TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2018) 42: 673-686

doi:10.3906/bot-1711-29

Gamma irradiation enhancement of biofilm production in *Bacillus amyloliquifaciens* and effect of mutants' inoculation on *APX1* expression in salt-stressed Egyptian barley

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Received: 19.11.2017 • **Accepted/Published Online:** 28.08.2018 • **Final Version:** 22.11.2018

Abstract: Gamma rays are known to induce random mutations, which are used to enhance the production of biofilms in bacteria. Therefore, to improve the activity of biofilm formation in wild *Bacillus amyloliquefaciens* (HM6), this bacterial strain was mutated using five doses of gamma irradiation. Sixteen mutants and their wild strain *B. amyloliquefaciens* (HM6) were tested for biofilm formation under two different (250 and 350 mM) NaCl concentrations. Only six mutants exhibited a significant increase in biofilm formation under 350 mM NaCl. Inoculation of salt-stressed Giza123 barley seedlings with the two mutants (M1 and M7), which showed distinguishable rep-PCR patterns, and their wild strain significantly decreased MDA content. In addition, such inoculation inhibited POX and CAT activities, while AsA content was increased. The lowest POX and CAT activities, as well as the highest AsA content, were recorded with mutant M7. The full length cDNA of Giza123 *APX1* was cloned, sequenced, and submitted to GenBank under accession number MF804856. In BLASTX analysis, its sequence exhibited 99% homology with the *Hordeum vulgare* peroxisome type ascorbate peroxidase. RT-PCR revealed variable *APX1* expression levels in the stressed and nonstressed Giza123 seedlings. Salt stress upregulated the *APX1* expression level in uninoculated–stressed seedlings compared to the uninoculated–unstressed ones. On the other hand, inoculation with M1 and M7 mutants and their wild strain modified the *APX1* expression level, where the wild and the M1 mutant downregulated the *APX1* expression level, while mutant M7 upregulated its expression level under salt stress.

Key words: Biofilm, *Bacillus amyloliquefaciens*, salt stress, *APX1* expression, Gamma irradiation

1. Introduction

Crop plants frequently undergo different adverse environmental conditions of which soil salinity represents the most serious abiotic stress threatening agriculture worldwide (Witzel et al., 2014). Plant growth promoting rhizobacteria (PGPR) were applied to alleviate salt stress, as well as to maintain a reasonable level of productivity under high salt concentrations (Singh et al., 2011; Nadeem et al., 2012). Alleviation of adverse effects of salt stress by various rhizobacteria in several crops was recently reviewed (Paul and Lade, 2014; Ilangumaran and Smith, 2017). Barley (*Hordeum vulgare* L.) is one of the most cultivated crops worldwide. Among cereals, it is the most salt-tolerant crop. It was described to get severely injured only after prolonged periods at salt concentrations higher than 250 mM NaCl (Munns et al., 2006). There are few studies that have investigated the growth promotion of barley using PGPR (Omar et al., 2009; Chang et al., 2014; Nabti et al., 2015). This is probably due to its inherent salt tolerance compared to other crops such as corn, soybean, wheat, tomato, and rice.

The success of PGPRs was ascribed to their effective colonization of plant roots (Bolwerk et al., 2003) and following growth to produce biofilms as a result of an effective plant–microbe interaction (Saleh-Lakha and Glick, 2006). These biofilms contain extracellular polymeric substances with high water content, which exhibit protection against desiccation, salt stress, and UV (Xiang et al., 2008). Recently, *Bacillus amyloliquefaciens* strains were found to serve as plant growth promoting bacteria (Ramesh et al., 2014; Choi et al., 2014; Kasim et al., 2016). The growth of rice plants increased under salinity when inoculated with *B. amyloliquefaciens* (SN13), where its salt tolerance (NaCl 200 mM) was attained via transcription regulation of a set of fourteen genes (Nautiyal et al., 2013).

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Ionizing radiation, such as gamma rays, was found to induce random mutations, which were used to improve antagonistic activity, enhance the production of biosurfactants and biofilms in bacteria, as well as increase disease resistance (Liu et al., 2005; Afsharmanesh et al., 2013). Afsharmanesh et al. (2013) reported that six gamma-irradiated mutants from *Bacillus subtilis* UTB1 revealed more profound biofilm compared to its wild type UTB1.

H_2O_2 is an endogenous ROS species that plays a double role in plants, where it is useful at low levels and lethal at higher concentrations (Petrov and Van Breusegem, 2012). To overcome H_2O_2-related cellular damage, aerobic organisms have developed various antioxidant systems with enzymatic and nonenzymatic components. Ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT) are the primary enzymes in charge of repressing the toxic levels of H_2O_2 (Apel and Hirt, 2004). Interestingly, APX also plays essential roles in ROS-scavenging, since even very low concentrations are adequate for breaking down H_2O_2 (Liu et al., 2013; Anjum et al., 2014; Sofo et al., 2015). APX (EC 1.11.1.11) is a member of the plant-type heme peroxidase superfamily (Lazzarotto et al., 2011). In rice, three genes encode chloroplastic isoforms and two genes produce cytosolic and peroxisomal forms, while only one gene is responsible for mitochondrial APX (Anjum et al., 2014). APX activity was also reported to increase (upregulate) under various stress conditions such as heavy metal, drought, water, salt, and heat stress (Shi et al., 2001; Sharma and Dubey, 2005; Koussevitzky et al., 2008; Anjum et al., 2014). In barley, it was demonstrated that the transcript level of peroxisomal *APX1* gene (*HvAPX1*) increased significantly under salt stress (Shi et al., 2001).

Usually, salinity gives rise to disruption in the expression of antioxidant genes by producing alterations in the transcriptional pattern in various plant species. Besides, the expression of a specific APX isoform leads to a redox homeostasis regulation in the cellular compartments (Caverzan et al., 2012). The main aims of our study were to enhance biofilm formation in *B. amyloliquefaciens* by induction of mutations using gamma rays and to understand the impact of coinoculation with *B. amyloliquefaciens* mutants on the tolerance of Egyptian salt-sensitive barley cultivar Giza123. Moreover, the aim was also to clone Giza123 peroxisomal *APX1* cDNA and to investigate the effect of coinoculation with *B. amyloliquefaciens* mutants on *APX1* expression under salinity stress in cultivar Giza123.

2. Materials and methods

2.1. Materials

Grains of salt-sensitive barley cultivar (*Hordeum vulgare* L.) Giza123 were obtained from the Barley Department, Agricultural Research Center (ARC), Giza, Egypt. The wild strain *Bacillus amyloliquefaciens* HM6 (Kasim et al., 2016) was used in this study.

2.2. Methods

2.2.1. Bacterial mutagenesis and mutant analysis

In order to enhance the activity of biofilm production, random mutagenesis with gamma irradiation was performed on *B. amyloliquefaciens* HM6 (wild strain). According to Afsharmanesh et al. (2013), five doses of gamma rays (1000, 1500, 2000, 2500, and 3000 Gray) were applied at Anshas Nuclear Research Reactor, Cairo, Egypt. For each dose of the gamma irradiation, three replicas were performed.

2.2.2. Assessment of salt tolerance of gamma-irradiated bacterial suspensions

After gamma-irradiation, each irradiated bacterial suspension was diluted 10^4 and 10^7-fold with NB medium and then incubated overnight at 30 °C. Afterwards, in order to assess their salt tolerance, 1 mL aliquots of each diluted sample were plated on LB plates supplemented with either 250 or 350 mM NaCl. Then, salt-tolerant mutant colonies were selected depending on the thickness of bacterial colonies as an indicator of active production of exopolysaccharides, growth vigor, and consequently salt tolerance.

2.2.3. Biofilm formation assay

Biofilm formation activity under salt stress of the wild and the selected salt-tolerant mutants was examined by growing them on LB medium supplemented with two different salt concentrations (250 and 350 mM NaCl) (Auger et al., 2006). The priming of barley cultivar Giza123 grains with bacterial inoculum was performed as described by Ibrahim and Omar (2015). Bacterial activities of biofilm formation were measured as described by Kasim et al. (2016). Depending on these results, only 6 mutant strains that showed higher activities of biofilm formation compared to the wild strain were selected for rep-PCR analysis.

2.2.4. ERIC and BOX repetitive elements-PCR analysis (rep-PCR fingerprinting)

Repetitive elements-PCR (rep-PCR fingerprinting) was carried out to uncover the genetic variability among the 6 bacterial mutants and the wild strain. Bacterial genomic DNA was extracted using CTAB method as described by Nishiguchi et al. (2002). For rep-PCR fingerprinting, primers ERIC1R, ERIC2 (ERIC-PCR), and BOX1R (BOX-PCR) were used (Table). The PCR conditions were as described by Mohapatra et al. (2007) with small modifications. A reaction mixture of 25 µL was used for rep-PCR; it contained the following: 12.5 µL of 2X PCR master mix (Takara, Japan), 1 µL of each ERIC1R and ERIC2 primers (10 pmol) or 2 µL of BOXA1R primer (10 pmol), 2 µL (25 ng) of bacterial genomic DNA, and 8.5 µL

Table. Primers used for BOX and ERIC fingerprinting and *APX1* expression analysis.

Primer name	Primer sequence (5`–3`)	Annealing temp.	Reference
*BOX1*R	5`-CTA CGG CAA GGC GAC GCT GAC G-3`	50 °C	Mohapatra et al. (2007)
*ERIC*2	5`-AAG TAA GTG ACT GGG GTG AGC G-3`	45 °C	Mohapatra et al. (2007)
*ERIC1*R	5`-ATG TAA GCT CCT GGG GAT TCA C-3`	45 °C	Mohapatra et al. (2007)
*HvAPX1*F	5`-CCA TGG TTC AAA TGC TGG CT-3`	59 °C	This study
*HvAPX1*R	5`-AAG CTT CGT ACA GGT AGC CC-3`	59 °C	This study
*HvActin*F	5`-TCG CAA CTT AGA AGC ACT TCC G-3`	50 °C	von Zitzewitz et al. (2005)
*HvActin*R	5`-AAG TAC AGT GTC TGG ATT GGA GGG-3`	50 °C	von Zitzewitz et al. (2005)

of ddH_2O. PCR amplifications were performed in thermal cycler T100™ BIO-RAD (Hercules, CA, USA) as follows: an initial denaturation step (95 °C for 5 min), followed by 30 cycles of denaturation (94 °C for 30 s), annealing (50 °C for BOX and 45 °C for ERIC for 30 s), extension (72 °C for 1 min), and a single final extension step (72 °C for 10 min).

Amplified PCR products were separated on a 1.5% agarose gel using 1X TAE buffer. 100 bp and 1 kb DNA ladders (Direct Load™, Sigma Chemicals Inc., Canada) were used. Electrophoresis was performed at room temperature using 100 V. The banding patterns of ethidium bromide-stained gels were photographed under UV light in BioImaging system (UVP Inc.). Depending on the results of the biofilm assay, ACC activity (data not shown), and the genetic diversity among the wild strain and the mutants, only two mutants (M1 and M7) were selected to investigate their effects on salt tolerance and *APX1* expression of the barley cultivar Giza123.

2.2.5. Evaluation of salt tolerance of barley cultivar Giza123 inoculated with bacterial mutants

In order to examine the potentiality of the wild and the two selected bacterial mutants (M1 and M7) for improving salt tolerance of barley cultivar Giza123 seedlings (30 days), a pot experiment was conducted in the greenhouse of the Wheat Department, Field Crops Research Institute, ARC, Giza, Egypt. Bacterial inoculation and grains coating were repeated immediately after sowing and after 7 days. In all treatments, 8 grains were sown in a plastic pot containing 10 kg of clay-sandy soil and 8 replicas were used for each treatment. The pots were irrigated every second day with either NaCl solution (250 mM) or with tap water (as control) and were leached with tap water once a week. From each treatment, 50 seedlings were frozen in liquid nitrogen and used for measurement of catalase and peroxidase activities, ascorbic acid and malondialdehyde (MDA) contents, and for *APX1* expression analysis as well.

2.2.6. Determination of catalase and peroxidase activities, ascorbic acid and malondialdehyde contents

Two antioxidant enzymes (catalase and peroxidase) were examined. First, total soluble proteins were extracted according to Beauchamp and Fridovich (1971). Then, catalase activity (EC 1.11.1.6) was measured according to Goth (1991) and enzyme activity was expressed as mmol H_2O_2/min/g FM. On the other hand, peroxidase activity (EC 1.11.1.7) was assayed as described by Kumar and Khan (1982), and the enzyme activity was expressed as the change in the absorbance by mmol/min/g FM.

Ascorbic acid (AsA), as a nonenzymatic antioxidant compound, was estimated according to Oser (1979). Determination of AsA content was done based on a standard curve prepared with ascorbic acid and the results were exhibited as mg/g DM. The level of lipid peroxidation was estimated by determining the concentration of malondialdehyde (MDA) as a product of peroxidation of unsaturated fatty acid (linolenic acid, 18:3) according to Hagege et al. (1990).

2.2.7. Expression analysis of *APX1* of barley cultivar Giza123

2.2.7.1. RNA extraction and cDNA synthesis

Expression analysis of ascorbate peroxidase (*APX1*) gene in barley cultivar Giza123 was performed using semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR). As a template for RT-PCR, cDNA synthesized from total RNA was used. Total RNA was extracted from 30-day-old leaves of barley cultivar Giza123 seedlings, which were grown under salt-stressed (250 mM NaCl) and unstressed conditions after their inoculation with the wild strain (*Bacillus amyloliquefaciens*) and two selected mutant strains (M1 and M7). Total RNA was extracted from barley cultivar Giza123 leaves using the TriPure reagent (Roche Molecular Biochemicals, Cat. No.1667165).

For the first cDNA strand synthesis, total RNA was used. The total reaction volume was 15 µL and it contained the following components: 1 µL Oligo (dT) primer, 2 µL RNA (2 µg/µL), and 12 µL DEPC H_2O. The mixture was heated to 70 °C for 5 min and then quickly chilled on ice for 5 min. The mixture was collected by brief centrifugation, then returned to ice and the rest of the reaction components were added as follows: 5 µL M-MLV

reaction buffer (5X), 1 µL dNTP (10 mM), 1 µL (200 units) M-MLV RT (Promega, USA), and 3 µL DEPC H_2O. Finally, the reaction tubes were incubated at 37 °C for 60 min.

2.2.7.2. RT-PCR of *APX1* of cultivar Giza123

For expression analysis of *APX1*, specific primers *HvAPX1*F and *HvAPX1*R (Table) were designed based on the CDS sequence (NCBI accession no.: BAB62533.1) and then used for PCR amplification reactions using Giza123 cDNA as template.

PCR was conducted in 25 µL reaction volume, which included the following constituents: 12.5 µL PCR master mix (2X), 1 µL forward primer (10 pmol), 1 µL reverse primer (10 pmol), 2.0 µL cDNA, and 8.5 µL ddH_2O. PCR was carried out in thermo-gradient PCR machine T100™ BIO-RAD. PCR amplification conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 30 cycles of 95 °C for 1 min, 50 °C for 30 s, and 72 °C for 90 s, with a final extension at 72 °C for 10 min. Various numbers of PCR cycles (25–35) were evaluated in order to detect the optimal number of cycles. Amplified *APX1* PCR fragments were electrophoretically separated on a 1.5% agarose gel. The anticipated *APX1* PCR amplicon size (665 bp) was confirmed with a 100 bp-Plus DNA ladder. In order to ensure equal amounts of cDNA, cDNA sample volumes for RT-PCR were first normalized for cDNA yield relative to a barley actin gene. Barley specific *HvActin* primers are shown in Table. PCR amplification conditions for *HvActin* gene were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 50 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 10 min.

2.2.7.3. Cloning and sequencing of *APX1* RT-PCR amplicon of barley cultivar Giza123

Amplified Giza123 *APX1* fragments were cloned into pGEM-T using TA Cloning Kit (PCRII) as described by the manufacturer (Promega Corporation, Madison, WI, USA). All the processes of molecular cloning, ligation, and transformation were done according to Sambrook et al. (1989). *E. coli* DH5a (Invitrogen, Cat. No. 18265–017, ThemoFisher Scientific, Waltham, MA, USA) was used for the transformation process.

Sequencing of the cloned Giza123 *APX1* cDNA was carried out using automatic DNA sequencer (Genetic Analyzer 310, Germany). Sequencing was conducted by Eurofins Genomics (Germany) using the method of Sanger et al. (1977). Sequencing data were provided as fluorimetric scans from which the sequences (positive and negative) were assembled using DNASTAR® software suite for sequence analysis (ver. 8.1).

2.2.7.4. Data and sequence analysis

All data were replicated three times and the presented data are the mean values. The results obtained in our study were statistically analyzed using ANOVA with Costat software (CoHort software, California, USA) to determine the significance between treatments. Cluster analysis of BOX and ECRIC was performed based on binary (1 or 0) data, using the UPGMA method, using NTSYS-pc, version 2.11 (Rohlf, 2005).

Quantification of *APX1* band intensities was performed using ImageJ software (version 1.49). The ratio between band intensities of treatment combinations and the *Actin* gene (control gene) was calculated to normalize any initial variation in cDNA concentration and as a control for reaction efficiency. Then, the relative *APX1* expression was calculated.

The consensus *APX1* sequence was used in NCBI for BLAST searches using BLASTX 2.6.0 (Altschul et al., 1997). The hits which showed an *E*-value higher than $3e^{-152}$ were selected for further alignment and phylogenetic relationship analysis. Multiple sequence alignment (MSA) analysis and phylogenetic tree analysis were performed using MacVector v. 11.0.4 software (MacVector, Inc., 2009). For MSA analysis, Gonnet similarity matrix of ClustalW (v. 1.83) was used, while the Neighbor Joining method and Distance: Poisson-correction was used for generating the phylogenetic tree.

3. Results

3.1. Bacterial mutagenesis and biofilm formation

Five gamma doses (1000, 1500, 2000, 2500, and 3000 Gray) were used to induce mutation in *B. amyloliquefaciens* HM6 (wild strain) in order to enhance its activity for biofilm formation under salt stress. After gamma mutagenesis, 39 colonies of mutant strains were selected according to thickness of colonies as an indicator of exopolysaccharides production, growth vigor, and consequently their salt tolerance.

The results showed that the biofilm formation activity of mutants was significantly increased in sixteen mutants (M1, M2, M6, M7, M15, M16, M20, M25, M28, M34, M36, M37, M40, M41, M42, and M44), while the other mutants did not show any significant change in their activity for biofilm formation when compared to the wild strain (Figure 1A).

Sixteen *B. amyloliquefaciens* mutants with enhanced potency for biofilm formation under unstressed condition were tested for biofilm formation activity under salt stress (250 and 350 mM NaCl). The results showed that under salt stress, *B. amyloliquefaciens* mutants exhibited variable activity for biofilm formation and the most significant increase in the activity of biofilm formation was observed in 6 mutants (M1, M2, M7, M28, M34, and M40), where their absorbance values were 0.275, 0.283, 0.258, 0.238, 0.375, and 0.233, respectively, under 350 mM NaCl compared to the wild strain (0.165) (Figure 1B).

Moreover, the result of ACC-deaminase-activity of the wild strain and these six mutants showed no differences; all of them were ACC-deaminase producers (data not shown).

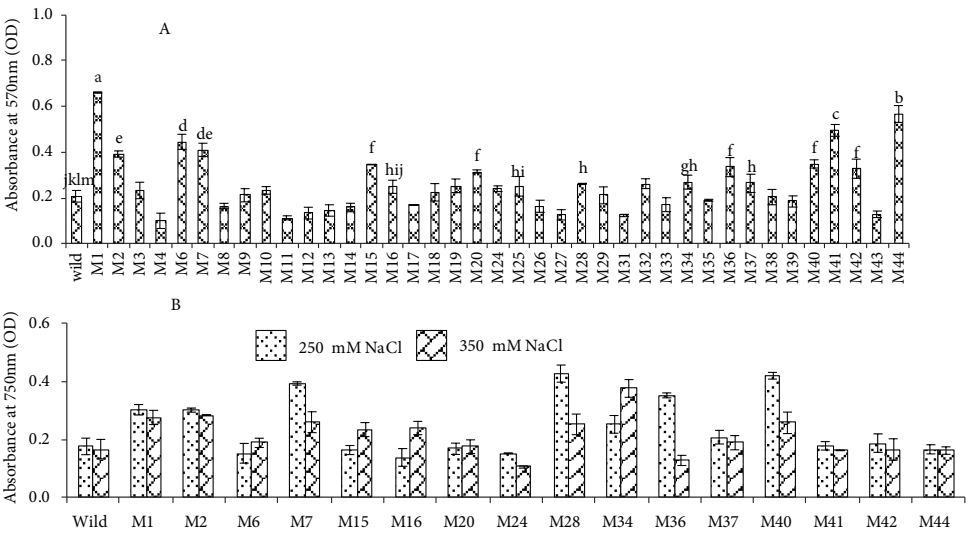

Figure 1. Optical density (OD) at 570 nm as a measure of biofilm formation activity. A) 39 *Bacillus amyloliquefaciens* mutants (unstressed condition), B) 16 selected *Bacillus amyloliquefaciens* mutants stressed with 250 and 350 mM NaCl. Error bars represent the standard deviation between 3 replicas. Treatments with different letters are significant at $P \leq 0.05$; identical letters are nonsignificant.

The genetic diversity and the presence of polymorphism between the wild strain and the six selected mutants were investigated using repetitive elements-PCR analysis (rep-PCR).

3.2. ERIC and BOX repetitive elements-PCR analysis

PCR amplification of the BOX and ERIC elements was performed on the wild strain and the selected six mutants in order to investigate their genetic diversity and to reveal the polymorphisms among the wild strain (*B. amyloliquefaciens*) and its six mutants. The PCR profiles showed that there was genetic diversity between the wild type and its six mutants. The polymorphism was in the form of variation in the number of bands (presence and absence) and in the molecular size of PCR bands compared with the wild type (Figure 2). Agarose gels showed variable changes in the PCR profiles of the wild strain and its six mutants. A total of 17 bands were observed in case of ERIC-PCR. Amplified band sizes ranged between 1400 bp and 160 bp (Figure 2A), while in BOX-PCR, 21 PCR bands were amplified. The molecular size of the bands ranged between 1400 and 200 bp. In the ERIC-PCR profiles, a sharp band with a molecular size of approximately 600 bp was observed in the wild strain and the two mutants, M7 and M40. In addition, mutants M7 and M40 showed two bands of the same molecular size of 600 and 400 bp, while mutant M1 showed one PCR band with high molecular size (1200 bp). Meanwhile, mutant M2 showed a unique band of molecular size of approximately 500 bp (Figure 2A). The highest numbers of PCR bands were observed in the PCR profiles of mutants M1 and M40, where 7 bands were amplified (Figure 2A).

Similarly, the BOX profile of wild strain exhibited a sharp band with a molecular size of approximately 950 bp. In addition, PCR profiles of mutants M7 and M40 showed a high degree of similarity, where 4 bands of molecular sizes 1250, 1050, 950, and 900 bp were observed (Figure 2B). Moreover, three bands with molecular sizes of 900, 600, and 500 bp were amplified in mutant M1 and M2. The highest number of BOX-PCR fragments (8 PCR bands) was observed in mutant M28, followed by 7 PCR bands in mutant M7 and the wild strain. The rep-PCR analysis using BOX primer failed to amplify any PCR bands in mutant M34.

BOX and ERIC phylogenetic cluster analysis revealed that the dendrogram is divided into three clearly separated groups (clusters); the first cluster included the wild strain and its two mutants M1 and M2. Meanwhile, the three mutants M7, M40, and M28 represented the second group; they showed similar banding patterns, particularly mutants M7 and M40. However, mutant M34 formed a third cluster (Figure 2C), which was completely separated from the other mutants. Depending on the results of BOX and ERIC-PCR analysis, two mutants (M1 and M7) were selected from groups one and two, which were distinguished from their parental strain (wild). These two mutant strains (M1 and M7) were used to investigate whether the increase in biofilm formation is due to genetic differences between the two selected mutants and their wild parent (*B. amyloliquefaciens* HM6).

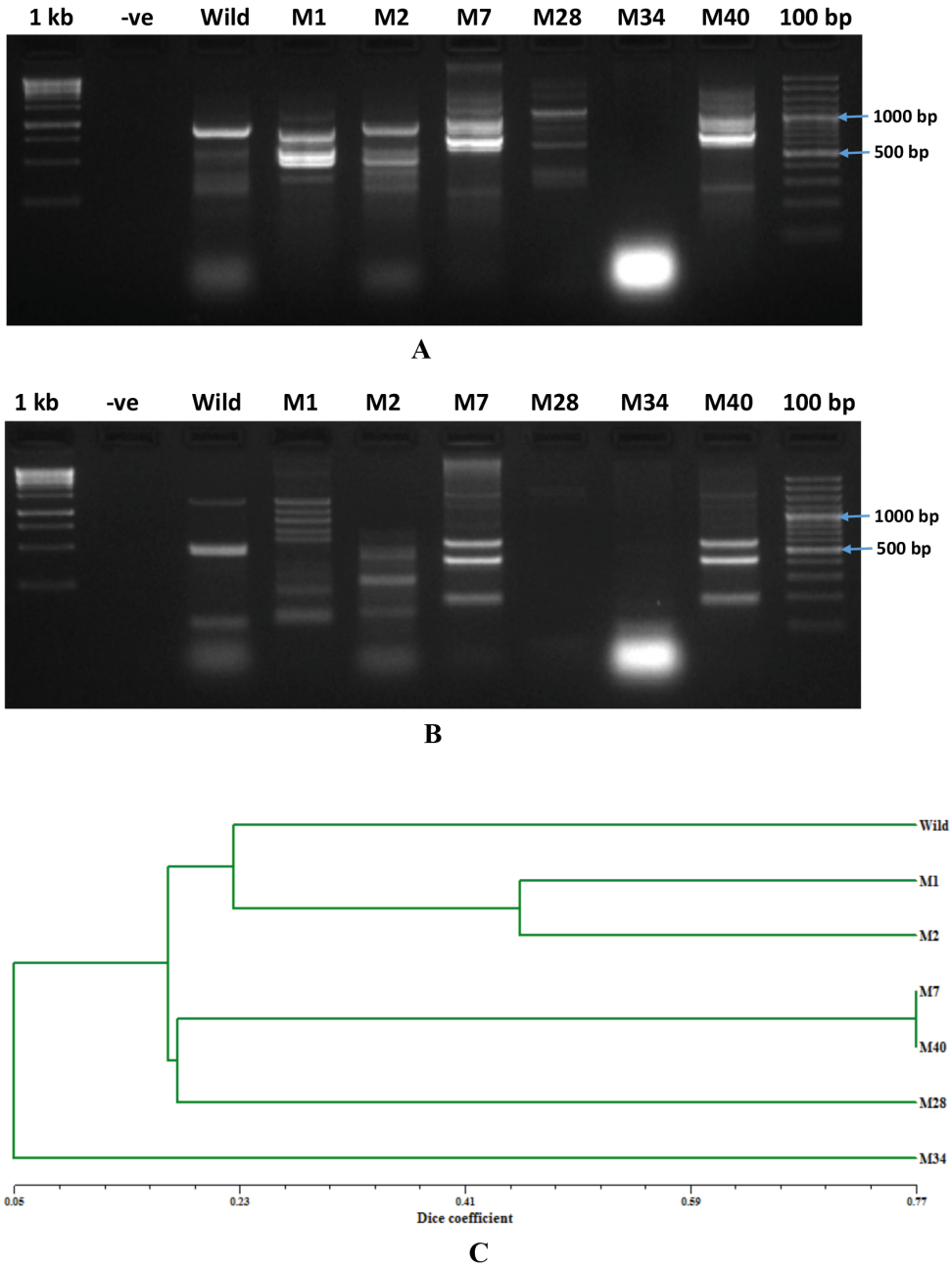

Figure 2. Rep-PCR fingerprinting of *Bacillus amyloliquefaciens* (wild) and M1, M2, M7, M28, M34, and M40 selected mutants on 1% agarose gels using BOX-PCR (A), ERIC-PCR (B), and BOX and ERIC phylogenetic cluster analysis using UPGMA method (C). DNA ladders: 1 kb (left) and 100 bp (right); -ve: water control.

3.3. Salt tolerance of barley cultivar Giza123 inoculated with *B. amyloliquefaciens* mutants

The effect of coinoculation with the wild strain (*B. amyloliquefaciens* HM6) and the two selected mutants (M1 and M7) on the tolerance of salt-sensitive barley cultivar Giza123 grown under 250 mM NaCl was studied.

Higher catalase activity was observed in the salt-stressed than in the unstressed Giza123 leaves. The highest catalase activity was recorded in the uninoculated–salt-stressed seedlings (553 mM/min/g FM) compared to the uninoculated–unstressed treatment (239 mM/min/g FM). However, inoculation with the two strains (wild and M1) reduced the catalase activity in the inoculated–salt-stressed Giza123 seedlings by 8%. Inoculation with M7 showed the highest reduction of catalase activity (3%) with respect to their uninoculated–salt-stressed counterparts (Figure 3A).

Peroxidase activity was also increased by salt stress; it was escalated in the uninoculated–salt-stressed leaves by about 2.2-fold compared to the uninoculated–unstressed counterparts (Figure 3B). Meanwhile, M7-inoculated–salt-stressed Giza123 leaves showed the highest reduction in peroxidase activity (22%) compared to their uninoculated–salt-stressed counterparts. By contrast, no significant differences in the activity of peroxidase were recorded among all the unstressed Giza123 leaves.

Ascorbic acid content was significantly decreased (13%) in the salt-stressed Giza123 seedlings compared to the uninoculated–unstressed ones (Figure 3C). Meanwhile, the accumulation of AsA was promoted by PGPR inoculation under salt stress, as shown in the M7-inoculated–stressed Giza123 seedlings, where it significantly increased by 31% compared to its content in the uninoculated–salt-stressed Giza123 seedlings. Under unstressed conditions, the highest value of ascorbic acid (17 mg/g FM) was recorded in the leaves of M1-inoculated Giza123 seedlings, compared to uninoculated–unstressed ones (15 mg/g FM).

Malondialdehyde (MDA) content significantly increased under salt stress in the Giza123 leaves in all bacterial inoculated and un-inoculated treatments (Figure 3D). On the other hand, bacterial inoculation with wild or mutant strains notably decreased the accumulation of MDA in the leaves of Giza123 seedling leaves under salt stress, without significant difference between strains. Under salt stress, the lowest MDA content (0.56 mmol/g FM) was recorded in the M1-inoculated seedlings, compared to 0.72 mmol/g FM in the uninoculated–salt-stressed ones. On the other hand, significant differences were recorded under unstressed conditions, where inoculation with wild and M7 mutant showed the highest MDA content. By contrast, strain M1 showed the lowest MDA content compared to its uninoculated–unstressed treatments.

3.4. *APX1* expression in the leaves of cultivar Giza123

The expression of barley *APX1* gene was monitored in the leaves of the 30-day-old seedlings of cultivar Giza123 that were grown under salt-stressed (250 mM NaCl) and unstressed conditions after inoculation with the wild strain (*B. amyloliquefaciens,* HM6) and the two selected mutants (M1 and M7). To study *APX1* expression, a semiquantitative RT-PCR using *APX1* gene specific primers was performed on cDNA of the treated and control Giza123 seedlings. To compare the abundance of *APX1* gene, a semiquantitative PCR was performed using specific primers of the *HvActin* gene (housekeeping gene). The same amount of cDNA templates was used in all RT-PCR reactions, and the same quantity of RT-PCR products was loaded on the agarose gels.

The results of RT-PCR demonstrated a variable change in the expression level of *APX1* in the leaves of barley Giza123 seedlings due to salt stress, bacterial inoculation, or their combinations (Figure 4A). Generally, in uninoculated–salt-stressed plants, the expression level of *APX1* increased by 0.3-fold compared to its expression level in the control (uninoculated–unstressed). Inoculation with the wild and M1 under stressed condition significantly decreased the *APX1* expression level by 0.64 and 0.84-fold, respectively, compared to the control (uninoculated–unstressed). However, in case of M7-inoculated–salt-stressed seedlings, the expression level of *APX1* recorded maximal upregulation of the gene by 0.31-fold compared with their uninoculated–unstressed control (Figure 4B).

Under unstressed condition, bacterial inoculation reduced the expression level of *APX1*, as shown in M1 and M7-inoculated–unstressed samples by approximately 0.46-fold, compared to the uninoculated–unstressed control. On the other hand, wild-inoculated–unstressed seedlings showed a higher *APX1* expression level by 0.1-fold compared to the uninoculated–unstressed control.

3.5. Sequencing of *APX1* cDNA of barley cultivar Giza123

In order to confirm that the expression analysis of *APX1* was performed on the correct gene fragment, amplified *APX1* fragments (obtained in RT-PCR) were cloned in pGEM-T7 vector. Then, the cloned fragments were sequenced using SP6 and T7 universal primers. The obtained forward and reverse sequences were 876 and 757 bp long, respectively. These two sequences were aligned with the sequence from NCBI (BAB62533.1) using SeqMan program of DNASTAR® ver. 8.1. The results showed that the Giza123 *APX1* sequences (forward and reverse) were perfectly integrated within one contig with BAB62533.1 sequence, indicating that these are accurate sequences.

Also, comparing these sequences revealed four transition (C-T) SNPs, where C presents in Giza123 cDNA sequence and T in CDS sequence (accession no. BAB62533.1). These four SNPs were found at positions 79, 477, 593, and 660 bp, respectively.

The consensus sequence (876 bp) of barley cultivar Giza123 was used to search for sequence homology in the NCBI database using the BLASTX program. The results showed that Giza123 cDNA sequence has conserved domains of the plant-peroxidase-like superfamily and has high homology with different plants' *APX* amino acids sequences. It has sequence identity of 97% to *Triticum aestivum* (AGG23553.1), 95% to *Stipa purpurea* (AJF34885.1), 93% to *Brachypodium distachyon* (XP_003574893.1), 91% to *Oryza sativa* Japonica group (XP_015650808.1), 90% to *Oryza brachyantha*, 89% to *Zea mays* (ACF84871.1) and *Sorghum bicolor*

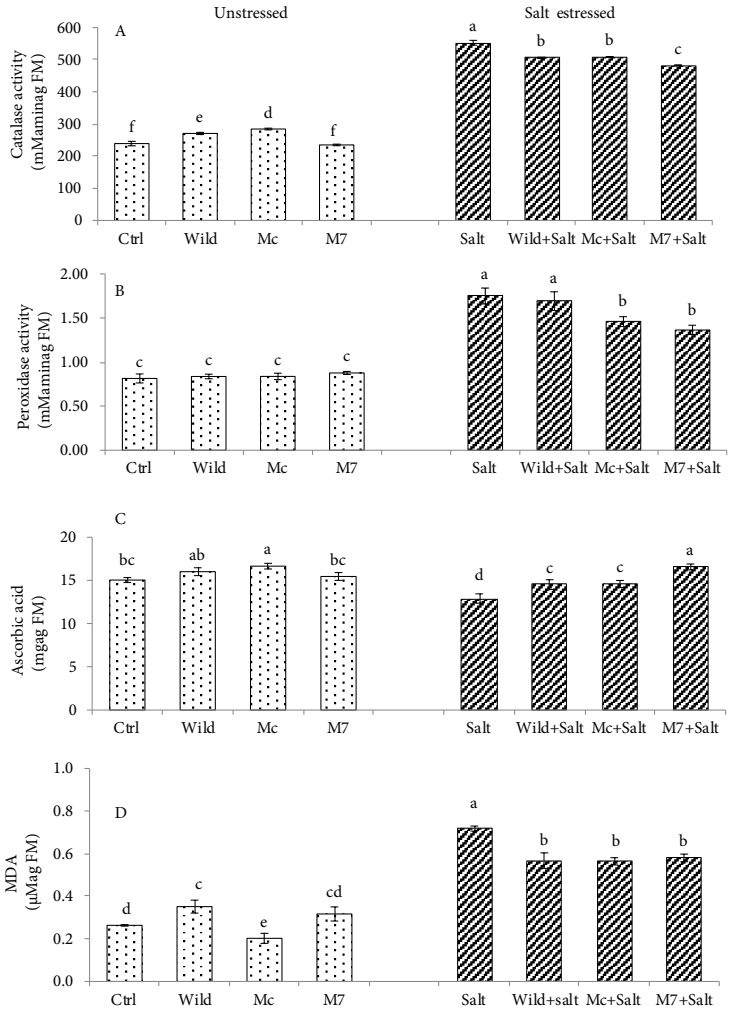

Figure 3. Effect of bacterial inoculation with wild strain (*Bacillus amyloliquefaciens*) and two selected mutants (M1 and M7) on catalase and peroxidase activities (mM/min/g FM) (**A** and **B**), ascorbic acid content (mg/g FM) (**C**), and malondialdehyde (MDA) content (mM/g FM) (**D**) in the leaves of 30-day-old seedlings of barley cultivar Giza123 grown in clay-sandy soil (2:1 w/w) under unstressed and salt-stressed (250 mM NaCl) conditions. Error bars represent the standard deviation between 3 replicas. Treatments with different letters are significant at $P \leq 0.01$; treatments with identical letters are nonsignificant.

(XP_002444620.1), and 81% to *Ananas comosus*. (XP_020112729.1). Significant *E*-values ranged from $4e^{-137}$ to 0.0. The highest *E*-value (similarity) of 0.0 was found to be with the peroxisome type ascorbate peroxidase of *Hordeum vulgare* subsp. *vulgare* (accession no. BAB62533.1). According to the open reading frame (ORF) analysis of the *APX1* cDNA sequence of barley cultivar Giza123, the protein length was 291 amino acid residues. The molecular weight of the deduced amino acid of the cloned *APX1* sequence was 31.5 KD, and its isoelectric point was 7.1. Amino acid analysis results of *APX1* protein revealed that it contains 47 positively charged (basic) amino acids, 39 negatively charged (acidic) amino acids, 151 hydrophobic amino acids, and 54 polar amino acids.

3.6. *APX1* alignment and phylogenetic analysis

Multiple sequence alignment of the predicted amino acid sequence of Giza123 *APX1* with other *APX1* proteins (selected based on *E*-value between 0.0 and $3e^{-152}$) identified several conserved regions, which are shown in Figure 5. Also, it showed highly conserved blocks (protein

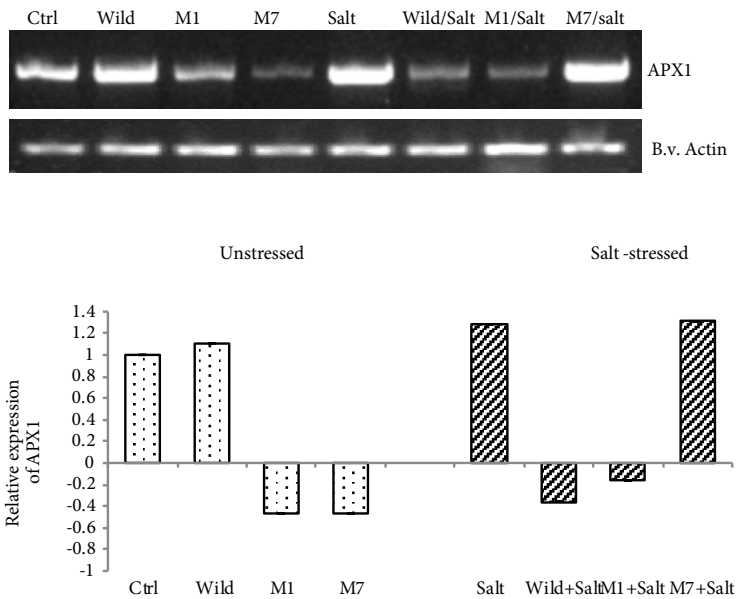

Figure 4. RT-PCR of *APX1* transcripts in the leaves of 30-day-old seedlings of barley cultivar Giza123 grown in clay-sandy soil (2:1 w/w) under unstressed and salt-stressed (250 mM NaCl) conditions after inoculation with wild strain (*Bacillus amyloliquefaciens*) and two selected *Bacillus amyloliquefaciens* mutants (M1 and M7). Relative *APX1* expression was normalized using *Actin* gene as a reference gene.

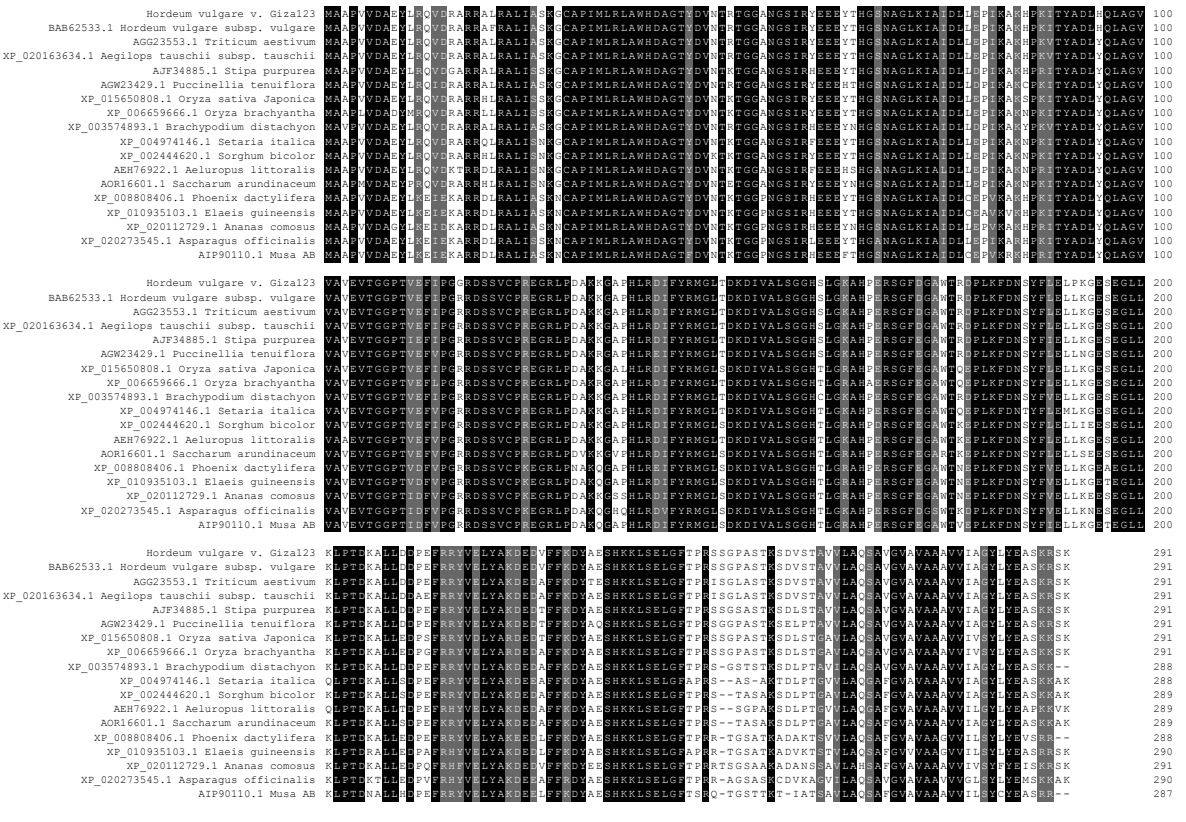

Figure 5. Multiple sequence alignment (MSA) of 17 APX1 homologs from 17 plant species. Identical and similar residues were shaded as black and grey, respectively.

residues) that appeared on the N-terminal regions, while weakly-conserved sequences existed on the C-terminal regions. From the results of the BLASTX sequence analysis, Giza123 APX1 protein sequence was shown to belong to a typical peroxidase heme-ligand superfamily. Multiple sequence alignment (MSA) clearly revealed that the peroxidase active site (APIMLRLAWHDA) of Giza123 APX1 protein is between 31 and 44, while peroxidase proximal heme-ligand site (DIVALSGGHTL) is located between 152 and 162. In addition, MSA of these 19 sequences revealed that 178 amino acid residues were identical, implying that their functions are evolutionarily conserved. Moreover, several conserved residues (Arg-36, His-40, His-160 and 166, Thr-110, Thr-177, Asn-185, and Ile-279) were observed that constitute a potential active site for metal binding.

A neighbor-joining phylogenetic tree was generated based on Giza123 APX1 amino acid sequence, and the 17 APX proteins from other plant species showed a high sequence homology (Figure 6). The phylogenetic tree was comprised of three major clusters: the first contained plant species with the most distal APX protein sequences (represented by *Asparagus officinalis*: XP_020273545.1), the second has fairly close sequences (*Sorghum bicolor*: XP_002444620.1), while the third cluster contained the closest APX1 protein sequences. The phylogenetic relationship between Giza123 APX1 protein and APX proteins of the other 17 plants showed that Giza123 APX1 is very close to *Hordeum vulgare* subsp. vulgare (BAB62533.1), *Triticum aestivum* (AGG23553.1), and *Aegilops tauschii* subsp. tauschii (XP_020163634.1), while the most distant relationship was with *Asparagus officinalis* (XP_020273545.1), *Ananas comosus* (XP_020112729.1), *Musa* AB (AIP90110.1), *Phoenix dactylifera* (XP_008808406.1), and *Elaeis guineensis* (XP_010935103.1) as they formed one major cluster (Figure 6).

4. Discussion

The use of biofilm producing bacteria is an alternative approach to ameliorate salinity tolerance in plants as salt stress can be alleviated by coinoculation of seeds and seedlings of crop plants with PGPR, which colonize the rhizosphere of plants and directly or indirectly promote their growth (Ilangumaran and Smith, 2017). In this study, the results revealed that the activity of biofilm formation of *B. amyloliquefaciens* HM6 (wild strain) was enhanced through random mutagenesis using gamma rays, where sixteen out of 39 *B. amyloliquefaciens* mutants showed high activity of biofilm formation compared to their

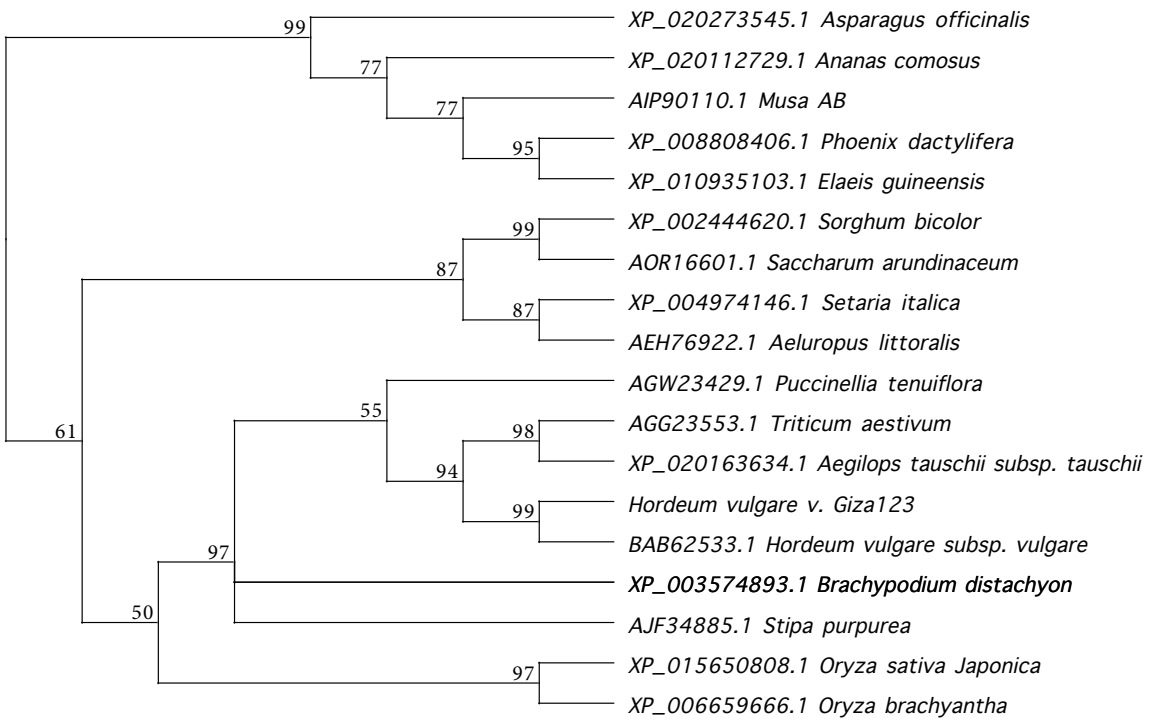

Figure 6. Phylogenetic tree of ascorbate peroxidase 1 (APX1) homologs from 17 plant species. The tree was constructed by MacVector v. 11.0.4 using neighbor-joining method with 1000 bootstraps.

parental wild strain. These results are comparable with those obtained by Afsharmanesh et al. (2013), as they used gamma rays to induce random mutations in *Bacillus subtilis* (UTB1) and found that six mutants showed enhanced production of biosurfactants and revealed more robust biofilm compared to the wild type UTB1. In addition, Liu et al. (2005) used random mutagenesis to create a *B. subtilis* mutant with a higher production of antifungal lipopeptides and faster growth over the parent strain.

Rep-PCR fingerprinting is a powerful technique in detecting polymorphism and genotyping of bacterial genomes (Mohapatra et al., 2007). It was used to study the genetic diversity between the genomes of gamma-irradiated and nonirradiated bacterial isolates (Afsharmanesh et al., 2013). In this study, rep-PCR using both BOX and ERIC primers showed a considerable genetic variability between the wild type *B. amyloliquefaciens* (HM6) and its six mutants. These results corresponded to the findings of Afsharmanesh et al. (2013), who applied rep-PCR using ERIC and BOX primers on *B. subtilis* (UTB1) wild type and its mutants to investigate the genetic variation between the wild type and irradiation-derived mutants. In our study, the two mutants M1 and M7 were selected for evaluating the effect of mutants' inoculation on the salt tolerance of Giza123 as these two mutants showed a distinct rep-PCR banding pattern compared to their parental wild strain (HM6). This was to ensure that the genome of these two mutants is different from that of the wild type parent (HM6). A similar approach was used by Afsharmanesh et al. (2013), who selected eight *Bacillus subtilis* UTB1 mutants out of the 45 depending on distinct polymorphism patterns produced by rep-PCR.

Interestingly, the results of the present study showed that inoculation of salt-stressed Giza123 seedlings with *B. amyloliquefaciens* (HM6) significantly decreased MDA content. The lowest MDA content was recorded with mutant M1. This result agrees with those obtained by Kakar et al. (2016), who demonstrated that inoculation of water-stressed and cold-shocked rice plants with *B. amyloliquefaciens* (Bk7) and *Brev. laterosporus* (B4) declined the leaf malondialdehyde (MDA) content.

APXs (particularly cytosolic APX) are believed to participate in protecting plants from oxidative stress damage. Under stress conditions, ROS are frequently created and accumulated in plant cells. Accumulation of ROS will generate an oxidative stress condition, which leads to suppression of plant growth. However, plants have established their protection systems (nonenzymatic and enzymatic ROS scavenging systems) against such oxidative stress (Sharma et al., 2012; Liu et al., 2013). A nonenzymatic system employs antioxidants to diminish ROS such as glutathione (GSH), AsA, and flavonoids, while an enzymatic system utilizes many enzymes, including ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GSH), and catalase (CAT).

Under salt stress, inoculation of Giza123 seedlings with *B. amyloliquefaciens* (HM6) significantly decreased POX and CAT activities, while AsA content increased. The lowest POX and CAT activity and the highest AsA content were recorded with mutant M7. This result indicates that Giza123 seedlings inoculated with PGPR do not face a severe salt stress condition. Therefore, antioxidant activities decreased in the presence of PGPR, while the increase of AsA may be considered an indication of Giza123 salt tolerance, which is attributed to AsA's increased ability to remove the excess of H_2O_2. However, *Bacillus amyloliquefaciens* SQR9 bacterial inoculation improved peroxidase and catalase activity in rice seedlings (Chen et al., 2016), which is opposite to the results obtained in this study.

The ascorbate–glutathione cycle is thought to be the major hydrogen peroxide detoxifying system in plants. Ascorbate peroxidase (APX) enzymes have a pivotal role in accelerating the change of H_2O_2 into H_2O, employing APX as a specific electron donor (Sofo et al., 2015). Previous studies indicated that APX activity was tightly associated with the expression of *APX* gene, which could be upregulated after the plants experience adverse stress conditions. After treatment with NaCl, APX activities increased in leaves of salt-tolerant pea cultivars (Hernández et al., 1995). Similarly, barley peroxisomal *APX1* gene (*HvAPX1*) expression level increased significantly under salt stress (Shi et al., 2001). In addition, *APX* expression in *Betula platyphylla* increased incredibly after treatment with NaCl (Wang et al., 2009). In *A. thaliana*, the combination of heat and drought stress caused the highest increase of *APX1* mRNA and protein (Koussevitzky et al., 2008). In our study, salt stress upregulated the expression level of Giza123 *APX1* in uninoculated–stressed plants compared to the uninoculated–unstressed ones. This increase of the Giza123 *APX1* expression level may have induced the activity of the antioxidant enzyme (APX) involved in the ascorbate–glutathione pathway in leaves of Giza123 seedlings, suggesting an elevated defense toward higher ROS levels (Omar et al., 2009).

On the other hand, our results demonstrated that inoculation with *B. amyloliquefaciens* (wild and mutant M1) downregulated the *APX1* expression level in Giza123 barley seedlings under salt stress. Similar results were reported by Omar et al. (2009), who found that bacterial priming resulted in a clear reduction in the expression of *APX1* and in the activities of various antioxidant enzymes, which are required in the ascorbate–glutathione redox pathway in the leaves of salt-stressed wheat. Moreover, our results agree with the findings of Gururani et al. (2013), who showed that inoculation of potato plants with PGPR

enhanced mRNA expression of various ROS pathway related genes under drought, heavy metal, and salt stress. In our study, reduction in the expression of *APX1* was accompanied by considerably lower antioxidant activities in the inoculated–stressed Giza123 leaves compared to their uninoculated–stressed counterparts, suggesting a possibility of low ROS level in the leaves of the *B. amyloliquefaciens* (HM6) inoculated plants. Induction of antioxidant level (a prerequisite for stress tolerance) suggests that *B. amyloliquefaciens* (HM6) can be considered an elicitor, which enhances defense enzyme activities and confers tolerance against salt stress (Nautiyal et al., 2013). *APX1* expression level in the stressed Giza123 barley seedling was downregulated with *B. amyloliquefaciens* (HM6) and mutant M1 inoculation, while its expression was upregulated when mutant M7 was used. This may be due to variation in the genomes of the two mutants, which was clearly revealed by rep-PCR fingerprinting.

MSA showed that highly conserved regions appeared on the N-terminal, while weakly conserved sequences existed on the C-terminal, as well as several conserved residues that constitute a potential active site for metal binding, were observed. These findings agree with the study of Liu et al. (2013). In addition, the presence of a significant degree of conserved residues in the main sites of the enzyme has been demonstrated (Ozyigit et al., 2016). These residues (Arg, His, Thr, Asn, and Ile) were reported to be conserved among the entire APX family and known to be essential in ligand (heme)-binding (Ozyigit et al., 2016). Moreover, this result indicates the presence of highly conserved structures of APX sequences at those sites, which are responsible for peroxidase activity in salt-tolerant plants.

Phylogenetic tree analysis of Giza123 APX1 amino acid sequence and the 17 APX proteins from other plant species revealed that the Giza123 APX1 sequence is closely related to that of three plants: *Hordeum vulgare* subsp. vulgare, *Triticum aestivum*, and *Aegilops tauschii* subsp. tauschii. APX1 of these three plants belongs to peroxisomal ascorbate peroxidase and has almost the same sequence length. Similarly, it has been found that the clustering of APX sequences at subbranches was primarily based on sequence length and monocot/dicot separation. However, considerable variation between sequences, even those belonging to the same subcellular localization, has been reported (Ozyigit et al., 2016).

In conclusion, gamma rays' mutagenesis has produced six *B. amyloliquefaciens* mutants, which showed high activity of biofilm formation under salt stress compared to their wild type parent. Inoculation of grains of barley cultivar Giza123 with *B. amyloliquefaciens* mutants reversed the inhibitory effects of salinity on the activity of POX and CAT, which decreased, while AsA content increased, particularly with mutant M7. Meanwhile, inoculation with wild type HM6 and M1 mutant downregulated *APX1* expression level, indicating that *B. amyloliquefaciens* M1 mutant has improved Giza123 salinity tolerance.

Moreover, sequence analysis of barley cultivar Giza123 *APX1* cDNA consensus sequence (876 bp) showed conserved domains of the plant-peroxidase-like superfamily and has high homology to different plants' *APX* amino acids sequences. MSA of 17 sequences revealed that 178 amino acid residues were identical, implying that their functions are evolutionarily conserved, and clearly revealed that the peroxidase active site (APIMLRLAWHDA) of Giza123 APX1 protein is between 31 and 44, while peroxidase proximal heme-ligand site (DIVALSGGHTL) is located between 152 and 162. Cluster analysis showed that Giza123 *APX1* is closely related to *Hordeum vulgare*, *Triticum aestivum*, and *Aegilops tauschii*, which belongs to the family *Poaceae*. These data revealed the importance of PGPR for improving salinity tolerance of salt-sensitive cultivar Giza123 and the possibility of using *APX1* overexpression in this cultivar to reveal the molecular mechanism of salinity tolerance in barley.

References

Afsharmanesh H, Ahmadzadeh M, Majdabadi A, Motamedi F, Behboudi K, Javan-Nikkhah M (2013). Enhancement of biosurfactants and biofilm production after gamma irradiation-induced mutagenesis of *Bacillus subtilis* UTB1, a biocontrol agent of *Aspergillus flavus*. Arch Phytopathol PFL 46: 1874-1884.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389-3402.

Anjum NA, Gill SS, Gill R, Hasanuzzaman M, Duarte AC, Pereira E, Ahmad I, Tuteja R, Tuteja N (2014). Metal/metalloid stress tolerance in plants: role of ascorbate, its redox couple, and associated enzymes. Protoplasma 251: 1265-1283.

Apel K, Hirt H (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55: 373-399.

Auger S, Krin E, Aymerich S, Gohar M (2006). Autoinducer 2 affects biofilm formation by *Bacillus cereus*. Appl Environ Microb 72: 937-941.

Beauchamp C, Fridovich I (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44: 276-287.

Bolwerk A, Lagopodi AL, Wijfjes AH, Lamers GE, Chin-A-Woeng TF, Lugtenberg BJ, Bloemberg GV (2003). Interactions in the tomato rhizosphere of two *Pseudomonas* biocontrol strains with the phytopathogenic fungus *Fusarium oxysporum* f. sp. radicis-lycopersici. Mol Plant Microbe In 16: 983-993.

Caverzan A, Passaia G, Rosa SB, Ribeiro CW, Lazzarotto F, Margis-Pinheiro M (2012). Plant responses to stresses: role of ascorbate peroxidase in the antioxidant protection. Genet Mol Biol 35: 1011-1019.

Chang P, Gerhardt KE, Huang XD, Yu XM, Glick BR, Gerwing PD, Greenberg BM (2014). Plant growth-promoting bacteria facilitate the growth of barley and oats in salt-impacted soil: implications for phytoremediation of saline soils. Int J Phytoremediat 16: 1133-1147.

Chen L, Liu Y, Wu G, Veronican Njeri K, Shen Q, Zhang N, Zhang R (2016). Induced maize salt tolerance by rhizosphere inoculation of *Bacillus amyloliquefaciens* SQR9. Physiol Plantarum 158: 34-44.

Choi SK, Jeong H, Kloepper JW, Ryu CM (2014). Genome sequence of *Bacillus amyloliquefaciens* GB03, an active ingredient of the first commercial biological control product. Genome Announcements 2: e01092-14.

Goth L (1991). A simple method for determination of serum catalase activity and revision of reference range. Clin Chim Acta 196: 143-151.

Gururani MA, Upadhyaya CP, Baskar V, Venkatesh J, Nookaraju A, Park SW (2013). Plant growth-promoting rhizobacteria enhance abiotic stress tolerance in *Solanum tuberosum* through inducing changes in the expression of ROS-scavenging enzymes and improved photosynthetic performance. J Plant Growth Regul 32: 245-258.

Hagege D, Nouvelot A, Boucaud J, Gaspar T (1990). Malondialdehyde titration with thiobarbiturate in plant extracts: avoidance of pigment interference. Phytochem Analysis 1: 86-89.

Hernández JA, Olmos E, Corpas FJ, Sevilla F, Río LA (1995). Salt-induced oxidative stress in chloroplasts of pea plants. Plant Sci 105: 151-167.

Ibrahim NAGAA, Omar MNAM (2015). Thermophilic *paenibacillus* nitrogen fixation bacteria increase cereal crops productivity. Journal of American Science 11: 39-54.

Ilangumaran G, Smith DL (2017). Plant growth promoting rhizobacteria in amelioration of salinity stress: a systems biology perspective. Front Plant Sci 8: 1768.

Kasim WA, Gaafar RM, Abou-Ali RM, Omar MN, Hewait HM (2016). Effect of biofilm forming plant growth promoting rhizobacteria on salinity tolerance in barley. Annals of Agricultural Sciences 61: 217-227.

Koussevitzky S, Suzuki N, Huntington S, Armijo L, Sha W, Cortes D, Shulaev V, Mittler R (2008). Ascorbate peroxidase 1 plays a key role in the response of *Arabidopsis thaliana* to stress combination. J Biol Chem 283: 34197-34203.

Kumar KB, Khan PA (1982). Peroxidase and polyphenol oxidase in excised ragi (*Eleusine corocana* cv. PR 202) leaves during senescence. Indian J Exp Biol 20: 412-416.

Lazzarotto F, Teixeira FK, Rosa SB, Dunand C, Fernandes CL, de Vasconcelos Fontenele A, Gomes Silveira JA, Verli H, Margis R, Margis-Pinheiro M et al. (2011). Ascorbate peroxidase-related (APx-R) is a new heme-containing protein functionally associated with ascorbate peroxidase but evolutionarily divergent. New Phytol 191: 234-250.

Liu HC, Tian DQ, Liu JX, Ma GY, Zou QC, Zhu ZJ (2013). Cloning and functional analysis of a novel *ascorbate peroxidase* (*APX*) gene from *Anthurium andraeanum*. J Zhejiang Univ-Sc B 14: 1110-1120.

Liu J, Liu M, Wang J, Yao JM, Pan RR, Yu ZL (2005). Enhancement of the *Gibberella zeae* growth inhibitory lipopeptides from a *Bacillus subtilis* mutant by ion beam implantation. Appl Microbiol Biot 69: 223-228.

Mohapatra BR, Broersma K, Mazumder A (2007). Comparison of five rep-PCR genomic fingerprinting methods for differentiation of fecal *Escherichia coli* from humans, poultry and wild birds. FEMS Microbiol Lett 277: 98-106.

Munns R, James RA, Läuchli A (2006). Approaches to increasing the salt tolerance of wheat and other cereals. J Exp Bot 57: 1025-1043.

Nabti E, Schmid M, Hartmann A (2015). Application of halotolerant bacteria to restore plant growth under salt stress. In: Maheshwari D, Saraf M, editors. Halophiles. Springer, pp. 235-259.

Nadeem SM, Zahir ZA, Naveed M, Nawaz S (2013). Mitigation of salinity-induced negative impact on the growth and yield of wheat by plant growth-promoting rhizobacteria in naturally saline conditions. Ann Microbiol 63: 225-232.

Nautiyal CS, Srivastava S, Chauhan PS, Seem K, Mishra A, Sopory SK (2013). Plant growth-promoting bacteria *Bacillus amyloliquefaciens* NBRISN13 modulates gene expression profile of leaf and rhizosphere community in rice during salt stress. Plant Physiol Bioch 66: 1-9.

Nishiguchi MK, Doukakis P, Egan M, Kizirian D, Phillips A, Prendini L, Rosenbaum HC Torres E, Wyner Y, DeSalle R Omar MNA, Osman MEH, Kasim WA, Abd El-Daim IA (2009). Improvement of salt tolerance mechanisms of barley cultivated under salt stress using *Azospirillum brasilense*. In: Ashraf M, Ozturk M, Athar HR, editors. Salinity and Water Stress. Dordrecht, the Netherlands: Springer, pp. 133-147.

Oser BL (1979). Hawk's Physiological Chemistry. New York, NY, USA.

Ozyigit II, Filiz E, Vatansever R, Kurtoglu KY, Koc I, Öztürk MX, Anjum NA (2016). Identification and comparative analysis of H_2O_2-scavenging enzymes (ascorbate peroxidase and glutathione peroxidase) in selected plants employing bioinformatics approaches. Front Plant Sci 7: 301.

Paul D, Lade H (2014). Plant-growth-promoting rhizobacteria to improve crop growth in saline soils: a review. Agron Sustain Dev 34: 737-752.

Petrov VD, Van Breusegem F (2012). Hydrogen peroxide—a central hub for information flow in plant cells. AoB Plants 2012: pls014.

Ramesh A, Sharma SK, Sharma MP, Yadav N, Joshi OP (2014). Plant growth-promoting traits in *Enterobacter cloacae* subsp. *dissolvens* MDSR9 isolated from soybean rhizosphere and its impact on growth and nutrition of soybean and wheat upon inoculation. Agr Res 3: 53-66.

Rohlf FJ (2005). NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System, Ver. 2.11. New York, NY, USA: Exeter Publishing Setauket.

Saleh-Lakha S, Glick BR (2006). Plant growth-promoting bacteria. In: van Elsas JD, Jansson JK, Trevors JT, Nannipieri P, editors. Modern Soil Microbiology, Boca Raton, FL, USA/UK: CRC/Thomson Publishing, pp. 503-520.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning: A Laboratory Manual (2). Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.

Sanger F, Nicklen S, Coulson AR (1977). DNA sequencing with chain-terminating inhibitors. P Natl Acad Sci USA 74: 5463-5467.

Sharma P, Dubey RS (2005). Modulation of nitrate reductase activity in rice seedlings under aluminium toxicity and water stress: role of osmolytes as enzyme protectant. J Plant Physiol 162: 854-864.

Sharma P, Jha AB, Dubey RS, Pessarakli M (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Journal of Botany 2012: 1-26.

Shi WM, Muramoto Y, Ueda A, Takabe T (2001). Cloning of peroxisomal ascorbate peroxidase gene from barley and enhanced thermotolerance by overexpressing in *Arabidopsis thaliana*. Gene 273: 23-27.

Singh JS, Pandey VC, Singh DP (2011). Efficient soil microorganisms: a new dimension for sustainable agriculture and environmental development. Agr Ecosyst Environ 140: 339-353.

Sofo A, Scopa A, Nuzzaci M, Vitti A (2015). Ascorbate peroxidase and catalase activities and their genetic regulation in plants subjected to drought and salinity stresses. Int J Mol Sci 16: 13561-13578.

von Zitzewitz J, Szucs P, Dubcovsky J, Yan L, Francia E, Pecchioni N, Casas A, Chen TH, Hayes PM, Skinner JS et al. (2005). Molecular and structural characterization of barley vernalization genes. Plant Mol Biol 59: 449-467.

Wang C, Yang CP, Wang YC (2009). Cloning and expression analysis of an *APX* gene from *Betula platyphylla*. Journal of Northeast Forestry University 37: 79-81.

Witzel K, Matros A, Strickert M, Kaspar S, Peukert M, Mühling KH, Börner A, Mock HP (2014). Salinity stress in roots of contrasting barley genotypes reveals time-distinct and genotype-specific patterns for defined proteins. Mol Plant 7: 336-355.

Kakar KU, Ren XL, Nawaz Z, Cui ZQ, Li B, Xie GL, Hassan MA, Ali E, Sun GC (2016). A consortium of rhizobacterial strains and biochemical growth elicitors improve cold and drought stress tolerance in rice (*Oryza sativa* L.). Plant Biol 18: 471-483.

Xiang W, Guo J, Feng W, Huang M, Chen H, Zhao J, Zhang J, Yang Z, Sun Q (2008). Community of extremely halophilic bacteria in historic Dagong Brine Well in southwestern China. World Journal of Microbiology and Biotechnology 24: 2297-2305.