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Isolation of partial genomic DNA sequence coding for Glyoxalase I in saline tolerant *Rizhophora mucuronata* (Indian mangrove)

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Abstract:

Glyoxalase pathway is normally induced and up regulated in response to salinity, drought and cold stresses in many plants. The mangroves are relatively saline resistant plant and it would be very interesting to know the saline tolerance mechanism of mangroves. In the current study we report, the isolation of partial glyoxalase I gene from *Rizophora mucuronata* (Mangrove) and its role in saline tolerance mechanism in mangroves. The various responses of the plant towards salinity have been utilized in genetic engineering to generate transgenic stress resistant crop plants either by transferring stress responsive genes or altering the expression of existing genes. Mangroves and associated plants are among the most salt tolerant plants and are ideal models for studying the salt tolerant mechanisms because of their ability to tolerate extremely high salinity. A partial genomic sequence coding for Glyoxalase I was PCR amplified using primers designed based on previously reported sequences on other species. Two specific primers were used. The 400 bp fragment was amplified from genomic DNA. The 400 bp genomic DNA fragment amplified was sequenced. After sequencing a 370 bp was obtained excluding the primer regions. Glyoxalase I gene from the present sequence shows significant similarity with several glyoxalase I genes isolated from different plant species. Maximum similarity for the sequenced portion of the gene was obtained with glyoxalase I from *Hevea brasiliensis* glyoxalase (100%), followed by *Manihot esculenta* glyoxalase (94.74%) and *Jatropha curcas* (93.61%). Distance tree also reveals relationship other glyoxalases from other species. The short protein sequence was also deduced from nucleotide sequences and it shows maximum similarity with *Hevea brasiliensis.*

Introduction:

Salinity is one of the most significant environmental challenges, limiting plant productivity, particularly in arid and semiarid climates. Salinity tolerance involves a complex of responses at molecular, cellular, metabolic, and whole plant level. Nowadays plant biology research works give an attention towards plant responses to salt stress and mechanism of salt tolerance. The various responses of the plant towards salinity have been utilized in genetic engineering to generate transgenic stress resistant crop plants either by transferring stress responsive genes or altering the expression of existing genes. Mangroves a are ideal models for studying the salt tolerant mechanisms because of their ability to tolerate extremely high salinity. Salinity is a major environmental stress impeding plant growth and productivity, thus affecting about 20% of the cultivable and about 50% of the irrigated lands worldwide. It imposes two kinds of stresses to plants; osmotic stress arising from the reduced water availability due to increased osmotic pressure, and ionic stress due to the increase in the levels of toxic ions like Na^+ and Cl^- leading to ionic imbalance. In this regard, mangrove plants are an important class of halophytes that grow in high saline environment.

Several mangrove trees have been shown to reach an optimal growth at salinities of 5–25% of standard seawater. To survive under saline condition arising from the fluctuating seawater levels, the mangrove plants have developed various morphological and physiological adaptations such as salt secretion via salt glands on the leaves, compartmentalization of salts, accumulation of osmolytes, and salt exclusion (ultrafiltration) by roots. Despite all these ecologically important characteristics, the *Rhizophora mucronata* is a salt-tolerant true mangrove which is widely distributed in Indian mangrove forest. Mangroves inhabit intertidal zones with high salinity water (Shan et al. 2008) and are able to tolerate a large range of salinities under natural conditions (Suarez et al. 1998). The growth and physiological mechanisms of mangroves vary in nature due to the complexity of structure and differences in flooding regime, tidal inundation, rapid influx of extra nutrients, as well as type of soil (Naidoo 1987). The plant aids in coastal stabilization and provides nursery areas for economically important fishes and crustaceans in the tropics and sub-tropics (Tomlinson 1986). The forest stature of this species is regulated by residual effects of tidal and wave energy, soil salinity, nutrient availability and flooding frequency.

Plants are constantly challenged by various biotic and abiotic stresses in nature. Abiotic stresses such as drought, salinity, cold, high temperature, chemical toxicity, high light intensity and oxidative stresses lead to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang et al., 1999). As a result, and the course of their evolution, plants have developed numerous unique adaptation and defense mechanisms to help them cope with unavoidable stresses that may be imposed upon them. One such defense mechanism is the development of an enzyme system for protection against potentially toxic effects of xenobiotics and reactive oxygen species generated during environmental stresses.

The glyoxalase system comprises the enzymes glyoxalase-I (Gly I; lactoylglutathione lyase; EC 4.4.1.5) and glyoxalase-II (Gly II; hydroxyacylglutathione hydrolase; EC 4.4.1.5). The two enzymes act co-coordinately to convert a variety of toxic-2-oxoaldehydes into less reactive 2-hyroxy acids, utilizing glutathione (GSH) as a cofactor. Methylglyoxal appears to be the primary physiological substrate for the glyoxalase system. Methylglyoxal is a potent cytotoxic found in all organisms which is formed primarily as a byproduct of carbohydrate and lipid metabolism (Thornelley 1990). Glyoxalase-I catalyzes the formation of S-D-lactoylglutathione (S-LG) from MG and GSH (Thornalley 1996). S-LG is further metabolized to D-lactate and GSH by glyoxalase-II. A high level of MG accumulation is toxic to cells, since it inhibits cell proliferation and results in a number of adverse effects such as increasing the degradation of proteins and inactivating the antioxidant defense system.

Recent investigations in plants have brought new developments in the involvement of the glyoxalase system in stress tolerance and its involvement with oxidative defense systems. Glyoxalase I have also been found to be one of the several genes induced in response to drought and cold stresses in Arabidopsis (Seki et al., 2001). In addition to the generation of ROS, the accumulation of methylglyoxal (MG) has also been reported under stress conditions, including drought (Singla-Pareek et al., 2006; Yadav et al., 2008; Hossain et al., 2009; Siraj et al., 2016). Because of the highly cytotoxic and reactive properties of MG, its concentrations must be kept under strict control. By maintaining GSH homeostasis and antioxidant enzyme levels, over expression of glyoxalase enzymes in plants has been found to limit the increase in ROS and MG levels under stress condition. To date the role of glyoxalase pathway in enhancing tolerance towards salinity and heavy metal stress has been documented.

Undoubtedly, the role of MG-scavenging systems in plant stress tolerance has increased through the use of gene transfer technology. A number of experiments clearly demonstrated that the enhancement of the MG-detoxification systems in plants provides partial protection from oxidative damage (Espartero et al., 1995., Veena et al., 1999, Yadav et al., 2005, Singla-Pareek et al., 2006, 2008,). Over-expression of glyoxalase pathway genes in transgenic plants has been found to keep a check on the MG and ROS levels under stress conditions, regulate glutathione homeostasis, allowing the transgenic plants to survive and grow under various abiotic oxidative stresses.). The present study investigates partial genomic DNA characterization of Glyoxalase I from *Rizhophora mucuronata*.

Materials and Methods:

Plant material

Young, healthy, 20 days old seedlings of Hevea brasiliensis clone RRII 105 were used for the study. Before use, the seedlings were removed from soil and thoroughly washed with deionized water.

Isolation of DNA

In the present study attempts have been made to sequence the partial Glyoxalase I gene from *Rizhophora mucronata*. Genomic DNA (**Fig.1**) was isolated according to the CTAB extraction procedure in good quality and concentration. In the present study the powdered tissue in presence of liquid nitrogen, was digested with CTAB buffer and aqueous phase was partitioned twice phenol: chloroform: isoamyl alcohol to eliminate all the contaminating components and macromolecules. The extracted DNA was precipitated with isopropanol and air-dried and dissolved the pellet in Tris Ethylene diamine tetra acetic acid buffer.

Agarose gel electrophoresis

The quality of the isolated DNA was checked by agarose gel electrophoresis. Two µl of loading buffer (0.25% Bromophenol blue, 30% Glycerol in TE buffer, pH=8) was added to 8 µl of DNA and the samples were loaded to 0.8% agarose gel prepared in 0.5X TBE buffer (Tris-Borate-EDTA). Electrophoresis was carried out at 70 V for nearly 2 h until the bromophenol dye front has migrated to the bottom of the gel. The molecular marker used was lambda DNA, double digested with *EcoRI* or *HindIII* restriction enzymes. Staining was carried out with 0.5 µg/ml of ethidium bromide. The gel was visualized using gel documentation system and was photographed under UV light

PCR amplification of glyoxalase I

The sequences coding for glyoxalase I in *Arabidopsis thaliana*, *Cicer arietinum*, *Lycopersicon esculentum* and *Avicenia marina* were downloaded and consensus sequences were identified using 'megalign' programme of lasergene software (DNASTAR, U.S.A). One set of specific primers 5'GATGAAGCAACTAAAGGTTA3' (forward) and 5'CCAATAGCCATCAGGATCTT3' (reverse) were used. The glyoxalase I partial sequences were PCR amplified from both genomic and cDNA. The PCR reactions were carried out in 20 µl reaction volumes containing 100 µM dNTPs, 250 nM of each primer, 10X Taq assay buffer and 0.75 U Taq DNA polymerase (Sigma, U.S.A) with 20 ng template DNA in a thermal cycler (Biorad, U.S.A). The PCR amplification profile consisted of first a denaturation at 94^0C for 4 min, followed by 35 cycles of 94^0C at 1 min, 45^0C for 2 min and 72^0C for 1 min. Amplified DNA fragments were electrophoresed in 1.5% agarose gel and observed under UV light. The PCR products from *Hevea* cDNA were gel purified and used for cloning.

Sequence analysis

The sequencing of the PCR amplified fragment was done at the DBT facility for DNA sequencing, Indian Institute of Science, Bangalore, India. The method was done in an automated DNA sequencer using the same forward and reverse primers used for PCR amplification. Sequence analysis was done through BLAST analysis at the NCBI (National Centre for Biotechnology information, USA) site.

Results and discussion

Under optimal PCR conditions, a prominent band of expected size (440 bp) was amplified from the genomic DNA (Figure. 1). The PCR product, purified from the gel, was sequenced with the same primers used for amplification. A partial DNA sequence of glyoxalase I, which was 379 bp long, was obtained from the sequencing results excluding the primer regions (Figure. 2). The genomic DNA sequence of glyoxalase I gene from Hevea, obtained in the present study was subjected to online BLAST analysis to find out the similarity with the already reported sequences. The present sequence showed significant similarity with several glyoxalase I genes isolated from different plant species. The present sequence shows significant similarity with several glyoxalase I genes isolated from different plant species. Maximum similarity for the sequenced portion of the gene was obtained with glyoxalase I from *Hevea brasiliensis* glyoxalase (100%), followed by *Manihot esculenta* glyoxalase (94.74%) and *Jatropha curcas* (93.61%). A comparative analysis of *Rhizophora mucronata* glyoxalase I with other sequences was given in Figure 3. In order to find the evolutionary relationship of *Rhizophora* glyoxalase I gene and other glyoxalases, a distance tree was also created from NCBI BLAST analysis. The distance tree analysis was given in the Figure 4. The short protein sequence was also deduced from nucleotide sequences and it shows maximum similarity with *Hevea brasiliensis* (Figure. 5). Molecular characterization of partial cDNA from *Hevea brasiliensis* has been carried out and the sequence shows high similarity with other glyoxalase sequences from plants (Siraj et al., 2016). Maximum similarity for the sequenced portion of the gene was obtained with glyoxalase I from *Ricinus communis* (90%), followed by expressed sequence tags from severe drought-stressed leaves (87%), *Glycine max, Cicer arietinum* (83%), *Cucurbita maxima* etc. It also showed similarities with Glyoxalase I mRNA isolated from *Arabidopsis* (82%), *Avecenia marina* (82%), *Solanum* (81%) and *Brassica* (80%) and several other mRNAs from drought-stressed leaves. According to phylogenetic analysis *Hevea brasiliensis* and *Ricinus communis* glyoxalase I are the closest in evolution. Also Cucurbita and Allium show a closer relationship with Hevea Glyoxalase I.A 126 amino acid long protein sequence was deduced from the cDNA sequence obtained. The protein sequence also showed high homology with the glyoxalases from different species. Maximum homology was obtained with the *Ricinus communis* glyoxalase protein (91%) (Siraj et al., 2016)

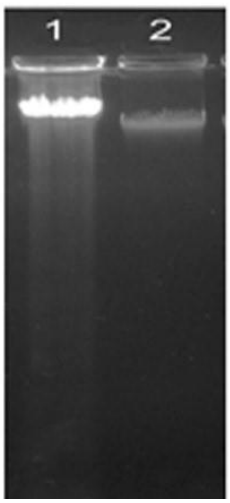

Figure 1. Genomic DNA isolated from *Rhizophora mucuronata*

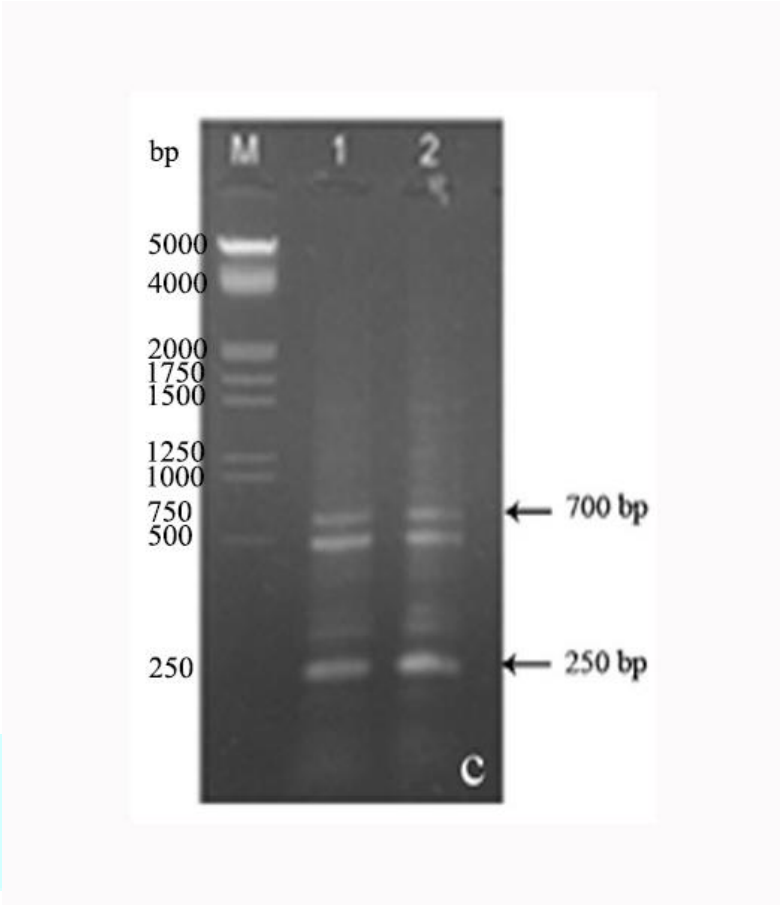

Figure 2. Amplification of glyoxalase I gene sequences from genomic DNA of *Rhizophora mucuronata* (Mangrove). M =100 bp DNA ladder, Lane 1 & 2 = 400 bp amplicon

ATTAAGGATCCAAAAATAAGTCTCGATTTTTATTCTCGCGTATTGGGCATGTCGTTGCTTAAGAGGTTGGATT
TTCCAGACATGAAGTTTAGCTTGTACTTTATGGGCTACGAGGATCCAGCATCAGCTCCAAGTGACCCAGTTG
AAAGAACTGTTTGGACCTTTGGTCAGAAGGCTACAATTGAATTAACTCATAATTGGGGTACTGAAAGTGAT
CCTGACTTCAAAGGATATCACAATGGAAATTCAGAACCTCGTGGCTTTGGACATATTGGTATTTCTGTGGAT
GATGTGTACAAGGCATGTGAGAGATTTGAACATCTAGGGGTGGAGTTCGCCAAAAAACCTGATGATGGAA
AAATGAAAGGAA

Figure 3. Partial genomic DNA sequences of glyoxalase I from *Rhizophora mucuronata* (Mangrove)

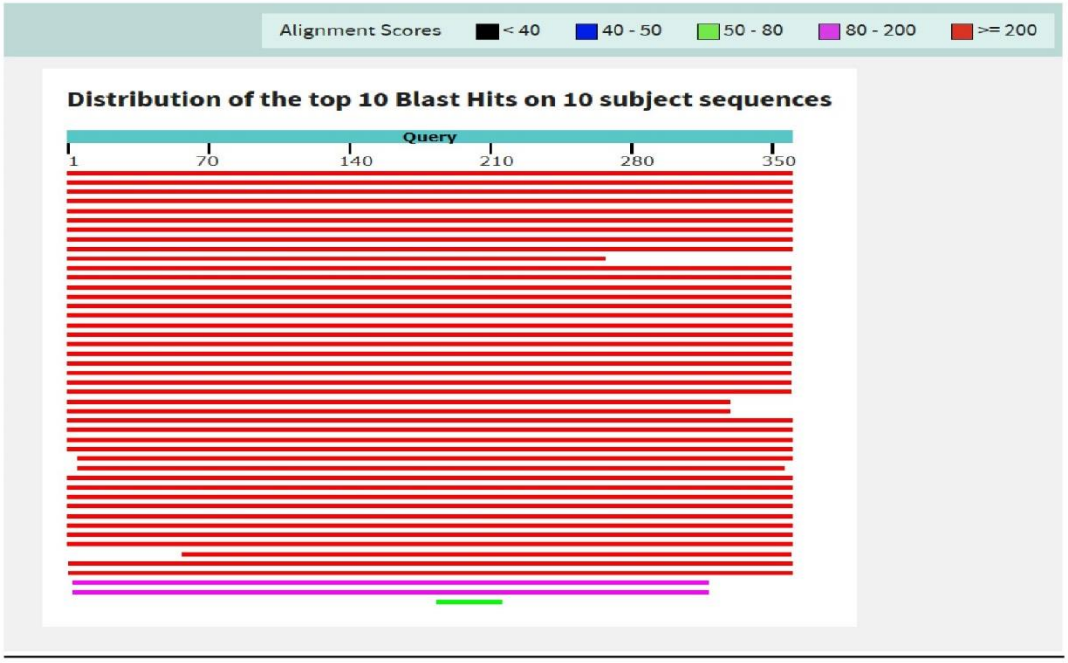

Figure 4. Distribution of the top 38 BLAST Hits on 38 subject sequences. The sequences with maximum similarity to *Rhizophora mucuronata* gene sequences was also shown

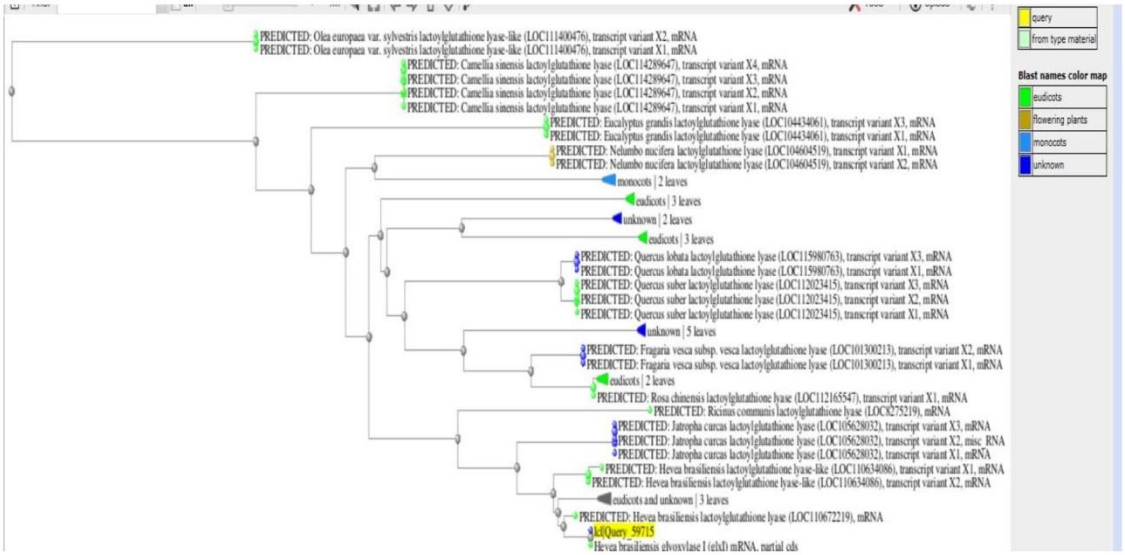

Figure 5. Blast Tree view. This tree was produced using BLAST pair wise alignment with *Rhizophora mucuronata* and other sequences

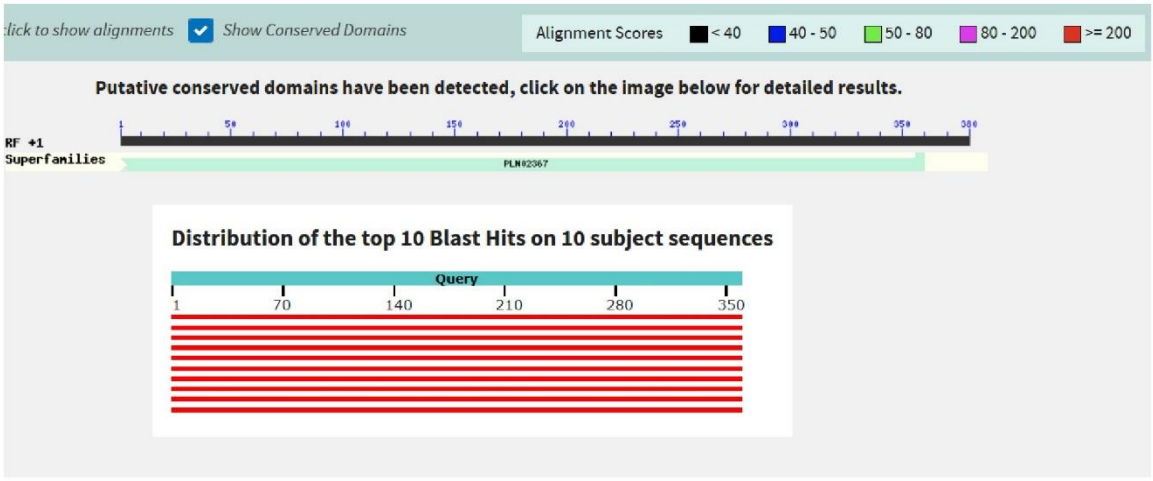

Figure 6. Distribution of the top 10 Blast Hits on 10 glyoxalase protein sequences (BLASTX). The sequence with maximum similarity to *Rhizophora* Glyoxalase I protein sequences was also shown.

In plants glyoxalase pathway considered to be associated with tolerance to various abiotic stresses. Recent investigations in plants have brought new developments in the involvement of the glyoxalase system in stress tolerance and its involvement with oxidative defense systems. Further insights into the biological function of the glyoxalase system came from the molecular cloning of their respective genes. The pioneering work of Dr. Sudhir Kumar Sopory and his associated co-workers (Veena et al., 1999, Singla-Pareek et al., 2003, Saxena et al., 2005, Yadav et al., 2005a, 2005b) provides a potential framework for interpreting the physiological roles of the glyoxalase system in higher plants against various abiotic stresses.

In general, salt tolerance is brought about by the interplay of multiple genes, which involves many physiological, biochemical, and molecular processes. Over the past decade, efforts have been made to understand this complex mechanism by profiling the global gene expression patterns in various plant species. In the beginning, most of the molecular insights were obtained using the glycophytic model plant *Arabidopsis*. Additional work with important crop plants such as rice and maize led to the identification and characterization of a number of salt-responsive genes. Such studies also unraveled various signaling pathways and the importance of regulation of expression of specific genes associated with salt tolerance.

Conclusion:

In the current study we report, the isolation of partial glyoxalase I gene from *Rizophora mucuronata* (mangrove) and its role in saline tolerance mechanism in mangroves. The various responses of the plant towards salinity have been utilized in genetic engineering to generate transgenic stress resistant crop plants either by transferring stress responsive genes or altering the expression of existing genes. Mangroves and associated plants are among the most salt tolerant plants and are ideal models for studying the salt tolerant mechanisms because of their ability to tolerate extremely high salinity. Further studies are needed to reveal the complete mechanism of saline resistance. Genetic variations and differential responses to salinity stress in plants differing in stress tolerance enable plant biologists to identify physiological mechanisms, sets of genes, and gene products that are involved in increasing stress tolerance and to incorporate them in suitable

species to yield salt tolerant varieties. Identified genes can be used for the study of the underlying biology of halotolerance. In this study, it is assumed that the tolerance towards salinity of mangrove plant may be due to over-expression of glyoxalase I gene, in the sense that under saline conditions more amount of glyoxalase enzyme was produced due to the increased activity of glyoxalase I gene, to detoxify MG and reactive oxygen species which lead to the increased tolerance of these plants against salinity stress.

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