Over Expression of the Carbonic Anhydrase Gene Confers Salinity Tolerance with Improved Yield in Rice

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ABSTRACT

Background: Abiotic and biotic stresses impact agricultural productivity. Rice being the stable food of India; requires more scientific intervention to boost productivity.

Methods: Cloning and transformation of carbonic anhydrase (CA) gene in IR64 rice, molecular analysis of T1 transgenic CA rice plants, salinity tolerance index, leaf disk senescence assay, chlorophyll content, biochemical analysis of antioxidant activities of marker-free CA transgenic lines and measurement of photosynthetic activities and agronomic characteristics of CA transgenic plants were conducted.

Result: We have raised two (2) lines of marker free CA overexpressing transgenic rice (*Oryza sativa* L. cv. IR64) plants. The overexpression of *CA* driven by CaMV35S promoter in transgenic rice confers high salinity (200 mM NaCl) stress tolerance in the rice. Photosynthetic characteristics such as net photosynthetic rate (PN), stomatal conductance (gs), intercellular CO_2 (Ci), as well as chlorophyll (Chl) contents were significantly (22-31%) higher in transgenic lines under salinity stress as compared to control plants. Ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR) and guaiacol peroxidase (GPX) were significantly higher (21-37%) in transgenics as compared with VC and WT plants. Overall, the present research is focused to develop marker free *CA* overexpressing transgenic lines for better tolerance against salinity stress, higher photosynthesis and better productivity.

Key words: Carbonic anhydrase, Marker-free rice, Mature seed-derived calli, Photosynthesis, Reactive oxygen species, Salinity stress, Stress tolerance.

INTRODUCTION

Agricultural productivity is severely affected by different abiotic and biotic stresses. To feed the ever-increasing population we have to overcome the limitations caused by different stresses. So it is now imperative to develop stress tolerant crop cultivars. Plant productivity is severely affected by soil salinity. Rice (*Oryza sativa*) is a staple food consumed and cultivated by more than half population of this world, particularly in Asian countries (Sen *et al*., 2020).

Carbonic anhydrase (CA) is the enzyme that accelerates the reversible hydration reaction of carbon dioxide converted to bicarbonate (Ren *et al*., 2021). They are essential in regulating carbon dioxide levels and ensuring optimal plant development and metabolic mechanisms (Vicente *et al*., 2022). The proper functioning of carbonic anhydrases (CAs) is crucial for integrating enzymes into various processes involved in regulating pH, CO_2 and HCO_3" concentrations and water and electrolyte balance and also essential in maintaining homeostasis in living organisms (Occhipinti and Boron, 2019). This particular enzyme assumes a crucial role in the process of C4 photosynthesis. The cytosolic carbonic anhydrase (CA) gene in *Flaveria bidentis* cells provides evidence for the enzyme's crucial role in the C4 photosynthetic pathway. Salt stress upregulates the expression of the OsCA1 gene encoding carbonic anhydrase in rice seedlings and total CA activity. Over-expression of OsCA in *Arabidopsis thaliana* mutant plants increase salt tolerance. This suggests that OsCA may enhance plants ability to cope with salt stress, providing a potential avenue for developing salt-tolerant crops (Kandoi *et al*., 2022).

In the present study, CA overexpressing transgenic rice plants (*Oryza sativa* L., cv. IR64) were developed which shows enhanced photosynthesis, tolerance to salinity stress with increased yield and improved biomass.

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MATERIALS AND METHODS

Cloning and transformation of *carbonic anhydrase (CA)* gene in IR64 rice

The carbonic anhydrase (*CA*) gene was used to develop transgenic rice (*Oryza sativa* cv. IR64) plants using the

tissue culture technique. The coding region of CA gene (0.8 kbp) from rice was PCR-amplified using forward and reverse primers (5'-ATGGGAAGTAAATCATATGAT-3' and 5'-TTTCACCTACTACCTCAGCATAA-3'). The gene (0.8 kb) was cloned in a reporter gene- free plant transformation vector pCAMBIA1300 in the place of hygromycin to generate a complete reporter and antibiotic marker-free plasmid pCAMBIA1300-*CA*. The above construct (pCAMBIA1300-*CA*) was used for the *Agrobacterium tumefaciens* (LBA4404) transformation method (Sahoo and Tuteja, 2012). After development of transgenic rice plants, all the analysis were compared with WT plant as control (C).

Molecular analysis (PCR, Southern blot and qRT-PCR) of T_1 transgenic *CA* rice plants

The genomic DNA was extracted from the healthy leaves of marker-free *CA* transgenic plants and used to check the integration of gene by PCR and Southern blot analysis. About 25 µg of genomic DNA was used for the Southern blot experiments. First the genomic DNA was digested with *Bam*HI restriction enzyme and resolved on 0.8% agarose gels, followed by transfer to a negatively charged nylon membrane (Hybond-N^+, Amersham, Inc.), as described by Sambrook *et al.* (1989). The probe was radiolabelled by the gene amplification method, using α–[^{32}P] dCTP. Hybridization with the probe was conducted using the method described by Sambrook *et al.* (1989).

The expression of *CA* gene in different transgenic lines were studied by qRT-PCR experiment. The 21 days old seedlings of transgenic (CA) and control (C) rice (*Oryza sativa* cv. IR 64) plants were used for this experiment. Total RNA was isolated from 100 mg of samples with TriZOL LS reagent (Invitrogen Life Technologies USA). The total RNA obtained was used as template for cDNA synthesis. The first strand cDNA was synthesized from 5 µg of total RNA using Superscript II Reverse Transcriptase (Invitrogen Life Technologies USA) with oligo (dT) primer according to the manufacturer's instructions. PCR reactions were performed on Step One Real-Time PCR system (Applied Biosystems). Using Power Syber Green PCR master mix (Applied BioSystems), a 20 µl reaction mixture containing 10 pM of each gene specific primer pair (α-tublin forward 5′-GGTGGAGGTGA TGATGCTTT-3′ and reverse 5′-ACCACGGGCAAAGTT GTTAG-3′ and with *CA* gene specific primers (Forward 5′-TTTCCAACCCGGTGAGG -3′ and Reverse 5'- AGCAGATA GTTGTAATT -3′). The PCR cycle was as follows: 95°C for 30 s, 58°C for 30 s and 72°C for 30 s. The quantitative variation between different samples was evaluated by the ΔΔCt method and the amplification of tubulin gene was used as internal control to normalize all data (He *et al*., 2021). To validate the qRT-PCR results, the experiments were repeated three times. The mean values for the expression levels of the genes were calculated from three independent experiments. The relative expression level was calculated as $2^{-\Delta\Delta CT}$. The $2^{-\Delta\Delta CT}$ values for the untreated transgenic were normalized to 1.

Salinity tolerance index (TI)

The TI of the 200 mM NaCl-treated CA transgenic (L7 and L9) and control plants were calculated using the following formula:

$$TI\ (\%) = \frac{\text{Plant dry weight with 200 mM NaCl}}{\text{Plant dry weight with water}} \times 100$$

Leaf disk senescence assay and chlorophyll content

Healthy and fully expanded rice leaf squares of 1 cm×1 cm dimension were taken from similar age transgenic lines (L7 and L9) of transgenic and control plants. The discs were floated in 100 and 200 mM solution of NaCl for 72 h. The experiment was performed at room temperature with three biological replicates as previously described (Tuteja *et al.,* 2013).

Biochemical analysis of antioxidant activities of marker-free *CA* transgenic lines

The seeds of *CA* transgenic (L7 and L9) and control (C) plants were placed in hydroponics for germination. After plants had grown for 21 days, they were shifted to another hydroponics filled with 200 mM NaCl for 24 h. All our experiments were conducted in the greenhouses of the Centurion University of Technology and Management, Bhubaneswar, Odisha, India, where 16 h light photoperiod and 8 h dark at 25°C was maintained. After 24 h salt stress, plant tissues were used for biochemical analysis of *e.g.*, catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), guaiacol peroxidase (GPX), proline, hydrogen peroxide and lipid peroxidation; in addition, we also measured the relative water content (RWC) as well as the electrolytic leakage. All the parameters were measured using the methods described earlier by Garg *et al.,* (2012).

Measurement of photosynthetic activities and agronomic characteristics of *CA* transgenic plants

The rate of photosynthesis, photosynthetic yield, intercellular CO_2 concentration, CO_2 release, stomatal conductance and the rate of transpiration were recorded using an infrared gas analyser (IRGA; LI-COR, http://www.licor.com) on sunny days between 11:00 AM and 12:00 noon. For these measurements, plants were grown in metal pots filled with soil and all the pots were kept inside another big horizontal tank filled with 200 mM NaCl solution. All the parameters were measured using the expanded leaves of mature (60 d old) plants. After 12 days of salt stress, different agronomic parameters (plant height, number of tillers/plant, number of panicle/plant, number of filled grain/panicle, number of chaffy grains/panicle, straw dry weight, 100 grain weight, root length, root dry weight, leaf area and plant dry weight) were measured using methods described earlier (Tuteja *et al.,* 2013).

Statistical analysis

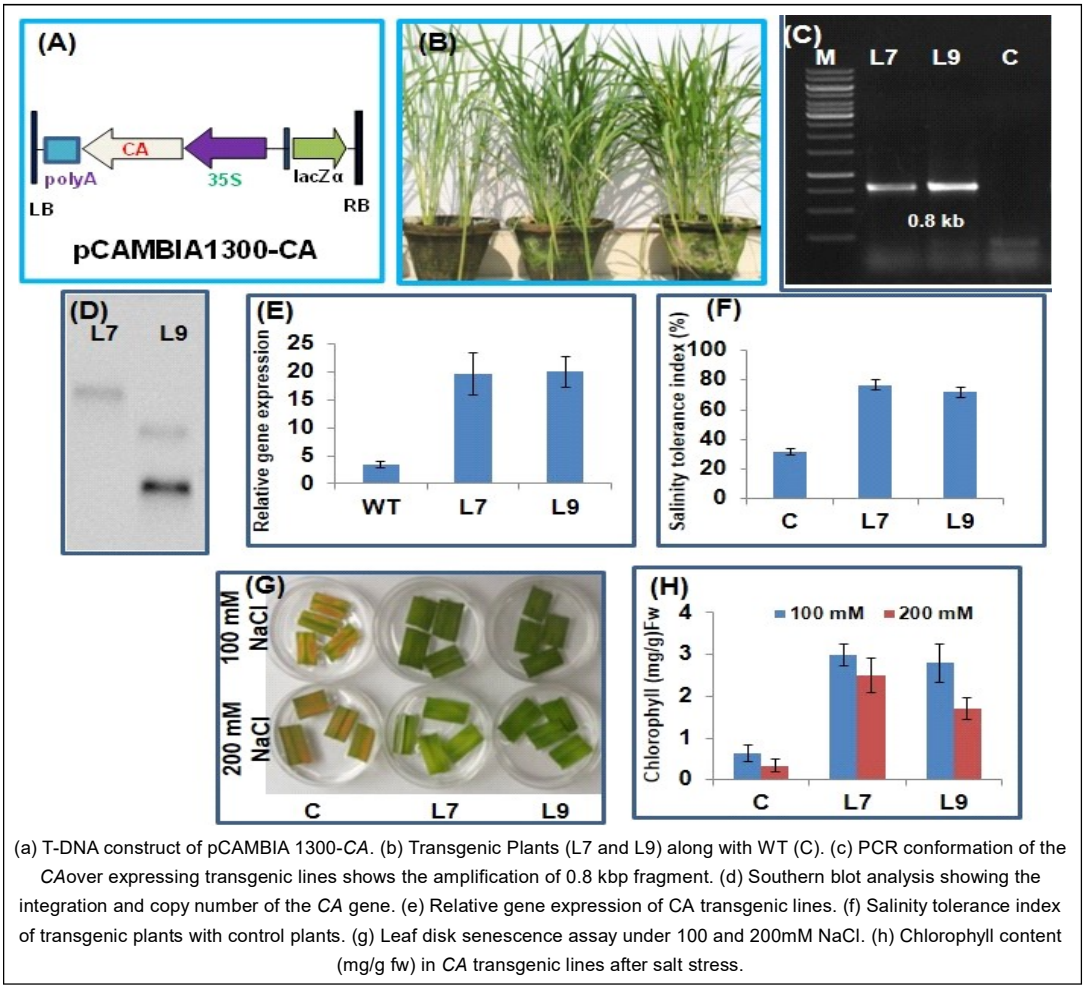
Data was obtained from at least three independent experiments. The mean values are presented and standard errors included. One-way analysis of variance (ANOVA) was

performed on the data using SPSS (12.0 Inc., USA) to determine the least significant difference (LSD). The means were interpreted by Duncan's multiple range tests (DMRT).

Results and Discussion

Molecular analysis of marker-free *PDH45* transgenic lines

The marker-free *CA* transgenic IR64 rice plants were developed using the pCAMBIA1300-*CA* gene construct (Fig 1a). Phenotypically the transgenic rice plants were not significantly different from control plants (Fig 1b). The desired *CA* gene (0.8 kb) fragment was detected by PCR (Fig 1c). The Southern blot results confirmed the integration of *CA* gene in transgenic rice plants in all the two transgenic lines (L7 and L9) (Fig 1d). The real-time PCR (qRT-PCR) provided ~5 fold induction in the transcript level of *CA* in transgenic lines (L7 and L9) (Fig 1e). The salinity tolerance index of CA transgenic rice lines was found to be higher (75.3 and 72.8% in L7 and L9 respectively) in comparison with control plants (30.2%) (Fig 1f). The damage caused in the leaf pieces by salinity stress was observed in all the plants after 72 h. The reduction of chlorophyll content in leaf tissues was lesser in transgenic lines as compared to C plants (Fig 1g). The lesser chlorophyll content in the leaf tissues of C plants as compared to transgenic lines provides strong evidence towards tolerance against salinity stress (Fig 1h). We have generated transgenic rice lines overexpressing carbonic anhydrase gene using *Agrobacterium*-mediated transformation with higher afinity for CO_2. Overexpression of CA gene under the control of 35S promoter resulted in higher gene expression, protein abundance and enzymatic activity of CA, in the transgenic lines. Two independent transgenic lines (L7 and L9) along with WT plants as control (C) were used for functional validation under salinity stress. This was indicated by the presence of higher chlorophyll content in the leaf disks of salinity-stressed transgenic plants, whereas WT plant leaves became yellow. These results indicate that the introduced trait is functional in transgenic plants and that it is also stable (Sahoo *et al*., 2022). Similar findings have been reported earlier (Amin *et al.,* 2012; Gill *et al.,* 2013; Tuteja *et al.,* 2013, 2015; Sahoo *et al.,* 2022).

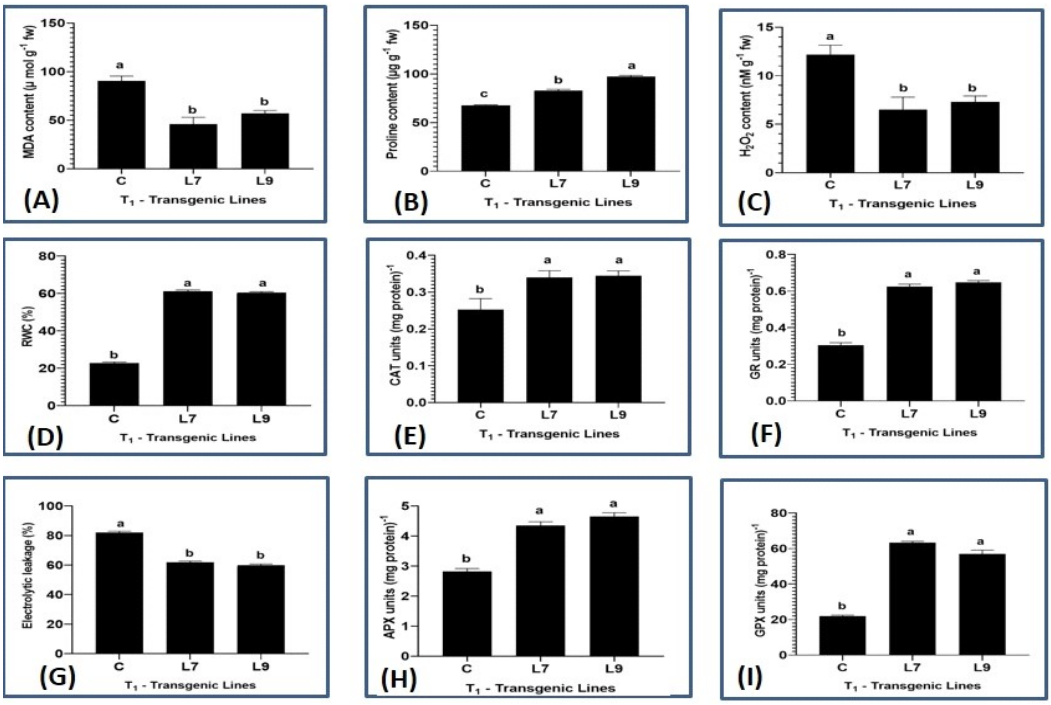


(a) T-DNA construct of pCAMBIA 1300-*CA*. (b) Transgenic Plants (L7 and L9) along with WT (C). (c) PCR conformation of the *CA*over expressing transgenic lines shows the amplification of 0.8 kbp fragment. (d) Southern blot analysis showing the integration and copy number of the *CA* gene. (e) Relative gene expression of CA transgenic lines. (f) Salinity tolerance index of transgenic plants with control plants. (g) Leaf disk senescence assay under 100 and 200mM NaCl. (h) Chlorophyll content (mg/g fw) in *CA* transgenic lines after salt stress.

Fig 1: Analysis of *CA* marker-free transgenic plants.

The antioxidant machinery of CA transgenic rice performed better than control plants

The salt-induced changes in the catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), guaiacol peroxidase (GPX), proline, hydrogen peroxide, ion leakage, accumulation of MDA (lipid peroxidation product), relative water content (RWC) were compared with C rice seedlings. Reduced levels of MDA, H_2O_2 and ion leakage and increased level of proline content in transgenic lines as compared to the C plants under salt (200 mM NaCl) stress were observed. The activities of CAT, APX, GPX, GR and RWC were increased in transgenic plants as compared to C plants (Fig 2 a-i). Under salinity stress, plants produce more ROS, which can cause serious damage to plasma membrane, chloroplasts and mitochondria through peroxidation and de-esterification of membrane lipids, as well as damage to nucleic acids and proteins (Hasanuzzaman *et al*., 2020). In the present study, lipid peroxidation, ion leakage and H_2O_2 production were found to be significantly decreased as compared to control plants under salinity stress. Previous studies have also shown decreased level of MDA, ion leakage and H_2O_2 production under salinity stress in different overexpression studies in rice (Yang *et al.,* 2012; Gill *et al.,* 2013; Tuteja *et al.,* 2013; Sahoo *et al.,* 2022). The damage caused due to salinity to membrane stability is a consequence of generation of H_2O_2 which is an important ROS that can cause oxidative damage to biomolecules including nucleic acids, proteins and lipids (Gill and Tuteja, 2010). To protect the plants from the injurious effects of H_2O_2, plants produce higher levels of APX through the AsA-GSH cycle, where APX uses ascorbate as hydrogen donor. Other antioxidant enzymes, such as GR, catalyse the NADPH-dependent reduction of GSSG (oxidized form) to GSH (reduced form) and maintain high ratio of GSH/ GSSG (Hasanuzzaman *et al*., 2019). In our investigation, the antioxidant enzymes APX, GPX and GR showed significantly higher activities under salinity stress in transgenic lines compared to control plants, suggesting that this provides better ROS scavenging during the stress. The exact mechanism on how salinity stress tolerance works in plants is still far from being understood. The excessive generations of ROS intermediates, such as superoxide radicals ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$), are the unfortunate consequences of salinity and other stresses in plants. Since the antioxidant enzyme machinery was found to be stronger in CA



(a) Lipid peroxidation expressed in terms of MDA content Ion leakage. (b) Level of proline accumulation. (c) Hydrogen peroxide (H_2O_2) content (d) Per cent relative water content (RWC). (e) Catalase (CAT) activity, one unit of enzyme activity defined as 1 µmol H_2O_2 oxidized min^{-1}. (f) Glutathione reductase (GR) activity, one unit of enzyme activity is defined as 1 ìmol of GS-TNB formed min^{-1} due to reduction of DTNB. (g) Electrolytic leakage (h) Ascorbate peroxidase (APX) activity, one unit of enzyme activity defined as 1 ммol of ascorbate oxidized min^{-1}. (i) Guaiacol peroxidase (GPX) activity. Error bars indicate the standard errors (SEs) calculated from three independent experiments.

Fig 2: Biochemical analysis of *CA* over expressing transgenic lines (L7 and L9) with WT (C) plants exposed to 24 h salinity stress (200 mM NaCl).

transgenic rice plants, hence, the salinity stress tolerance might be due to better detoxification of ROS. Generally, plants adapt to stress by accumulating amino acids and/or amino acid derivatives, sugar alcohols and several other substances. These were found to be accumulated at higher levels in CA transgenic rice plants and may help plants to better adapt to changing environment. The salinity stress tolerance could also be due to the interaction of CA with the components of different signalling pathways (Mishra *et al*., 2023).

Agronomic performance of marker-free *PDH45* transgenic plants under stress

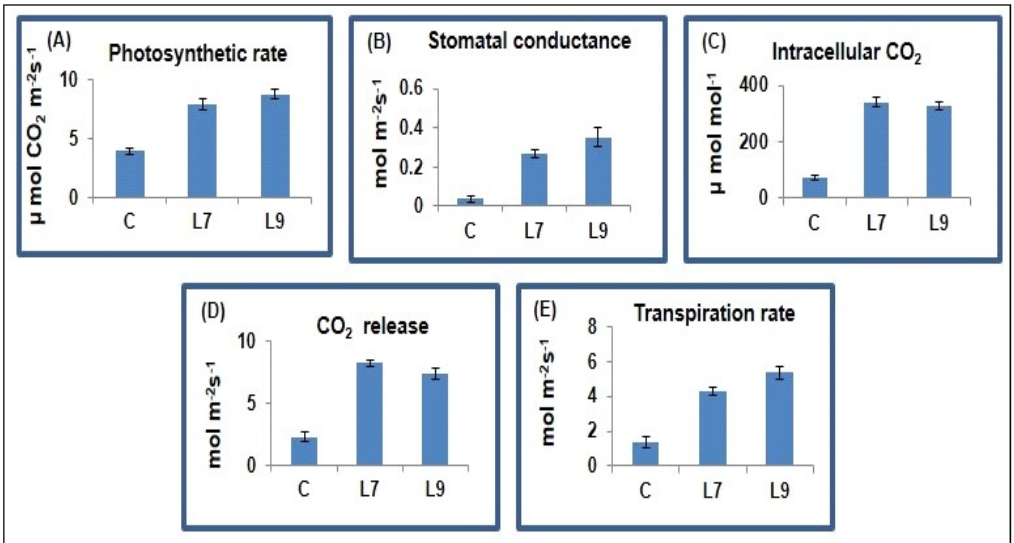
The agronomic performance of CA transgenic (under 200 mM NaCl stress) was compared with C plants under 0 mM NaCl (water) stress. Better agronomic characteristics were observed in *CA* transgenic plants as compared to C plants (Table 1). Several phenotypic characteristics of transgenic plants were recorded and found to be almost similar to the C plants grown in water (0 mM NaCl). Similar studies have been reported in rice (Tuteja *et al.,* 2013; Sahoo *et al.,* 2022).

Photosynthetic characteristics of marker-free CA transgenic plants under stress

The photosynthetic characteristics of transgenic plants were observed and compared to WT and VC plants after 12 d of induction of salt (200 mM NaCl) stress. The photosynthetic rate declined by 33% in C plants as compared to *CA* transgenic lines. The photosynthetic rate, stomatal conductance and intracellular CO_2, CO_2 release and transpiration rate was also higher in transgenic lines as compared to the C plants (Fig 3a-e). The better photosynthetic activities like photosynthetic yield, photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, CO_2 release and transpiration rate were observed in *CA* transgenic lines as compared to the control plants. The retention of chlorophyll content in transgenic lines indicates the better control over photosynthetic apparatus under salinity stress. Our data are in agreement with the earlier reports on *PDH45*, *SUV3* and *BAT1* over expressing rice plants in stress (Sahoo *et al.,* 2012; Gill *et al.,* 2013; Tuteja *et al.,* 2013, 2015; Sahoo *et al.,* 2022).

Table 1: Agronomic parameters of control (C) and CA overexpressing transgenic lines (L7 and L9) of rice.

Agronomic parameters	Control (C)	L7	L9
Time required for flowering (days)	90±2.1	90±2.6	90±2.3
No. of tillers/plant	20±1.0	22±0.17	23±1.0
No. of panicle/plant	15±1.2	20±1.1	21±0.5
No. of filled grain/panicle	80±3.6	87±2.1	82±3.1
No. of chaffy grains/panicle	10±0.51	08±0.27	10±0.15
Straw dry weight (g)	50±1.5	51±3.2	51±2.2
100-grain weight	2.27±0.13	2.42±0.1	2.38±0.1
Seed weight per plant	45.5±1.5	60.21±1.3	57.2±1.0



(a) Photosynthetic rate. (b) Stomatal conductance. (c) Intracellular CO_2 (d) CO_2 release (e) Transpiration rate.

Fig 3: Measurement of photosynthetic characteristics of control and *CA* marker-free transgenic rice lines (L7, L9) under 200 mM NaCl stress.

CONCLUSION

Photosynthesis and CO_2 availability are the ultimate determinants for crop growth and yield. The overall photosynthetic performance was found to be higher in marker free transgenic rice compared to the controlled ones. The yield and yield attributing factors *viz*; time required for flowering (days), no. of tillers/plant, no. of panicle/plant, no. of filled grain/panicle, no. of chaffy grains/panicle, straw dry weight (g), 100-grain weight, seed weight per plant were also enhanced in transgenic rice compared under salinity stress. Thus the current piece of work provides evidence of a unique function of CA in providing salinity stress tolerance in transgenic rice without affecting yield. It also provides a profound example for the exploitation of C4 components in C3 plants like rice for enhanced photosynthesis as well as agricultural production that have the ability to withstand extreme climatic conditions.

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Conflict of interest

The authors declare that they have no conflict of interest.

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