Overexpression of the Glyoxalase II Gene Leads to Enhanced Salinity Tolerance in *Brassica Juncea*

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Abstract: Engineering of salinity tolerance in agronomically important crop plants is required to increase their productivity by enabling them to grow in saline soils, which are otherwise left uncultivated. Since an increase in the enzymes of glyoxalase system has been shown to impart salinity tolerance in the model plant tobacco, we used the glyoxalase II gene for engineering salinity tolerance in an important oil yielding crop, *Brassica juncea*. The transgenic plants of *B. juncea* overexpressing the glyoxalase II gene showed higher salinity tolerance as compared to the untransformed control plants as observed by delayed senescence in leaf discs at 400 mM and 800 mM NaCl in T1 generation. The percentage of germination of the T2 transgenic seeds was higher at 150 mM and 200 mM NaCl as compared to the seeds of untransformed plants. This for the first time demonstrates the applicability of utilizing the glyoxalase II gene for enhanced salinity tolerance in an oilseed crop plant *B. juncea*.

Keywords: *Brassica juncea*, glyoxalase II, salinity tolerance, methylglyoxal, NaCl.

1. INTRODUCTION

As the population of the world is increasing, new means of improving crop productivity must be found to increase the resources available. In contrast to monogenic traits for engineered resistance to the pests and herbicides, the genetically more complex responses to abiotic stresses are difficult to control and engineer. In the recent years, because of an increase in the level of salt in water and soil, salinity has become a major threat to agricultural productivity worldwide. Present engineering strategies for salinity tolerance rely on the transfer of one or more genes that are either involved in the signaling pathways or that encode enzymes required for the functional and structural protectants, such as osmolytes and antioxidants or that encode the proteins that confer stress tolerance [1-3]. However, considering the complex metabolic response of the plants under stress, there is still need to show that the other candidate genes also function equally well in conferring salinity tolerance.

The glyoxalase system consists of two enzymes-glyoxalase I (EC 4.4.1.5, lactoylglutathione lyase) and glyoxalase II (EC 3.1.2.6, hydroxacylglutathione hydrolase). These enzymes act coordinately to convert cyotoxic methylglyoxal and other 2-oxoaldehydes to their 2-hydroxyacids using glutathione as a cofactor [4]. Methylglyoxal is a potent cytotoxic compound and its cytotoxicity is mediated by the inhibition of protein synthesis and interaction with nucleic acids. It has been shown to inhibit the activity of various enzymes of glycolytic pathway under *in vitro* conditions [5-8]. It has also been shown to arrest growth in G1 phase of the cell cycle through inhibition of DNA synthesis [9, 10]. However, glutathione is a well known important antioxidant involved in the detoxification of reactive oxygen species.

In higher plants, glyoxalase I activity was found to be correlated with the mitotic index [11]. An inverse correlation between glyoxalase I activity and differentiation *in vitro* was also observed in plants [12, 13]. Glyoxalase I from tomato and *Brassica juncea* has been shown to be upregulated under different abiotic stresses [14, 15]. Moreover; the over-expression of glyoxalase I from *B. juncea* has been shown to impart tolerance in tobacco plants under salinity and heavy metal stress [15].

Unlike *glyoxalase I*, *glyoxalase II* is a less studied component of glyoxalase system in plants. The expression of the *glyoxalase II* gene has only been studied in *Arabidopsis* [16], *B. juncea* [17], and rice [18]. Reports in rice and *B. juncea* showed an upregulation of the transcript under stress condition. Interestingly, the overexpression of *glyoxalase II* in *glyoxalase I* overexpressing lines of tobacco showed improved tolerance as compared to the plants overexpressing *glyoxalase I* or *gyoxalase II* [19]. We transferred *glyoxalase II* cDNA of rice in the important oil yielding crop, *Brassica juncea*. The transgenic plants overexpressing *glyoxalase II* showed significant tolerance to high concentration of NaCl, suggesting that the *glyoxalase II* gene plays a vital role in salt stress alleviation. This is the first report of salt stress alleviation by overexpression of the glyoxalase II gene in an oilseed crop.

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MATERIALS AND METHODS

Reagents and Supplies

The enzymes and chemicals used for DNA manipulation were purchased from NEB, England. The oligonucleotides were obtained from Microsynth, Switzerland. Random probe labeling kit was obtained from Amersham, USA. Plant hormones, antibiotics, methylglyoxal, MOPS, etc were purchased from Sigma Chemicals,St. Louis, USA, and rest of the chemicals were from Qualigens, India.

Plant Transformation and PCR Analysis

The GV3101 strain of *Agrobacterium tumefaciens* was transformed with the construct *pCAM-glyII* [19] containing the *glyoxalase II* cDNA from rice (*Os-glyII*) under the control of a 35S CaMV promoter (Fig. **1a**). These cells carrying the construct were subsequently used to transform *Brassica juncea* cv Varuna according to Pental *et al.* [20]. Transformed shootlets regenerated on MSB_1N_1 medium (MS medium with 6-benzyl-aminopurine and α-naphthaleneacetic acid each at a concentration of $1mgl^{-1}$) containing hygromycin (20 mg l^{-1}) and rooted on MSI_2 medium (MS medium with Indole-butyric acid at a concentration of 2 mgl^{-1}). Primary transformants were screened by PCR analysis using *glyII* specific primers (5'-ATGCGGATGCTGTCCAAGGCG-3' and 5'-TAAAGTT-ATCCTTCGCTCG-3'). These PCR positive plants were hardened and grown to maturity. They flowered and set seeds. The T1 generation were collected and germinated on MSO medium. The seedlings were rooted in selection medium (MSI_2 medium containing hygromycin at the concentration of 20 mgl^{-1}). Genomic DNA was isolated from the fully-grown plants [21] and used for Southern blot analysis.

Southern Blot Analysis

Southern blot analysis was done according to a modified protocol of Sambrook *et al.* [22]. Genomic DNA (20 μg) was digested with *Nco*I and *Pst*I for insert release and copy number analysis respectively, fractionated on 1% agarose gel and transferred onto a nylon membrane (MDI, India). The ~1 kb insert of *Os glyII* was used as probe. The hybridized signals were visualized using Phosphoimager (FLA-500, Japan).

Northern Blot Analysis and Enzyme Assays

Total RNA was isolated [23] and 30 μg of total RNA was fractionated in a 1.5% agarose-formaldehyde gel, transferred on nylon membrane (MDI, India) by capillary flow method and UV cross-linked. The blot was stained with methylene blue to check for equal loading. Northern blots were hybridized with [α-^{32}P] dCTP labeled (random primed), purified *glyII* cDNA probes. The signals were detected by Phosphorimager.

For enzyme assays, protein extract was prepared by homogenizing leaf tissue in liquid nitrogen and then resuspending the powder in 1 vol (wt/vol) of extraction buffer (0.1 M sodium phosphate buffer {pH 7.0}, 50% glycerol, 16 mM $MgSO_4$, 0.2 mM PMSF and 0.2%PVPP). The glyoxalase II enzyme activity was determined as described [16]. Three different enzyme extractions were done per sample for all independent transgenic lines. The specific activity is expressed in units per mg^{-1} of protein.

Leaf Disc Assay for Tolerance Against MG and Salinity Stress

Healthy and fully expanded leaves (of same age) from wild type untransformed and transgenic plants were briefly washed in deionized water and leaf discs of 1 cm diameter were punched out and floated in 5 ml solution of methylglyoxal (15 mM) for 4 days or NaCl (400, 800 mM) for 8 days. The chlorophyll content was measured as described [24].The experiments were done in triplicates.

Salinity Tolerance of Transgenic Seeds

T_2 seeds from the wild type untransformed and transgenic plants showing higher glyoxalase II activity were germinated on MS medium containing 100, 150, 200 mM NaCl and also on MS medium without additional NaCl. Percentage of seeds germinated was observed after 5 days. The experiments were done in triplicates of 30 seeds each.

RESULTS AND DISCUSSIONS

Agrobacterium tumefaciens GV3101 containing pCAM-gly II was used to transform hypocotyls of *B. juncea*. The T0 transgenic plants were checked by PCR using the *glyII* specific primers. The PCR positive plants were hardened and grown to maturity. The T1 seeds from five independent PCR positive lines were germinated on MS medium and selected on hygromycin containing medium. The integration of the *glyoxalase II* gene in these plants was checked by PCR and Southern blot analysis. All these plants showed the amplification of ~1kb band corresponding to the *glyoxalase II* gene. (Fig. **1b**). These plants were hardened and their DNA was isolated and digested with *Nco*I and subjected to Southern blot analysis. The plants showed the presence of ~1kb insert which corresponded to the *Os glyI* gene (Fig. **1c**). To check the copy number of the transgene, the genomic DNA was also digested with the *Pst*I enzyme which is an unique site within the T-DNA. All the transgenic plants showed a single insertion of the T-DNA within the genome (Fig. **1d**). The expression of the transgene in these plants was also checked by northern blot analysis. The fusion transcript (*glyII-gfp-gus*) was detected in four out of five transgenic lines (Fig. **1e**). In one transgenic plant, low expression of the fusion transcript was also detected. The wild type untransformed control plants did not show the presence of the fusion transcript. In both Southern and northern blotting, the blots were probed with the ^{32}P labeled *glyII* cDNA. All these plants were also analyzed for the glyoxalase II activity. The specific activity of glyoxalase II in T1 transgenic plants was 3-6 times higher than the activity in wild type untransformed plants and it could be correlated well with the expression level of the fusion transcript (Fig. **1f**).

The sensitivity of transformed plants towards NaCl was checked by leaf disc assays.The leaf discs of equal diameter from fully grown mature leaves of the T1 transgenic lines with higher (line 2) and lower expression (line 4) of the *glyoxalase II* gene and wild type untransformed plants were floated on NaCl (400 mM and 800 mM). Incubation of the leaf discs of these transgenic lines and wild type untransformed plants in NaCl (400 mM and 800 mM) showed delayed senescence in the transgenic plants at both the

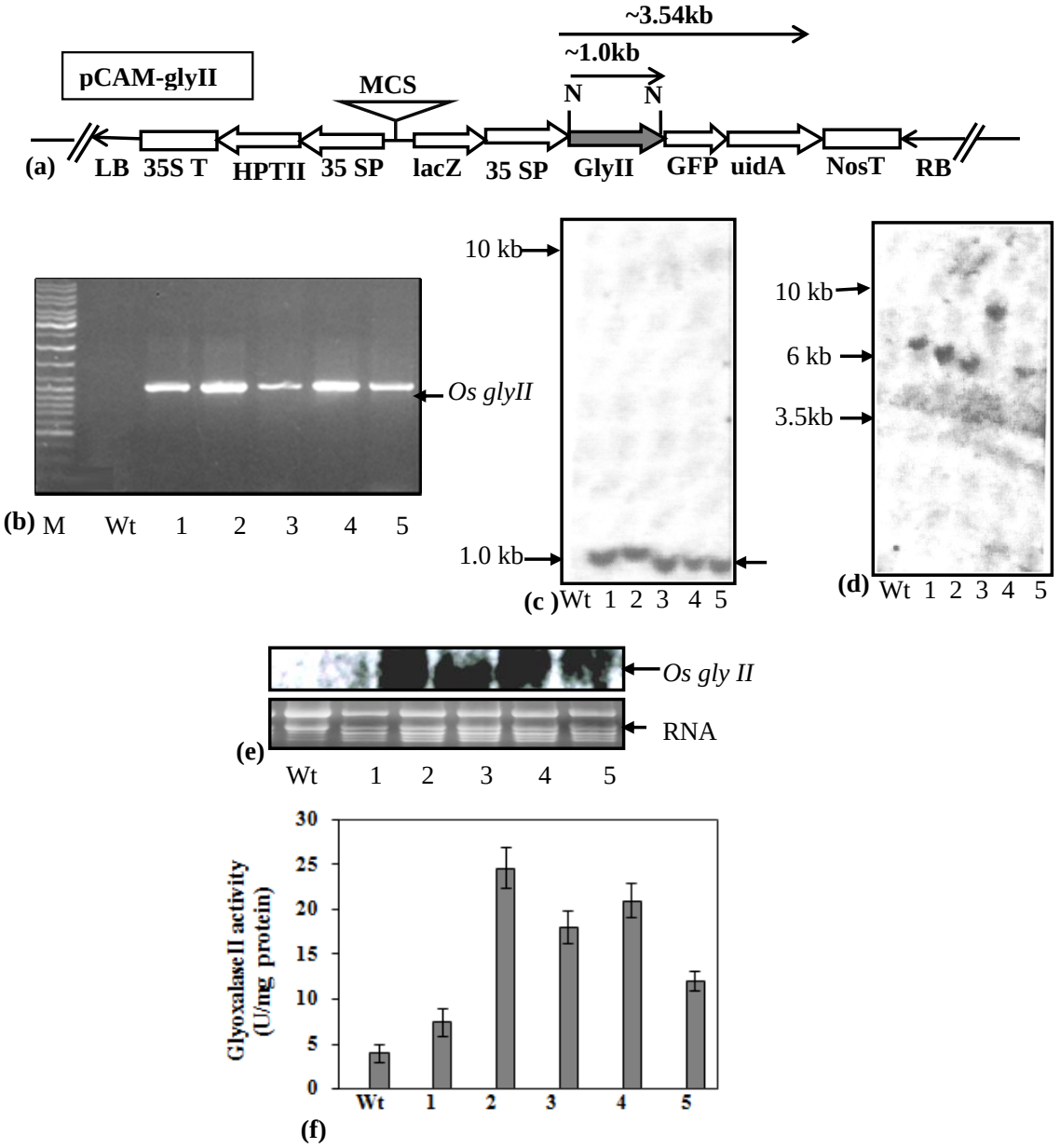

Fig. (1). (a) Schematic representation of glyoxalase II construct used for *Brassica juncea* transformation. **(b)** PCR analysis of glyoxalase II transgenics in T1 generation showing the presence of the transgene. **(c)** Southern blot analysis of the glyoxalase II transgenic plants vs. untransformed control plants for the presence of the transgene. **(d)** Southern blot analysis of the glyoxalase II transgenic plants vs. untransformed control plants for the copy number of the transgene. **(e)** Expression studies of the glyoxalase II transgenic plants in T1 generation by Northern analysis. **(f)** Specific activity of the glyoxalase II enzyme in transgenic plants vs. untransformed control plant. Total protein isolated from the transgenic as well as untransformed control plants was used for the Glyoxalase II assays in Tris buffer (pH7.2) containing 150 µM SLG. Wt: Wild type untransformed plants 1-5: transgenic plants.

concentrations (Fig. **2a**) as compared to wild type untransformed plant after 8 days of incubation. At both the concentrations of NaCl (400 and 800 mM), chlorophyll content in the leaf discs of transgenic plants was higher as compared to those from the wild type untransformed plants. Moreover, the chlorophyll content in the leaf discs from the plants with higher expression of the *glyII* gene (line 2) was also more than in the leaf discs of the plants having lower expression of the *glyII* gene (line 4) at 400mM and 800mM NaCl concentration. The chlorophyll content in the leaf discs of T1 transgenic plants at 400 mM was 88 µg/ g F.wt in line 2 and 55 µg/g F.wt in line 4 respectively which was ~6.0 and 3.5 fold higher as compared to the chlorophyll content in the leaf discs from untransformed control plants which was 15 µg/ gF.wt. However, at 800 mM NaCl, the chlorophyll content in line 2 (35 µg/ gF.wt) and line 4 (26 µg/ gF.wt) was ca. 3.0 and 4.5 fold greater as compared to the untransformed control lines in which it was 8.0 µg /g F.wt (Fig. **2b**). The leaf discs of these lines also showed enhanced tolerance against methylglyoxal. Significant difference was observed among the wild type and transgenic plants after four days of 15 mM methylglyoxal treatment (Fig. **2c**). Leaf discs from line 2 with a higher expression of the *glyII* gene, were greener when compared to the leaf discs of the line 4

which had a comparatively lower expression of the transgene. The chlorophyll content in these plants further confirmed the observed delay in the senescence of the leaf discs (Fig. **2d**). The chlorophyll content retained in the leaf discs of T1 transgenic plants exposed to 15 mM MG was 12 µg/ g F.wt in line 4 and 22.5 µg/g F.wt in line 2 which was ~2.5 fold and ~4.5 fold more as compared to that in the leaf discs of wild type untransformed plants (4.95 µg/ g F.wt).

Salt tolerance of the glyoxalase II transgenic seeds (Line 2) was tested by their germination on MS medium containing different concentrations of NaCl for 5 days (Fig. **3**). There was 100% germination in medium with and without 50 mM NaCl, in seeds from untransformed control plants as well as transgenic plants. At higher concentrations of NaCl, a gradual decrease in the germination percentage was observed in the transgenic as well as wild type seeds. However, the percentage germination in transgenic seeds was always higher as compared to the untransformed control seeds. The germination of transgenic seeds at 100 mM NaCl was 1.4 folds while at 150 mM it was twice as compared to the controls. At 200 mM NaCl, wild type seeds showed only 3% germination as compared to 40% germination of the transgenic seeds (Table **1**).

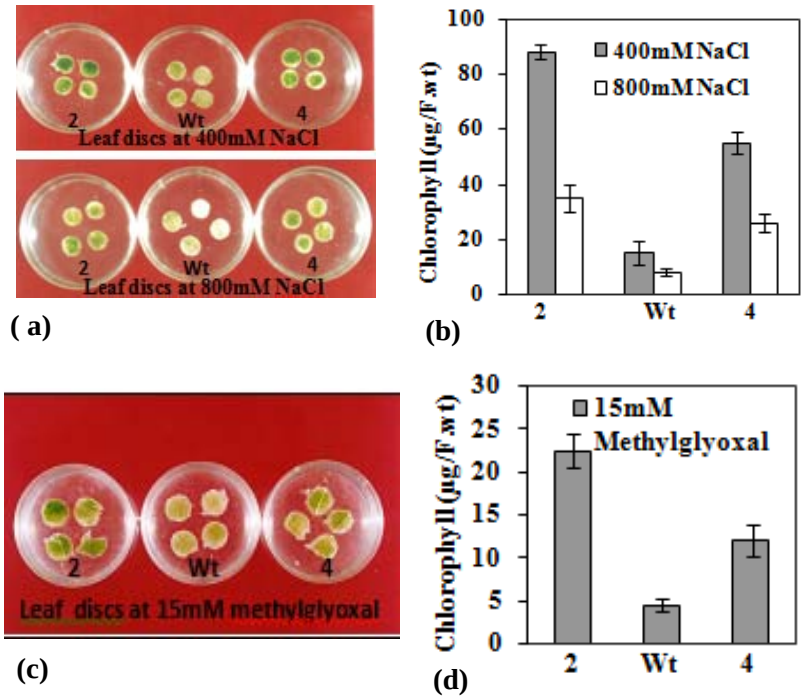

Fig. (2). Retardation of salinity and methylglyoxal induced senescence in the glyoxalase II transgenics in T1 generation. **(a)** Leaf discs of untransformed control plant (Wt) and transgenic plants (2 and 4) were floated on 400 mM and 800 mM NaCl. Visible differences were observed in the leaf discs of transgenic plants when compared to the untransformed control plant after 8 days. **(b)** Chlorophyll estimation of the leaf discs of these plants. This is in accordance with the visible effects. **(c)** Leaf discs of untransformed control plant and transgenic plants (2 and 4) were floated on 15 mM methylglyoxal. Visible differences were observed in the leaf discs of transgenic plants when compared to untransformed control plant. **(d)** Chlorophyll estimation of the leaf discs of the above mentioned plants.

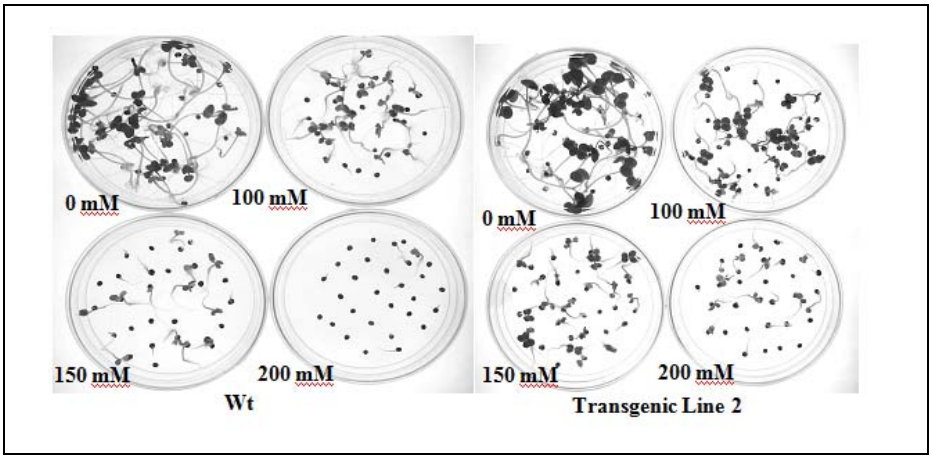

Fig. (3). Germination of T2 seeds of transgenic *Brassica juncea* (Line 2) vs. untransformed control plant on MS medium containing different concentrations of NaCl (0, 100,150,200 mM) after 5 days.

Table 1. Percentage Germination of Wild Type vs. Transgenic Seeds at Different Concentration of NaCl

Concentration of NaCl	0 mM	100 mM	150 mM	200 mM
Wt	100	58.8.±3.86	36.6±3.35	4.44±1.92
Line 2	100	82.2±1.90	72.3±1.84	39.96±3.35

Three different mechanisms have been suggested to be involved in stress tolerance. These include (i) ion and osmotic homeostasis, (ii) regulating cell division and growth and (iii) detoxification and cellular repair. Glyoxalase system appears to be operating through the mechanism of detoxification and cellular repair. Methylglyoxal, besides its own cytotoxicity, lowers the level of glutathione, by the formation of hemithioactal with glutathione. Under stress conditions, the increased level of methylglyoxal could further lower the glutathione level required for scavenging of reactive oxygen species, which increase under stress conditions [25]. Since the *glyoxalase I* has been shown to be upregulated under different abiotic stresses [14, 15] and an increase in the *glyoxalase II* transcript has been observed under various abiotic stresses in *Brasssica juncea* [17] and in rice [18], the overexpression of the enzymes of the glyoxalase system is presumed to be involved in stress tolerance by detoxifying methylglyoxal and elevating the level of glutathione. This has been recently demonstrated in transgenic tobacco plants overexpressing the *glyoxalase I* and *glyoxalase II* gene. These plants were able to maintain higher level of reduced glutathione and could tolerate the increased level of methylglyoxal under salinity stress [26]. In another study, enhanced salinity tolerance was observed in transgenic tobacco overexpressing both the *glyoxalase I* and *glyoxalase II* genes [19]. These plants were also shown to have increased heavy metal tolerance [27]. In this investigation, the overexpression of the *glyoxalase II* gene in the oilseed crop, *B. juncea* resulted in successful engineering of salinity tolerance. This work might prove to be beneficial in devising future strategies for the recovery of *B. juncea* with enhanced abiotic stress tolerance.

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