cloning vector, pGEM®-T Easy Vector (Promega®, USA). Finally, *OsBADH1* cDNA fragment was commercially sequenced in both forward and reverse directions using M13 primer. The sequencing data were analyzed using vector NTI computer program. The *OsBADH1* nucleotide and amino acid sequence alignment was performed using ClustalX and ClustalW program, respectively.

Northern blot analysis

Total RNA was extracted from leaves of plants after stress treatment as described above. 20 µg of total RNA was subjected to 1.5% formaldehyde gel electrophoresis. After transferring and fixing to nylon membrane (Hybond N, Amersham Japan, Tokyo), hybridization was carried out with ^{32}P-labeled *OsBADH1* fragment which was amplified from pGEM/*OsBADH1* plasmid. Expression of mRNA was observed after exposing membrane to X-ray film. The intensity of mRNA band on X-ray film was measured and calculated using gel tool program of Gel Doc™ 2000 system (BioRad, USA). The relative expression level of mRNA was calculated by comparing the intensity of mRNA on x-ray film to the intensity of loaded 28S rRNA band. To normalise mRNA levels in the different samples, the value of the band corresponding to each mRNA level was divided by the intensity of the corresponding 28S rRNA band used as an internal standard.

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