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Overexpression of *Setaria italica* phosphoenolpyruvate carboxylase gene in rice positively impacts photosynthesis and agronomic traits

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ABSTRACT

C_4 plants have the inherent capacity to concentrate atmospheric CO_2 in the vicinity of RuBisCo, thereby increasing carboxylation, and inhibiting photorespiration. Carbonic anhydrase (CA), the first enzyme of C_4 photosynthesis, converts atmospheric CO_2 to HCO_3^-, which is utilized by PEPC to produce C_4 acids. Bioengineering of C_4 traits into C_3 crops is an attractive strategy to increase photosynthesis and water use efficiency. In the present study, we isolated the *PEPC* gene from the C_4 plant *Setaria italica* and transferred it to C_3 rice. Overexpression of *SiPEPC* resulted in a 2-6-fold increment in PEPC enzyme activity in transgenic lines with respect to non-transformed control. Photosynthetic efficiency was enhanced in transformed plants, which was associated with increased ΦPSII, ETR, lower NPQ, and higher chlorophyll accumulation. Water use efficiency was increased by 16–22% in PEPC transgenic rice lines. Increased PEPC activity enhanced quantum yield and carboxylation efficiency of PEPC transgenic lines. Transgenic plants exhibited higher light saturation photosynthesis rate and lower CO_2 compensation point, as compared to non-transformed control. An increase in net photosynthesis increased the yield by (23–28.9%) and biomass by (24.1–29%) in transgenic PEPC lines. Altogether, our findings indicate that overexpression of C_4-specific SiPEPC enzyme is able to enhance photosynthesis and related parameters in transgenic rice.

1. Introduction

Rice is a principal source of carbohydrates consumed by nearly fifty percent (50%) of the global population. To feed the increasing global population, the current rate of crop yields per unit area seems inadequate. In rice, atmospheric CO_2 assimilation is carried out in mesophyll cells through the C_3 photosynthetic pathway. Photosynthetically, C_3 plants are underachievers because of the loss of fixed CO_2 during photorespiration. High CO_2 favours the carboxylase activity of the primary photosynthetic enzyme, RuBisCo, and subsequently net CO_2 assimilation, whereas high O_2 promotes the oxygenase activity directing photorespiration. To subdue photorespiration, some tropical plants like maize, foxtail millet, and sorghum have judiciously evolved a CO_2-specific biochemical pump (C_4 photosynthetic cycle), favouring the concentration of CO_2 around the micro-environment of RuBisCo, ultimately encouraging its carboxylation activity. At the current atmospheric CO_2 level (380 ppm), C_4 photosynthesis has higher efficiency to convert solar energy into biomass (6%) than the C_3 photosynthetic pathway (5%) (Zhu et al., 2008). C_4 plants have the capability to utilize solar energy more efficiently than C_3 plants. Net assimilation of CO_2 under elevated conditions of drought, salinity, and temperature is more efficient in the C_4 photosynthetic system, as compared to C_3 system. C_4 photosynthesis drives higher productivity and improved water and nitrogen use efficiency in several economically important crops, such as maize (*Zea mays*), sugarcane (*Saccharum officinarum*), and sorghum (*Sorghum bicolor*). So far, traditional breeding approaches to introduce C_4 traits in C_3 crops have not been successful due to their sexual incompatibility and un-relatedness. Therefore, the transgenic approach for the introduction of C_4 traits in C_3 rice has appeared to be an appealing way to accelerate the photosynthetic rate.

Carbonic anhydrase (CA) acts as a primary enzyme for the conversion of atmospheric carbon dioxide to bicarbonate in the C_4 photosynthetic cycle (Hatch and Burnell, 1990). This bicarbonate is further fixed

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Abbreviation

BS	Bundle sheath
ETR	Electron transport rate
IRGA	Infra-Red Gas Analyzer
M	Mesophyll
NPQ	Non photochemical quenching
PAR	Photosynthetically active radiation
PEPC	phospho*enol* pyruvate carboxylase
q*P*	Photochemical quenching
iWUE	instantaneous water use efficiency
ΦPSII	Quantum yield of photosystem II
PPN	*SiPEPC* expression cassettes
PVP	polyvinylpyrrolidone

by PEP carboxylase (PEPC), resulting in the production of C_4 acids. Phosphoenolpyruvate carboxylase (PEPC) (EC:4.1.1.31), the primary carboxylating enzyme of the C_4 cycle, plays a significant role in maintaining equilibrium between CO_2 and HCO_3^-. PEP is carboxylated by the presence of PEPC and HCO_3^- to produce a four-carbon compound, oxaloacetate (OAA) (Izui et al., 2004). Upon entering the bundle sheath cell, OAA is decarboxylated to produce CO_2 that is subsequently consumed in the Calvin cycle (Kajala et al., 2011). PEPC is confined to the cytoplasm of mesophyll cells of C_4 and CAM plants, where it plays a significant role in the carbon metabolism (Masumoto et al., 2010). In C_3 plants, PEPC maintains OAA and malate, the intermediates of the citric acid cycle that are essential for nitrogen assimilation and biosynthesis of amino acids (Miyao and Fukayama, 2003; Masumoto et al., 2010). PEPC is present in all photosynthetic organisms and isoforms play significant housekeeping metabolic roles, which are non-photosynthetic (O'Leary et al., 2011).

Many physiological changes have been accomplished with the transformation of C_4 *PEPC* genes in rice. Earlier reports have revealed that overexpression of *ZmPEPC* increased the net photosynthesis rate leading to a higher yield of transgenic rice (Ku et al., 1999, 2000). Transgenic plants expressing C_4 genes showed significant resistance to various abiotic stresses viz. drought (Gu et al., 2013; Ding et al., 2015; Qian et al., 2015; Zhang et al., 2017; He et al., 2020; Liu et al., 2021), salinity (Yadav and Mishra, 2020; Kandoi et al., 2016), high temperature (Qi et al., 2017; Muthusamy et al., 2019), heavy metal (Zhang et al., 2018) and high light intensity (Jiao et al., 2002; Zhang et al., 2021). Engineering of C_4 pathway into C_3 crops upgrades photosynthetic ability with increased yield, biomass, and increases water use efficiency (Ermakova et al., 2020). Nearly all rice transformation studies with intact *PEPC* gene (with promoter) from maize, showed a significant enhancement of relative gene expression than that with PEPC cDNA (Matsuoka et al., 2001; Agarie et al., 2002). However, earlier studies also reported no enhancement in net photosynthesis rate in maize PEPC expressing transgenic rice (Agarie et al., 2002; Fukayama et al., 2003). It has been noted that overexpression of sorghum *PEPC* improved the photosynthetic efficiency of transgenic rice by lowering photorespiration and CO_2 compensation point (Zhang et al., 2003). C_4 type PEPC from different sources has been ectopically overexpressed in rice with variable effects on photosynthesis and other growth and agronomic parameters (Bandyopadhyay et al., 2007; Lian et al., 2014; Ding et al., 2015). Over expression of *Zm-PEPC* multiplied the carbon level in transgenic rice lines that influence the regulation of photorespiratory pathway, showing tolerance to low-N stress and increased grain yield per plant (Tang et al., 2018). Similarly, overproducing the principal carbon fixing enzyme of C_3, Rubisco, in transgenic rice resulted in increased yield and N_2 use efficiency (Yoon et al., 2020; Raines, 2022).

Setaria italica, domesticated from the wild ancestor *Setaria viridis*, is a model C_4 grass with a diploid genome. Despite being an important crop in the semi-arid tropical area and an excellent model for C_4 photosynthesis (Li and Brutnell, 2011; Yang et al., 2020), *S. italica* has not been utilized well in the effort to bioengineer C_4 traits into C_3 crops. A pertinent question is whether overexpression of C_4 PEPC from *Setaria italica* in rice can increase photosynthesis and water use efficiency. In this study, we isolated the C_4 *PEPC* gene from *Setaria italica* and ectopically overexpressed it in Indica rice variety IR64 to study its impact on photosynthesis and associated physiological parameters. Our results revealed that the overexpression of the *SiPEPC* gene positively influences the photosynthesis rate and other agronomic features, including yield of transgenic plants.

2. Materials and methods

2.1. Plant material

Setaria italica and *Oryza sativa* L.ssp. *indica* var. IR64 were used as plant materials for this study. *Setaria italica* seeds (collection GS-1384; NBPGR accession no. IC479929) were collected from NIPGR, New Delhi. The seeds were treated with Bavistin, surface sterilized, washed with distilled water, and germinated in Petri plates containing a sterile filter paper soaked with water. The germinated seeds were transferred to the soil. *Setaria italica* was used for PEPC cDNA (Si005789m) isolation and *Oryza sativa* L. ssp. *indica* var. IR64 was used for genetic transformation.

2.2. Isolation and cloning of SiPEPC gene

Total RNA was isolated from the leaves of 21 days old seedlings of *Setaria italica* using RNeasy® Plant Mini Kit (Qiagen, Germany). cDNAs were synthesized from RNA using Maxima cDNA synthesis kit with dsDNase (Thermo Fisher Scientific, USA). cDNA was used as the template for PCR amplification of the *SiPEPC* gene using specific primers pair employing HotStar HiFidelity DNA polymerase (Qiagen, Germany). *Xba*I and *Kpn*I sites were used in forward and reverse primer sequences of *SiPEPC*. The sequences of primer pair used to amplify 2895 bp *PEPC* gene were as follows- F-5′-GCT CTA GAG CAT GGC GTC CAA GCC CGT GGA-3′and R-5′-GGG GTA CCC CCT AGC CAG TGT TCT GCA TGC CGG-3'. The 2895 bp PCR product was cloned in the *pTZ57*R/T-P_{ZmPPDK} vector and Sanger sequenced for verification. The sequence was submitted to NCBI GenBank (Accession no. MF967570).

2.3. Binary vector construction

The *pCAMBIA1301-*P_{ZmPPDK}*-SiPPDK-nos* (Swain et al., 2021) was digested by *Bam*HI, treated with Klenow fragment, and then digested by *Xba*I to release the *SiPPDK* fragment and make the vector (*pCAMBIA1301-*P_{ZmPPDK}*-nos*) with one blunt and one sticky end (*Xba*I). The *pTZ-SiPEPC* was digested by *Acc*651 followed by Klenow treatment. Subsequently, *Xba*I digestion released the *SiPEPC* fragment having one blunt end and one *Xba*I sticky end. Then, the *SiPEPC* was cloned in *pCAMBIA1301-*P_{ZmPPDK}*-nos* to produce *pCAMBIA1301-*P_{ZmPPDK}*-SiPEPC-nos* (abbreviated as PPN) for rice transformation.

2.4. Rice transformation

Embryos were isolated from surface-sterilized mature seeds of rice cultivar 'IR64' and plated on Callus Induction Medium (CIM) (N6 medium supplemented with 2.5 mg/L 2,4-D, 30 gm/L sucrose, and 8 gm/L agar) (Swain et al., 2018; Behera et al., 2019) and kept in dark at 26 ±2 °C for 10 days. Emerging radicles and plumules were excised from the calli and discarded. Calli were then transferred to fresh CIM. PPN binary vector was transformed to *Agrobacterium tumefaciens* strain LBA4404 by freeze-thaw method. *pCAMBIA-1301* containing *Agrobacterium* was used as empty vector control. Overnight culture of LBA4404 harbouring vector construct was resuspended in liquid MS

medium containing acetosyringone (150 μM) with an $OD_{600} < 0.1$. 21 days old embryogenic calli were placed in bacterial suspension for 20 min under vacuum infiltration. The calli were then blot dried on sterile filter paper and cultured on the co-cultivation medium (MS medium supplemented with 2.0 mg/L 2, 4-D, 30 gm/L sucrose, 8 gm/L agar, 150 μM acetosyringone) for 3 day at 28 °C in dark. After washing with sterile water and liquid MS+200 mg/l Timentin, the infected calli were transferred to the selection medium (MS medium supplemented with 2.0 mg/L 2, 4-D, 30 gm/L sucrose, 8 gm/L agar, 50 mg/L hygromycin B and 250 mg/L Timentin) and maintained in the dark at 27 °C. After three selection cycles of 15 days each, healthy, proliferated calli were transferred to the regeneration medium (MS with 3 mg/L BAP, 1.5 mg/L kinetin, 0.5 mg/L NAA) and kept in light under a 16-h photoperiod for 2–3 weeks at 28 °C. The regenerated plantlets were transferred to the rooting medium (1/2 MS with 0.5 mg/L NAA) (Behera et al., 2019). Rooted plants were transferred to the soilrite for a week, and then to the soil pot under the greenhouse condition. The growth condition was as follows: photoperiod of 14 h light and 10 h dark, day/night temperature regime of 28°/24°-C, and relative humidity 80%. The pots were fertilized with the N:P:K with a ratio of 80:40:40.

2.5. PCR-based screening

Genomic DNA was extracted from transgenic and control plants by using Qiagen Plant DNA Isolation Kit (Qiagen, Germany). The presence of *HPT*, and *SiPEPC* genes were screened by PCR using respective pairs of primers. Primer sequences are as follows: hpt-F-5′-TCA ATG ACC GCT GTT ATG-3′ and hpt-R-5′- CGC CGA TGG TTT CTA CAA AGA-3’; PEPC-F-5′-GCT CTA GAG CAT GGCGTCCAAGCCCGT GGA -3′ and ScPEPC-R-5′-GCTCCGACTCCTGACGGATGTCC-3’. Amplification of *HPT* was carried out in a thermocycler (Eppendorf, Germany) following the PCR cycle of initial 30-s incubation at 98 °C for complete denaturation, followed by 35 cycles of 98 °C for 10s, 60 °C for 30s, 72 °C for 60s and final extension at 72 °C for 10 min. All the conditions for amplification of the *SiPEPC* gene were the same except that the annealing temperature was 58 °C for the *HPT* gene. PEPC screening was done following a PCR cycle of initial 30-s incubation at 98 °C for complete denaturation, followed by 35 cycles of 98 °C for 10s, 58 °C for 30s, 72 °C for 60s, and final extension at 72 °C for 10 min.

2.6. Southern blot analysis

The PCR-positive plants were selected for Southern blot analysis. Genomic DNA was isolated from the leaves of transgenic and wild-type plants following a modified Dellaporta method (Dellaporta et al., 1983). For each line, 15 μg of genomic DNA were digested with the restriction enzyme *Sal*I (Promega, US). The fragmented DNA was separated in 1% Agarose (w/v) gel by electrophoresis. DNA was transferred to a nylon membrane (Amersham Hybond-N+), hybridized, and washed following standard protocols described earlier (Deininger, 1990). A 1.1 Kbp restriction digested fragment of the *HPT* gene was used as a probe and was labeled with a Dig-labeled DNA Labelling Kit (Roche Applied Science, Germany). Results were documented by photography.

2.7. RNA extraction and cDNA synthesis

Total RNA from the leaves of transgenic and non-transgenic wild-type control rice plants was isolated by using the RNeasy® Plant Mini Kit (Qiagen, Germany). The RNA sample was treated with DNase I (Sigma) to remove all traces of genomic DNA. cDNA was prepared using a Maxima cDNA synthesis kit (Thermo Fisher, U.S.).

2.8. Quantitative real-time PCR analysis

Quantitative real-time PCRs were carried out in a Realplex real-time system using Maxima qPCR Master Mix with SYBR green (Thermo Fischer, USA) following the manufacturer’s instructions. PCR cycling conditions were followed as: initially, DNA was denatured at 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The primers were synthesized to quantify the *SiPEPC* transcript level (F, 5′- CAC ACC TTG GCT TTC GTT CA-3’; R, 5′- ACA TGC CAA TAG TTT GTG GTC T-3′). Rice *tubulin* gene (F, 5′-GGA GTC ACA TGC TGC CTA AGG TT-3′; R, 5′-TCA CTG CCA GCT TAC GGA GG-3’; accession no. X78143) was used as a reference to normalize all data (Molla et al., 2016). The $^{\Delta\Delta}$CT method was used to determine quantitative variation among different samples (Livak and Schmittgen, 2001). The mean values for the expression levels of the genes were calculated from three independent experiments.

2.9. PEPC enzyme assay

Phosphoenolpyruvate carboxylase activity was measured following an earlier report (Ku et al., 1999). 100 mg of leaf samples were harvested from the fully expanded leaves and grounded with 1 ml of extraction buffer (50 mM Tris-HCl (pH 7.0), 1 mM EDTA, 10 mM $MgCl_2$, 5 mM dithiothreitol, 5% insoluble PVP, and 10% glycerol) and centrifuged at 13000×*g* for 10 min. The supernatant was used for protein estimation and PEPC assay. The enzyme assay was carried out spectrophotometrically at room temperature. The assay mixture contains 50 mM HEPES-KOH (pH-8), 5 mM $MgCl_2$, 10 mM $NaHCO_3$, 3 units of NAD-MDH, 0.2 mM NADH and 100 μl of enzyme extract. The reaction was initiated by adding 2 mM PEP and OD was measured at 340 nm for 120 s. The activity of PEPC was expressed as μmol/min/mg.

2.10. Photosynthetic pigment estimation

Chlorophyll content of leaves of transgenic, control and vector control plants were estimated according to an earlier described protocol (Arnon, 1949). 25 mg leaf samples (fully matured leaf) were dipped in 10 ml of 80% acetone and incubated in dark at 4 °C for 48 h. Then the absorbance was taken at different wavelength (480, 510, 645 and 663 nm) in the UV–visible spectrophotometer (Thermo Scientific, USA). Total chlorophyll, chlorophyll ‘a’, chlorophyll ‘b’ and carotenoid content from the 80% acetone extract was quantified by following formulae:

$$\text{Total chlorophyll (μg/ml)} = \{20.2(A_{645}) + 8.02\ (A_{663})\}\ \{V/1000) \times W\}$$

$$\text{Chlorophyll } a \text{ (μg/ml)} = \{12.7\ (A_{663}) - 2.69\ (A_{645})\}\ \{V/1000) \times W\}$$

$$\text{Chlorophyll } b \text{ (μg/ml)} = \{22.9\ (A_{645}) - 4.68\ (A_{663})\}\ \{V/1000) \times W\}$$

$$\text{Carotenoid} = \{7\ (A_{480}) - 1.47\ (A_{510})\}\ \{V/1000) \times W\}$$

Where A_{480}, A_{510}, A_{645}, A_{663} are the solution absorbance at 480, 510, 645 and 663 nm, respectively.

2.11. Gas exchange measurements

Gas exchange parameters were measured in the fully expanded first leaf at 50% flowering stage using a portable Infrared Gas Analyzer (IRGA) (LI-COR 6400 XT portable photosynthetic system; Lincoln, NE) under an imposed light intensity (PAR of 1200 μmol m^{-2} s^{-1}), at an ambient CO_2 concentration of 400 μmol mol^{-1} and a chamber temperature of 25 °C at a flow rate 500 μmol. The parameters like net photosynthetic rate (*A*), transpiration rate (*T*), stomatal conductance (g_s), and instantaneous water use efficiency (iWUE) were calculated. In addition, we measured light response curves and CO_2 response curves following the IRGA manual.

2.12. Photosynthesis at different light intensities

Photosynthetic responses of attached fully expanded flag leaves to different light intensities were measured by IRGA (LICOR 6400 XT) at

ambient CO_2 (400 μmol mol^{-1}). Leaf photosynthesis was measured with a portable photosynthetic system between 9 a.m. and 11 a.m. in controlled environmental conditions. During the measurement, block temperature was maintained at 30 °C with a constant flow rate of 500 $\mu mols^{-1}$. For photosynthetic light response of plants, net CO_2 assimilation was measured for 2 min at 0, 25, 50, 75, 100, 200, 400, 600, 800, 1000, 1200, 1500, 2000 μmol $m^{-2}s^{-1}$ of photon. Data were taken at 50% flowering stage (90–92 days old plants).

2.13. Photosynthesis at different concentrations of carbon dioxide

Net photosynthesis (*A*) was measured from the uppermost, fully expanded leaf of transgenic and control plants over a range of CO_2 concentrations. Measurements were carried out after leaves were equilibrated at 400 μmol of CO_2, the flow rate at 500 $\mu mols^{-1}$, leaf temperature at 30 °C, and irradiance at 1200 μmol photons $m^{-2}s^{-1}$. CO_2 response curves were measured in a stepwise increase in CO_2 partial pressure at an interval of 180 s. Data were taken at 50% flowering stage (90–92 days old plants).

2.14. Chlorophyll fluorescence measurement

We used an LI-6400XT with an integrated leaf chamber fluorometer (LCF) (LI-6400-40; LI-COR, Inc., Lincoln, NE, USA), to measure leaf-based chlorophyll fluorescence. The actinic light of 1200 $\mu molm^{-2}\ s^{-1}$ was used in the measurements. The rapid and non-destructive leaf chlorophyll fluorescence was conducted on the fully expanded first leaf from the top. The leaf was dark-adapted for 20 min before measurement (Demmig et al., 1987). After the dark-adapted leaves were transferred to light, the maximum quantum efficiency (Fv/Fm) of PSII was measured, where Fm is the maximum fluorescence, and Fv is variable fluorescence (Schreiber and Berry, 1977). The actual photochemical efficiency of photosystem II was calculated as ΦPSII which is used to calculate ETR (electron transport rate). ETR was calculated following the formula: ETR = PPFD*ΦPSII*0.5*0.84, where PPFD is photosynthetic photon flux density. 0.5 was used as the fraction of excitation energy distributed to PSII and 0.84 is the fraction of light absorption by the leaf. Additionally, photochemical quenching (qP) and non-photochemical quenching (NPQ) were also measured.

2.15. Total sugar and nitrogen content

For estimation of total sugar, dried leaf samples were collected before anthesis and during the harvesting stage, digested with an acid and then assayed spectrophotometrically by using the anthrone reagent method (Hedge, J.E. and Hofreiter, 1962). Dried leaf samples (0.5 gm) of control and transgenic lines before anthesis were used to determine the total nitrogen content by the micro-Kjeldahl method (Kjeldahl, 1883).

2.16. Agronomic evaluation of transgenic plants

At the 50% flowering stage, the plant height of the control, vector control, and transformed plants were measured. After maturity (118 days-old-plant), plants were harvested and yield attributing parameters such as tillers/plant, panicle weight, fertility percent, yield/plant, and dry biomass were recorded.

2.17. Statistical analysis

The data were analyzed using Graphpad prism 9.0 software, USA. One-way and two-way analyses of variance (ANOVAs) and Dunnett's Multiple Comparison Test were used to compare the differences between the non-transgenic control and the transgenic plants. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Isolation of C_4 specific PEPC from Setaria italica

We have amplified PEPC cDNA from *Setaria italica* (GS-1384) by RT PCR. The sequencing result revealed that the length of the open reading frame is 2895 bp. The sequence information of PEPC from *Setaria italica* was deposited in NCBI Genbank with the accession number: MF967570. C_4-specific PEPC has increased kinetic efficiency and reduced sensitivity to feedback inhibitors (e.g. malate) compared to C_3 PEPC. An earlier study showed that a single amino acid difference accounts for increased kinetic efficiency (Ala773Ser) and reduced inhibitor sensitivity (Arg885Gly) in C_4 PEPC (Paulus et al., 2013). We have performed a multiple sequence alignment of C_3 and C_4 determining region of PEPC sequence from different C_3 and C_4 species., including the sequence of the PEPC isolated in this study. *Sorghum bicolor* has six PEPC genes, of which *SbPEPC1* is C_4 type and *SbPEPC2–5* are C_3-type PEPCs (de la Osa et al., 2022). Here, we included SbPEPC3 in our analysis as a C_3-type PEPC. We have observed the isolated SiPEPC has C_4-specific serine at 773 position, while the 884 region has glutamine instead of C_4-specific Gly (Fig. 1). We have also compared the sequence obtained in our study with two earlier published high-quality genome sequences from Yugu1 (Bennetzen et al., 2012) and *xiaomi* (Yang et al., 2020). The comparison revealed 99.79% identities (962/964) with two amino acid mismatches (H455R and A664V) between GS-1384 and Yugu1/*xiaomi*.

3.2. Generation of SiPEPC-transgenic rice lines

The constructed cassette *ZmPPDK-SiPEPC-Nos* was abbreviated as PPN [Fig. 2a]. The cassette was introduced into embryogenic calli of rice variety IR64 using the *Agrobacterium* mediated transformation method. Hygromycin-resistant putative transgenic plants for PEPC were screened by *HPT* and *SiPEPC* screening primer [Fig. 2b and c]. Expected bands were observed in the lane corresponding to transgenic plants, whereas no band was seen in the wild-type (WT) control plant at the specific position. To verify the stable integration of transgene, PCR-positive plants were selected for Southern hybridization. The hybridization profile showed positive signals in transgenic PEPC lines 2, 4, 7, 8, [Fig. 2d]. No bands were detected in WT control plants. For PEPC lines, P4, P7, and P8 showed integration of two transgene copies and P2 exhibited one copy integration.

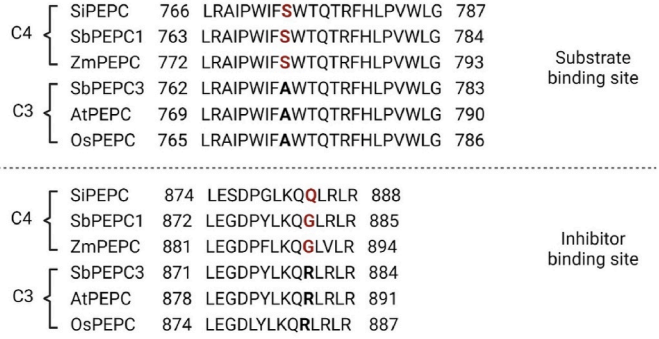

Fig. 1. Multiple sequence alignment of different C_3 and C_4 isoforms of PEPC. C_3/C_4 determining regions are shown here. Amino acids that determine C_3/C_4 specific function are highlighted in bold. In the substrate-binding site, Ala773 (*Oryza sativa* numbering) governs C_3 specificity, whereas Ser773 determines increased PEP saturation kinetics in C_4 PEPC. In the inhibitory site, Arg883 (*Oryza sativa* numbering) controls malate binding in C_3 PEPC, while Gly883 in C_4 PEPC mediates increased tolerance to feedback inhibitors like malate and aspartate. However, SiPEPC harbours a Gln instead of Gly. SbPEPC1 (*Sorghum bicolor* P15804), $SbPEPC_3$ (*Sorghum bicolor* XP_002451855) ZmPEPC (*Zea mays* P04711), OsPEPC (*Oryza sativa* subsp. indica Q84XH0), AtPEPC (*Arabidopsis thaliana* Q84VW9), and SiPEPC (*Setaria italica* AWD90035.1; this study).

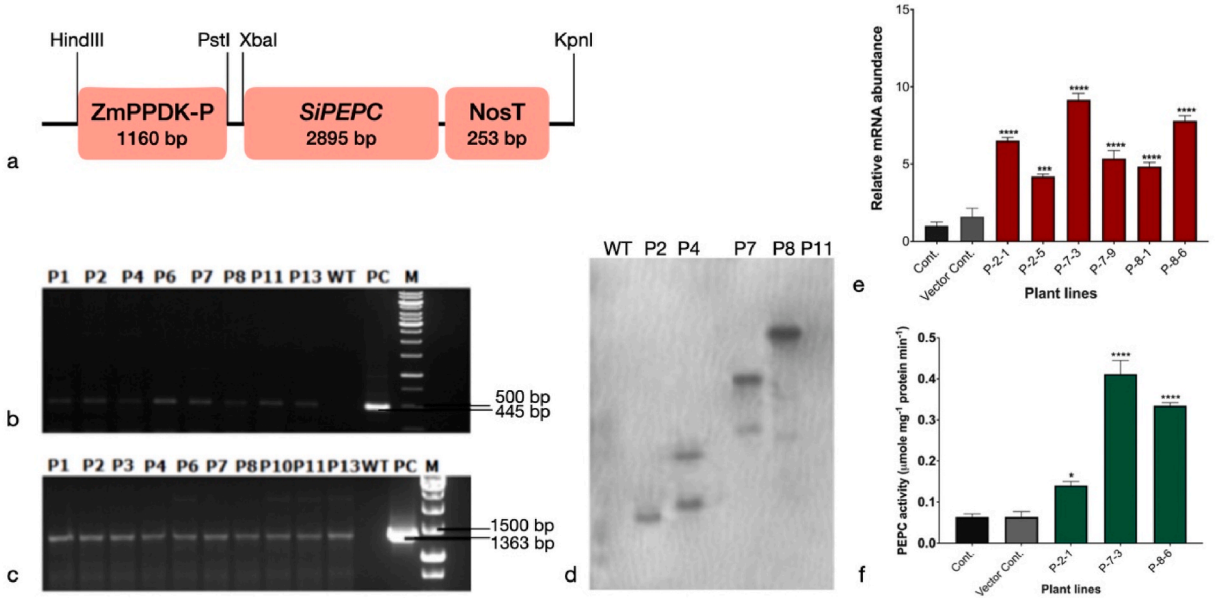

Fig. 2. Molecular characterization of transgenic plants. (a) Schematic diagram of the gene construct PPN (P_{ZmPPDK}-*SiPEPC-nos*) (b) Agarose gel image showing PCR products amplified from putative transgenic plants for HPT gene-specific PCR (445 bp) from putative PPN transgenic plants (c) Partial *SiPEPC* gene-specific PCR (1363bp) from putative PPN transgenic plants. PC- positive control, WT-wild type (negative control), M- 1 kb DNA marker (d) Southern hybridization analysis of transgenic PEPC plants. Genomic DNA was digested with *SalI* and hybridized with a 1.1 kbp *HPT* gene fragment probe. WT represents wild-type control (e) Quantitative real-time PCR analysis of transgenic and control plants. The relative quantity of *SiPEPC* mRNA in leaves of transgenic plants. Results are the mean ± SE of three independent biological replicates (f) PEP carboxylase enzyme activity was measured with respect to change in NADH in control and transgenic lines. Level of significance is denoted by *($P < 0.05$), ** ($P < 0.01$) *** ($P < 0.001$) and **** ($P < 0.0001$). Each data point is the average of three replicates ± SE.

3.3. Elevated expression of SiPEPC and increased PEPC enzyme activity in transgenic rice lines

Quantitative real-time PCR (qRT-PCR) was used to study the expression of *SiPEPC*. The result showed that *SiPEPC* was expressed at the mRNA level in all transgenic plants to a varying degree (Fig. 2e). The expression of *SiPEPC* transgene was significantly higher ($P < 0.0001$) in all transformed lines compared to wild-type. As compared to the control plant, maximum expression of *SiPEPC* was observed in P-7-3 (9.1-fold) followed by P-8-6 (7.8-fold) and P-2-1 (6.5-fold) transgenic lines, whereas P-2-5 line showed relatively lower expression (2.06-fold of control).

PEPC enzyme activity was measured from total protein isolated from the leaf of transgenic and control plants. The expression of transgene led to an increment in PEPC activity in three transgenic lines. The activity of the PEPC enzyme was found to increase 2.2–6.4-fold higher in transgenic lines as compared to control. The increment in PEPC activity was statistically significantly ($P = 0.03$, $P < 0.0001$) (Fig. 2f).

3.4. Analysis of different physiological parameters in transgenic rice lines

3.4.1. Photosynthetic pigment content

Based on higher expression of transgene and enzyme activities, PPN lines (P-2-1, P-7-3, and P-8-6) were selected for physiological studies.

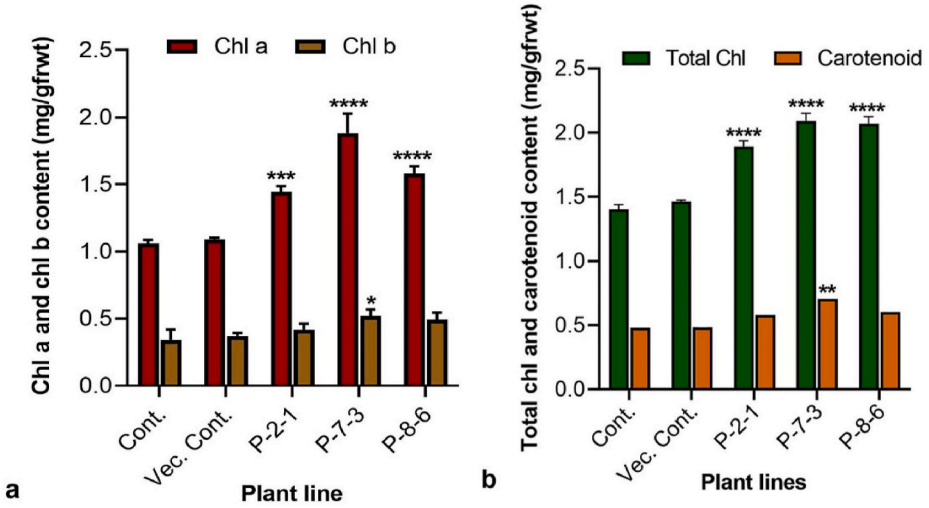

Fig. 3. Variation in chlorophyll *a*, *b*, total chlorophyll, and carotenoid accumulation in transgenic and control plants. (a) Chlorophyll *a* and *b* accumulation; (b) total chlorophyll and carotenoid accumulation at the flowering stage. **** ($P < 0.0001$), *** ($P < 0.001$) * ($P < 0.05$) and * ($P < 0.05$). Each bar represents three independent replicates ± SE.

Variation in chlorophyll *a*, *b*, total chlorophyll and carotenoid content was recorded at the flowering stage. A significant difference in pigment content was not detected in the control and vector control. Chlorophyll-a contents of PPN transgenic lines were significantly higher with respect to control plant ($P < 0.001$). PPN lines exhibited 36.3–73% more chlorophyll-a content than that of control plants (Fig. 3a). Although chlorophyll-b content increased slightly in transgenic lines, the difference was not significant compared to control except for P-7-3 lines ($P = 0.02$) (Fig. 3a). On the other hand, control and transgenic lines had similar carotenoid content with no significant difference (Fig. 3b). Interestingly, total chlorophyll content in PPN lines (1.35–1.7 times) was higher than in control. The difference between the control and all the transgenic lines was statistically significant ($P < 0.0001$).

3.4.2. *Chlorophyll fluorescence analysis of transgenic lines*

The chlorophyll fluorescence measurements are used as the indicator of the photosynthetic activity of a plant. Observations were taken on Fv/Fm, photochemical quenching coefficient (qP), electron transport rate (ETR), the effective quantum yield of photosystem II (ΦPSII), and non-photochemical quenching (NPQ). Fig. 4 showed that the values of qP, ETR, and ΦPSII in transgenic rice lines were higher than those in the control plants. Dark-adapted values of Fv/Fm estimate the quantum efficiency of PSII. Data showed that Fv/Fm did not change much in transgenic lines as compared to controls (Fig. 4a). The fluorescence parameter, qP measures the photochemistry of PSII. qP value of transgenic lines was higher than control, but the difference was not statistically significant (Fig. 4b). Compared to the control, ETR increased significantly ($P = 0.0193$, $P = 0.00109$ and $P = 0.0066$ by 25.4%, 36%

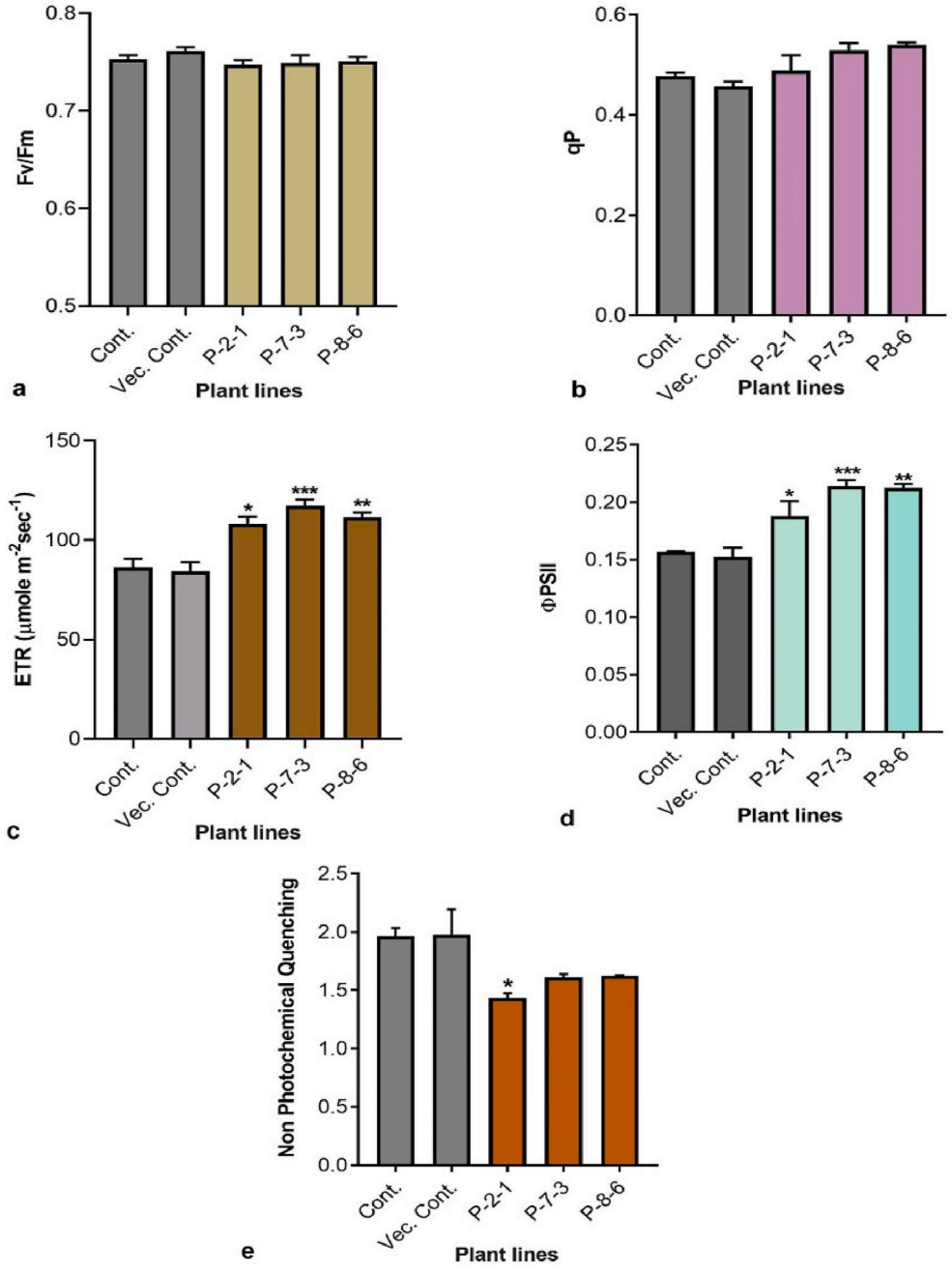

Fig. 4. Chlorophyll fluorescence measurements of transgenic and control rice plant at flowering stage. (a) Fv/Fm ratio (b) photochemical quenching (qP) (c) electron transport rate (d) quantum efficiency of PSII (ΦPSII) (e) non-photochemical quenching of chlorophyll. Level of significance is denoted by *($P < 0.05$), ** ($P < 0.01$) *** ($P < 0.001$) and **** ($P < 0.0001$). Each bar represents mean ± SE of three independent biological replicates.

and 29.2% in P-2-1, P-7-3 and P-8-6 lines, respectively) (Fig. 4c). The ΦPSII was significantly higher in all transgenic lines than in control and vector control (Fig. 4d). The ΦPSII value was highest in P-7-3 among the *SiPEPC* expressing lines. The increase in quantum yield efficiency of PSII in PPN transgenics ranged from 19.8 to 36.3%. There was no significant difference observed in NPQ among transgenic and control/vector control though the value slightly decreased in transgenic lines (Fig. 4e). Only the PEPC line P-2-1 had significantly lower NPQ ($P = 0.0143$) than the control. NPQ was decreased by 17.2–18% in PEPC transformed lines than in control.

3.4.3. Transgenic rice lines exhibited increased photosynthesis

Data for photosynthesis rate (*A*), stomatal conductance (g_s) transpiration rate (*E*) and instantaneous water use efficiency (iWUE) were recorded by the Portable Photosynthesis System (LI-6400XT, LICOR, USA). Based on the data from IRGA, the photosynthesis rate was significantly higher in PPN transgenic lines, where net photosynthesis increased by 15.9%, 20.06%, and 21.9% in P-2-1, P-7-3, and P-8-6 lines ($P < 0.0001$), respectively (Fig. 5a). Differences in stomatal conductance (Fig. 5b) and transpiration rate (Fig. 5c) between transgenics and control were statistically insignificant. Instantaneous water use efficiency (iWUE) (Photosynthesis/transpiration) was calculated from the data obtained during the observation of photosynthesis and related parameters. PPN rice lines P-7-3 and P-8-6 exhibited higher iWUE than control plants. Fig. 5d showed that the water use efficiency was increased by 15.9–21.9% in PPN transgenic rice lines. The difference between control and PEPC transgenic lines (P-7-3 and P-8-6) was statistically significant ($P = 0.0065$ and $P = 0.0037$).

3.4.4. Increment in quantum yield of PEPC transgenic lines highlights positive co-relation with photosynthesis

Photosynthetic response to different light intensities was measured by IRGA (LI-6400XT). Data revealed that the photosynthesis rate increased in *SiPEPC* transgenic with increasing light intensity (Fig. 6a). The Quantum yield of transgenic PEPC lines was more (18%) than the control plants. Light compensation points in vector control and non-transformed control plant were at ~24 μmole photons $m^{-2}s^{-1}$, which reduced to 21 μmole photons $m^{-2}s^{-1}$ in the *SiPEPC* line [Supplementary Fig. S1a]. Compared to the control, transgenic PEPC exhibited a higher rate of photosynthesis (24–27%) at high light intensity (1600–2000 μmol $m^{-2}s^{-1}$).

The CO_2 Response curve of control and transgenic plants revealed differences in photosynthetic response at different CO_2 concentrations (Fig. 6b). Carboxylation efficiency of PPN lines was 7.23% higher than

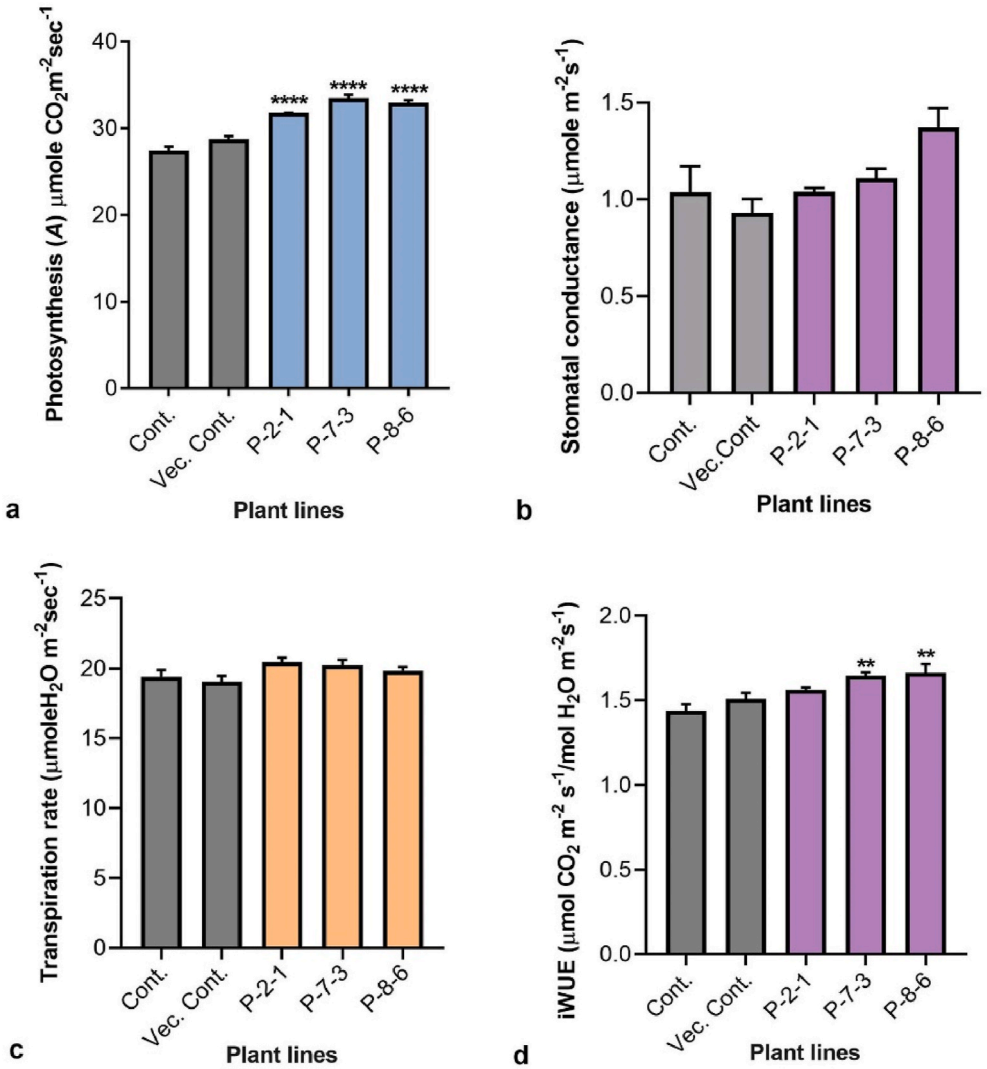

Fig. 5. Photosynthetic behaviour of transgenic plants at flowering stage. (a) Net photosynthetic rate (b) stomatal conductance (c) transpiration rate (d) intrinsic water use efficiency. Level of significance is denoted by *($P < 0.05$), ** ($P < 0.01$) *** ($P < 0.001$) and **** ($P < 0.0001$). Each bar represents mean of three independent replicates ± SE.

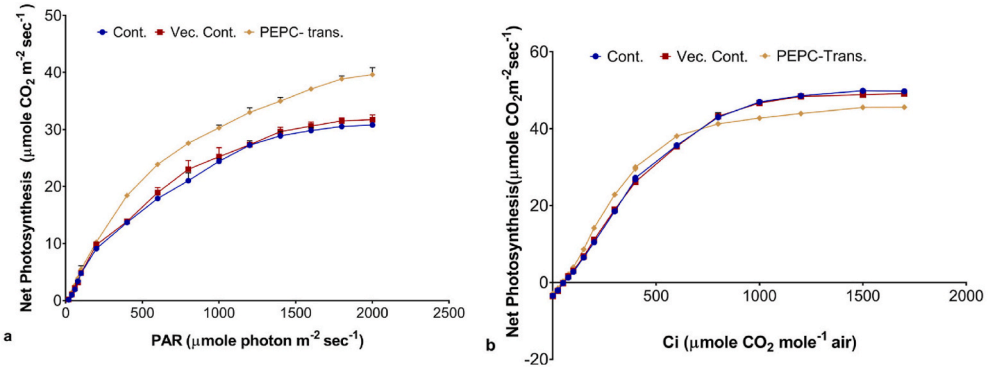

Fig. 6. Light and CO_2 response curve. (a) Photosynthesis light response curve. The rate of photosynthesis was measured in transgenic, control, and vector control plants with respect to different light intensities in ambient CO_2 (b) The rate of photosynthesis was measured at different CO_2 concentrations at a constant light intensity of 1200 µmol m^{-2} s^{-1}. Each data point is an average of three replicates ± SE.

control plants. The compensation point was found to be reduced to 44 ppm in PPN from 53 ppm in control (Supplementary Fig. S1b). The ratio of quantum efficiency of PSII (ΦPSII) and quantum yield of CO_2 assimilation (ΦCO_2) was found to be reduced in transgenic lines as compared to non-transformed control plants (Supplementary Fig. S3). The ratio is normally decreased when the carboxylation of Rubisco is higher than the oxygenation (Häusler et al., 1999). Transgenic lines had a higher photosynthesis rate at lower pCO_2 (<800) than non-transformed control and vector control. Net photosynthetic rate showed an increment in all plants in response to increasing CO_2 concentration up to 1200 µmole and then plateaued.

3.5. *Transgenic leaves showed an increment in sugar and nitrogen content*

A significant difference was noted in total soluble sugar (TSS) content between transgenic and control plant leaves. At the vegetative stage, TSS was found to be significantly increased in transgenic leaves compared to non-transgenics (Fig. 7a). However, at maturity, the total sugar content in flag leaves of transgenic lines was reduced significantly than those of control. The reduction was found to be 15.5–52.8% in PPN

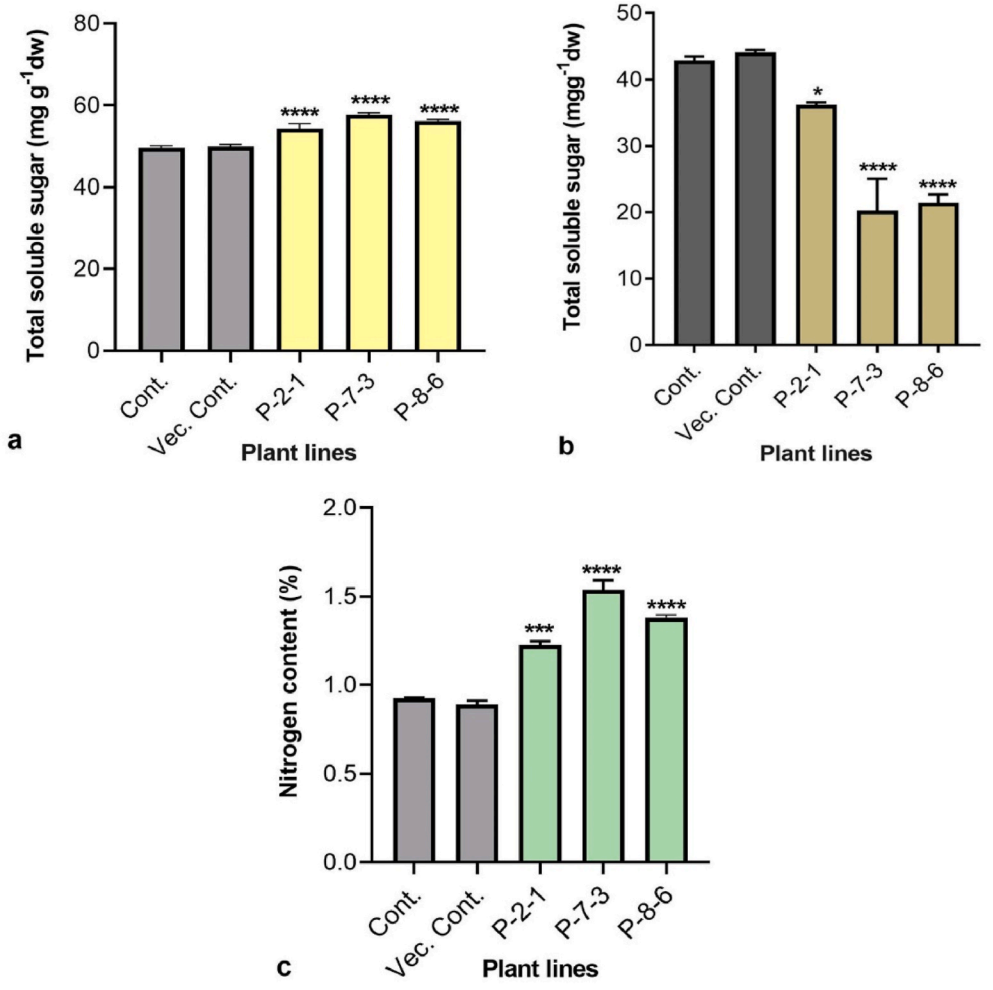

Fig. 7. Sugar and nitrogen content in transgenic leaves. (a) Total Soluble sugar (TSS) content in leaves of transgenic and control plants before anthesis. Each bar represents mean ± SE of three independent biological replicates. (b) TSS content of leaves at harvesting stage (c) Total nitrogen contents in the leaves of transgenic and control plants before anthesis. Each bar represents the mean of three replicates ± SEM. Asterisks indicate a significant difference between control and transgenic lines. Level of significance is denoted by *($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$) and **** ($P < 0.0001$).

rice lines (Fig. 7b). The lowest reduction was observed in P-2-1 ($P = 0.0148$). The TSS reduction at maturity might be due to sugar remobilization in the grain. Total nitrogen content in the leaves of PPN lines increased by 33–66% at the vegetative stage (Fig. 7c). The line P-7-3 showed the highest increment in N_2 content among the plant lines analyzed.

3.6. Changes in yield attributes and yield

Significant changes in plant height, tiller number, panicle weight, fertility %, yield/plant, and dry biomass were observed in PEPC transgenic lines (Fig. 8). Significant differences were observed between control and transformed rice lines for most of the traits studied. These results highlighted that the integration of the *SiPEPC* gene is likely to have an influence in enhancing the yield and biomass in transgenic lines (Fig. 9).

4. Discussion

Enhancement of photosynthesis in changing climate is crucial to increase the yield potential of rice. C_4 plants are superior to C_3 plants in photosynthetic efficiency, as well as N_2 and water use efficiency. Bioengineering C_4 photosynthetic traits into C_3 crop rice is one of the attractive strategies to reduce photorespiration and increase the radiation use efficiency to enhance photosynthesis and yield in changing climate (Shen et al., 2019). Improvement in photosynthesis was achieved in transgenic rice plants expressing C_4 photosynthetic genes (Karki et al., 2013; Tang et al., 2018; Ermakova et al., 2020; Swain et al., 2021). Installation of C_4 PEPC enzyme in transgenic rice showed enhancement in yield potential under high light (Ku et al., 2000; Zhang et al., 2009; Ding et al., 2013). Various modifications are required to install C_4-like characters in C_3 plants such as engineering leaf anatomy and vein density leading to photosynthetic pigment accumulation in the proximity of bundle sheath cells. Integration of a two-cell C_4 metabolic pathway into C_3 rice leaves may lead to an increase in photosynthetic efficiency under the current ambient CO_2 concentration (Wang et al., 2017). The establishment of appropriate leaf anatomy in addition to C_4 gene expression is important for the functioning of the C_4 pathway in rice.

Phosphoenolpyruvate carboxylase (PEPC) is one of the key enzymes of the C_4 pathway that acts at the beginning of the C_4 cycle. CA converts atmospheric CO_2 to HCO_3^-. Subsequently, PEPC carboxylates phosphoenolpyruvate (PEP) utilizing the HCO_3^- produced by CA. In this study, we have made an effort to explore the effectivity of the C_4-specific *Setaria italica PEPC* gene in enhancing photosynthetic efficiency in transgenic rice lines. We have isolated C_4-specific *PEPC* from *S. italica* GS-1384 and sequenced it to confirm its C_4-specificity. A comparative analysis revealed that the isolated *SiPEPC* gene is C_4 specific, as it has the codon for serine773, unlike C_3-specific alanine (Fig. 1). We also compared the GS1384 PEPC sequence with the available high-quality sequence of *S. italica* cv. Yugu1 and a recently developed mutant *xiaomi* (Yang et al., 2020). We have found a high level of similarities with the C_4-specific PEPC of *xiaomi* and Yugu1 except for two amino acids: H455R and A664V. Those mutations are less likely to have a significant impact, as histidine and arginine are both with positively charged R groups, while alanine and valine are both nonpolar and with an aliphatic R group.

For generating transgenic lines, we constructed the transformation cassette, where PEPC was cloned downstream of the green tissue-specific *ZmPPDK* promoter (Fig. 2a). Here, we used the *ZmPPDK* promoter for PEPC expression as it was earlier reported that constitutive expression of PEPC has a negative impact on plant development, resulting in retarded growth (Rademacher et al., 2002; Chen et al., 2004). The *SiPEPC* expression cassette was abbreviated as PPN.

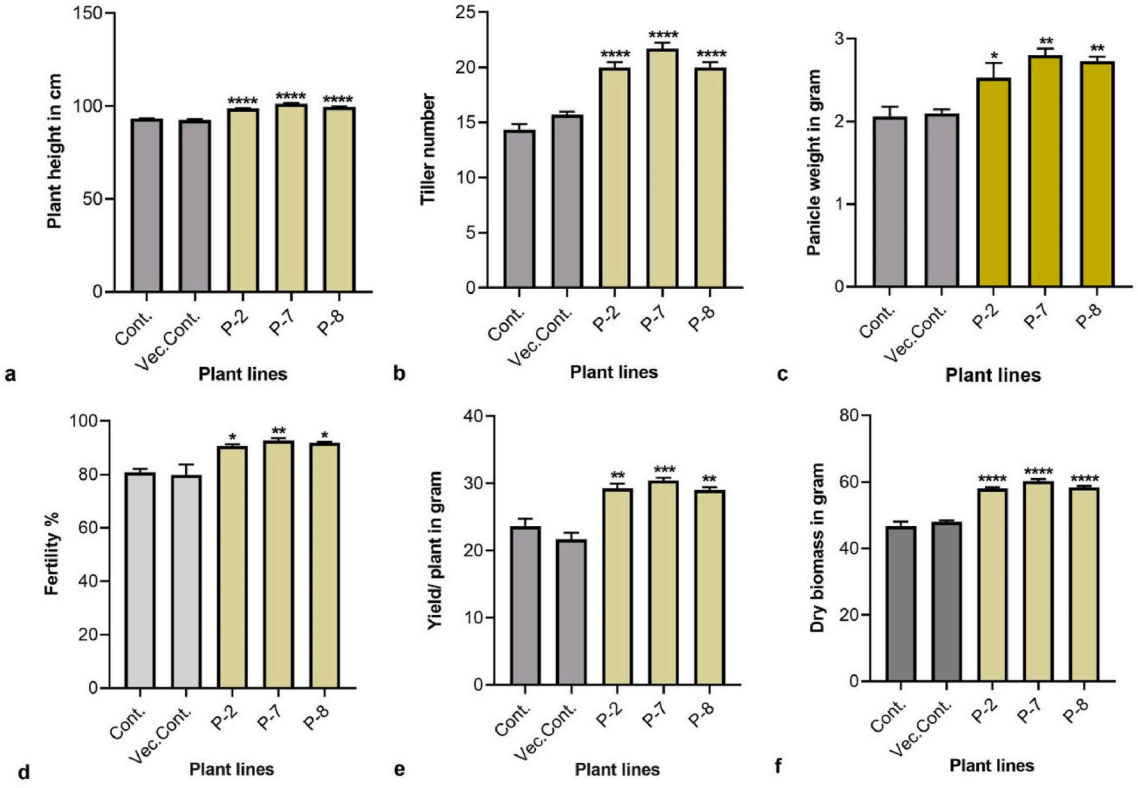

Fig. 8. Agronomical parameters of transgenic plant lines carrying *Setaria italica PEPC* gene (a) Average plant height of transgenic plant lines (b) Average tiller number of transgenic plant lines grown in a greenhouse (c) Average panicle weight (d) Fertility % of transgenic plant lines (e) Total grain yield of plant lines (f) Dry biomass of plant lines. All observations were recorded in three biological replicates. Asterisks indicate the significant difference among plant lines. *($p < 0.05$), **($p < 0.001$), ***($p < 0.0001$), and ****($p < 0.00001$).

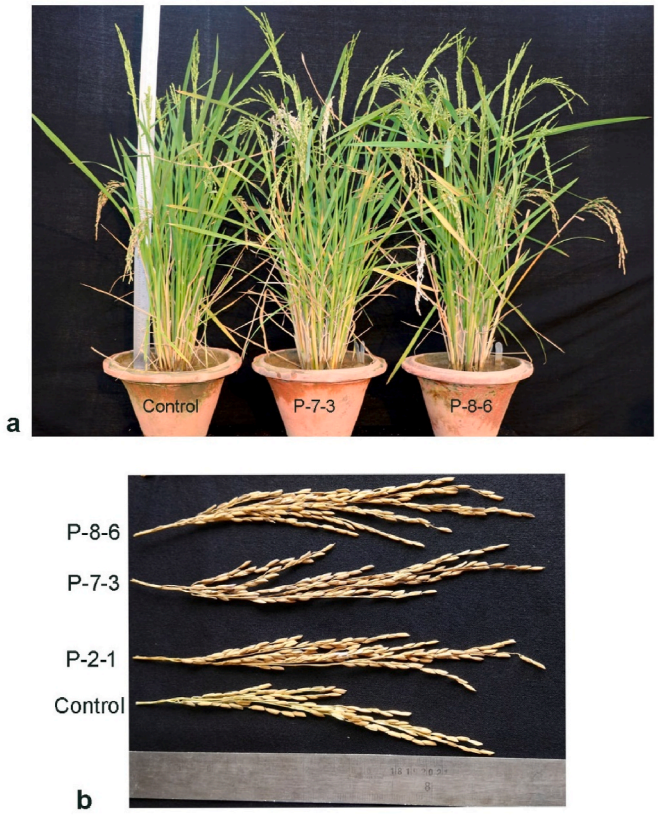

Fig. 9. Representative morphological variations in control (WT) and transgenic plants. (a) The appearance of transgenic (P-7-3 and P-8-6) and wild-type plants after 90 days of germination (b) Representative panicles from WT and transgenic plants.

Hygromycin-selected putative transformants were subjected to PCR screening (Fig. 2b and c). Southern hybridization of PCR-positive plants revealed stable integration of transgene cassette (Fig. 2d). When the PPN expressing rice lines were evaluated by quantitative real-time PCR, they all showed variable levels of increased mRNA expression. PEPC expression ranged from 6.5 to 9.1 in PPN lines (Fig. 2e). The level of expression varies among the transgenic lines due to the distinct insertion sites and other related factors (Molla et al., 2016). Transgenic lines overproduced the enzyme PEPC as evidenced by the activity analysis (Fig. 2f). The PEPC enzyme activity was elevated by 2.2–6.4-fold in PPN lines, with respect to control plants. PEPC enzyme activity measured here is a cumulative activity of the endogenous protein in wild-type rice and the transgene encoded protein. It was not possible to distinguish between the two types of activities. Similar to our result, several reports with *ZmPEPC* transgenic rice narrated elevated enzyme activity (Suzuki et al., 2006; Lian et al., 2014; Chen et al., 2017; Ermakova et al., 2021). Three lines from PPN (P-2-1, P-7-3, and P-8-6) transformants that showed higher enzyme activity was selected for further downstream physiological investigations.

In our experiment, PPN rice plants showed significant accretion in total chlorophyll and chlorophyll *a* in comparison to control and vector control plants (Fig. 3a and b). This finding is dissimilar from that of earlier studies (Xia and Cao, 2013; Ding et al., 2015). They showed that the chlorophyll content did not change in PEPC overexpressing rice lines under control conditions but it was higher under stress conditions. The increment in chlorophyll content had a statistical correlation with that of photosynthetic rate (Supplementary Fig. S2b). The rise in chlorophyll content is probably due to the increasing leaf area in transgenic plants with respect to control (Supplementary Fig. S4).

The chlorophyll fluorescence is a highly sensitive and non-destructive way to illustrate photosynthesis (Govindjee, 2004). It also gives an idea about the capability of PSII to use the absorbed light energy by chlorophyll (Maxwell and Johnson, 2000). Integration of transgene can affect the electron transport chain by increasing the electron transport rate (ETR) and quantum efficiency of PSII (Φ_{PSII}) (Genty et al., 1989). Earlier studies were reported no difference in qP, ΦPSII, and NPQ values between the control and the transformed PEPC rice lines in control conditions (Ding et al., 2013, 2015). Our finding is contrary to those of previous studies. We observed that qP, ETR and ΦPSII were higher in *SiPEPC*-lines than in the control plants (Fig. 4b, c, and 4d). The observed difference between our study and the studies by Ding et al. (2013) and Ding et al. (2015) could be due to the use of Indica and Japonica cultivars, respectively. A higher electron transport rate coupled with increased quantum yield elevated the photosynthesis rate in transgenic rice plants. Transgenic rice lines had NPQ lower than the control (Fig. 4e), suggesting that PSII of transgenic plants utilizes light energy more efficiently, and a lesser amount of absorbed light is dissipated as heat than control. This NPQ data is well supported by other data. For example, total chlorophyll and carotenoid contents were found to be significantly increased and unchanged, respectively, in transgenic plants compared to control. Chlorophyll is directly involved in photochemical quenching, while carotenoid especially xanthophylls is responsible for NPQ. We also found a positive correlation between Φ_{PSII} and photosynthesis rate (Supplementary Fig. S2a). Photosynthesis rate increased significantly with increasing ΦPSII, supporting the notion that photosynthesis rate is closely associated with PSII efficiency. Higher expression of *SiPEPC* produces noticeable changes in the photosynthetic attribute of transgenic rice plants. Here, we observed that increased activity of SiPEPC improved the photosynthesis rate (*A*) of transgenic PPN lines (Fig. 5a). An earlier study reported an increase of about 18% in *A* in transgenic *Arabidopsis* with *ZmPEPC* gene in comparison to wild type (Kandoi et al., 2016). Similarly, *ZmPEPC* transgenic wheat showed 26% higher *A* than control (Hu et al., 2012). Indica rice expressing *ZmPEPC* showed an increment of *A* as compared to control (Bandyopadhyay et al., 2007). Likewise, another study was demonstrated 30% increase in PEPC-transgenic rice plants (Ku et al., 2000). On contrary, an earlier study revealed no enhancement of *A* in transgenic rice, though the *in vitro* PEPC activity was higher in the transgenics with respect to the control (Taniguchi et al., 2008). Results from these studies highlight the need for further investigation to understand why *in vitro* activity and *in vivo* activity of C_4 enzymes in transgenic rice is different. This might be due to the unavailability of adequate substrate in *in vivo* conditions.

As compared to the control plant, nitrogen accumulation was increased in the leaves of transgenic lines collected at pre-anthesis. This finding was concurrent with a previous study (Lian et al., 2014), where nitrogen accumulation increased significantly in the leaf of some of the transgenic rice lines expressing sugarcane *PEPC*. The results further support the finding of Fukayama et al. (2001), where an overproduction of C_4 PPDK induced nitrogen accumulation in japonica rice. Similarly, overexpression of sugarcane *PEPC* brings about significant changes in gene expression pattern, enzyme activity, metabolites, availability of phytohormones and nitrogen uptake at different transgenic rice lines (Lian et al., 2021). The report highlighted higher total nitrogen content in transgenic rice at different growth stages and under different nitrogen source concentrations.

C_4 species have higher radiation use efficiency (RUE) than C_3 plants (Ehleringer and Monson, 1993). For a C_4 plant, the light saturation point is higher than a C_3 plant. However, the light compensation point is lower in C_4 than in C_3 plants. Our investigation showed that photosynthesis in transgenic lines were saturated at higher light intensities as compared to the control and vector control (Fig. 6a), which indicates higher radiation use efficiency of transgenic lines. PPN lines showed lower light compensation points than the control plants.

The quantum yield of transgenic PEPC lines was more than that of control plants (Fig. 6b). PPN lines showed lower CO_2 compensation points than control plants (Supplementary Fig. S1b). It is evident from

the result that transgenic lines maintained a high photosynthetic rate when the availability of CO_2 is reduced. From the CO_2 response curve, *SiPEPC* transgenic lines performed a higher rate of photosynthesis at lower (50–400 μmol/mol) CO_2 than the control (Fig. 6b). However, at higher CO_2, the transgenic lines exhibited slightly reduced CO_2 assimilation than the controls (Fig. 6b). This result accords with the findings of an earlier study, in which expression of Z*mPEPC* enhanced the carboxylation and radiation use efficiency of transgenic wheat (Hu et al., 2012). PEPC transgenic rice had a higher (55%) light saturation photosynthesis rate and lower (27%) CO_2 compensation point than untransformed rice (Jiao et al., 2002). This finding is consistent with that of an earlier study (Zhang et al., 2003), where the overexpression of the Sorghum *PEPC* gene improved the carboxylation efficiency of transgenic rice by lowering the CO_2 compensation point.

We also examined whether the expression of transgene brought about any changes in the morphology of PPN lines. Indeed, the transgenic plants exhibited increment in plant height (Fig. 8a), tiller number (Fig. 8b), and panicle length (Fig. 9b). *SiPEPC* plants were taller than the control untransformed plant (Figs. 8a and 9a). Previous report suggested that the expression of maize *PEPC* increased height and leaf size (Sen et al., 2017). In our study, plant height and tiller number per plant increased by 6–8% and 39–46% in PEPC lines. According to Giuliani et al. (2019), overexpression of *ZmPEPC* has not consistently affected photosynthesis and plant growth of transgenic rice. It was thought that the expression of single C_4 gene is insufficient to boost the CO_2 assimilation rate in rice. Therefore, a quadruple line was developed with four C_4 genes from maize (i.e., *PEPC, NADP-MDH, NADP-ME and PPDK*). Additive expressions of these genes magnified the enzyme activity, still had no effect on CO_2 assimilation rate (Lin et al., 2020). In a similar way, when transgenic Kitaake plants were transformed with five transgenes, *ZmCA, ZmPEPC, ZmNADP-MDH, ZmPPDK and ZmNADP-ME* in a single construct (Ermakova et al., 2021), only *ZmPEPC* expressing rice lines that having higher malate and aspartate content showed more efficient CO_2 assimilation than WT. Superior phenotypic traits with increased photosynthetic rate and biomass production was reported in the transgenic rice lines expressing C_4-PPDK and NADP-ME from *Setaria italica* (Swain et al., 2021).

As compared to C_3 crops, C_4 crops have greater productivity because of the high rate of photosynthesis (Sales et al., 2021). Several reports suggested that the overproduction of maize PEPC enzyme increased net photosynthesis and yield in transgenic plants (Ku et al., 1999; Jiao et al., 2002; Bandyopadhyay et al., 2007). Similarly, tiller number, panicle number, total grain number/hill, and grain yield/plant were significantly higher in transgenic rice plants expressing maize C_4-*PEPC* gene (Tang et al., 2018). However, in rice, a correlation has not been found frequently between photosynthesis and yield (Takano and Tsunoda, 1971). We have observed yield enhancing attributes in *SiPEPC*-transgenic lines (Fig. 8). In our research, a positive correlation between photosynthesis and yield was observed (Supplementary Fig. S2c) which resembles the previous study that has also reported a positive relationship between yield and photosynthesis (Ambavaram et al., 2014). In rice, grain yield is influenced by three components: number of panicles per plant (associated with tiller number), number of filled grains per panicle (fertility %), and grain weight (Xing and Zhang, 2010). Yield potential of plant is determined by the efficiency of CO_2 assimilation in source and utilization of that assimilated carbon by the sink (grain) (McCormick et al., 2006; Yang and Zhang, 2010). During the grain filling stage, carbon as total soluble sugar (TSS) is transferred directly to the grain from the source leaf. *SiPEPC* rice lines showed a 24–29% increase in biomass over the control plant. The fertility in transgenic plants was 12–15% higher than in WT counterparts. The increase in fertility percentage evident in *SiPEPC* lines can be due to the efficient translocation of photosynthates into the grain. It has been reported that mobilization of photo-assimilates from source to sink is essential for high yield, provided the source and sink are not limiting (Asseng and van Herwaarden, 2003). If the sink is small and the source is large, the yield cannot be high. Similarly, if the source capacity is limited, the yield cannot be high; even though the sink is large. The sink is expanded by profuse tillering, and increased grain size and number. In this study, during harvest, leaf total soluble sugar content in transgenic was found less than that of control (Fig. 7b). A large portion of assimilated carbon is transported to panicles to produce more filled grains. Depletion of sugar content in flag leaves is because of the large sink size that uses source efficiently, likely ensuring strong source-sink interaction, leading to high grain yield per plant in transgenic lines. However, total soluble sugar (Fig. 7a) contents were significantly higher in the leaves of transgenic lines as compared to the control before anthesis. The panicle number was also increased (Supplementary Fig. S5), implying that both source and sink were more active in transgenic PEPC rice plants with respect to WT control. This indicates that an increase in photosynthesis in the source organ directly influences the yield of the plant. Our data showed that an increase in net photosynthesis (15.9–21.9%) increased the grain yield by 23–28.9% and biomass by 24.1–29% in transgenic PEPC lines as a result of the increased tiller and panicle number per plant than control. This result is consistent with the findings of Ding et al. (2013), where overexpression of C_4 *SiPEPC* in Japonica rice improved photosynthesis rate and yield under upland field cultivation. (Qihua et al., 2006) reported that during grain filling stages total soluble sugar mobilizes from the leaf as the main source of assimilation for grain yield in rice. As a result, grain yield and fertility percentage increased in transgenic rice lines compared to WT.

Taken together, results in this study suggest that the introduction of the C_4 photosynthesis enzyme SiPEPC into indica rice has the potential to improve photosynthetic capacity, water and radiation use efficiency, and ultimately productivity in transgenic rice. These findings will be of interest to improve the strategy to engineer a C_4 pathway in rice.

Authors contribution statement

MJB and KAM conceived the idea and designed the experiments. DB, AS, and KAM generated the constructs. DB and AS carried out the experiments and collected data. MD helped in collecting data. DB performed data analysis and prepared figures and tables. SK helped in southern hybridization. DB wrote the manuscript. KAM, SK, PS and MJB edited the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2022.11.011.

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