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MOLECULAR CLONING AND EXPRESSION ANALYSIS OF BETAINE TRANSPORTER GENE IN MAIZE (*ZEA MAYS* L.)

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Abstract

During exposure to stress, betaine content was reported to increase in maize (*Zea mays* L.), but the exact mechanism of this increase is not clearly understood. In the current study, we attempted to identify a novel betaine transporter gene and investigated the expression patterns under salt and drought stresses. The betaine transporter gene, *ZmBetProt*, was cloned by RACE technique from Maize, which covered 1538 bp with a 1299 bp open reading frame encoding 432 amino acids, protein molecular weight was 46.986 kDa and theoretical isoelectric point of 8.90.The *Bet/ProT* homologous genes were obtained from various plant species. The highest similarity was observed with proline transporter of *Arabidopsis thaliana (AtProT3)*. Under salt stress, betaine gene expression was not induced significantly, but induced significantly under mannitol stress. Full-length sequence data for cDNA of the betaine transporter gene was submitted to the GenBank with accession number: KX013323.1. This study is designed to provide in-depth understanding of the roles of *ZmBetProt* in regulation of betaine transporter under abiotic stress.

Key words: Maize, Betaine transporter gene, Expression analysis, Gene cloning.

Introduction

Maize (*Zea mays* L.) is a world widely cultivated crop, playing a vital role to support the increasing world population. The production process of maize is highly significantly influenced by environmental stress factors (Li *et al.,* 2017). In China, approximately 60% of the maize are cultivated in arid areas, which is subjected to 20-30% yield loss due to drought or salinity. (Gong *et al.,* 2014; Zhang *et al*., 2011; Liu *et al*., 2018). Abiotic stresses are considered as the vital limitations for maize growth and productivity. Adversity stress can influence physiological, metabolic, molecular processes, also disturb a series of cellular and metabolic processes, thus, affecting the plant growth and survival (Zélicourt *et al.,* 2016). Multitude of abiotic stress factors can be mitigated by using salt and drought tolerant genotypes that contribute to resilience (Vaughan *et al.,* 2017). One of the defense mechanisms against salt and drought stress is induction of osmoregulation mediated by betaine and proline (Singh *et al.,* 2008). It acts as an osmoregulator to stabilize the intracellular ion homeostasis and maintains the membranes integrity against the negative effect of salt, cold, heat or freezing (Sakamoto & Murata, 2002).

Although mechanism of betaine synthesis is not fully understood, it is believed to be crucial factor in deterring plant tolerance to abiotic stress. Some earlier published reports have indicated the transporters for betaine in different plants. For example the betaine transporter genes have been isolated from *Avicennia marina* (*mangrove*), which is considered as betaine accumulator (Waditee *et al.,* 2002). Betaine transporters are believed to be actively involved in transport of proline as well as betaine, and their activity is induced by saline stress (Hoque *et al.,* 2008). Homologous transporters have been identified in two betaine non-accumulator plants, tomato and *Arabidopsis,* which are capable to transport both betaine and proline (Grallath *et al.,* 2005; Schwacke *et al.,* 1999). In addition, the betaine transporters in model plants have been characterized in some betaine non-accumulators, such as *LeProT1-3* in tomato (Schwacke *et al.,* 1999), *OsProT1* in rice (Igarashi *et al.,* 2000), and *AtProT1–3* in *Arabidopsis* (Rentsch *et al.,* 1996). Moreover, they were characterized also in betaine accumulating plants such as *AmBet/ProT1–2* in *A.marina* (Waditee *et al.,* 2002), *BvBet/ProT1* in sugarbeet (Yamada *et al.,* 2009), and *HvProT1* and *HvProT2* in barley (Ueda *et al.,* 2001; Fujiwara *et al.,* 2010). *HvProT1* from barley was reported to be involved in the transport of proline only, but not betaine (Ueda *et al.,* 2001). However, previous study has suggested that *Bet/ProTs* from dicots are capable of transporting proline as well as betaine (Bregoff & Delwiche, 1955). In monocots, it is not yet reported if *Bet/ProTs* can transport both osmotica betaine and proline. Therefore, the purpose of this study was to provide an evidence as a basis for investigation on the function of betaine transporter gene on abiotic stress. Betaine transporter gene was isolated and cloned from maize (*Zea mays* L.). Further, expression of betaine transporter gene was evaluated under salt and osmotic stress.

Materials and Methods

Plant growth and stress treatment: Sterilized maize seeds were allowed to germinate for 7 days at 25°C. The

seedlings were subjected to Hoagland's nutrient solution and placed in a growth chamber at 25°C maintained at 16 h daily photoperiod with 200 µmol m^{-2} s^{-1} and 60-70 % humidity. The daily dark period was set for 8 h at 22°C and 60-70% humidity. The salt treatment, 0, 150, 200 and 300 mM NaCl in Hoagland's nutrient solution was initiated when the seedlings were at three-leaf stage. However, mannitol treatment was applied using Hoagland's nutrient solution containing 0, 200, 400, 500 and 600 mM mannitol. After 6 h of above treatments, leaves and roots collected from plants were immediately frozen at −80°C for further experiments.

Cloning of *ZmBetProt* cDNA: The total RNA was extracted from the leaves according to acid guanidinium phenol chloroform method (Chomczynski *& Sacchi*, 1987). For determining the quality of RNA in the leaf samples, ethidium bromide (EB) stained agarose gel electrophoresis was performed, and concentrations of RNA was determined spectrophotometrically. Extracted RNA samples were stored at -80°C prior to RACE and RT-PCR analysis. For isolating *ZmBetProt* cDNA, first strand cDNA was synthesized following the manufacturer's protocol using a cDNA synthesis SuperMix (TRANS, Beijing, China). cDNA fragments of the *BetProt* homologous gene were isolated by degenerate primers forward, GGGG TACCATGGACGCCGCCGCCACG and reverse, TCA GGATTTGGTT GGGGTTCT.

5´RACE gene fragments of *ZmBetProt* were obtained using Invitrogen RACE Kit following the prescribed guidelines of the manufacturer and they were further completely sequenced to verify the sequences. In the first round of PCR, AGCCTAGAGGGACCAT was used as the forward primer. Two nested reverse primers, ACGTATGCGC TGTTGACC and GGTTGTGAGGACG AAGCC. 3´-RACE-forward primer, ATCAGGCTACCT CCTCAACAGTGT CACA and 3´-RACE-reverse, TCTGGGTAAAGCGGTTGCAAATCT. PCR product was cloned into pMD18-T vector (TaKaRa) for sequencing.

Bioinformatics analysis: The molecular weight and isoelectric point of *ZmBetProt* were analyzed by using BioXM 2.6 software. The protein secondary structure and tertiary structure were predicted by using GOR IV (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html) and SWISS-MODEL (https://www. swissmodel. expasy. org/), respectively. The transmembrane domains were predicted with the TMHMM Server 2.0 (http://www.cbs. dtu.dk/services/ TMHMM-2.0/). The homology of the full-length cDNA sequence of the *BetProt* cDNA gene was compared with the GenBank database using BLAST (https://blast.ncbi. nlm.nih.gov/Blast.cgi). The DNAMAN software was employed to determine the amino acid sequence. The phylogenetic tree was generated by using software MEGA 5.1 (http://mega.software.informer.com/5.1b/).

Expression profile analysis through quant itative real-time PCR: For comparing the expression pattern of *ZmBetProt* in the roots and leaves at various concentrations of NaCl and mannitol stress treatments. RNA was isolated from the roots and leaves after each treatment. The primers used for RT-PCR were as follows: *ZmBetProt* gene with a pair of specific primers, forward, TCAGGATTTGGTTGGGTTCT and reverse, CTGCGA TGATTTGGGGATG, Actin gene (a housekeeping gene) forward, AACTGCCCAGCAATGTATG and reverse, CATCAGGTTGT CGGTAAGGT. The formula $2^{-\Delta\Delta Ct}$ was used for calculating the relative expression level of the target gene.

Statistical analysis

The Data of three replicates in each parameter were presented as mean ± SD ($p \leq 0.05$). Statistical significance of the treatments was evaluated by Analysis of variance test (ANOVA) performed by mean separation by Duncan's multiple range test using by SPSS 19.0.

Results

Cloning and analysis of the full-length cDNA of *ZmBetProt*: The full-length cDNA sequence of *ZmBetProt* was attained by splicing the sequences of 5'RACE and 3'RACE.The primers for cloning were designed based on the conserved region in *BetProt* genes. 5'RACE and 3'RACE amplification products of betaine transporter gene were shown in (Fig. 1). Sequence analysis indicated that the full-length cDNA of *ZmBetProt* was1538 bp in length and contained 1299 bp ORF encoding a polypeptide of 432 amino acids with a calculated molecular weight of 46.986 kDa. The cDNA sequence of *ZmBetProt* from *Zea mays L.* was submitted to the GenBank with accession number: KX013323.1.

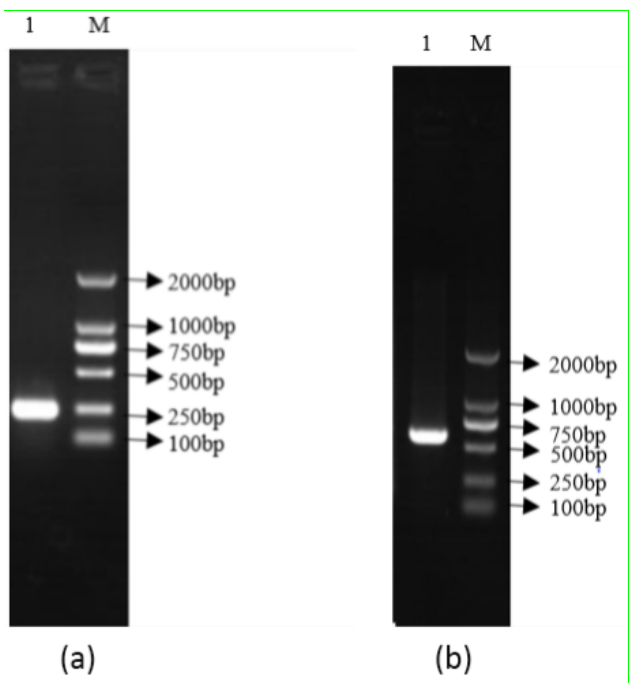


Fig. 1. The electrophoresis results after obtaining 5'sequence and 3'sequence. (a) amplification of 5'RACE. M: DL2000 marker; (b) amplification of 3'RACE. M: DL2000 marker.

Based on amino acid sequence results, protein physicochemical properties, conservative motif, transmembrane domain, helical structure and potential physiological function were predicted by the corresponding multiple bioinformatics approaches. Amino acid sequence analysis showed that in the sequence there existed an obvious transmembrane and a hydrophobic region (Fig. 2a). The number of alpha helixes and beta sheets in the secondary structure were predicted to be 23 and 10, respectively. The spatial structure that *zmBetProt* protein contained (Fig. 2b). Regions of all sequences predicted to be located inside or outside the membrane are shown in blue and pink, respectively. The structural prediction of membrane domains showed that the protein was characterized by10 transmembrane domains (Fig. 2c). The conserved histidine residue, which may be responsible for betaine binding in transport processes.

Alignment analysis of *zmBetProt* with other betaine transporter genes: The amino acid sequences of *zmBetProt* was compared with that of other different species in the GenBank using online protein BLAST (Fig. 3a). Results revealed that *BetProt* amino acid sequences were highly conserved in all species and contained a highly conserved motif. The deduced amino acid sequences of *ZmBetProt* as well as *BetProt* from other species, including *Atriplex hortensis (AhBet/ProT), Beta vulgaris (BvBet/ProT), Arabidopsis thaliana* (*AtProT1-3*), *Lycopersicon esculentum* (*LeProT1-3*), *Elaeis guineensis (EgProT), Oryza sativa* (*OsProT*), *Hordeum vulgare (HvProT)* were presented in (Fig. 3a). A phylogenetic tree was constructed to evaluate the phylogenetic relationship of the *ZmBetProt* from different species. As shown in (Fig. 3b), sequence alignment suggested that the sequence of *ZmBetProt* is a full-length version. *ZmBetProt* showed the highest similarity to proline transporter of *Arabidopsis thaliana (AtProT3)* and the lowest similarity with proline transporter for *Beta vulgaris (BvBet/ProT)* and *Oryza sativa* (*OsProT*).

***ZmBetProt* expression in the roots and leaves after mannitol and saline stress:** Under mannitol stress, the expression of *ZmBetProt* significantly improved in roots at the mannitol levels up to 400 mM, while it did not change at the two higher mannitol levels, 500 and 600 mM (Fig. 4a). While, the expression of *ZmBetProt* was up-regulated at 500 and 600 mM treatments in leaves, which revealed that *ZmBetProt* expression had a tissue-specificity. These results indicated that betaine transporter gene expression could be induced in roots and leaves. Based on these results, it could be deduced that *ZmBetProt* expression were induced dramatically at high concentration of mannitol 400 and 500 mM in root and leaf, respectively. It could be concluded that *ZmBetProt* expression may be involved in the acquisition of plant tolerance to a varying intensity of drought stress. However, no significant difference of betaine transporter gene expression were observed in leaf or root of maize under varying concentrations of NaCl (Fig. 4b).These results revealed that the expression of betaine transporter gene was not induced under salt stress.

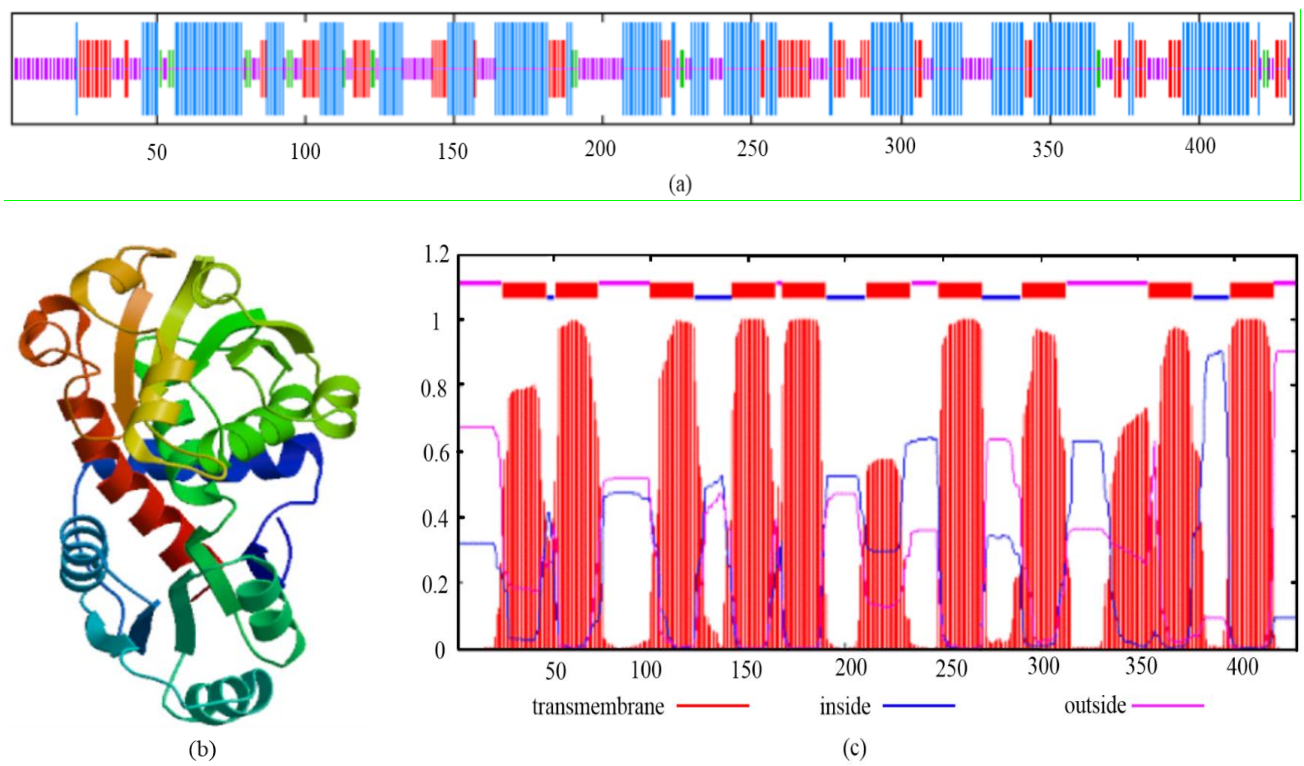
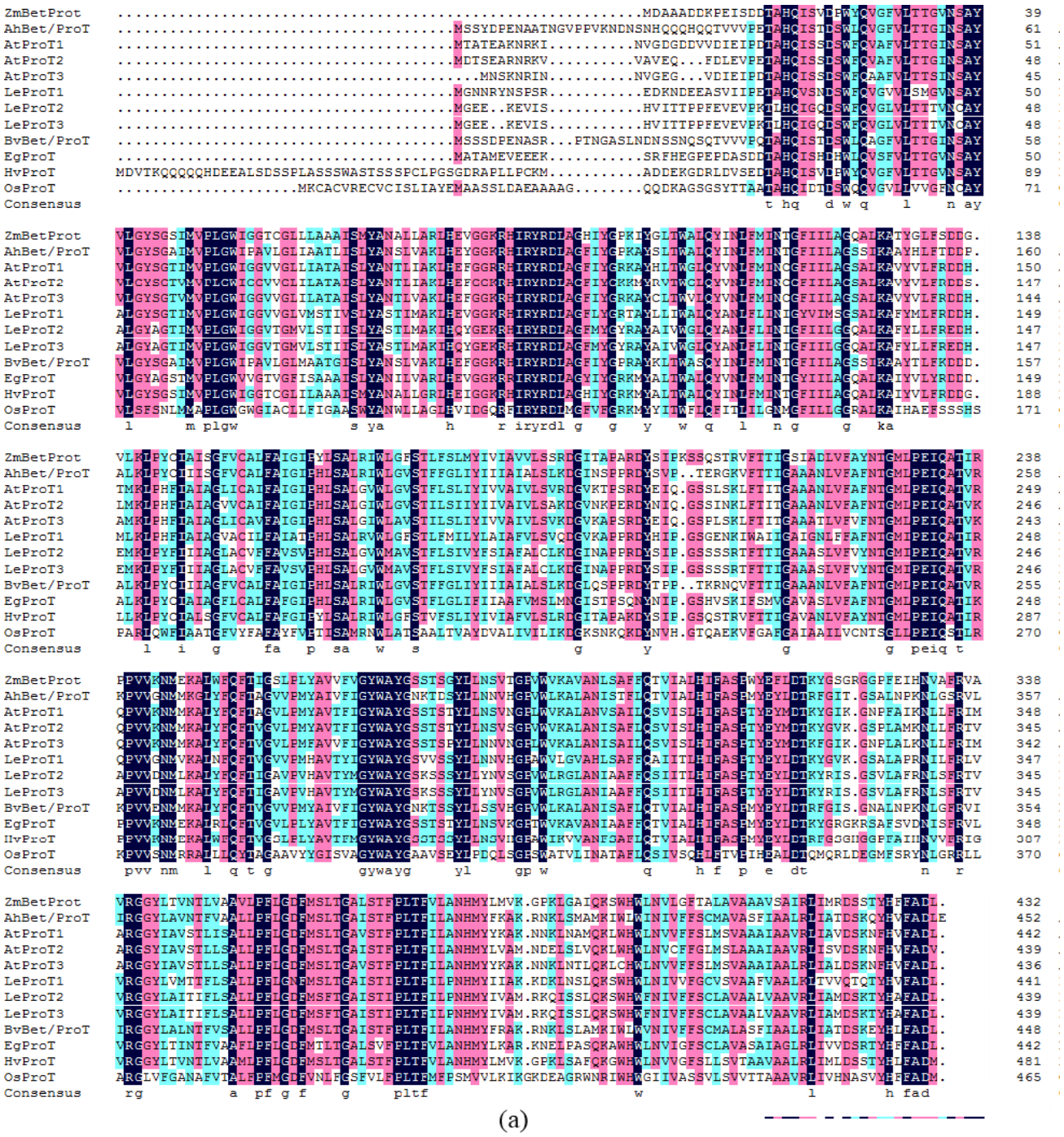
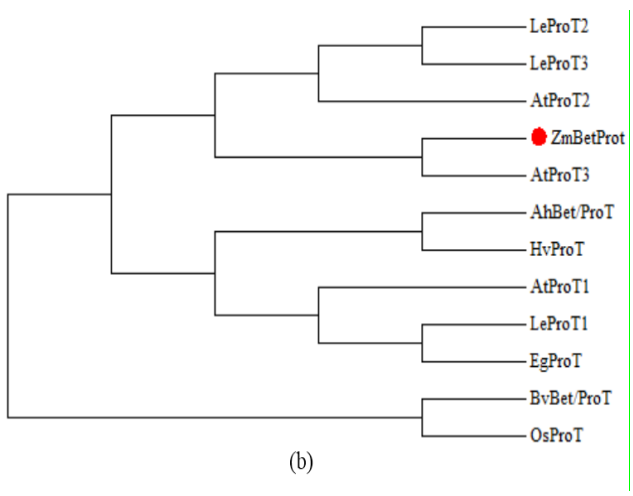


Fig. 2. Predicted secondary and 3D structure of *ZmBetProt*. (a) secondary structure; (b) tertiary structure; (c) the structural predication and membrane domain analysis of ZmBetProt. Blue: α-helix. Purple: Random coil .Red: extended strand.



(a)



(b)

Fig. 3. Sequence alignments and phylogenetic relationships of *ZmBetProt* other betaine transporter gene. (a) the alignment between amino acid sequence of the prediction ORF of target gene and other betaine transporters' amino acid sequences. (b) the alignment of deduced amino acid sequences of Maize. *ZmBetProt*, *Zea mays*(ANG58469.1); *AhBet/ProT* , *Atriplex hortensis* (AAF76897.1); *AtProT1*, *Arabidopsis thaliana* (CAA65052.1); *AtProT2*, *Arabidopsis thaliana* (CAA65053.1); *AtProT3*, *Arabidopsis thaliana* (NP_181198.1); *LeProT1*, *Lycopersicon esculentum* (AAD25160.1); *LeProT2*, *Lycopersicon esculentum* (NP_001233990.1); *LeProT3*, *Lycopersicon esculentum* (NP_001233990.1); *BvBet/ProT*, *Beta vulgaris* (BAH95859.1); *EgProT*, *Elaeis guineensis* (XP_010905371.1); *HvProT*, *Hordeum vulgare* (BAK03150.1); *OsProT*, *Oryza sativa* (XP_015616483.1).

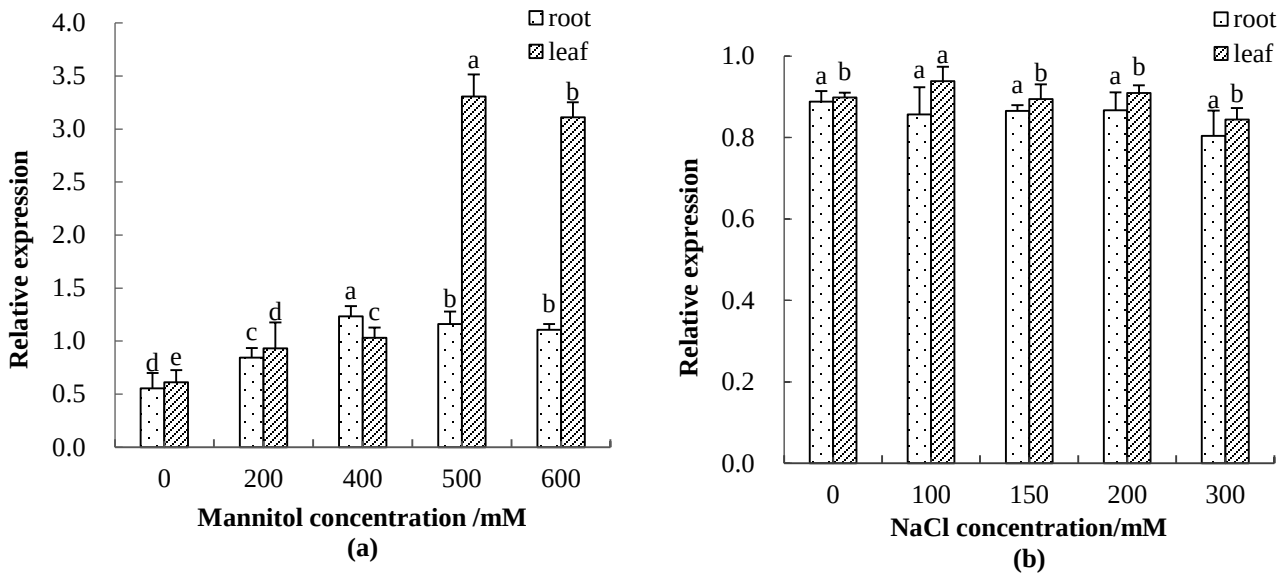


Fig. 4. Analysis of relative expression for *ZmBetProt* in roots and leaves under (a) mannitol stress and (b) salt stress. The data are expressed as the means of three replicates and error bars represent the standard deviation.

Discussion

Salt and drought stress are the vital restrictions to obtain high maize yield and quality. Although maize is generally considered as sensitive crop to both drought and salt stress (Sun *et al.,* 2015), it strives to resist to a certain degree of a stress using various metabolic processes. Plants including maize subjected to water stress essentially require some adaptive mechanisms for keeping their cells and tissues functional under water deficit conditions (Setter & Flannigan, 2001). One such adaptive mechanism to prevent cellular desiccation is to abundantly accumulate low molecular weight organic osmolytes such as proline and glycine betaine, which can effectively play an effective role in balancing cellular osmolarity (Takabe, 2012). Betaine is a major osmoprotectant in plants, animals, bacteria and algae (Rhodes & Hanson, 1993). In plants, the main site of betaine synthesis is the chloroplast where in choline undergoes a two-step oxidation forming betaine, which in turn is subjected to a long-distance transport with the plant exposed to stressful environments (Lamark *et al.*, 1996; Rathinasabapathi *et al.,*1997). However, no much information exists in literature on the betaine transport, because so far betaine transporter genes are not well characterized in plants. In this study, one betaine transporter gene was isolated from maize seedlings after salt stress and mannitol stress (Figs. 1 and 2). The levels of *ZmBetProt* expression were up-regulated at high concentration (500 and 600) mM in leaves, whereas, the *ZmBetProt* expression was up-regulated at concentration of 400 mM in roots under mannitol stress (Fig. 4a). It would have been interesting to clarify the *ZmBetProt* expression can be induced at mannitol stress. From the results presented here, it is amply clear that *ZmBetProt* may play a critical role in plants under drought stress conditions to maintain the osmotic pressure and could be potentially involved in growth and development. *ZmBetProt* gene was isolated from the cDNA libraries constructed from the leaf samples. It was of considerable value to appraise the role of betaine transporter in osmotic (mannitol) stress tolerance. Undoubtedly, more research is needed to fully elucidate the mechanism of betaine transport in maize. Therefore, it could be of great value to incorporate betaine transporter gene as well as the genes involved in betaine synthesis to improve the resistance of plants against environmental cues. Under salt stress, the support was achieved by proline mediated by salt inducible *HvProT1*. The expression patterns of *HvProT2* is unique, since expression of other *ProT* genes, such as *HvProT1*, *AtProT2*, *BvBet/ProT1*, *AmBet/ProT1-3* is clearly induced by salt stress (Waditee *et al.,* 2002; Rentsch *et al.,* 1996; Ueda *et al.,* 2001). Accumulation of betaine in plants is considered as one of the potential strategies of counteracting the injurious effects of saline environments (Fujiwara *et al.,* 2010). Although it is widely known that barley (a salt tolerant plant) is capable of accumulating large amount of betaine in response to saline conditions (Siththisarn *et al.,* 2009), the mechanism of betaine transport is not fully elucidated. However, our findings clearly demonstrated that a maize betaine transporter gene was not induced by salt stress (Fig. 4b), while it was induced by mannitol stress. These results were similar to a previous study where Chaum *et al.,* (2010a & 2010b) found that proline transporter gene expression in oil palm increased up-regulation abiotic stresses such as salt and drought. The different expression of betaine transporter gene under saline stress and drought stress might be due to the active involvement of toxic ions, i.e., Na^+ and Cl^- in the signal transduction pathway under saline stress, which might have hindered the expression of the gene. This argument can be supported by Ressl *et al.,* (2009) who studied the expression of various genes in sunflower with different levels of tolerance to drought and salt stresses, discovered that some of the genes significantly responded to both stresses while others were regulated

differently under different stresses, which was ascribed to the nature of a stress or plant tissue. Our study confirms the link between the expression of *ZmBetProt* gene and tolerance to abiotic stress (salt and drought), which suggests that *ZmBetProt* gene might play a crucial role in inducing tolerance to drought and salt stresses in maize and perhaps other crops. Overall, these findings provide evidence that *ZmBetProt* gene transporter was involved in abiotic stress resistance of maize.

Conclusion

In the present study, a full-length cDNA of *ZmBetProt* was cloned from maize (*Zea mays* L.). Different tissues revealed differential expression profiles of *ZmBetProt* genes in response to abiotic stresses (salt and drought), which suggested that *ZmBetProt* might play an important role in betaine transport in maize plants exposed to stress. The present study indicated that the expression of *ZmBetProt* gene as a functional betaine transporter helped to reduce the harmful effects of abiotic stresses on maize seedlings. This novel finding will help to better understand the functional diversity of *ZmBetProt* gene transporter under abiotic stress and exploring genes of interest for genetic improvement of betaine in maize.

Acknowledgments

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