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Glyoxalase I expression pattern in *Hevea brasiliensis* seedlings under varied stress conditions

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Drought is one of the most important stress factors which adversely affect plants' growth and productivity. Global climate change may make this situation more serious in the years ahead. Considering the long time span required for the generation of drought resistant genotypes in Rubber (*Hevea brasiliensis*) through conventional breeding, molecular interventions to engineer plants to have either drought responsive genes or genes expected to alter osmotic regulation would be very attractive. The glyoxalase pathway involving glyoxalase I and glyoxalase II enzymes is required for glutathione-based detoxification of methylglyoxal. In this study the effects of various abiotic stresses on the up-regulation of methylglyoxal levels and glyoxalase I activities in *Hevea brasiliensis* seedlings were investigated. Most of the stresses caused significant increase in methylglyoxal level and glyoxalase I activity, among which drought caused the highest induction of glx I followed by salinity, 2, 4-D, ABA, methylglyoxal, white light and $CdCl_2$. The stress-induced increases in methylglyoxal and glyoxalase I activity found in the present study suggest an important role of glyoxalase I in conferring drought tolerance. The up-regulation of glyoxalase I under drought stress indicates its future utility in developing tolerance to drought stress in *Hevea brasiliensis*. In the present study, a partial cDNA sequence coding for glyoxalase I was amplified by PCR using specific primers. The 440 bp cDNA amplicon obtained was sequenced and subjected to online BLAST analysis. The sequence of *Hevea brasiliensis* glyoxalase I (GenBank Acc. No: GU598520) had six open reading frames. The ORF finder revealed the longest ORF of 336 bp. Glyoxalase I from *Ricinus communis* had the highest nucleotide sequence homology (90%) compared to the amplified gene. BLASTP analysis also showed high homology between the deduced protein sequence of the amplified gene and glyoxalases from other species. Our results suggest that the multi-stress inducibility of glyoxalase I in the present study may be due to the fact that it might protect the plants against MG that is formed under various stresses including drought and confers tolerance by increasing the GSH-based detoxification system and decreasing lipid peroxidation

Key words: Glyoxalase I, *Hevea brasiliensis*, Methylglyoxal, drought tolerance, abiotic stress.

INTRODUCTION

Nature has not provided any other industrial raw material of plant origin as flexible as natural rubber. *Hevea brasiliensis*, the Para rubber tree accounts for more than 99% of the world's natural rubber production. In recent

years, the global consumption of natural rubber is steadily increasing and the production has to be increased so as to meet the demand. Many of the recently developed high yielding *Hevea brasiliensis* clones including RRII 105 are susceptible to drought. One of the most severe environmental stresses, drought, is a major constraint for plant productivity worldwide. Thus, it is among the worst scourges of agriculture. The acclimation of plants to drought is often associated with increased levels of reactive oxygen species (ROS) such as superoxide radical. ROS attack the most sensitive biological macromolecules in cells and impair their functions. If drought stress is prolonged to a certain extent, ROS production will overwhelm the scavenging action of the antioxidant system, which results in extensive cellular damage and death.

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 cytotoxin found in all organisms, which is formed primarily as a byproduct of carbohydrate and lipid metabolism. Glyoxalase-I catalyzes the formation of S-D-lactoylglutathione (S-LG) from MG and GSH. 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. Apart from MG, pathway intermediate S-LG (substrate for glyoxalase II) has also been found to be cytotoxic at high concentrations in that it inhibits DNA synthesis (Thornalley et al., 1996). The genes encoding glyoxalase I and glyoxalase II have been isolated and characterized from microbial and animal systems and found to have significant protein sequence homology (Rhee et al., 1987; Lu et al., 1990; Ray et al., 2001; Ranganathan et al., 1993).

Glyoxalase I activity has been studied in several higher plant species, and in some cases the enzyme has been further characterized (Deswal et al., 1993; Paulus et al., 1993; Umeda et al., 1994). In tomato (*Lycopersicon esculentum*), an 848-bp cDNA clone was identified by differential screening of salt induced genes, and glyoxalase I activity was confirmed by expression in yeast (Gody et al., 1990; Espartero et al., 1995). Using a similar approach in the resurrection grass *Sporobolus staphianus*, a 1.2-kb cDNA clone was found in desiccated plants (Blomstedt et al., 1998). In addition, a cDNA clone encoding a 186-residue short glyoxalase I has been isolated from epicotyls of *Cicer arietinum* grown under osmotic stress conditions (Romo et al., 1998), but glyoxalase I cDNA from *Brassica juncea* was cloned and found to confer resistance to stress when expressed in *E.coli* and tobacco. Over-expression of glyoxalase I resulted not only in improved tolerance against MG, but interestingly, the transgenic plants tolerated higher levels of salinity as compared with the non-transgenic plants. Over-expression of glyoxalase I and glyoxalase II together conferred improved salinity tolerance, thus offering another effective strategy for manipulating stress tolerance in crop plants. 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 (Hossain et al., 2009; Yadav et al., 2005; Bhomkar et al., 2008). Glyoxalase I has also been found to be one of the several genes induced in response to drought and cold stresses in *Arabidopsis* (Seki et al., 2001).

The present study investigates the methylglyoxal levels and glyoxalase I activity under different abiotic stresses in *Hevea brasiliensis,* and provides sequence information on glyoxalase I from rubber.

MATERIALS AND METHODS

Plant material

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

Stress treatments

The seedlings were subjected to different stress treatments for the estimation of methylglyoxal and Glyoxalase I levels. For drought stress treatment, seedlings were placed in a pot without water and kept at 25°C. Seedlings were placed in 20 ml of 300 mM NaCl solution for salt stress. 1 mM $CdCl_2$ solution was also used as heavy metal stress. To study the effect of white light, seedlings were placed in a beaker containing 20 ml of distilled water and exposed to white light (60 µmol photon $m^{-2}s^{-1}$) and illuminated for 18 and 24 h at 25°C. To observe hormonal effects on *Hevea brasiliensis* glyoxalase I, 50 µM ABA and 50 µM 2, 4-D solutions were used. Four seedlings were used in each treatment and were incubated for 24 h in the dark. 25 mM MG solution was used for chemical stress. Seedlings incubated in 20 ml of distilled water in the dark at 25°C were used as controls.

Sample preparation for MG estimation

Methylglyoxal was estimated according to the method of Hossain et

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al., (2009). In brief about 0.5 g hypocotyl tissue was homogenized in 3 ml of 0.5 M perchloric acid. After incubating 15 min on ice, the mixture was centrifuged at 4°C for 10 min at 11,000 g. The supernatant was decolorized by adding charcoal (10 mg/ml), kept for 15 min at room temperature, and centrifuged at 11,000 g for 10 min. Before using this supernatant for MG assay, it was neutralized by keeping for 15 min with saturated solution of potassium carbonate at room temperature and centrifuged again at 11,000 g for 10 min. The neutralized supernatant was used for MG estimation.

Methylglyoxal assay

Methylglyoxal assay was carried out according to the method of yadav et al. (2005). In a total volume of 1 ml, 250 µl of 7.2 mM 1, 2-diaminobenzene, 100 µl of 5 M perchloric acid, and 650 µl of the neutralized supernatant were added in that order. The absorbance at the 335 nm of the derivatized MG was read after 25 min in a spectrophotometer. The final concentration of the MG was calculated from the standard curve and expressed in terms of µg/ml.

Preparation of crude enzyme solution

The cotyledon and roots were removed from the stress treated seedlings, and hypocotyls were homogenized in an equal volume of 50 mM potassium phosphate buffer (pH 7.0) containing 100 mM KCl, 1% (w/v) ascorbate and 10% (w/v) glycerol. The homogenates were centrifuged at 11, 500 g for 10 min and the supernatant was used as a crude enzyme solution. For glyoxalase I assay, proteins were precipitated by ammonium sulphate at 65% saturation from the crude enzyme solution and centrifuged at 11,500 g for 10 min. The precipitate was dissolved in a minimum volume of buffer and transferred to a dialyzed membrane, dialyzed against 10 mM potassium phosphate buffer (pH 7.0) overnight, and then used for glyoxalase I assay.

Glyoxalase I enzyme assay

Glyoxalase I assay was carried out according to Hossain et al. (2009). Briefly, the assay mixture contained 100 Mm potassium phosphate buffer (pH 7.0), 15 Mm magnesium sulphate, 1.7 Mm reduced glutathione and 3.5 Mm methylglyoxal in a final volume of 0.8 ml. The reaction was started by the addition of MG and the formation of thioester was measured by observing the increase of absorbance at 240 nm for 1 min in a spectrophotometer. Concentration of Glyoxalase I was calculated from the standard curve and expressed in µg/ml.

Statistical analysis

All data obtained was subjected to one-way analysis of variance (ANOVA) and the significance of difference between the mean values was compared by Duncan's multiple range tests using MSTAT-C. Differences at $P \leq 0.05$ were considered significant

RNA isolation

Total RNA was isolated from leaves by LiCl precipitation method (Sambrook et al., 1989). Around 200 mg of leaf tissue was ground to a fine powder in liquid nitrogen and 2 ml of extraction buffer (0.2 M NaCl, 0.1M Tris-HCl, pH 8.5, 0.01M EDTA, pH 8.0, 1.5% SDS, 0.1% 2-mercaptoethanol and 1% insoluble PVPP) was added. Following extraction with an equal volume of extraction buffer-saturated phenol (centrifugation for 15 min at 10,000 g), the aqueous phase was transferred and re-extracted twice with equal volumes of chloroform. The RNA was precipitated overnight in 1/3 volume of 8M LiCl at -20°C. The precipitated RNA was recovered by centrifugation for 10 min at 10,000 g, washed with ice-cold 2M LiCl and dissolved in 250 µl sterile RNase free water. The RNA was then re-precipitated by adding 0.1 volumes of 3M sodium acetate and 2.5 volums of ethanol at -20°C. Following centrifugation for 10 min at 10,000 g, the pellet was re-suspended in 100 µl RNase free water. Ten microgram (10 µg) of Total RNA was resolved in 1% formaldehyde agarose gel.

cDNA synthesis

First strand cDNA was synthesized from total RNA by reverse transcription with oligo (dT) primer using the kit Super Script III first-strand synthesis system for RT-PCR (Invitrogen U.S.A) according to the manufacturer's protocol. 2 µl of the first strand cDNA was used to amplify the glyoxalase I sequences.

PCR amplification of glyoxalase I

The sequences coding for glyoxalase I in *Arabidopsis thaliana*, *Cicer arietinum*, *Lycopersicon esculentum* and *Avicenia marina* were compared and consensus sequences were identified using 'megalign' programme of lasergene software (DNASTAR, USA). One set of specific primers 5'GATGAAGCAACTAAAGGTTA3' (forward) and 5'CCAATAGCCATCAGGATCTT3' (reverse) were used. 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, USA) with 20 ng template DNA in a thermal cycler (Biorad, USA). The PCR amplification profile consisted of first a denaturation at 94°C for 4 min, followed by 35 cycles of 94°C at 1 min, 45°C for 2 min and 72°C for 1 min. Amplified DNA fragments were electrophoresed in 1.5% agarose gels stained under UV light. The PCR products were gel purified and used for cloning.

Cloning of glyoxalase I

The ligation reaction was set up with 100 ng of eluted PCR products. 1 µl (50 ng) of pGEM-T easy vector (Promega corporation, USA), 1 µl of T4 DNA ligase and 5 µl 2X rapid ligation buffer in a 10 µl total reaction volume kept at 16°C till transformation. The ligation product was transformed into competent cells of *Escherichia coli* JM109 prepared by $CaCl_2$ treatment and plated on LB/ampicillin plates with lPTG (7µl) and X-gal (40µl) and incubated overnight at 37°C. Recombinants were selected through blue-white screening on Luria agar. The presence of insert in single white colonies was confirmed by PCR with the same primer combination.

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 and sequence alignment was done using Multlign programme (http://multalin.toulouse.inra.fr/multalin/cgi-bin/multalin.pl)

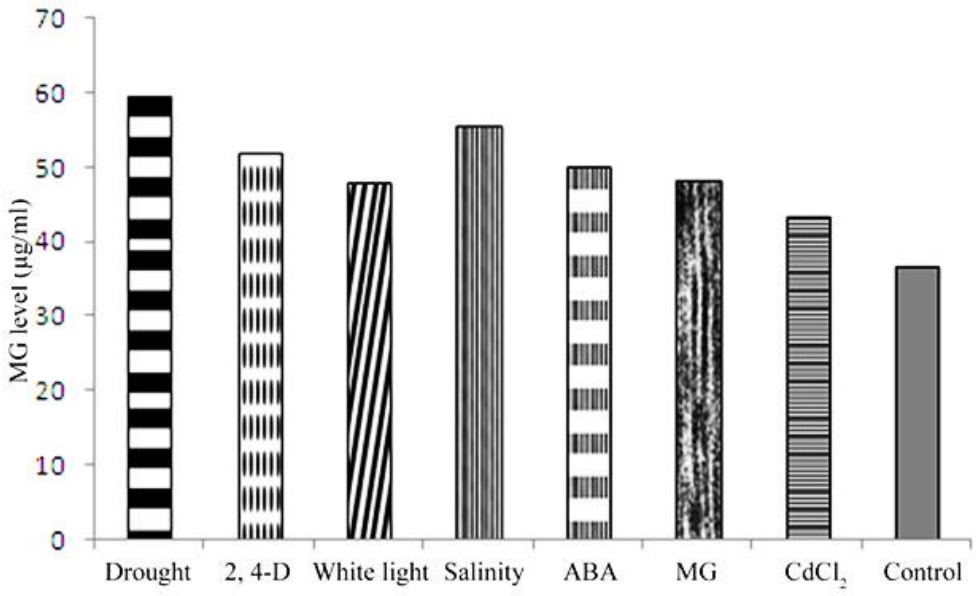

Figure 1. Effects of various stresses on MG levels in Hevea seedlings. Hevea seedlings were treated with drought, 2, 4-D (50 µM), white light, Salinity (300 mM NaCl), ABA (50 µM), MG (25 mM) and heavy metal ($CdCl_2$ (1mM)).

RESULTS AND DISCUSSION

Up-regulation of Methylglyoxal levels in *Hevea brasiliensis* under normal and stress conditions

The up- regulation of MG in rubber in response to various abiotic stresses was measured in young *Hevea brasiliensis* seedlings under control and various stresses like salt, drought, white light, MG, 2,4-D, ABA and heavy metal ($CdCl_2$) stresses. It was observed that methylglyoxal levels increased significantly under different stress treatments within 24 h and the levels ranged from 28 to 66.8 µg/ml under control and various stress conditions (Figure 1). Drought caused the highest induction (1.62 fold) of methylglyoxal followed by salinity, 2, 4-D, ABA, MG, White light and $CdCl_2$ stresses. Elevated levels of MG due to stress treatments have also been reported recently in plants (Yadav et al., 2005; Singla-Pareek et al., 2006; Hossain et al., 2009). Under stress conditions, cells become metabolically active, which is mirrored by upregulation of enzymes involved in glycolysis and TCA cycles (Umeda et al., 1994; Espartero et al., 1995). It is reported that MG levels increased under stress conditions. It may be that MG could act as a signal for plants to respond to stress (Hossain et al., 2009).

Up-regulation of glyoxalase I activity in *Hevea brasiliensis* under drought and other stress conditions

The concentrations of glyoxalase I in *Hevea brasiliensis* seedlings under normal and stress conditions were estimated. There was a significant increase in the enzyme concentration under the various stress treatments (Figure 2). Highest increase of glyoxalase I concentration was observed due to drought stress, followed by salinity, 2, 4-D, white light, ABA and MG stresses. Several research groups have reported that the activity of glyoxalase I was affected by various exogenous factors and abiotic stress treatments including salt, water, white light, ABA and heavy metal stresses (Chakravarty and Sopory, 1998; Espartero et al., 1995; Veena et al., 1999; Hossain et al., 2009). In the present study we also observed a significant increase of glyoxalase I activity due to different stress treatments especially drought and the results were in accordance with the MG levels.

Molecular characterization of glyoxalase I

In the present study we have isolated and cloned the sequence coding for glyoxalase I from *Hevea brasiliensis*. RNA in good quality and quantity without much degradation and DNA contamination was obtained by the LiCl precipitation method. After electrophoresis, the RNA was observed as a clear patch with bands of 18S and 28S rRNA. The mRNA in the isolated RNA was found to be intact as it could be successfully used for the cDNA synthesis and subsequent amplification of the gene from cDNA. The first strand cDNA from mRNA present in the total leaf RNA was synthesized through reverse transcription using the kit Super Script III first-strand

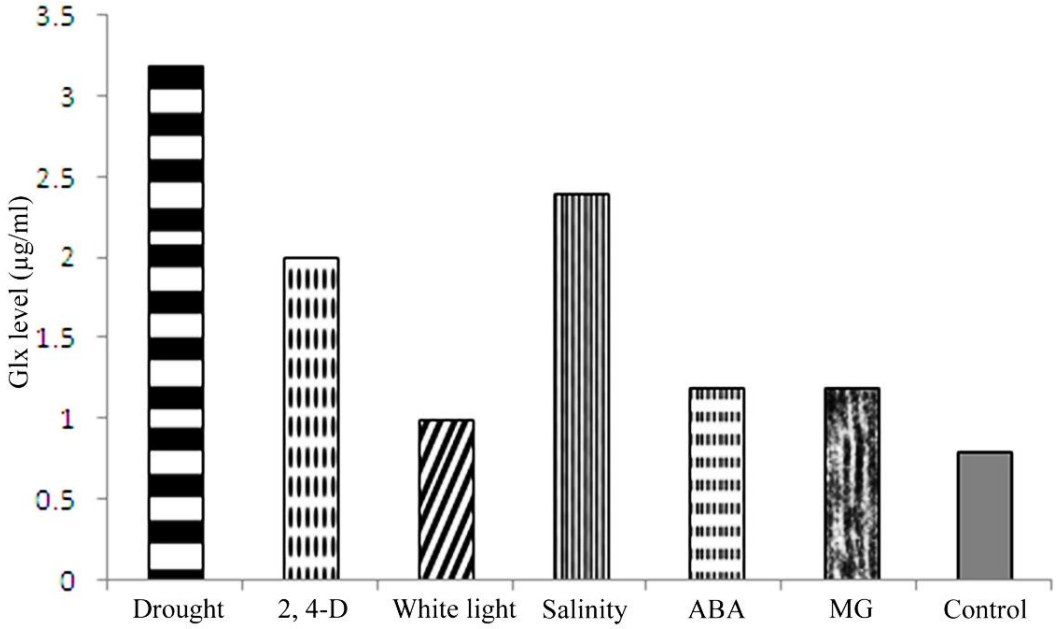

Figure 2. Effects of various stresses on glyoxalase I activities in Hevea seedlings. Hevea seedlings were treated with drought, 2, 4-D (50 µM), white light, salinity (300 mM NaCl), ABA (50 µM) and MG (25 mM).

synthesis system for RT-PCR. The glyoxalase I sequence was amplified using primers designed on the available sequence information in other species.

Under optimal PCR conditions, a prominent band of expected size (440 bp) was amplified from the cDNA (Figure 3a). The band was purified from gel for cloning and further sequence analysis. Cloning was confirmed by agarose gel electrophoresis and PCR analysis of the putative recombinant plasmids (Figure 3b and c). The PCR product, purified from the gel, was sequenced with the same primers used for amplification.

A partial cDNA sequence of glyoxalase I, which was 379 bp long, was obtained from the sequencing results excluding the primer regions (Figure 4).The sequence of *Hevea* glyoxalase (GenBank Acc. No: GU598520) had six open reading frames. The ORF finder revealed the longest ORF of 336 bp. The cDNA 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 and multiple sequence alignment was done through Multalign programme. The present sequence showed significant similarity with several glyoxalase I genes isolated from different plant species (Figure 5). Maximum similarity for the sequenced portion of the gene was obtained with the glyoxalase I from *Ricinus communis* (90%), followed by populus EST from severe drought-stressed leaves (87%), *Glycine max,Cicer arietinum* (83%), *Cucurbita maxima* etc. It also shows similarities with glyoxalase I mRNA isolated from *Arabidopsis* (82%), *Avecenia marina* (82%), *Solanum* (81%) and *Brassica* (80%) and several other mRNA from drought-stressed leaves. Further a distant tree was constructed (figure not shown) to visualize the evolutionary relationship of glyoxalase I sequences of plants. Phylogenetic analysis indicated that *Hevea brasiliensis* and *Ricinus communis* glyoxalase I are closest in evolution. *Cucurbita* and *Allium* also showed 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%). A comparative analysis of *Hevea brasiliensis* glyoxalase I with protein sequence from *R.communis, G.max, C. arietinum and C. maxima* showed regions of high homology. Conserved domain analysis of amino acid residues detected active site, metal binding site, glutathione binding site and dimer interface.

As global demand for natural rubber increases, a major challenge for the cultivation of rubber plants is their capacity to withstand the unfavorable environmental conditions in the context of global climate change. Drought remains one of the most biologically damaging and ecologically limiting factors among all environmental constraints. Drought stress can occur at any stage of growing process, and can cause complete loss of crops or serious damage to yield. The major limiting factor that prevents its cultivation of *Hevea brasiliensis* is drought or semi-arid conditions. Therefore development of drought

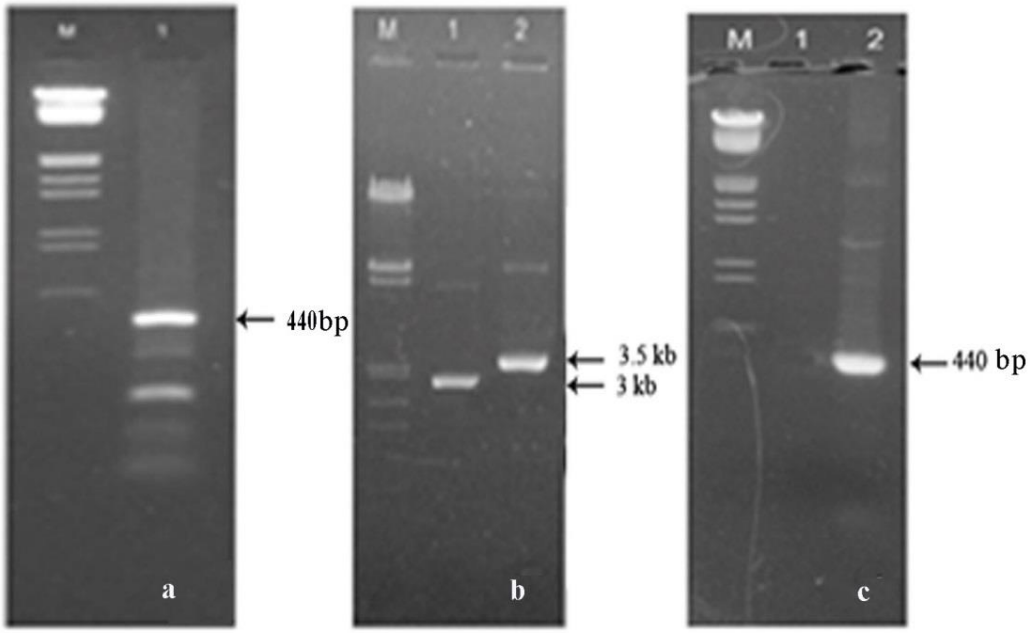

Figure 3. **(a)** The 440 bp fragment amplified with cDNA template with glyoxalase I specific primers. M=Molecular weight marker; Lane 1= 440 bp amplicon. **(b)** Recombinant plasmid isolated from E.coli. M=Molecular weight marker; lane 1=plasmid without insert (3 kb); lane 2=Plasmid with insert (3.5 kb). **(c)** 440 bp fragment amplified from recombinant plasmid with glyoxalase I specific primers. M= Molecular weight marker; lane 1= Plasmid DNA without insert; lane 2= Plasmid DNA with insert.

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1   attaaggatccaaaaataagtctcgattttattctcgcgtattgggcatgtcgttgctt  60
1   I  K  D  P  K  I  S  L  D  F  Y  S  R  V  L  G  M  S  L  L    20
61  aagaggttggatttccagacatgaagtttagcttgtactttatgggctacgaggatcca  120
21   K  R  L  D  F  P  D  M  K  F  S  L  Y  F  M  G  Y  E  D  P   40
121 gcatcagctccaagtgacccagttgaaagaactgtttggacctttggtcagaaggctaca  180
41   A  S  A  P  S  D  P  V  E  R  T  V  W  T  P  G  Q  K  A  T   60
181 attgaattaactcataattggggtactgaaagtgatcctgacttcaaaggatatcacaat  240
61   I  E  L  T  H  N  W  G  T  E  S  D  P  D  F  K  G  Y  H  N   80
241 ggaaattcagaacctcgtggctttggacatattggtatttctgtggatgatgtgtacaag  300
81   G  N  S  E  P  R  G  F  G  H  I  G  I  S  V  D  D  V  Y  K  100
301 gcatgtgagagatttgaacatctaggggtggagttcgccaaaaaacctgatgatggaaaa  360
101  A  C  E  R  F  E  H  L  G  V  E  F  A  K  K  P  D  D  G  K  120
361 atgaaaggaatagctttct                                          379
121  M  K  G  I  A  F                                            126
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Figure 4. Nucleotide sequence of Hevea glyoxalase I cDNA and deduced amino acid sequence.

important. The drought tolerant clones of rubber have been found to possess a more efficient osmoregulation mechanism.

In this study we observed increases in both glyoxalase I and methylglyoxal and enhanced accumulation of glyoxalase I protein in response to drought, salinity, tolerant genotypes of *Hevea brasiliensis* is very heavy metal, 2-4 D, ABA and white light. Our results here on the induction of glyoxalase I and methylglyoxal due to drought stress clearly suggest the role of this pathway in

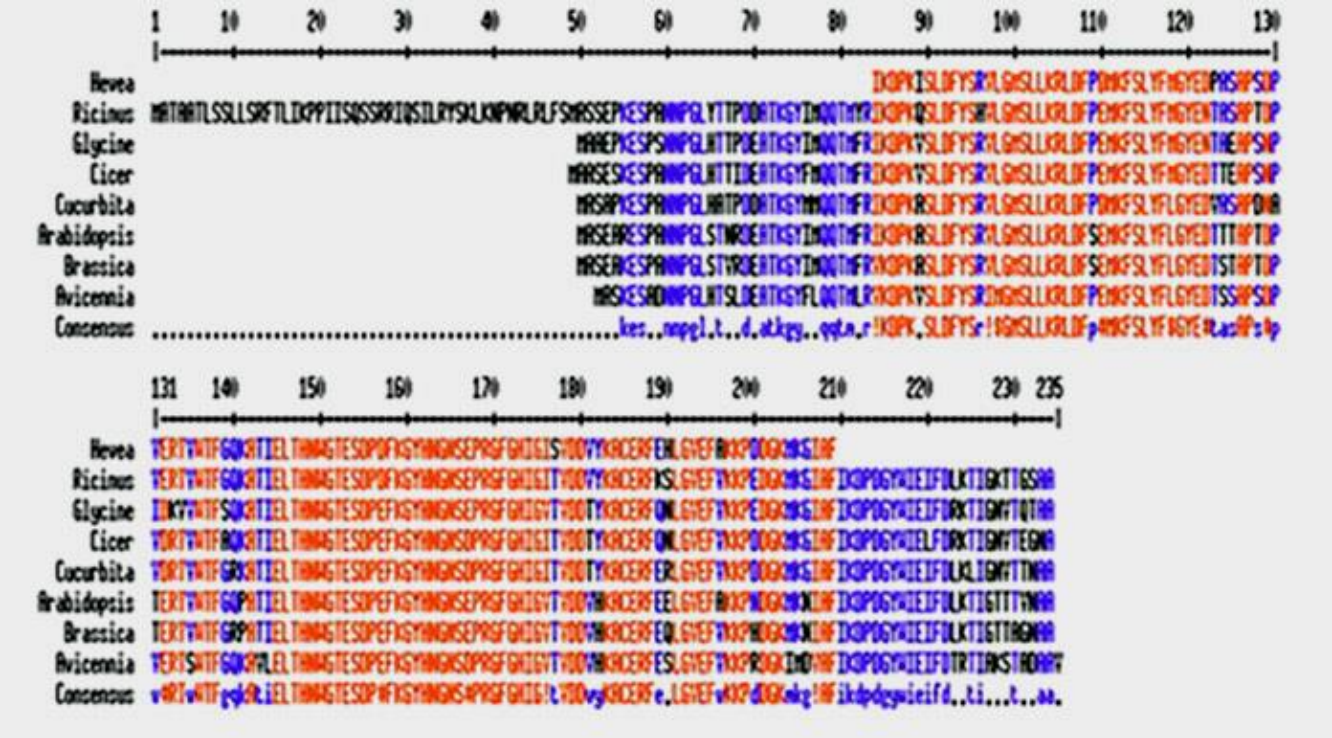

Figure 5. Multiple sequence alignment of nucleotide sequence of *Hevea* glyoxalase I and previously reported sequences in the database.

plants under drought stress. The higher glyoxalase I activity suggested that it might protect the plants against MG, which was formed under drought stress and confers tolerance by increasing the GSH-based detoxification system and decreasing lipid peroxidation. In view of the up-regulation of methylglyoxal and glyoxalase I in drought stressed seedlings of *Hevea brasiliensis* as well as from the sequence information, cloning of the glyoxalase I gene and incorporating the same through genetic transformation would be useful in improving the abiotic stress tolerance especially drought in *Hevea brasiliensis*.

Conflict of interests

The authors have not declared any conflict of interests..

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Abbreviations

ABA, Abscisic acid; **$CdCl_2$,** cadmium chloride; **2, 4-D,** 2, 4-dichlorophenoxyacetic acid; **Glx,** glyoxalase; **MG,** methylglyoxal; **GSH,** glutathione; **BLAST,** basic local alignment search tool; **BLASTP,** basic local alignment search tool for protein sequences.

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