Evaluation of Cd^{2+} Stress Tolerance in Transgenic Rice by Overexpressing *PgGPx* Gene that Maintains Cellular Ion and Reactive Oxygen Species Homeostasis

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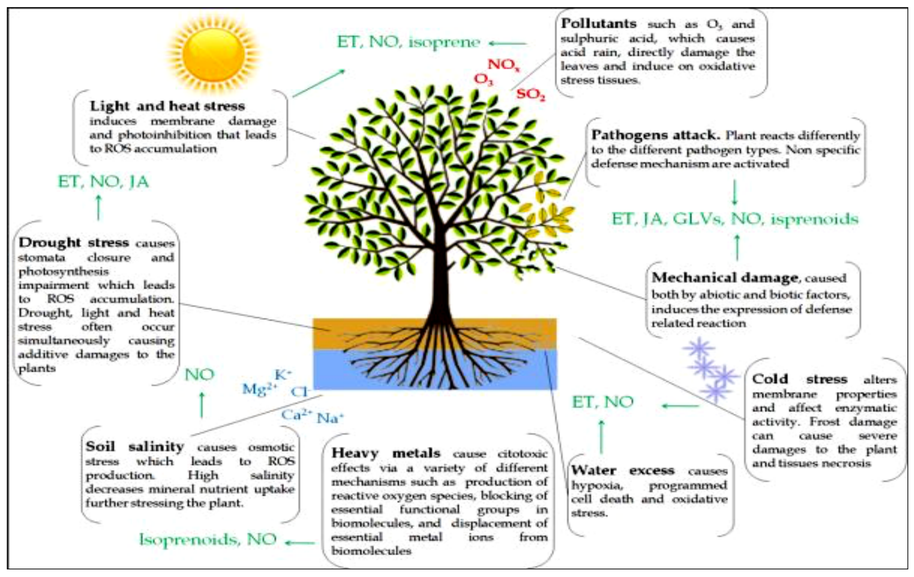
Abstract

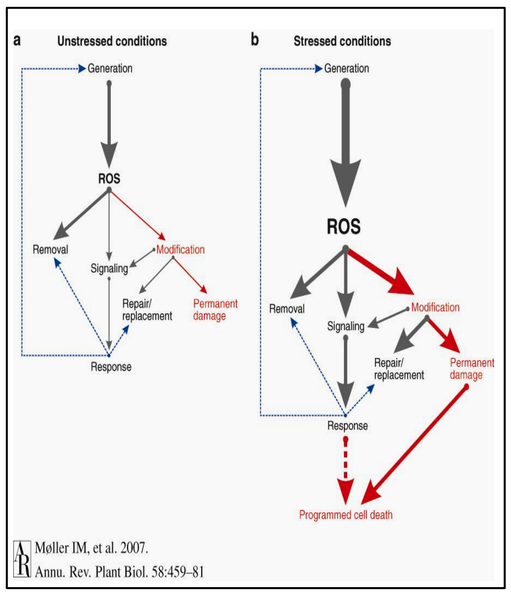
Increasing contamination and higher enrichment ratio of non-essential heavy metals induce various toxic responses in plants when accumulated above the threshold level. These effects and growth responses are genotype and heavy metal level dependent. Cadmium, a non-essential toxic heavy metal, interferes with the plant growth and development. It reaches the leave through xylem and may become part of the food chain, thus causing a detrimental effect to human health. Therefore, there is an urgent need to develop strategies for engineering plants for Cd^{2+} tolerance and less accumulation. Plant species generate a range of defense mechanisms to resist Cd^{2+} induced toxicity and to recover the subsequent damages eliciting their genotype based biochemical responses. To counter damages plants have an efficient system of stress enzymes and antioxidant non-enzyme molecules that is termed as antioxidant system. The members of peroxidase family of antioxidant system, transport metal ions including Cd^{2+}, and thus play important role an ion homeostasis. The present study elucidates the role of a Pennisetum glutathione peroxidase (PgGPx) in Cd^{2+} stress tolerance. Transgenic rice expressing *PgGPx* showed tolerance towards Cd^{2+} stress as demonstrated by several physiological indices including root length, biomass, chlorophyll, malondialdehyde and hydrogen peroxide content. Roots of the transgenic lines accumulated more Cd^{2+} as compared to shoot. *PgGPx* expression in rice also protected the transgenic plants from oxidative stress by enhancing the activity of antioxidant enzymatic (SOD, CAT, APX, GR) machinery. Thus, overexpression of *PgGPx* confers Cd^{2+} stress tolerance in transgenic lines by maintaining cellular ion homeostasis and modulating reactive oxygen species (ROS)-scavenging pathway. Thus, the present study will help to develop strategies for engineering Cd^{2+}stress tolerance in economically important crop plants.

Effects of stress on Plants



Results

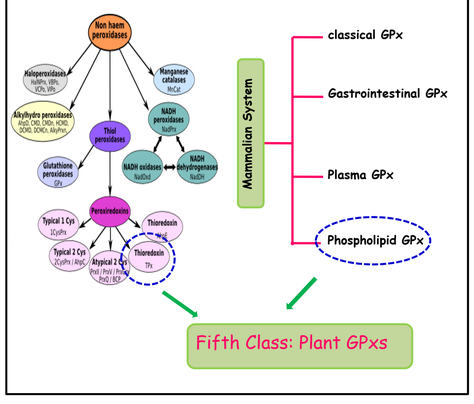
1. Plant responses towards stress



2. Glutathione peroxidase: A versatile antioxidant enzyme

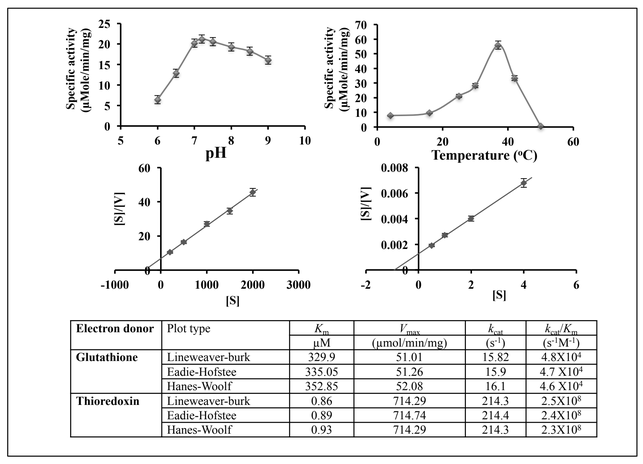
GPX (1.11.1.9)

- First enzyme catalytic activity was reported by Mills (1957) in the protection of red blood cells against haemolysis by oxidation. Also reported by Schwarz and Folz (1957).
- Plant GPXs first footprints has been reported by Criqui (1992).



3. PgGPx showed Thioredoxin dependent peroxidase activity

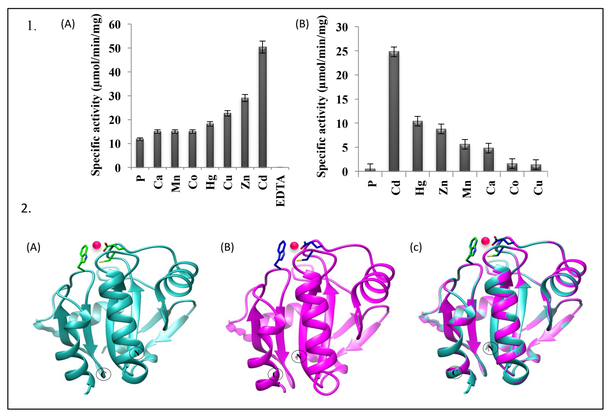
- Pearl millet, *Pennisetum glaucum* (L.) ranks as the fifth cereal in the world in order of economic importance after wheat, rice, maize, and sorghum.
- Well adapted to growing areas characterized by drought, low soil fertility, high temperature, high salinity and low pH.



Electron donor	Plot type	K_m (µM)	V_{max} (µmol/min/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)
Glutathione	Lineweaver-burk	329.9	51.01	15.82	$4.8X10^4$
	Eadie-Hofstee	335.05	51.26	15.9	$4.7 X10^4$
	Hanes-Woolf	352.85	52.08	16.1	$4.6 X10^4$
Thioredoxin	Lineweaver-burk	0.86	714.29	214.3	$2.5X10^8$
	Eadie-Hofstee	0.89	714.74	214.4	$2.4X10^8$
	Hanes-Woolf	0.93	714.29	214.3	$2.3X10^8$

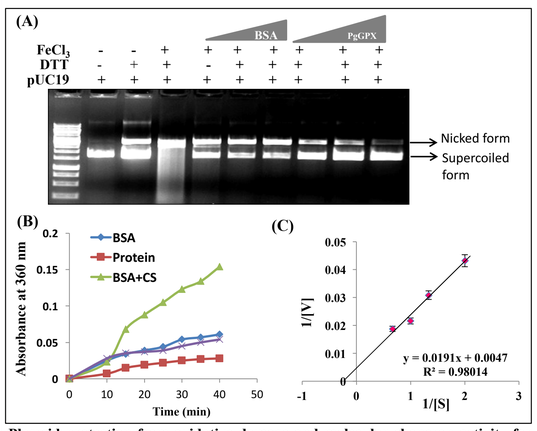
Determination of enzyme kinetic parameters for PgGPx enzyme using varying concentration of t-BHP. Km and Vmax values were calculated by extra plotting Michaelis-Menten hyperbola. PgGPX showed Thioredoxin dependent activity.

4. PgGPx showed Cd^{2+}-dependent peroxidase activity



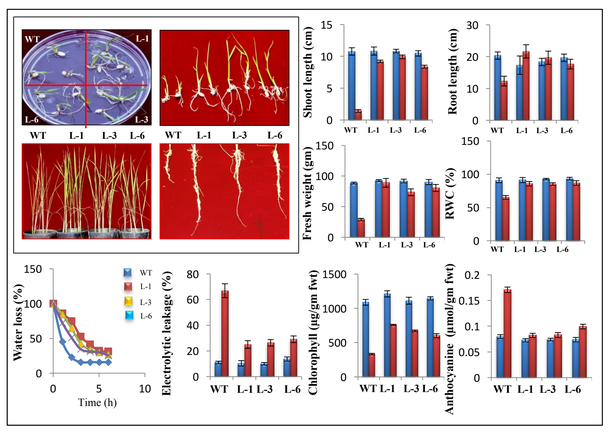
***In vitro* GPx assay for and dependence of metal for the maximum activity of recombinant PgGPx and Homology model of PgGPx depicting predicted active site.** 1. (A) Determination of specific GPx activity of recombinant PgGPX without addition of any metal ion (marked as "Protein") and after exogenous addition of various divalent metal ions (name of metal mentioned below of each bar), and EDTA (to chelate the metals). Note that the maximum specific activity was achieved with$Cd2+$. (B) Reactivation of PgGPx activity by adding exogenous metal to the metal chelated form of protein. Maximum specific activity was observed with again Cd2+. Experiment was repeated thrice with 3 replicates each time; error bars represent standard error. 2. (A) Template of poplar glutathione peroxidase 5 (2p5q), (B) Homology model of PgGPx was build based on Poplar GPx structure(2p5q). (C) Superimposed structure of both the proteins onto each other.

5. Plasmid protection against oxidative damage, molecular chaperon and Glutathione S-Transferase activity of PgGPx



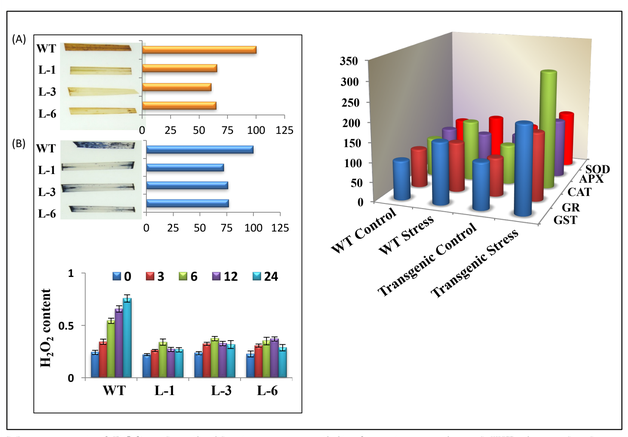
Plasmid protection from oxidative damage and molecular chaperon activity for PgGPx. (A) Protection of plasmid DNA from oxidation by the presence of PgGPx. pUC19 plasmid DNA was incubated with FeCl3 and 10 mM DTT with different concentration of BSA and PgGPx protein. After treatment, plasmid DNA was analyzed on ethidium bromide-stained 1% agarose gels to visualize the intensity of DNA damage. (B) Molecular chaperone activity of PgGPx on citrate synthase(CS). The chaperone function was measured as a function of the thermal aggregation of CS induced by incubating at 45 °C for 40 min. Protein aggregation was monitored by measuring the absorbance at A360 nm after every 10 min interval.

6. Analysis of transgenic lines under heavy metal stress (Cd^{2+})



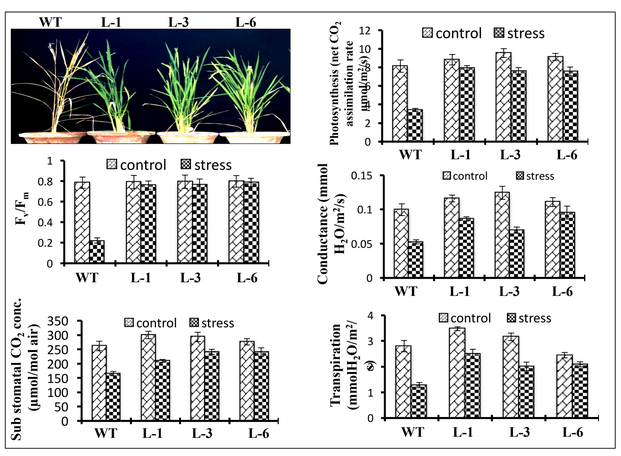
Germination and Measurement of various physiological parameters from the transgenic and WT plants under excess cadmium stress. Seeds from WT and three transgenic lines (L-1, L-3, L-6) were germinated on MS media supplemented with 10 mM $CdCl_2$ to mimic cadmium toxicity. WT seeds were unable to grow properly, whereas transgenic seedlings were able to survive and grow. Different physiological parameters such as shoot length, root length, fresh weight and stress tolerance index were calculated by comparing the growth of seedlings under stress with that of control condition. Different important physiological parameters such as relative water content, water loss, electrolyte leakage, chlorophyll content and anthocyanin content were measured from the heavy metal stressed seedlings and compared with that of control seedlings.

7. Measurement of ROS and antioxidant enzymes activity from transgenic and WT rice under heavy metal toxicity



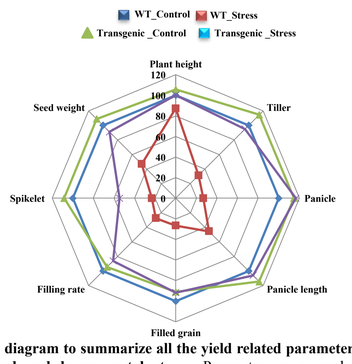
Measurement of ROS and antioxidant enzymes activity from transgenic and WT rice under heavy metal toxicity. H_2O_2 and superoxide from the leaves of WT and PgGPx overexpressing rice under heavy metal toxicity were measured. Leaves from WT and three transgenic lines (L-1, L-3, L-6) grown under $CdCl_2$ stress were collected and stained with DAB to detect the H_2O_2 (A) and the intensity of the staining were measured. Similarly, superoxide level was stained in the leaves from WT and five transgenic lines by staining with NBT (B) and the intensity of the staining were measured. Changes in the level of total H_2O_2 were measured from the WT and PgGPx transgenic seedlings at various time points of 24 h. Various enzyme activities were measured from the leaves under stress and compared with that of control values.

8. Effect of Cd^{2+} toxicity on photosynthetic machinery



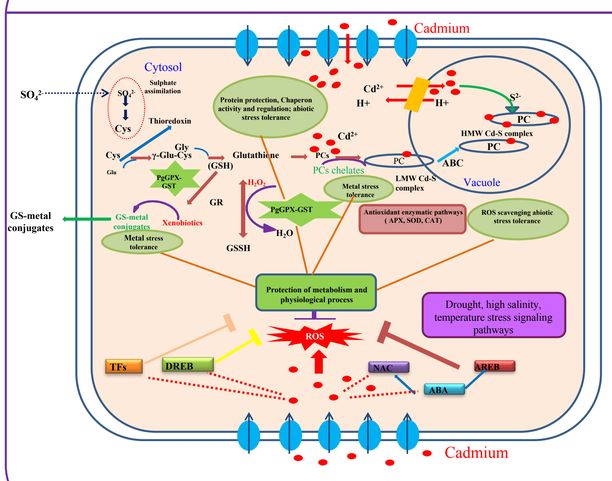
Assessment of photosynthetic efficiency for PgGPx overexpressing rice transgenics vis-a-vis wild type plants under oxidative stress. Various photosynthetic parameters were measured from the stressed and control plant and compared. The parameters are photosynthesis rate, Photo system II efficiency, stomatal conductance, transpiration rate, and sub-stomatal CO2 concentration.

9. Yield potential of the transgenic and WT plants under Cadmium stress



Web diagram to summarize all the yield related parameters analyzed under control and heavy metal stress. Parameters were analyzed for WT and ectopically expressing PgGPx transgenic plants. Different parameters such as plant height, number of tiller, number of panicle, panicle length, number of spikelet, number of filled grain, grain filling rate, and average seed weight of transgenic lines were compared with that of WT plants under control and heavy metal stress conditions. Average data of three independent transgenic lines (L-1, L-3, L-6) has been plotted here.

10.Proposed hypothesis



Hypothetical model for cadmium-induced ROS generation and PgGPx mediated cadmium tolerance in transgenic rice

Conclusions

1. PgGPx is a functional 2-cys thioredoxin dependent peroxidase.
2. PgGPx showed DNA protection, molecular chaperon and Glutathione S-Transferase activity apart from peroxidase activity.
3. Overexpression of *PgGPx* transgenic seedlings showed better membrane stability under stress as compared to WT plants.
4. *PgGPx* transgenic plants showed less H_2O_2 and O_2^- accumulation under stress.
5. *PgGPx* transgenic plants were able to maintain better photosynthetic and anti-oxidant machinery under stress.
6. *PgGPx* over expressing plants showed less yield loss as compared to WT plants under heavy metal toxicity.

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