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Comparison between transgenic maize with exotic betaine aldehyde dehydrogenase (*BADH*) gene and its untransformed counterpart

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Abstract

We investigated the performance of a transgenic maize (*Zea mays* L) line with an exotic betaine aldehyde dehydrogenase (*BADH*) gene and its untransformed counterpart under drought and normal water conditions. Membrane permeability, osmoprotectant contents, and antioxidant enzyme activities of the maize lines as well as plant height and biomass were compared. The results showed that, under drought stress, compared with the untransgenic line, the contents of glycine betaine (GB), soluble sugars, soluble proteins and proline of the transgenic line were significantly higher, so was the peroxidase (POD) activity; the contents of superoxide anion free radical, malondialdehyde (MDA) and the electrical conductivity of the transgenic line were lower; plant height and the biomass of the transgenic line were significantly higher. Under normal water conditions, the contents of soluble protein and MDA content of the transgenic line were significantly lower; but it was not the case for the content of superoxide anion free radical, electrical conductivity and superoxide dismutase (SOD) activity. No significant difference was observed in GB content and, the plant height and the biomass between the 2 lines. We conclude that the transgenic maize with exotic *BADH* gene was superior over its untransformed counterpart under drought stress and they performed similarly under normal water conditions.

Keywords: *BADH* gene, maize, physiology, growth

Introduction

Water shortage in the world is becoming increasingly severe (Chaves and Oliveira, 2004). Plants are often subjected to soil-salinization and drought stresses during their life cycle in many areas of the globe. It is estimated that 6% of the world's land and 30% of the world's irrigated areas already suffer from salinity problems (Munns and Tester, 2008). Plants have evolved protective mechanisms to maintain normal cellular metabolism and prevent damage to cellular components during a long evolution process. One common metabolic adaptation to salinity stress is the accumulation of compatible solutes (Bohnert et al, 1995), such as soluble sugars, soluble proteins, and dissociated proline to regulate osmosis within cells. However, their ability to tolerate osmotic stress is limited when osmotic stress is severe, which will cause plant yield reduction. Maize (*Zea mays* L) is an important food and forage crop as well as a raw material for producing ethanol in industry. As an annual crop, maize productivity is largely affected by drought. Due to its poor salt tolerance and the more and more common salinization of land, scientists have been trying to improve salt tolerance of maize by various strategies including genetic engineering (Türkan and Demiral, 2009; Hu et al, 2012). Previous studies have demonstrated that under stressed conditions, the biosynthesis of glycine betaine (N,N,Ntrimethylglycine, GB) is up-regulated in many plants as barley (*Hordeum vulgare* L), sorghum (*Sorghum bicolor* L Moench), wheat (*Triticum aestivum* L), and spinach (*Spinacia oleracea* L) (Rhodes and Hanson, 1993; Ashraf and Foolad, 2007; Chen and Murata, 2008), wherein GB has been shown as an effective ameliorating agent against stresses. Exogenous application of GB or introduction of GB biosynthesis-related genes enhanced stress tolerance in a number of plant species as, maize (Nawaz and Ashraf, 2009; Di, 2015), wheat (Mahmood et al, 2009) and potato (*Solanum tuberosum* L; Ahmad et al, 2014). In the majority of biological systems, including plants, GB is synthesized by a two-step oxidation of choline via an intermediate form of betaine aldehyde, where the *BADH* gene is a key regulator for GB synthesis. Since *BADH* genes were isolated and purified from spinach in 1981, BADH genetic engineering has been increasingly valued (Pan et al, 1981). The exotic BADH genes have been introduced into many plant species, such as rice (*Oryza sativa* L; Guo et al, 1997), tobacco (*Nicotiana tabacum* L; Mcneil et al, 2001) and maize (Wu, et al, 2008). Studies on the performances of transgenic plants with the *BADH* gene under osmotic stress have been conducted (Hattori et al, 2009; Ahmad, et al, 2013), which suggest that the *BADH* gene plays a

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definite role in stress tolerance. But some questions still remain. For instance, what is the performance of BADH maize when cultured under normal water conditions? Will the BADH expression affect physiology or development of maize under normal water conditions? Those are interesting issues to investigate and also indispensible aspects to fully understand BADH maize. In the present study, the performance of a transgenic maize line, with its untransformed counterpart, under drought and normal water conditions was investigated, where the physiological characteristics such as osmo-regulation substances contents, cell membrane permeability, and antioxidant enzyme activities were measured, and their development and biomass accumulation were studied as well.

Materials and Methods

Plant material and treatments

A *BADH* gene was cloned from *Atriplex hortensis* L and the plasmid pBIN438 harboring the *BADH* gene with 2×35S CaMV promoter (35S), tobacco mosaic virus(TMV) enhancer and a NOS terminator (Li et al, 2000) was introduced into maize lines. A transgenic maize line with the exotic *BADH* gene (treatment) and its untransformed counterpart, Zheng-58 (control) were provided by the Biotechnology Research Center, Shanxi Academy of Agricultural Sciences. The seeds from the 2 lines were grown in an open experimental plot with sufficient irrigation when needed. The field management was carried out according to the guideline provided for the breed. Their leaves were collected at the nine-leaf stage that was coincident with the jointing stage; drought stress was imposed on the seedlings grown in a controlled growth chamber (BOXUN BSG-800, Shanghai, China) with ambient temperature of 25°C for 14 h photoperiod at 40% relative air humidity and a photosynthetic photon flux density (PPFD) of 150 μmol m^{-2} s^{-1} light intensity. Relative pot water capacity equal to 70% of the maximum soil capacity was maintained by daily watering until the third-leaf stage; then water supply was decreased for seven days, and irrigation was withheld for the next seven days. Pot water capacity was equal to 31%-36% of the maximum soil capacity at the time of sampling. The leaves were collected at the five-leaf stage. All analyses were performed with the newest leaves. Leaf samples were flash frozen and stored in liquid nitrogen until measuring.

Measurement and analysis

Osmo-regulation substances contents and cell membrane permeability measurement

Five seedlings were randomly taken from the treatment and the control, and the content of soluble sugars, proteins, and free proline in leaves were measured according to Zhang et al (1990); glycine betaine (GB) content was determined by spectrophotometry according to Wang et al (2008). Relative conductivity, which represents cell membrane permeability, was determined by a conductivity meter (LEICI DDSJ-308A, Shanghai, China). The leaves were soaked in deionized water and incubated at 25°C for 20 min, conductivity of the bathing solution (C1) was recorded. Samples were then boiled in a water bath for 20 min and the conductivity reading (C0) was obtained upon equilibration at 25°C. The relative conductivity was expressed as (C1 / C0) × 100 %.

Antioxidant enzyme activity and malondialdehyde content measurement

Superoxide dismutase (SOD) and peroxidase (POD) activities, superoxide anion free radical content, and malondialdehyde (MDA) content were measured with leaves according to Lee (2000). SOD activity was determined by a colorimetric method that based on the inhibition of nitroblue tetrazolium (NBT). SOD active unit (U) was defined as the amount of enzyme inhibiting 50% of the auto-oxidation reaction; POD activity was assayed spectrophotometrically at 470 nm using guaiacol as a phenolic substrate with hydrogen peroxide. The active unit (U) was defined as the amount of enzyme when absorbance had been reduced by 0.1 per minute at 470 nm. Superoxide anion free radical content was determined by the hydroxylamine oxidation method and MDA content was measured using thiobarbituric acid (TBA) method at absorbance of 532 nm.

Plant development and biomass measurement

Seedling height, fresh weight, and dry weight of 60 seedlings with 3 replications from the 2 lines were measured, and means and standard deviations were calculated.

Statistical analysis

All data were expressed as means ± standard deviation. Differences among the two lines were evaluated using an Independent-Samples T-test, SPSS (17.0) program.

Table 1 - Contents of osmo-regulation substances in 2 lines under drought stress.

Treatment	Soluble sugar (μg μg^{-1} FW)	Soluble proteins (mg g^{-1} FW)	Free proline (mg g^{-1} FW)	Glycine betaine (mg g^{-1} FW)
CK	39.69 ±8.88	6.66 ±1.42	1.22 ± 0.15	1.42 ± 0.06
T	52.97 ±1.37	12.84 ± 0.62	9.59 ± 0.20	3.14 ± 0.02
P	0.001	0.033	<0.001	<0.001

P values less than 0.05 indicate significant difference at 95% level; n=5. CK: the transgenic maize line with the exotic *BADH* gene; T: the untransformed counterpart.

Table 2 - Contents of osmo-regulation substances in maize seedlings under normal water conditions.

Treatment	Soluble sugar (μg μg^{-1} FW)	Soluble proteins (mg g^{-1} FW)	Free proline (mg g^{-1} FW)	Glycine betaine (mg g^{-1} FW)
CK	31.88 ± 9.05	9.13 ± 0.80	3.87 ± 0.37	2.13 ± 0.05
T	30.47 ± 7.16	2.95 ± 0.32	3.03 ± 0.13	1.66 ± 0.02
P	0.843	0.001	0.733	0.216

P values less than 0.05 indicate significant difference at 95% level; n=5. CK: the transgenic maize line with the exotic *BADH* gene; T: the untransformed counterpart.

Results

The osmo-regulation substances contents in 2 lines

Under drought stress, the contents of soluble sugars, soluble proteins, proline, and GB from the two lines were all significantly different ($P < 0.05$) (Table 1); they were 33.5%, 92.8%, 686.1%, and 121.1% higher in the transgenic line than in the control, respectively.

Under normal water conditions, contents of soluble sugars, proline and GB from the two lines were not significantly different ($P > 0.05$) (Table 2). However, the contents of soluble proteins from the transgenic line were 67.69% lower than the control ($P < 0.05$), and the GB was slightly less than the control. The results suggested that drought stress triggered the accumulation of GB in transgenic plants which contributed to osmotic equilibrium.

The cell membrane permeability and antioxidant enzyme activity in 2 lines

Under drought stress, relative conductivity, the content of superoxide anion free radical, and MDA in the transgenic line were significantly lower than those of untransformed control ($P < 0.05$) (Table 3), while POD activity were significantly higher ($P < 0.05$).

Under normal water conditions, relative conductivity and superoxide anion free radical content in the transgenic line were significantly higher than those of untransformed control ($P < 0.05$) (Table 4), while MDA content was significantly lower. SOD activity was higher in the transgenic line ($P < 0.05$), and the POD activity was not significantly different ($P> 0.05$) between the 2 lines.

Plant development and biomass accumulation

Under drought stress, seedling height, dry weight, and fresh weight in the transgenic line were significantly higher than those of the untransformed control ($P < 0.05$) (Table 5); they were 112.2%, 105.4%, and 53.1% higher, respectively.

In an experimental plot with normal water supply, no significant difference ($P > 0.05$) was observed between the transgenic line and its untransformed counterpart in plant height and biomass accumulation (Table 6), though the plant height of the transgenic line was 7.18% higher, while dry weight and fresh weight of the transgenic line were 4.26% and 0.60% lower than the untransformed control, respectively.

Discussion

When plants suffer from drought, osmotic balance and homeostasis of plants are disrupted; plants have adopted various mechanisms to alleviate osmotic stress. Osmo-protectants play vital roles to keep osmotic equilibrium. Chemically, they are categorized into three types: betaines and allied compounds, polyols and sugars, and amino acids such as proline (McNeil et al, 1999). It is reported that with the increase of soluble sugar content, the water potential will decrease so as to regulate cell osmotic pressure and ensure normal cell status (Hakimi et al, 1995). Soluble proteins, especially some stress-related proteins, with strong hydrophilic ability, can obviously enhance water-possessing ability of cells (Hsiao, 1973). Higher plants accumulate GB in response to both water stress and salinity stress (Wyn Jones and Storey, 1981). Wu (2008) stated that the maize seedlings harboring *BADH* gene showed higher GB than wild-type seedlings under salt stress. Our results demonstrated that under the drought stress, the transgenic maize line, which is a GB-accumulator plant because one of its ALDH10 isoenzymes has BADH activity (Muñoz-Clares, et al, 2014), accumulated remarkablely higher GB compared with their untransformed counterparts, so were the osmo-protectants, such as soluble proteins, sugars and free proline, which account for maintaining the osmotic equilibrium under drought. Upon stressed environment, anion free radicals produced accordingly, and plants usually exhibit increased peroxidase and superoxide dismutase activities (Talbi et al, 2015) to control the level of reactive oxygen species (ROS), thus to protect the integrity of cells membrane. It has been well documented that GB stabilizes the structures and activities of enzyme and maintains the in-

Table 3 - Membrane permeability and antioxidant enzyme activities in seedlings under drought stress.

Treatment	Relative conductivity (%)	Superoxide anion free radical (μg g^{-1} FW)	MDA (mmol g^{-1} FW)	SOD activity (U ml^{-1})	POD activity (U g^{-1} FW min^{-1})
CK	37.63 ±1.01	3.11 ±1.54	8.43 ± 0.58	0.03 ± 0.001	48.33 ± 7.54
T	22.69 ±1.20	1.31 ± 0.38	3.70 ± 0.46	0.04 ± 0.005	65.46 ± 2.89
P	0.001	0.002	<0.001	0.090	0.039

P values less than 0.05 indicate a significant difference at 95% level; n=5. CK: the transgenic maize line with the exotic BADH gene; T: the untransformed counterpart.

Table 4 - Membrane permeability and antioxidant enzyme activities in seedlings under normal water conditions.

Treatment	Relative conductivity (%)	Superoxide anion free radical (μg g^{-1} FW)	MDA (mmol g^{-1} FW)	SOD activity (U ml^{-1})	POD activity (U g^{-1} FW min^{-1})
CK	25.66 ± 4.39	0.34 ± 0.09	8.43 ± 0.58	0.05 ± 0.003	467.50 ± 45.68
T	73.23 ± 8.58	4.44 ± 0.66	3.70 ± 0.46	0.09 ± 0.024	435.60 ±26.21
P	0.013	0.019	<0.001	0.022	0.295

P values less than 0.05 indicate a significant difference at 95% level; n=5. CK: the transgenic maize line with the exotic BADH gene; T: the untransformed counterpart.

tegrity of membranes against the damaging effects of excessive salt, heat and freezing (Papageorgiou and Murata 1995), which agree with our observation where higher activities of POD and SOD exhibited in BADH maize compared with its untransformed counterparts, therefore, the improved potential to quench H_2O_2 and less damage in membrane. The present study agreed with prior reports where BADH maize showed lower electrical conductivity under salt stress (Wu, 2008) and less cell leaf damage upon drought (Quan, et al, 2004). In conclude, with more GB accumulation in BADH maize, the osmotic stress alleviated, the structures and activities of enzyme were maintained, so was the membrane integrity, and, the plant height and biomass enhanced accordingly.

The plant performance of transgenic maize with over-expressed *BADH* gene and its untransformed counterparts under normal water conditions has been poorly investigated. A field experiment with transgenic maize seedlings was conducted at jointing stage which is closely associated with yield in the present study. Interestingly, we observed no significant difference in GB generation between the 2 groups, which disagreed with Liang et al (2009) and Zhang et al (2010) where higher GB concentration presented in transgenic wheat than wild type under non-stressed condition. And the issue need to be further investigated with more species. We speculated that under normal water conditions, there is no need for plants to produce extra osmo-protectants to maintain osmotic equilibrium, therefore, the total GB content in BADH maize is similar as the untransformed counterpart. We believe self-regulation mechanism is involved there, by which plants maintain normal metabolism by re-establishing the cellular homeostasis. The lower soluble protein in BADH maize also reflected the varied responses of different osmolytes to altered environments. There was no significant difference in seedling height and biomass between the 2 lines under normal water conditions; this was also the case when plants were harvested (data not shown), indicating that the transgenic maize performed the same as the control under normal water conditions.

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References

Ahmad R, Hussain J, Jamil M, Kim MD, Kwak, SS, Shah MM, Hendawy SE, Suhaibani NA, Rehman SU, 2014. Glycinebetaine synthesizing transgenic potato plants exhibit enhanced tolerance to salt and cold stresses. Pak J Bot 46: 1987-1993

Ahmad R, Lim CJ, Kwon SY, 2013. Glycine betaine: a versatile compound with great potential for gene pyramiding to improve crop plant performance against environmental stresses. Plant Biotechnol Rep 7: 49-57

Ashraf M, Foolad MR, 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ Exp Bot 59: 206-216

Bohnert HJ, Nelson DE, Jensen RG, 1995. Adaptations to environmental stress. Plant Cell 7:1099-1111

Chaves MM, Oliveira MM, 2004. Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. J Exp Bot 55: 2365-2384

Chen TH, Murata N, 2008. Glycinebetaine: an effective protectant against abiotic stress in plants. Trends Plant Sci 13: 499-505

Di H, Tian Y, Zu H, Meng X, Zeng X, Wang Z, 2015. Enhanced salinity tolerance in transgenic maize plants expressing a *BADH* gene from *Atriplex mi-*

Table 5 - Plant height and biomass of maize seedlings under drought stress.

Treatment	Plant height (cm)	Dry weight (g)	Fresh weight (g)
CK	10.33 ± 2.005	0.037 ± 0.006	0.49 ± 0.12
T	21.92 ± 5.059	0.076 ± 0.014	0.75 ± 0.13
P	0.049	0.030	0.044

Seedlings were at five-leaf stage cultivated in growth chamber, P value less than 0.05 indicates significant difference at 95% level; n=60. CK: the transgenic maize line with the exotic BADH gene; T: the untransformed counterpart.

Table 6 - Plant height and biomass of maize seedlings under normal water conditions.

Treatment	Plant height (cm)	Dry weight (g)	Fresh weight (g)
CK	46.67 ± 1.53	0.47 ± 0.018	8.39 ± 0.56
T	50.02 ± 5.29	0.45 ± 0.093	8.34 ± 0.97
P	0.468	0.923	0.579

Seedlings were at five-leaf stage cultivated in growth chamber, P value less than 0.05 indicates significant difference at 95% level; n=60. CK: the transgenic maize line with the exotic BADH gene; T: the untransformed counterpart.

crantha. Euphytica 206: 775-783

Guo Y, Zhang L, Xiao G, Cao SY, Gu DM, Tian WZ, Chen SY, 1997. Expression of betaine aldehyde dehydrogenase gene and salinity tolerance in rice transgenic plants. Sci China Ser C 40: 496-501

Hakimi AA, Monneveux P, Galiba G, 1995. Soluble sugars, proline and relative water content (RWC) as traits for improving drought tolerance and divergent selection for RWC from *Triticum polonicum* into *Triticum durum*. J Genet Breed 49: 237-244

Hattoria T, Mitsuya S, Fujiwara T, Jagendorf AT, Takabe T, 2009. Tissue specificity of glycinebetaine synthesis in barley. Plant Sci 176: 112-118

Hsiao TC, 1973. Plant responses to water stress. Annu Rev Plant Physiol 24: 519-570

Hu S, Tao H, Qian Q, Guo L, 2012. Genetics and molecular breeding for salt tolerance in rice. Rice Genom Genet 3: 39-49

Lee HS, 2000. Principles and Experimental Techniques of Plant Physiologyl and Biochemistry, 1st ed. Higher Education Press, Beijing (in Chinese)

Li YX, Chang FQ, Du LQ, Guo MH, Li HJ, Zhang JS, Chen SY, Zhu ZQ, 2000. Genetic transformation of watercress with a gene encoding for betaine-aldehyde dehydrogenase (*BADH*). Acta Bot Sin 42: 480-484

Liang C, Zhang XY, Luo Y, Wang GP, Zou Q, Wang W, 2009. Over accumulation of glycine betaine alleviates the negative effects of salt stress in wheat. Russ J of Plant Physiol 56: 370-376

Mahmood T, Ashraf M, Shahbaz M, 2009. Does exogenous application of glycinebetaine as a presowing seed treatment improve growth and regulate some key physiological attributes in wheat plants grown under water deficit conditions? Pak J Bot 41:1291–1302

McNeil SD, Nuccio ML, Hanson AD, 1999. Betaines and related osmoprotectants: Targets for metabolic engineering of stress resistance. Plant Physiol 120: 945-949

McNeil SD, Nuccio ML, Ziemak MJ, Hanson AD, 2001. Enhanced synthesis of choline and glycine betaine in transgenic tobacco plants that overexpress phosphoethanolamine N-methyltransferase. Proc Natl Sci USA 98:10001–10005

Munns R, Tester M, 2008. Mechanisms of salinity tolerance. Annu Rev Plant Biol 59: 651-681

Muñoz-Clares RA, Riveros-Rosas H, Garza-Ramos G, González-Segura L, Mújica-Jiménez C, Julián-Sánchez A, 2014. Exploring the evolutionary route of the acquisition of betaine aldehyde dehydrogenase activity by plant ALDH10 enzymes: implications for the synthesis of the osmoprotectant glycine betaine. BMC plant biology 14:149

Nawaz K, Ashraf M, 2009. Exogenous application of glycinebetaine modulates activities of antioxidants in maize plants subjected to salt stress. J Agron Crop Sci 196: 28-37

Pan SM, Moreau RA, Yu C, Huang AHC, 1981. Betaine accumulation and betaine-aldehyde dehydrogenase in spinach leaves. Plant Physiol 67: 1105-1108

Papageorgiou GC, Murata N, 1995. The unusually strong stabilizing effects of glycinebetaine on the structure and function in the oxygen-evolving photosystem II complex. Photosynthesis Reasearch 44: 243-252

Quan R, Shang M, Zhang H, Zhao Y, Zhang J, 2004. Engineering of enhanced glycine betaine synthesis improves drought tolerance in maize. Plant Biotechnol J 6: 477- 486

Rhodes D, Hanson AD, 1993. Quaternary ammonium and tertiary sulfonium compounds in higher plants. Annu Rev Plant Physiol 44: 357-384

Talbi S, Romero-Puertas MC, Hernández A, Terrón L, Ferchichi A, Sandalio LM, 2015. Drought tolerance in a Saharian plant *Oudneya africana*: Role of antioxidant defences. Environ Exp Bot 111: 114-126

Türkan I, Demiral T, 2009. Recent developments in understanding salinity tolerance. Environ Exp Bot 67: 2-9

Wang JH, Wang LL, Wu YM, Zhou Q, 2008. Measure of the amount of betaine in fodder beet by colorimetry. China Beet & Sugar 11: 5-7 (in Chinese with English abstract)

Wu W, Su Q, Xia XY, Wang Y, Luan YS, An LJ, 2008. The *Suaeda liaotungensis* kitag betaine aldehyde dehydrogenase gene improves salt tolerance of transgenic maize mediated with minimum linear length of DNA fragment. Euphytica 159: 17-25

Wyn Jones RG, Storey R, 1981. Betaines, pp. 171-204. In: The physiology and biochemistry of drought resistance in plants. Paleg LG and Aspinal D eds. Academic Press, Sydney

Zhang XY, Liang C, Wang GP, Luo Y, Wang W, 2010. The protection of wheat plasma membrane under cold stress by glycine betaine overproduction. Biologia Plantarum 54: 83-88

Zhang ZL, 1990. Plant Physiology Experiment Instruction, 2nd ed. Higher Education Press, Beijing (in Chinese)