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Salt tolerance of potato genetically engineered with the *Atriplex canescens* *BADH* gene

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Abstract

Potato (*Solanum tuberosum* L.) is among the top staple foods in the world, and salinity adversely affects its yield and quality. To improve salt tolerance in potato, the present study is focused on the *Agrobacterium*-mediated transformation of potato by the *Atriplex canescens* betaine aldehyde dehydrogenase (*BADH*) gene driven by single, double, and triple CaMV 35S promoters. The study led first to the detection of seven lines containing the *BADH* gene followed by the identification of T-DNA insertions *via* DNA hybridization and enzyme-linked immunosorbent assays. The salt tolerance was found to be promoter dependent, as the lines with triple promoters showed a higher resistance than those transformed with single and double promoters. The transgenic lines showed lower content of H₂O₂ and malondialdehyde and a lower relative electrical conductivity than wild-type plants. Furthermore, these lines also showed higher proline and chlorophyll content. *In silico* analysis confirmed that the *A. canescens* *BADH* protein had a remarkable tendency to interact with sodium ions and water molecules like other *BADH* proteins. Taken together, the overexpression of *BADH* under triple CaMV 35S promoters enhanced salt tolerance of potato.

Additional key words: *Atriplex canescens*, CaMV 35S promoter, chlorophyll, malondialdehyde, proline, *Solanum tuberosum*.

Introduction

Potato is ranked high among the leading staple foods in the world. Cultivated potatoes are susceptible to various biotic and abiotic stresses (Axel *et al.* 2012). Among abiotic stresses, salt stress is the most lethal one and causes enormous economic loss worldwide (Sudharsan *et al.* 2012, Levy and Tai 2013). The incorporation of resistant genes in the host plant and organized breeding are the most effective approaches to developing new lines with increased salt tolerance and higher yield (Krivosheeva *et al.* 2014). To protect and maintain cellular metabolism and cellular components, plants use different mechanisms (Jbir-Koubaa *et al.* 2014). Osmoprotectant accumulation is the most common mechanism against salinity stress in plants (Si *et al.* 2012).

Glycinebetaine is a bipolar quaternary ammonium compound. It can help to maintain osmotic balance with the environment and to stabilize complex quaternary

structure of proteins; thus it protects cells against salt stress (Giri 2011). In plants, glycinebetaine is synthesized in two steps: conversion of choline to betaine aldehyde and then betaine aldehyde to glycine betaine (Mitsuya *et al.* 2011, Rajaeian *et al.* 2017). Betaine aldehyde dehydrogenase (*BADH*) is the enzyme that catalyzes the second step in the synthesis of glycine betaine. The *BADH* gene has been cloned from many species, such as *Spinacia oleracea* (Luo *et al.* 2012), *Atriplex hortensis* (Xiao *et al.* 2013), *Sorghum bicolor* (Wood *et al.* 2013), *Avicennia marina* (Díaz-Sánchez *et al.* 2012), and *Beta vulgaris* (Luo *et al.*, 2012). *BADH* is a homodimer of nuclear-encoded subunits and has a molecular mass of 60 kDa (Rathinasabapathi *et al.* 1997). Betaine aldehyde dehydrogenase (*BADH*) belongs to the aldehyde dehydrogenase super family (Yu *et al.* 2017).

There is a wide diversity in the structure of enzymes with *BADH* activity; however, in the majority of plants *BADH* is a dimeric protein (Weretilnyk and Hanson 1989).

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Abbreviations: *BADH* - betaine aldehyde dehydrogenase; *MDA* - malondialdehyde; *MS* - Murashige and Skoog; *REC* - relative electrical conductivity; *SIM* - sulfide indole motility.

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The conversion of betaine aldehyde to glycine betaine catalyzed by BADH is dependent on the oxidation of the cofactors NAD⁺ or NADP⁺ (Fujiwara *et al.* 2008) which predominantly accumulate in the leaves and stems of a diverse range of dicotyledons and some monocotyledons (Ishitani *et al.* 1993). Plant abiotic stress resistance is often mediated by increasing BADH activity (Nakamura *et al.* 2015). The presence of different *BADH* genes has been reported in plants and they show minor sequence differences. However, some plant species (tomato, *Arabidopsis thaliana*, tobacco, and potato) accumulate low amounts of glycinebetaine. We used *BADH* gene from *Atriplex canescens* because it has been well documented that this species provides better salt tolerance compared with many other species, and this *BADH* also has promoter-dependent activity (Jia *et al.* 2002).

The current research was designed with the following five objectives: 1) to induce salt resistance through incorporating the *BADH* gene into potato lines; 2) to investigate the glycinebetaine biosynthesis pathway; 3) to assess promoter efficiency in enhancing salt tolerance; 4) to check the frequency of the *BADH* gene, followed by T-DNA insertion identification, DNA hybridization, and enzyme-linked immunosorbent assays; and 5) to monitor the *BADH*-induced biosynthesis of glycinebetaine in transgenic plants using single, double, and triple *CAMV 35S* promoters and correlate promoter frequency with salt tolerance.

Materials and methods

Plants: *Atriplex canescens* Nutt. (salt bush) plants were collected from district Muzaffargarh, South Punjab, Pakistan. The potato (*Solanum tuberosum* L. cv. AGB-Red) tubers were collected from district Lahore, Central Punjab, Pakistan. For experiments we used the wild-type lines (WT1, WT2, WT5, WT7, WT8, WT9, and WT10) and transgenic plants with inserted *BADH* gene under *CAMV 35S* promoter (lines T1, T5a, T5b, T5c, T7a, T7b, T7c, T9, and T10) (Figs. 1 and 2 Suppl.).

Extraction of DNA, cDNA synthesis, and PCR: Genomic DNA from *A. canescens* was isolated using a DNA isolation kit (Fermentas, Burlington, Canada), and cDNA was synthesized using a *Super-Script III* kit (Invitrogen, Carlsbad, USA). PCR was performed using 1 μ m³ of cDNA (100 ng) in a final volume of 30 μ m³ along with 10 pmol of each primer, 0.25 mM dNTP, and 1 μ m³ of Taq polymerase with its respective buffer. The PCR was performed with up to 40 cycles according to the following

program: denaturation at 95 °C for 1 min and again at 94 °C for 2 min, annealing at 45 °C for 1 min, extension at 72 °C for 1.5 min and a final extension at 72 °C for 10 min.

Gene construct and genetic transformation of potato tubers: Total cDNA from *Atriplex* was used for full-length amplification of the *BADH* gene (1.5 kb) according to the procedure of Zhang and Shi (2013). The gene was positioned successively under the control of triple, double, and single *CAMV 35S* promoters in the *pCAMBIA1301* plant expression vector under *Hind*111, *Bam*H1, and *Kpn*1 restriction sites, respectively, and confirmed *via* restriction digestion with the aforementioned restriction enzymes (Fig. 1). The constructs were transformed into *Solanum tuberosum* *via* the *Agrobacterium*-mediated transformation method. The surface sterilization of potato tubers was performed using 0.2 % (m/v) HgCl₂ for 20 s and 75 % (v/v) ethanol for 5 min, followed by rinsing with distilled water ten times. After rinsing, the tubers were kept on filter paper overnight to dry. Murashige and Skoog (1962; MS) medium was used for the germination of sterilized seeds under a 18-h photoperiod and a temperature of 25 °C. After 2 weeks, nodes were again precultured in the dark and 25 °C on MS and callus induction medium (pH 5.8) for 2 d. The medium was supplemented with 3 % (m/v) sucrose, 2 g dm⁻³ casein acid, 4.0 mg dm⁻³ hydrolysate, and 1.0 mg dm⁻³ 6-furfuryl-aminopurine (kinetin) and 2,4-dichlorophenoxyacetic acid (2,4-D). *Agrobacterium tumefaciens* was grown in YEP medium (pH 7.2; 5 g dm⁻³ NaCl, 10 g dm⁻³ yeast extract, 10 g dm⁻³ peptone, 2 mM MgCl₂) supplemented with 50 mg dm⁻³ rifampicin and 50 mg dm⁻³ kanamycin. The murky medium was subjected to continuous agitation at 25 °C until the *Agrobacterium* attained an absorbance (A₆₀₀) of 0.8. The *Agrobacterium* suspension was collected by centrifugation (1 000 g for 10 min) and was re-suspended in a Schenk and Hildebrand liquid medium [0.1 MS medium supplemented with 1 % (m/v) glucose, 4 % (m/v) sucrose, 0.1 mg dm⁻³ 6-benzylaminopurine, 5 mg cm⁻³ 2,4 D] to absorbance at 600 nm of 0.8 was achieved. Explants remained in the *Agrobacterium* suspension for 30 min in the dark. Afterward, the explants were blotted on filter paper and then cultivated in callus induction medium that was supplemented with both 50 mg cm⁻³ kanamycin and 100 mg cm⁻³ cefotaxime for 2 weeks. After two weeks, the resistant explants were transported to drug-free MS medium.

Identification of transgenic plants *via* PCR and reverse transcription PCR: Transgenic and wild-type potato plants were used for DNA isolation; total genomic DNA

Fig. 1. Construction of plant expression vector carrying the betaine aldehyde dehydrogenase (*BADH*) gene. LB - left border, RB -right border, 35S - cauliflower mosaic virus 35S promoter, Nos - nopaline synthase terminator.

was isolated using a *K0513* DNA isolation kit (*Fermentas*, Waltham, MA, USA). Integration was confirmed using the following full-length primers: *BADH*-forward, ATGGCGTTTCCTATTCTGTTC, and *BADH*-reverse, TTAAGGAGACTTGTACCAGCC. A 1.5-kb product was revealed and separated on 0.8 % (m/v) agarose gel. Ribonucleic acid was extracted using a *GeneJET* plant RNA purification kit *K0801* (*Ambion*, Vilnius, Lithuania), and complementary DNA was synthesized using a *Super-Script III* kit (*Invitrogen*, Waltham, MA, USA). First-strand cDNA was synthesized from 1 µg of total RNA using reverse transcriptase. Of that, 2 mm³ was used as a template in a total reaction volume of 25 mm³ along with a *SYBR Green PCR Master Mix* reaction mixture (*Applied Biosystems*, Waltham, MA, USA). The expression of *BADH* transcripts was assessed using primers (*BADH*-SF, GTTAGCTGGTGCCGTGTTT, *BADH*-SR, ATACCCCATCCCCAAGTTC) under the following conditions: denaturation at 94 °C for 3 min, 94 °C for 30 s, annealing at 55 °C for 45 s, and 72 °C for 1 min. This was followed by a final cycle at 72 °C for 10 min to allow the completion of polymerization. *Actin* was used as an internal control using primers (*Actin*-F: 5'-ATGTTCCCGGGTATTGCTGACAGA-3' and *Actin*-R: 5'-CTGCCTTTGCAATCCACATCTGCT-3').

Hybridization of DNA: The presence of the *BADH* gene in the potato genome was confirmed through DNA hybridization. Gene-specific probes were used for binding and were detected using their proper substrates. By hybridization of DNA on nitrocellulose membrane (*Fermentas Biotin DecaLable*TM DNA labeling kit), biotin-labeled *BADH* probe was estimated. Genomic DNA was digested with *Bam*H1 and *Kpn*1 restriction enzymes and then transformed on to a *Hybond-N*⁺ nylon membrane after depurination and neutralization. To fix DNA, the membrane was exposed to UV radiation for 2 - 3 min. Diluted 20 mm³ of streptavidin-alkaline phosphatase conjugate was applied to the membrane for 30 min after removing blocking solution. The membrane was washed twice with 1× blocking/washing buffer. After removal of washing buffer, the membrane was treated with detection buffer for half an hour. Enzymatic reaction was performed overnight by applying freshly prepared 1 × 5-bromo-4-chloro-3-indolyl phosphate (BCIP) / nitro blue tetrazolium (NBT) in the dark.

Relative electrical conductivity assay and *BADH* activity of transgenes: After multiplication and rooting on MS medium with NaCl and a sulfide indole motility (SIM) medium (Jia *et al.* 2002), the transgenic potato plants were transplanted into pots and watered with MS solution containing NaCl at 0 mM and 180 mM MS medium. The SIM medium was designed for the detection of sulfide production, indole formation, and motility of *Agrobacterium*. The medium contained ferrous ammonium sulfate and sodium thiosulfate, which together serve as an indicator for the production of hydrogen sulfide (H₂S). The hydrogen sulfide production was detected when ferrous sulfide, a black precipitate, was produced due to

the result of ferrous ammonium sulfate reacting with H₂S. Casein peptone, another component of the SIM medium, is rich in tryptophan. The organism that possesses the enzyme tryptophanase degrades tryptophan in to indole. Indole is usually detected upon the addition of Kovacs reagent (Hemraj *et al.* 2013) to the incubation medium. Indole combines with *p*-dimethylaminobenzaldehyde and produces a red band at the top of the medium. A negative indole test produces no color change upon the addition of Kovacs Reagent. The small amount of agar added to the medium provides a semi-solid structure allowing for the detection of bacterial motility. Motile organisms extend from the stab line and produce turbidity or cloudiness throughout the medium. Non-motile organisms grow only along the stab line and leave the surrounding medium clear. The potato plants were watered with MS nutritional solution until they reached the 4-to-5 leaf stage. The third leaf from the top was used for relative electrical conductivity (REC) and *BADH* activity assays. Following the procedure of Guo *et al.* (1997), *BADH* activity was measured. Consuming 1 nM NAD per minute in the reaction volume is defined as 1 unit of *BADH* activity. Each measurement was the average of three replicates. A *Thermo Scientific Orion StarA112* (*Thermo Electron LED*, Illkirch Cedex, France) conductivity benchtop meter was used for REC testing. The transgenic lines and controls were stressed in 0, 90, 180 or 270 mM NaCl solution for 24 h. Immediately after stress treatments, the content of chlorophyll and malondialdehyde (MDA) was measured according to the procedure of Zhang and Shi (2013).

Proline content assay: Proline content was measured according to the procedure suggested by Bates *et al.* (1973). After homogenizing, 3 % (m/v) sulfosalicylic acid (3 cm³) was used for digesting ~150 mg of leaves at 100 °C for 25 min. The mixture was then centrifuged at 3 000 g for 5 min and 2 mm³ of glacial acetic acid and 3 cm³ of ninhydrin were added to the 2 cm³ of supernatant. Samples were extracted with 5 cm³ of toluene after boiling for 40 min. Proline content was calculated from a standard curve after determining the absorbance at 520 nm.

Determination of glycine betaine: Glycine betaine content was measured according to Xing and Rajashekar (2001). Liquid nitrogen-freeze-dried samples of potato tubers were ground to a powder and incubated in redistilled water at 4 °C for 24 h. Potato leaves were frozen in liquid nitrogen and thawed for expressing sap using a glass injector. The extracts of leaf samples and potato powders were centrifuged at 12 500 g and 4 °C for 12 min, and the supernatant was dried under a stream of nitrogen. The dried fractions were dissolved with D₂O, in which 1 mM sodium dodecyl sulfate (SDS) acted as the internal reference for quantification, and ¹H spectra were determined using a *Bruker AM500* NMR spectrometer (Beijing, China).

Protein analysis: The *BADH* protein content was detected by an enzyme linked immunosorbent assay *ELISA* which is an accurate, sensitive, and a rapid detection method for proteins. It is especially an effective method when large

numbers of plant samples have to be assayed, and when results for protein test are needed rapidly even when the suitable indicator like plants and/or greenhouse facilities are even not available. For the detection of our BADH proteins *ELISA* was used as described by Wood *et al.* (2013).

In silico analysis: The *ExPASy* translate tool (Gasteiger *et al.* 2003) was used to determine the amino acid sequence of the *BADH* gene. This sequence was further used to predict the 3-D model for the *BADH* protein using *MODELLER* (Pieper *et al.* 2006) and viewed with *Chimera* (Hou and Bartels 2014). The predicted structure was verified using various analyses, *e.g.*, *ERRAT* (<http://servicesn.mbi.ucla.edu/ERRAT/>), *PROVE* (<http://servicesn.mbi.ucla.edu/PROVE/>), *WHATCHECK* (<https://servicesn.mbi.ucla.edu/WHATCHECK/>), *VERIFY3D* (<http://servicesn.mbi.ucla.edu/Verify3D/>), and Ramachandran plots (<http://mordred.bioc.cam.ac.uk/~rapper/rampage.php>), and the 2-D structure annotation was calculated using the protein data bank (*PDB*) database (<http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/GetPage.pl?pdbcode=index.html>). The subcellular localization of the *BADH* protein was carried out *in silico* using multiple online protein localization prediction programs to eliminate the possibility of any false positive predictions. The tools used for this analysis included *Prot Comp v. 9.0* (<http://linux1.softberry.com/berry.phtml>), *MultiLoc2* (Zhang *et al.* 2014), *CELLO v. 2.5* (Yu *et al.* 2006), and *Euk-mPLoc 2.0* (Chou and Shen 2007). Additionally, the theoretical pI and Mr values were computed using the *Expassy Compute pI/Mw* tool (web.expasy.org/compute_pi/). The *3D-BLAST* was performed using the *BioXGEM 3D-Blast* protein structure search, available at <http://3d-blast.life.nctu.edu.tw/>, to identify the most functionally related protein. *NCBI Genome Workbench v 2.9.0* was used to build alignment with *Muscle* of different *BADH* sequences of various species. These sequences were retrieved from *NCBI* protein database using domain enhanced lookup time accelerated *BLAST*. A neighbor-joining tree was built using the Poisson algorithm.

Results

After the amplification of *Atriplex* cDNA, *BADH* was ligated into the pCAMBIA1301 plant expression vector under single, double, and triple CaMV 35S promoters, and their ligation was confirmed through restriction digestion with *Hind*III, *Bam*HI, and *Kpn*I restriction enzymes, as shown in Fig. 2B. The construct was transformed in potato, and the putative transgenic shoots were sorted out using PCR with the gene-specific primers mentioned above. To test the expression of the *BADH* gene in transgenic potato (Figs 1 and 2 Suppl.), reverse transcription PCR was performed with T2 transgenic and wild-type plants (Fig. 6). Different *BADH* expressions were observed in T2 *BADH* transgenic lines with single, double, and triple CaMV 35S promoters. Two lines (5 and 7) were positive for three constructs and were tested under all the three promoter constructs. Potential transgenic shoots were surface-sterilized to eliminate false positives arising from consistent *Agrobacterium* and transferred to a cefotaxime sodium-free SIM medium. No *Agrobacterium* appeared after 10 - 12 d. PCR analysis detected a 1.5-kb band in all 10 shoots obtained from the SRM medium (NaCl rich MS medium), while the wild type, which was used as control, did not show this band (Fig. 5A). The incorporation of the *BADH* gene in transgenic potato plants was confirmed through DNA hybridization. After binding, the probes were detected using proper substrates. Genomic DNA was digested using *Bam*HI and *Kpn*I restriction enzymes. Binding with gene-specific probes and the detection of a 1.5-kb gene together confirmed the successful integration of the *BADH* gene in transgenic potato plants, as shown in Fig 5B.

An ELISA was used to evaluate *BADH* protein content in transgenic potato plants (Wood *et al.* 2013). Lines 5 and 7 were also subjected to ELISA with three different construct-isolated proteins. Total crude protein was isolated from other transgenic potato lines. The colour development and detection of the *BADH* protein, after applying gene-specific antibodies from *Sigma Life*

Fig. 2. *A* - Amplification by PCR of the *betaine aldehyde dehydrogenase (BADH)* from genomic cDNA of *Atriplex*. Lane 1 - 1-kb molecular mass marker, lane 2 - wild-type plants (without DNA), lanes 3—6 - *BADH*-positive samples. *B* - Construct confirmation via restriction digestions. 1 - 1-kb ladder, 2 - complete cassette (pCAMBIA1301 + Triple CaMV 35S promoter + *betaine aldehyde dehydrogenase*), 3 - cassette digested with *Hind*III and *Kpn*I restriction enzymes contained in the pCAMBIA1301 vector (above) and triple promoter + the *BADH* (below), 4 - cassette digested with *Bam*HI and *Kpn*I containing only *BADH*.

Fig. 3. Salt tolerance of potato transgenic lines. *A* - transgenic lines rooted normally in Murashige and Skoog medium containing 90 mM NaCl; *B* - wild-type plants, which could form only a swelling such as a callus mass.

Science (Saint Louis, MO, USA), were performed using a 5-bromo-4-chloro-3-indolyl phosphate (BCIP) / nitro blue tetrazolium (NBT) substrate. The content of *BADH* protein was from 0.255 to 0.742 $\mu\text{g g}^{-1}(\text{f.m.})$ (Fig. 3 Suppl.).

To test whether H_2O_2 accumulation was affected by increased activity of *BADH*, the H_2O_2 content was measured in both *BADH* transgenic lines and wild type plants. After salt stress, H_2O_2 content increased by 3.6 to 4-fold in control plants and by 1.7 to 2-fold in *BADH* transgenic lines. Lines 5 and 7 had lower *BADH* activity under the triple CaMV 35S promoter than under double and single promoter (Fig. 5 Suppl.). Higher chlorophyll content was observed in *BADH* transgenic lines compared to that in wild type plants (Fig. 5 Suppl. and Table 7 Suppl.).

The proline content increased with increasing NaCl stress in both *BADH* transgenic and wild type plants compared to untreated plants (Fig. 6 Suppl.). The accumulation of proline was higher in transgenic plants compared to that in wild type plants. Malondialdehyde content also increased with increasing salt stress. The MDA content was higher in *BADH* transgenic plants,

especially in lines 5 and 7, than in other transgenic lines and wild type plants (see Fig. 6 Suppl. and Table 7 Suppl.), respectively.

The T3 generation of the elite transgenic potato inbred line AGB-Red was used for the measurement of glycine betaine content in potato tubers and leaves by proton nuclear magnetic resonance ($^1\text{H-NMR}$) spectroscopy. Glycinebetaine content in transgenic lines with *BADH* gene under a single promoter was 2 to 2.5-fold higher compared with that of the wild type and 3 to 3.5-fold higher under the double promoter, and 4 to 4.5-fold higher under the triple promoter.

The 3-D structure of the *BADH* protein was predicted and validated using multiple *PDB* validation tools (Fig. 7 Suppl.). *ERRAT* analysis predicted an overall quality factor of 81.504. *VERIFY3D* tool results showed that 83.83 % of the residues had an average 3D-1D score ≥ 0.2 . The Ramachandran plot showed that, of the 501 residues, almost 100 % of phi and psi bond angles of the residues were in the favourable region, whereas those of only 1 residue were in the unfavourable region (Fig. 7 Suppl.). The 2-D structure annotation for helices, sheets, turns, and loops was determined using the *PDB* sum generation tool (Fig. 8 Suppl.). Furthermore, *BADH* was found to be a multilocalized protein as predicted by various computational tools that use different strategies for predicting protein localization. The *BADH* protein was found to be present mainly in the cytoplasm and to a lesser extent in peroxisomes and mitochondria. The theoretical pI and Mr values were 5.03 and 123614.45 Da. A protein *3D-Blast* structure similarity search found that our queried protein was 83.0 % identical to the crystal structure of aminoaldehyde dehydrogenase 2 from *Pisum sativum* (psamadh2) and 67.3 % identical to the crystal structure of aminoaldehyde dehydrogenase 1 from *Pisum sativum* (psamadh1). Finally, we analyzed the phylogenetic lineage of the *BADH* protein by building phylogenetic tree. A total of 72 sequences, having an identity of more than 70 %, were retrieved from *NCBI*. These sequences included flowering plants and comprised both eudicots and monocots (Fig. 9 Suppl.).

Based on Tables 1–6 Suppl., significant differences

Fig. 4. Comparison of glycine betaine content in control (wild-type) plants and betaine aldehyde dehydrogenase (*BADH*) transgenic potato plants. Means $\pm$ SEs of three experiments; different lowercase letters indicate significant differences (the Tukey's test, $P < 0.05$).

Fig. 5. *A* - Molecular assay of transformed potato lines. M - 1-kb DNA ladder, N - wild-type plant, 1—10 - independent transgenic lines. *B* - DNA hybridization with biotin-labeled *betaine aldehyde dehydrogenase* (*BADH*) probes; genomic DNA was digested with *Bam*HI and *Kpn*I restriction enzymes; N - wild-type plant, 1,5,2,7,8,9,10 - PCR-positive transgenic lines. *C* - Enzyme-linked immunosorbent assay with *BADH* gene-specific antibodies, N - wild-type plant 1,5(a,b,c), 2,7(a,b,c),8,9,10 - independent transgenic lines.

Fig. 6: Reverse transcription PCR analysis of *betaine aldehyde dehydrogenase* (*BADH*) expression in transgenic potato plants. N - wild-type plants, 1—10 - transgenic plants; 5a and 7a contain the triple *CaMV* 35S promoter; 5c and 7c have a single *CaMV* 35S promoter; the others have the double *CaMV* 35S promoter.

were observed among salt stress treatments, transgenic plants, and interactions between stress treatments and transgenic plants for all studied traits. A strong and significant correlation was found between all studied traits except REC with MDA, H_2O_2 , and proline. Experimental data show that due to the increased salt stress, the concentration of H_2O_2 increased, which caused a reduction in chlorophyll content. Activity of *BADH* increased proline, chlorophyll and MDA content while decreased H_2O_2 content (Table 8 Suppl.). The results show *BADH* activity positively influenced MDA, chlorophyll, and proline production (Table 9 Suppl.). The regression equation was calculated as Y (*BADH* activity) = $41.999 + 0.2402$ (MDA) $- 0.2979$ (REC) $+ 5.0961$ (chlorophyll contents) $- 0.00289$ (H_2O_2) $+ 0.46527$ (proline).

Discussion

Salinity stress is a major problem for potato and other

crops as it limits their yield (Boyer 2004). To provide salt tolerance and to identify quantitative trait loci with associated markers to enhance salt tolerance and to improve yield and quality, extensive rounds of breeding are needed (Foolad 1999). For maintaining optimum cellular metabolism and preventing cellular damage to cellular components, plants employ a number of protection mechanisms (Wood and Lenne 1997). The accumulation of osmoprotectants is the most common metabolic adaptation against salinity stress, and among these osmoprotectants proline and glycinebetaine are very important (Rhodes and Hanson 1993).

Potato does not possess a glycinebetaine metabolic synthesis pathway and so it is a weak or non-accumulator of glycinebetaine (Sakamoto and Murata 2000). Therefore, in engineered potatoes, the accumulation of stress-induced glycinebetaine can be essential (Rathinasabapathi 2000). It has been reported that the amount of *BADH* transcripts increases 2-fold in salt-stressed spinach and 3- and 4-fold in the taproots and leaves of sugar beet, respectively (Ma *et al.* 2012, Zhu *et al.* 2014). It has also been reported that *BADH* mRNA content increases up to 8-fold in barley leaves when exposed to salt, drought, and water stress, and the content decreased when the stresses are reduced (Saneoka *et al.* 2001). In salt-stressed plants, the accumulation of betaine is regulated by changes in betaine biosynthetic pathway genes, and the stress response of the *BADH* gene occurs via cis-regulatory elements (Ghosh *et al.* 2015).

In the current investigation, *BADH* was firstly subjected to an *in silico* analysis revealing this protein was structurally homologous to the queried protein (*BADH*) playing a vital role in salt tolerance of various plants.

Both plant aminoaldehyde dehydrogenases-2 and plant aminoaldehyde dehydrogenases-1 have been reported to interact with sodium-isolated plant aminoaldehyde dehydrogenases-2, which is comparatively 83 % identical to the *BADH* protein and contains 2 chains (A and B), 2 Na⁺, 7 glycerol molecules, 290 water molecules, and the ADP parts of two NAD⁺ molecules (Tylichova *et al.* 2010). This protein is present mainly in the cytoplasm, as predicted by most of the protein localization prediction tools. It can interact with sodium ions and prevent the outflow of water from inside the cell. A phylogenetic tree was computed using 72 proteins with similar sequences from 11 monocot and 61 eudicot plants.

It was found that salt stress tolerant *BADH* gene could be amplified with gene specific primers and successfully cloned in adenine and thymine plant expression vectors (Fig. 2A) (Kiani *et al.* 2013). The *BADH* gene was introduced into potato plants with plant expression vector pCambia 1301 under triple, double, and single CaMV 35S promoters to enhance the constitutive expression (Jia *et al.* 2002, Kiani *et al.* 2013, Rao *et al.* 2013) (Fig 2B). Its integration into the potato genome was confirmed *via* DNA hybridization. *BADH*-positive plants were subjected to DNA hybridization analysis with biotin-labeled gene-specific probes, and the results were found to be remarkably consistent with the PCR results, as shown in Fig. 5A,B. No matter whether salt stress was present or not, transcripts of *BADH* gene were detectable, but when subjected to *ELISA*, the expression patterns were different under single, double, and triple CaMV 35S promoters (Fig. 5C) (Rao *et al.* 2013, Muzaffar *et al.* 2015). This phenomenon could be due to the difference between the insertion points throughout the genome of each transformant (Guo *et al.* 1997, Liu *et al.* 2011, 2013, Van Leeuwen *et al.* 2014). Regarding the destructive effects of excess ROS on plant growth and development, they contributed to the adverse effects of environmental stresses. By using ascorbate as reducing power, it is known that a scavenger (superoxide dismutase) of superoxide radicals produce H₂O₂ and that is eliminated and broken down directly to form water and oxygen *via* *BADH* (Babakhani *et al.* 2013).

The functions of whole cells and plants depend on cell membranes. The physiological function of a cell is dependent on cell membrane permeability. Harsh conditions such as drought, salinity, and low and high temperatures initially damage the cell membrane and later affect its function; this phenomenon first causes leakage of the membrane and ultimately the loss of intracellular content. When nutritional solution without NaCl was used, small differences were observed in REC values between wild type and transgenic plants, but REC values increased when NaCl was applied. Despite the increase, the REC values of transgenic lines were significantly lower than those of the wild type plants (Fig. 4 Suppl.). These findings are consistent with those of Jia *et al.* (2002). Thus, the activation of glycinebetaine protects membranes of plant cells during salt stress (Hasegawa *et al.* 2000). APX activity increased and was maintained at a very high level in transgenic *Arabidopsis* plants by the overproduction of *BADH*. During treatment with NaCl, *BADH* expression

in transgenic plants triggers the activation of oxygen-scavenging system and protects plants from salt stress by equilibrating H₂O₂ metabolism (Tang *et al.* 2014 and Fig. 5 Suppl.) leading to increased chlorophyll content (Fig. 5 Suppl.).

Membrane protection against reactive oxygen species in plant cells is provided by proline, which acts as an electron acceptor (Rejeb *et al.* 2014). Unlike in wild-type plants, proline content was high in *BADH* transgenic plants (Fig. 6 Suppl.). Under stress conditions, MDA is the final product of lipid peroxidation (Puniran-Hartley *et al.* 2014). Before salt treatment, the MDA content was similar in both wild-type and transgenic plants, but after salt treatment, it was increased only in transgenic plants (Fig. 6 Suppl.). Glycine betaine content was dependent on promoter used and transgenic potato with the *BADH* gene inserted under triple promoter exhibited the highest glycine betaine content, which indicates that *BADH* expression was promoter dependent (Fig. 4). Even with a single promoter, glycine betaine content was higher compared with wild type plants but lower than with the double promoter. MDA, chlorophyll, and H₂O₂ content corresponded to glycinebetaine content.

Transgenic and wild-type plant shoots were put in MS medium supplemented with 90 mM NaCl. All transgenic shoots rooted normally within 2 weeks, but in wild-type shoots, only stunted growth and callus-like formation were observed instead of rooting (Fig. 3). These findings are consistent with Jia *et al.* (2002) and Farace *et al.* (2015). The rooted potato shoots were transplanted to pots, after which they were watered with MS or MS with 90 mM NaCl. During the vegetative stage, the transgenic plants grew well even under 120 mM NaCl solution, while the wild types either died or turned yellow (Fig. 6 Suppl.).

Most of the work relating to metabolic engineering has been focused on grasses and tobacco; hence, published reports on vegetables, such as tomato and potato, are scarce. For glycine betaine biosynthesis, *BADH* or other genes have been introduced from different plant species to increase salt stress tolerance (Rathinasabapathi 2000, Cookson *et al.* 2013). The results from our correlation and regression analyses showed that *BADH* activity plays an important role in the increased production of proline and chlorophyll (Ali *et al.* 2013, 2014). The reduction of H₂O₂ content was also carried out in transgenic potato plants. Hence, the correlation and regression analyses provide an opportunity for the selection of salt-resistant potato genotypes (Ali *et al.* 2016). This is perhaps the first report on introducing *Atriplex BADH* in Pakistan potato cultivars.

Conclusions

Transgenic potato plants that harbored the *BADH* gene under the triple CaMV 35S promoter could tolerate higher salt stress than those harbored the *BADH* gene under the single and double CaMV 35S promoter and wild type plants. The triple CaMV 35S promoter boosted the expression of *BADH* in transgenic potato plants leading to 4 to 4.5-fold higher glycine betaine content compared

with the wild type and also higher chlorophyll and proline content under salt stress.

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