



Research article

Targeting prokaryotic choline oxidase into chloroplasts enhance the potential of photosynthetic machinery of plants to withstand oxidative damage

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ABSTRACT

Chloroplasts from plants of transgenic lines expressing prokaryotic choline oxidase gene (the *codA_{ps}* gene; GenBank accession number-AY589052) and wild-type of chickpea and Indian mustard were evaluated for their efficacy to withstand photoinhibitory damage, by exposing them to high light intensity ($\sim 1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density) at 10 and 25 °C. Western analysis confirmed presence of choline oxidase in chloroplasts of only transgenic lines. The loss in PS II activity in chloroplasts of wild-type exposed to high light intensity was significantly higher than that in chloroplasts of transgenic chickpea as well as Indian mustard. Although, chloroplasts of both wild-type and transgenic chickpea as well as Indian mustard were more sensitive to photoinhibitory damage at 10 than at 25 °C, the damage recorded in chloroplasts harboring choline oxidase was significantly lower than those of wild-type. High light promotes H₂O₂ production in chloroplasts more significantly at low temperature (10 °C) than at 25 °C. We compared low temperature accelerated photoinhibition of chloroplasts with that caused due to exogenously applied H₂O₂. Although exogenous H₂O₂ accelerated high light intensity induced loss in PS II activity of chloroplasts of wild-type, it caused only a little alteration in PS II activity of chloroplasts from transgenic lines of both chickpea and Indian mustard, demonstrating that the chloroplasts harboring choline oxidase are better equipped to resist photoinhibition. We hypothesize that H₂O₂ produced by choline oxidase as a byproduct during synthesis of glycinebetaine is responsible for building stronger antioxidant system in chloroplasts of transgenic lines compared to that of wild-type.

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1. Introduction

Abiotic stresses in particular drought, frost/low temperature, salinity are amongst the significant and refractory constraints to productivity of crops like chickpea and Indian mustard [1–6]. Transgenic technology has become an inevitable component of modern agriculture for enhancing tolerance of crop plants to abiotic stresses. Advancement in genetic engineering has facilitated the introduction of desired gene(s) from vast variety of living systems into various plant species even across extreme sexual boundaries, which is otherwise impossible through conventional breeding [1,7]. This technology made it possible to enhance tolerance of crop plants such as Indian mustard, rice, tomato etc. against abiotic stresses by introducing bacterial/prokaryotic genes such as the *codA* gene from *Arthrobacter globiformis*, the *mtlD* and *CDH* genes from *Escherichia coli* [1,8–14]. The *codA* gene from *A. globiformis* is most well tested amongst the genes that are known to enhance

abiotic stress tolerance of crop plants. Amongst the four glycinebetaine biosynthetic pathways, the one mediated by choline oxidase is the simplest one as it is a single gene/enzyme mediated pathway. Choline oxidase, the translated product of the *codA* gene, is a FAD-dependent enzyme that catalyzes the four electron oxidation of choline into glycinebetaine in presence of O₂ [1,15]. Glycinebetaine is a compatible solute that protects cellular machinery which includes membranes, proteins, nucleic acids, electron transport complexes etc. against various abiotic factors [1,6,8,10,12].

Photosynthesis is the single most vital metabolic process that not only regulates plant growth and development, but also directly controls overall productivity. Photosynthetic machinery is highly sensitive to abiotic stresses such as low temperature, drought, salinity and high light intensity [1,6,8,10]. Crop plants such as chickpea and Indian mustard are prone to photoinhibition, especially under abiotic stresses in particular low temperatures [1,6]. Therefore, during present investigations we have tested if the chloroplasts of the transgenic lines of chickpea and Indian mustard developed by our research team possess any enhanced potential to withstand photoinhibitory damage compared to the chloroplasts of

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respective wild-type plants. In this communication, we are reporting for the first time that the H_2O_2 produced by choline oxidase as a byproduct during synthesis of glycinebetaine is responsible for the enhanced potential of the chloroplasts of *codA_{ps}* transgenic lines of both chickpea and Indian mustard to resist photoinhibition.

2. Results and discussion

2.1. Raising transgenic lines

Transgenic lines of both chickpea and Indian mustard were raised using *Agrobacterium tumefaciens* harboring pSG vector. pSG vector was constructed by cloning 2.5 kb *codA_{ps}* gene cassette [consisting of the *codA_{ps}* gene (AY589052) of *A. globiformis* joined to transit peptide sequence of small subunit of Rubisco of chickpea and poly A signal of nopaline synthase with CaMV 35S promoter] in pPZP200 vector having *npt II* gene cassette between the *loxP* sites [13].

Explants of chickpea consisting of single cotyledon with half embryonal axis [as detailed in Anwar et al. [16]] were incubated on MS medium supplemented with 2 μ M kinetin, 10 μ M 2-iP and 4 μ M TDZ for 6 days and then co-cultivated with *Agrobacterium* harboring pSG vector for 48 h. After washing with 250 mg/l cefotaxime these explants were transferred to MS medium supplemented 20 mg/l kanamycin and 250 mg/l cefotaxime. After 14 days, healthy adventitious shoots arising from the explants were transferred to fresh MS medium supplemented with 2 μ M kinetin, 5 μ M 2-iP and 50 mg/l kanamycin. Healthy shoots arising from different portions of the same explant or from different explants were carefully marked as independent putative transgenic lines and were sub-cultured on MS medium with 2 μ M kinetin and 5 μ M 2-iP at regular interval of 14 d by gradually increasing the concentration of kanamycin to 100 mg/l. Strong shoots of independent putative transgenic lines were rooted by exposing their cut ends to 10 s pulse treatment with 100 μ mol/ml IBA as detailed in Anwar et al. [16] in presence of 50 mg/l kanamycin. A number of putative transgenic lines obtained through this protocol were evaluated for

the presence of the *codA* gene through PCR and Western analysis. Through this transformation protocol 21 independent and healthy transgenic lines were selected. Southern analysis indicated that seven of these transgenic lines possessed single insert of the *codA* gene. By advancing these transgenic lines to T3 and T4 generations, it was possible to select the homozygous lines of each of the independent transgenic lines. One of the seven independent homozygous transgenic lines at T4 generation, with relatively superior features like over all growth and superior seed yield compared to other lines, was chosen for the present study. Details of the way transgenic lines of Indian mustard were raised are given in Gupta et al. [13]. One of the 15 independent homozygous transgenic lines of Indian mustard (at T4 generation) with single insert of the *codA* gene that was relatively superior to other lines in terms of over all growth was chosen for present studies.

2.2. Localization of choline oxidase in chloroplast

Immunoblot analysis of crude enzyme extract of the leaves of transgenic lines of both chickpea and Indian mustard showed the presence of choline oxidase, whereas the extracts from respective wild-type plants lacked choline oxidase (Fig. 1). This confirmed that wild-type chickpea and Indian mustard do not possess choline oxidase or any protein/polypeptide that could cross react with the antibodies against choline oxidase. As expected no band of choline oxidase was observed when the crude enzyme extract was treated with proteinase K. Intact chloroplasts isolated from leaves of transgenic lines showed the presence of choline oxidase even if they were treated with proteinase K (Fig. 1). However, disrupted chloroplasts treated with proteinase K failed to show any choline oxidase band (Fig. 1). These results convincingly demonstrated the presence of choline oxidase within the isolated chloroplasts.

2.3. Chloroplasts of transgenic lines resist high light intensity induced photoinhibition

The chloroplasts isolated from the leaves of wild-type and transgenic lines of both chickpea and Indian mustard were subjected to

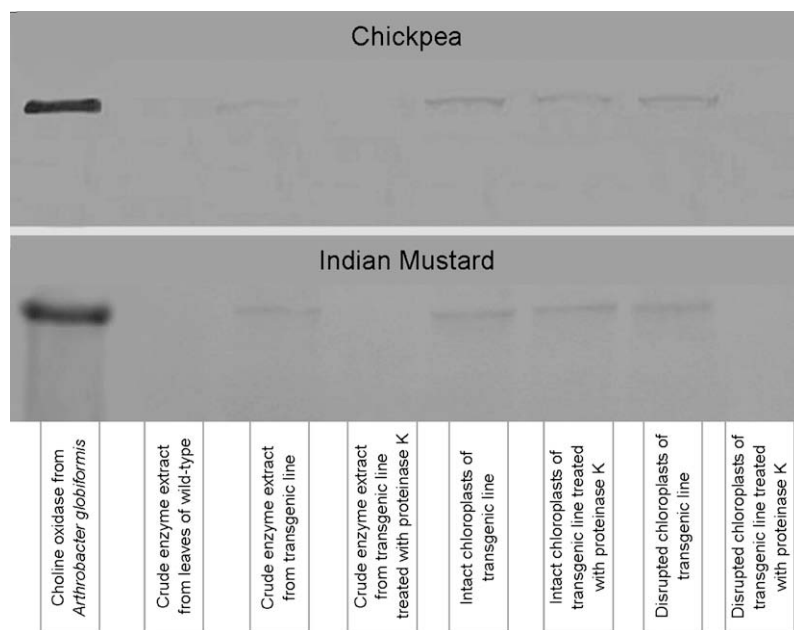


Fig. 1. Western blot analysis to detect the presence of choline oxidase in the chloroplasts of transgenic lines of chickpea and Indian mustard. Please note the presence of choline oxidase in intact chloroplast, intact chloroplasts treated with proteinase K, and disrupted chloroplasts. These observations along with the absence of choline oxidase in disrupted chloroplasts treated with proteinase K, demonstrate the localization of choline oxidase in the chloroplasts.

high light intensity at 10 and 25 °C. In general, intact chloroplasts of both wild-type and transgenic lines of chickpea as well as Indian mustard showed continuous decline in PS II activity with time of exposure to high light intensity. However, the decline in PS II activity was significantly higher at 10 °C compared to that recorded at 25 °C. Intriguingly, the transgenic lines of both chickpea and Indian mustard showed significantly lesser loss in PS II activity in comparison to that of wild-type both at 10 °C and 25 °C (Figs. 2 and 3). Upon exposure to high light intensity for 60 min at 25 °C, the chloroplasts of *codA_{ps}* transgenic lines of chickpea and Indian mustard showed only 5 and 8 percent loss in PS II activity in comparison to respective wild-types which showed 10 and 30 percent loss in PS II activity, respectively. The chloroplasts of wild-type chickpea and Indian mustard exposed to high light intensity at 10 °C for 60 min showed ~50 and 66 percent lower PS II activity (over initial value), respectively. In contrast, the chloroplasts of the *codA_{ps}* transgenic lines of chickpea and Indian mustard exposed to high light intensity at 10 °C for 60 min showed only ~5 and 24 percent decline in PS II activity over the respective initial values (Fig. 2).

As the enhanced tolerance of chloroplasts of transgenic lines to photoinhibition recorded during present investigations could be

due to the presence of glycinebetaine, which is well documented to protect several cellular components including photosynthetic machinery, membranes, DNA and enzymes [1,6,12,17], performance of isolated chloroplasts of wild-type and transgenic lines of both chickpea and Indian mustard, under high light intensity was evaluated in the presence of glycinebetaine. As is evident from Fig. 2, glycinebetaine could significantly curtail high light intensity induced loss in PS II activity of chloroplasts from leaves of wild-type chickpea and Indian mustard at both 10 and 25 °C. These findings confirmed that glycinebetaine protects photosynthetic machinery against photoinhibition both in chickpea as well as in Indian mustard. However, exogenous application of glycinebetaine did not bring about any significant alteration in high light intensity induced alteration in PS II activity of chloroplasts from transgenic plants at both 10 and 25 °C (Fig. 2). It is likely that the intact chloroplast of transgenic lines might be possessing threshold level of glycinebetaine bound to its photosynthetic machinery including PS II units. Quaternary ammonium cations such as glycinebetaine possess potential to bind to indole and phenyl groups from tryptophan, tyrosine, and phenylalanine which have an evenly negative surface potential Schiefner et al. [18]. It is quite likely that glycinebetaine

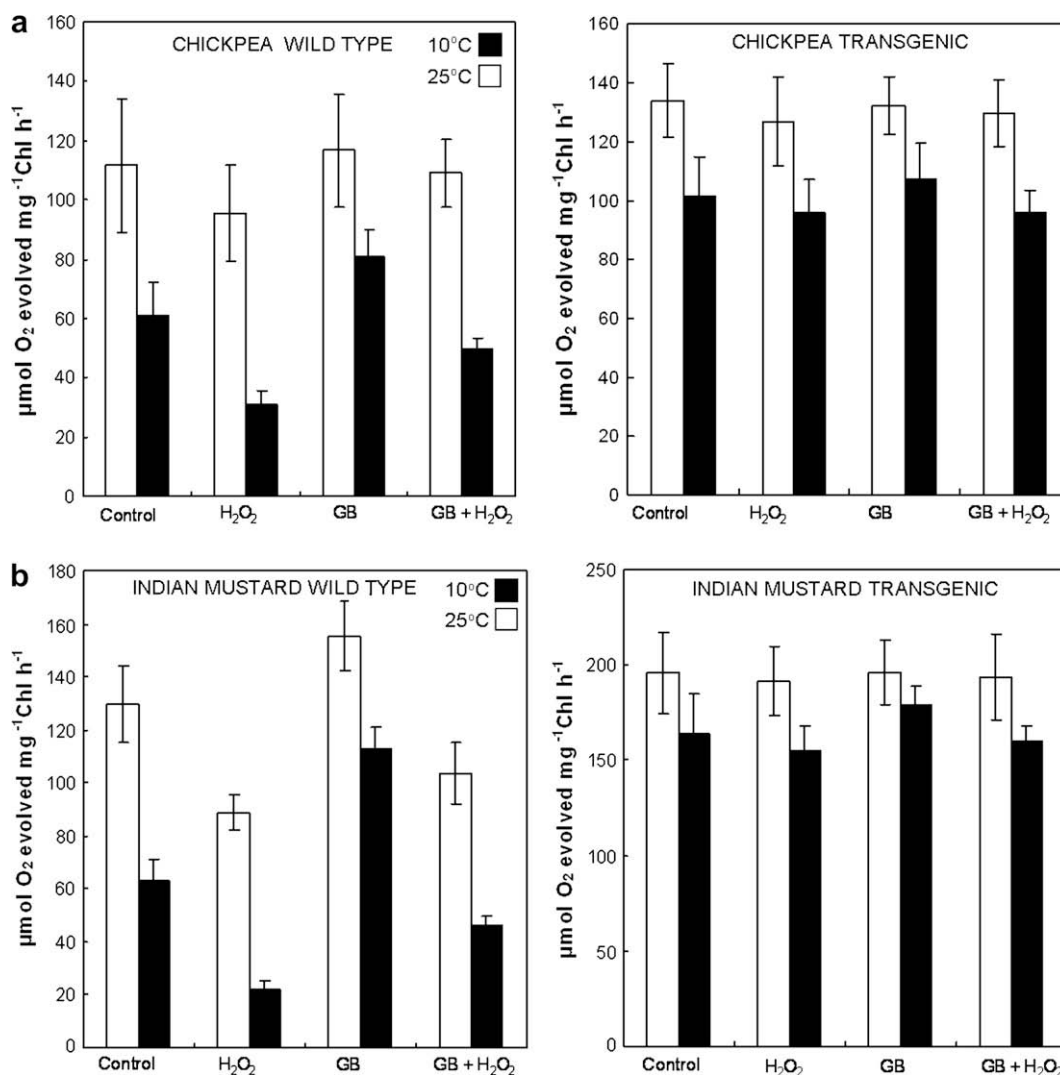


Fig. 2. PS II activity in intact chloroplasts, of wild-type and the *codA_{ps}* transgenic lines of chickpea and Indian mustard, incubated in suspension buffer supplemented with 0 and 250 μM H₂O₂ in the presence and absence of 100 mM glycinebetaine, exposed to high light intensity (1200 μmol m⁻² s⁻¹) for a duration of 60 min at 25 and 10 °C. The initial PS II activities of intact chloroplast of wild-type and *codA_{ps}* transgenic lines of (a) chickpea were 124 ± 20 and 141 ± 18 μmol O₂ evolved mg⁻¹ Chl h⁻¹; and (b) Indian mustard were 185 ± 28 and 213 ± 38 μmol O₂ evolved mg⁻¹ Chl h⁻¹, respectively. The data represent mean ± standard error of mean values from five independent experiments.

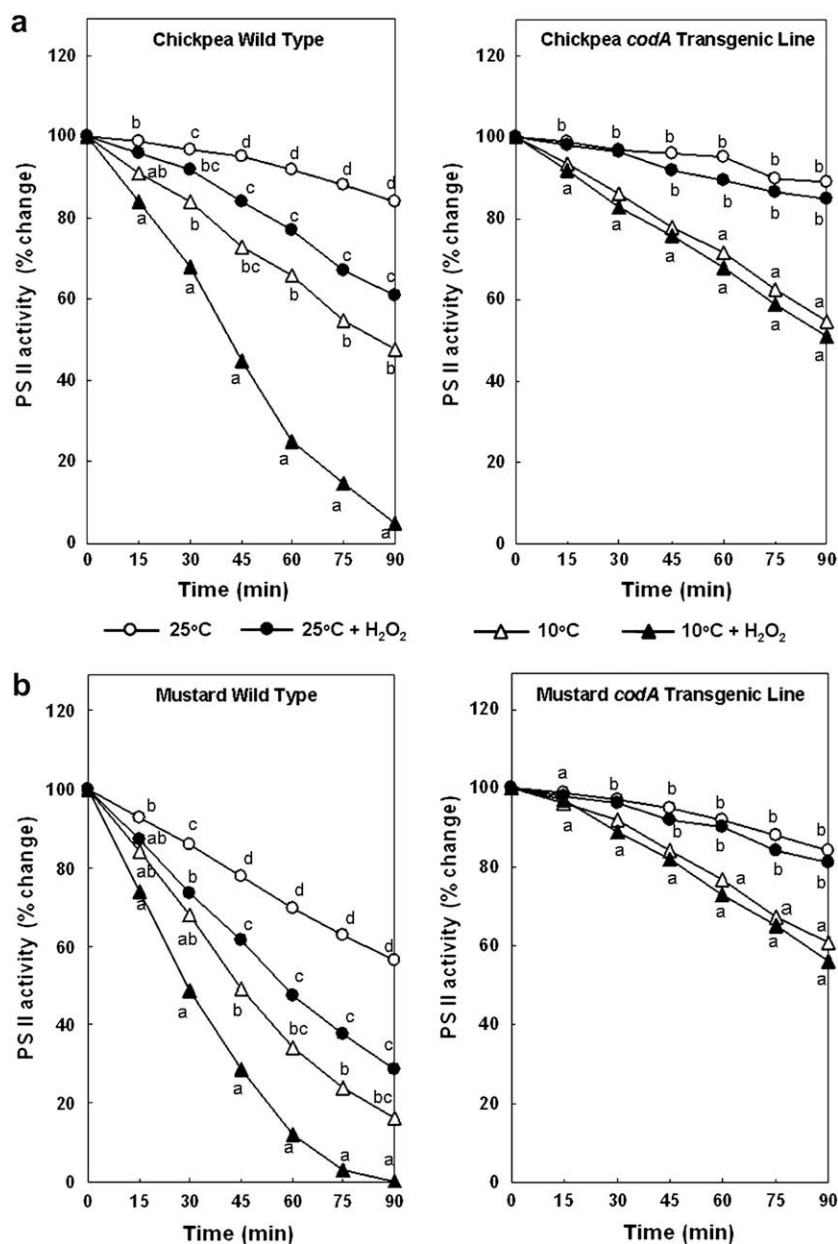


Fig. 3. Time dependent decline in PS II activity in intact chloroplasts of wild-type and the *codA_{ps}* transgenic lines of chickpea and Indian mustard, exposed to high light intensity ($1200 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 25 and 10 °C in the presence of 0 and 250 μM H_2O_2 . The initial PS II activities of intact chloroplasts of wild-type and *codA_{ps}* transgenic lines of (a) chickpea were 127 ± 14 and $138 \pm 25 \mu\text{mol O}_2 \text{ evolved mg}^{-1} \text{Chl h}^{-1}$; and (b) Indian mustard were 176 ± 31 and $220 \pm 43 \mu\text{mol O}_2 \text{ evolved mg}^{-1} \text{Chl h}^{-1}$, respectively. The data represent mean of values from five independent experiments. Values designated by different small letters between different treatments at any given time interval are significantly different from each other at $P \leq 0.05$ level [Duncan's multiple range test [26]].

that remained bound to the tryptophan, tyrosine, and phenylalanine of proteins/polypeptides of photosynthetic machinery in the chloroplast of the transgenic lines of chickpea and Indian mustard, is helping PS II units to retain their activity by resisting photodamage.

2.4. Chloroplasts of transgenic lines withstand H_2O_2 induced photoinhibition

Choline oxidase generates 2 mol of H_2O_2 for each mole of glycinebetaine synthesized by dehydrogenating/oxidizing 1 mol of choline using 2 mol of O_2 [1,6,15]. H_2O_2 is one of the forms of reactive oxygen species that has been well known to cause oxidative damage to various cellular components resulting in the

disruption of general cellular metabolism [19,20]. Therefore, during present investigations the effect of exogenous H_2O_2 on high light intensity promoted loss in PS II activity in chloroplasts of wild-type as well as transgenic lines of both chickpea and Indian mustard were evaluated. The exogenous application of H_2O_2 accelerated high light intensity induced suppression in PS II activity of chloroplasts from wild-type chickpea as well as Indian mustard at 10 as well as 25 °C, but the drop in activity was sharper at 10 than at 25 °C (Fig. 3). Amazingly, as is apparent from Fig. 3, low temperature promoted decline in PS II activity was significantly lesser in chloroplasts of the *codA_{ps}* transgenic lines of both chickpea and Indian mustard compared to respective wild-type. Upon 60 min exposure to high light intensity in presence of exogenously applied H_2O_2 , the chloroplasts of wild-type chickpea showed a decline in

their PS II activity by 75 and 23 percent at 10 and 25 °C, respectively. In contrast, the chloroplasts from transgenic line showed loss in PS II activity by only ~32 and 10 percent at 10 and 25 °C, respectively. As is clear from Fig. 3, even in case of Indian mustard, the chloroplasts from transgenic lines showed significantly higher potential to withstand H₂O₂ promoted enhancement in high light induced decline in PS II activity compared to that of wild-type (Fig. 3).

In order to see if H₂O₂ accelerated enhancement in the loss of PS II activity of wild-type chloroplasts to high light intensity can be curtailed by glycinebetaine, the chloroplasts were exposed to high light intensity in the presence of H₂O₂ and glycinebetaine. Interestingly, glycinebetaine could only partially curtail H₂O₂ accelerated high light intensity induced loss in PS II activity of chloroplasts from wild-type plants of both chickpea and Indian mustard at 10 °C (Fig. 2). Astonishingly, no significant variation in high light intensity induced loss in PS II activity of chloroplasts from transgenic lines of either of the plant systems, when the chloroplasts were incubated with exogenous glycinebetaine and H₂O₂ individually or in combination (Figs. 2 and 3). This ability of the chloroplasts of the *codA_{ps}* transgenic lines of both chickpea and Indian mustard to resist H₂O₂ and low temperature promoted photoinhibition can be linked to the presence of superior antioxidant system in them as compared to the chloroplasts from wild-type. The transgenic plants of *Arabidopsis* and Indian mustard expressing the *codA* gene possessed relatively higher activities of anti-oxidant enzymes linked to ascorbate–glutathione cycle [10,21]. H₂O₂ is considered to be the most important ROS member that serves as a signal molecule in the transduction of stress signals and acts as a regulator for the expression of genes encoding antioxidants, cell rescue/defense proteins, and signaling proteins such as transcription factors [22,23]. The H₂O₂ generated as byproduct during synthesis of glycinebetaine by choline oxidase [1,6,15] in the *codA_{ps}* transgenic lines of chickpea as well as Indian mustard must have switched on genes involved in developing machinery necessary for resisting oxidative stress. Accordingly, the chloroplasts of the *codA_{ps}* transgenic lines are well equipped to counter H₂O₂ and high light intensity induced photodamage.

3. Conclusion

The transgenic lines of both chickpea and Indian mustard expressing the prokaryotic *codA_{ps}* gene with the choline oxidase specifically targeted into chloroplasts possessed enhanced potential to withstand low temperature and H₂O₂ promoted decline in PS II activity compared to their respective wild-type plants. Our results convincingly demonstrated that the chloroplasts of crop plants harboring choline oxidase possess potential to withstand photo-inhibitory damage. We hypothesize that this inbuilt ability of chloroplasts of the *codA_{ps}* transgenic lines to withstand photo-inhibitory damage could be due to the presence of stronger antioxidant system compared to the chloroplast of wild-type.

4. Materials and methods

The *codA_{ps}* transgenic lines of chickpea (*Cicer arietinum*) cv. PG114 and Indian mustard (*Brassica juncea*) cv. Varuna used for present investigations were raised through *Agrobacterium* mediated transformation using pSG vector harboring the *codA_{ps}* gene (AY589052) from *A. globiformis* (MTCC 944). The chloroplast targeting transit peptide coding sequence from *C. arietinum* was fused in frame with the coding sequence of the *codA_{ps}* gene. pSG vector was constructed by cloning 2.5 kb *codA_{ps}* gene cassette [consisting of the *codA_{ps}* gene (AY589052) of *A. globiformis* joined to transit peptide sequence of small subunit of Rubisco of chickpea and poly A

signal of nopaline synthase with CaMV 35S promoter] in pPZP200 vector having *npt II* gene cassette [consisting of the *npt II* gene with poly A signal of octopine synthase driven by P-nos (nopaline synthase promoter)] sandwiched by the *loxP* sites of Cre-*loxP* site specific recombination system of bacteriophage P1 [13]. These transgenic lines at T₄ generation were homozygous with the *codA_{ps}* gene present at single locus.

4.1. Isolation of intact chloroplasts

Leaves from three-month-old plants of both chickpea and Indian mustard were used for isolation of intact chloroplasts. Freshly harvested leaves were dipped in ice-cold isolation buffer {(pH 7.6) containing 330 mM sorbitol, 5 mM NaCl, 1 mM MgCl₂, 2 mM EDTA and 50 mM HEPES}. After 30 min incubation in dark at 6 ± 2 °C, these leaves were homogenized in the pre-chilled mortar and pestle in ice-cold isolation buffer in dark. The homogenate was filtered through 4 layers of Mira cloth and the filtrate was centrifuged at 3000 × g for 10 min at 4 °C. The pellet was washed and re-suspended in a minimal volume of isolation buffer. This chloroplast suspension was laid on sucrose gradient (discontinuous) and centrifuged at 25,000 × g for 45 min. The layer containing intact chloroplasts was dispensed into a fresh tube, washed twice with isolation buffer and pelleted. The chloroplasts were suspended in small volume of suspension buffer (pH 7.5) consisting of 330 mM sorbitol, 5 mM NaCl, 1 mM MgCl₂, 2 mM EDTA, 1 mM sodium ascorbate and 50 mM HEPES. Chlorophyll content of the chloroplast preparation was determined according to the method of Arnon et al. [25] in 80% acetone.

4.2. Determining the presence of choline oxidase in chloroplasts

In order to ascertain the localization and normal targeting of choline oxidase into chloroplasts, experiment was performed using proteinase K according to the procedure of Kavanagh et al. [24]. The proteinase treatment was performed by incubating intact/sheared chloroplasts, corresponding to 10 µg protein along with proteinase K (1 mg/ml) at 37 °C for 30 min. The treatment was terminated by addition of 10 mM PMSF (Phenyl methyl sulphonyl fluoride) to the reaction mixture. The reaction mixture was loaded on SDS (10%) discontinuous gel and electro-blotted on to PVDF membrane which was probed with primary antibodies raised against pure choline oxidase (Sigma Chemical Co.) and immunological detection was carried out as detailed in Prasad et al. [8]. Western blot analysis using antibodies against pure choline oxidase were also carried out with crude enzyme extracts from the leaves of transgenic lines and wild-type plants of chickpea and Indian mustard.

4.3. Determination of choline oxidase activity

Choline oxidase (EC 1.1.3.17) is a bifunctional enzyme capable of catalyzing glycinebetaine biosynthesis from choline via betaine aldehyde. Choline oxidase activity in the leaves and chloroplasts of transgenic plants was determined according to the procedure of Gadda et al. [15]. Enzyme activity was measured polarographically by monitoring the rate of oxygen consumption with a Hansatech oxygen electrode (UK) thermostated at 25 °C.

4.4. Measurement of PS II activity

Photochemical activities of the chloroplasts were assayed polarographically using Clark-type oxygen electrode (Hansatech, UK). The photosystem (PS) II dependent O₂ evolution was assayed with para-benzoquinone (pBQ) as an electron acceptor using saturating white light (~500 µmol m⁻² s⁻¹) provided by a 300 W

projector lamp. The assay mixture for measuring PS II activity contained suspension buffer (pH 7.5) and 50 μM pBQ. Chloroplasts equivalent to 20 μg chlorophyll were used for each assay. Intactness of the chloroplasts was determined by estimating PS II activity in terms of oxygen evolution using FeCN and pBQ as electron acceptors. For the assay of PS II mediated photoreaction $\text{H}_2\text{O} \rightarrow \text{FeCN}$, reaction mixture contained in addition to the suspension buffer, 2 mM FeCN.

4.5. Effect of high light on PS II activity in intact chloroplasts

Tolerance of intact chloroplasts, isolated from wild-type and transgenic lines, to high light intensity at 10 and 25 °C was evaluated. For this the chloroplasts (equivalent to 2 mg/ml chl) were exposed to strong white light (1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$) in a jacketed glass cuvette with slow stirring. The temperature of the cuvette was maintained at 10 and 25 °C with circulating water bath (Techne Cambridge Ltd., UK). A set of chloroplast suspension was also maintained in dark at 10 and 25 °C. After specified intervals of time, aliquot of 10 μl chloroplast suspension was taken from samples from each treatment and PS II activity was determined in terms of $\mu\text{moles oxygen evolved mg}^{-1} \text{chl h}^{-1}$.

4.6. Effect of glycinebetaine and H_2O_2 on the PS II activity in intact chloroplasts

Chloroplast suspension (equivalent to 2 mg/ml chl) were exposed to high light intensity in the presence of 100 mM glycinebetaine and 250 μM H_2O_2 individually and in combination at 10 and 25 °C. After specified time intervals a suitable aliquot was taken from the incubation mixture and PS II activity was determined in terms of $\mu\text{moles oxygen evolved mg}^{-1} \text{chl h}^{-1}$.

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