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ORIGINAL ARTICLE

Towards efficient photosynthesis: overexpression of *Zea mays* phosphoenolpyruvate carboxylase in *Arabidopsis thaliana*

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Abstract Plants with C4 photosynthesis are efficient in carbon assimilation and have an advantage over C3 photosynthesis. In C4 photosynthesis, the primary CO_2 fixation is catalyzed by phosphoenolpyruvate carboxylase (PEPC). Here, we show that overexpression of *Zea mays* PEPC cDNA, under the control of ^{35}S promoter, in *Arabidopsis thaliana* resulted in ~7–10 fold higher protein abundance and ~7–10 fold increase in PEPC activity in the transgenic lines than that in the vector control. We suggest that overexpression of PEPC played an anaplerotic role to increase the supply of 4-carbon carboxylic acids, which provided carbon skeletons for increased amino acid and protein synthesis. Higher protein content must have been responsible for increased metabolic processes including chlorophyll biosynthesis, photosynthesis, and respiration. Consequently, the PEPC-overexpressed transgenic plants had higher chlorophyll content, enhanced electron transport rate (ETR), lower non-photochemical quenching (NPQ) of chlorophyll *a* fluorescence, and a higher performance index (PI) than the vector control. Consistent with these observations, the rate of CO_2 assimilation, the starch content, and the dry weight of PEPC-overexpressed plants increased by 14–18 %, 10–18 %, and 6.5–16 %, respectively. Significantly, transgenics were tolerant to salt stress as they had increased ability to synthesize amino acids, including the osmolyte proline. NaCl (150 mM)-treated transgenic plants had higher variable to maximum Chl *a* fluorescence (F_v/F_m) ratio, higher PI, higher ETR, and lower NPQ than the salt-treated vector controls. These results suggest that expression of C4 photosynthesis enzyme(s) in a C3 plant can improve its photosynthetic capacity with enhanced tolerance to salinity stress.

Keywords *Arabidopsis thaliana* · C4 photosynthesis · Chl *a* fluorescence · CO_2 assimilation · Phosphoenolpyruvate carboxylase · Salt stress

Abbreviations

APT	Adenine phosphoribosyl transferase
CaMV	Cauliflower mosaic virus
Chl	Chlorophyll
EDTA	Ethylenediaminetetraacetic acid
ETR	Electron transport rate, see text for details
F_m	Maximum Chl fluorescence

The authors honor George C. Papageourgiou, who was a good friend of Late Prasanna Mohanty, B. C. Tripathy's mentor; both Papageorgiou and Mohanty were Ph.D. students of one of us (Govindjee).

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F_o	Minimum Chl fluorescence
F_v	Variable fluorescence, $F_m - F_o$
gs	Stomatal conductance
LED	Light-emitting diode
MDA	Malondialdehyde, $CH_2(CHO)_2$
MDH	Malate dehydrogenase
ME	Malic enzyme
MS	Murashige and Skoog medium
NPQ	Non-photochemical quenching (of Chl fluorescence)
nptII	Neomycin phosphotransferase (kanamycin resistance gene)
OAA	Oxaloacetic acid
PAR	Photosynthetically active radiation
PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate carboxylase
PFD	Photon flux density
PI	Performance index, see text for details
PMSF	Phenylmethylsulfonyl fluoride
PSI	Photosystem I
PSII	Photosystem II
PVP	Polyvinylpyrrolidone
qE	Energy-dependent quenching of the excited state of Chl *a*
qI	Photoinhibitory quenching of the excited state of Chl *a*
qT	State transition quenching of the excited state of Chl *a*
RC/ABS	Density of reaction centers per PSII antenna chlorophyll (see text for details)
RC	Reaction center
ROS	Reactive oxygen species
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
TAE	Tris-base, acetic acid and EDTA
TEM	Transmission electron microscopy
VC	Vector control
WT	Wild type
WUE	Water use efficiency

Introduction

Oxygenic photosynthesis is the conversion of light energy into chemical energy on a massive scale: water is oxidized to molecular oxygen and CO_2 is reduced to organic matter (see Blankenship 2014; Eaton-Rye et al. 2012). Life on our planet depends on photosynthesis. For our own food, we depend on many crop plants, and several of them, e.g., wheat and rice, assimilate atmospheric CO_2 by the C3 pathway (Calvin–Benson cycle) of photosynthesis. Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is the primary carboxylation enzyme of C3 plants. However, O_2 promotes oxygenase reaction of RuBisCO leading to photorespiration that reduces net carbon assimilation and thus productivity of C3 plants, often by 30–40 % (Ehleringer et al. 1991). Thus, under ambient CO_2, C3 plants have reduced photosynthetic efficiency. In contrast, C4 plants (e.g., maize, sorghum, sugarcane) have evolved a "CO_2 pump," the Hatch & Slack pathway of photosynthesis, to concentrate atmospheric CO_2, thus overcoming photorespiration. As a consequence, C4 plants have higher rates of photosynthesis, faster growth, and increased water use efficiency (Long 1999). A specialized leaf anatomy, composed of mesophyll and bundle sheath photosynthetic cell types, i.e., Kranz anatomy (Haberlandt 1904), is generally required for C4 photosynthesis. In addition to the C3 pathway, these plants use the C4 photosynthetic cycle to increase CO_2 concentration at the site of RuBisCO. The initial fixation of CO_2 (rather bicarbonate) in the C4 pathway occurs, in the mesophyll cell cytosol, on PEP (phosphoenol pyruvate), using PEPC (PEP carboxylase) to form the 4-carbon oxaloacetate (OAA), which is rapidly converted to the more stable malate or aspartate. These diffuse to the bundle sheath cells where CO_2 is released with the help of decarboxylating enzymes. The released CO_2 is re-fixed by RuBisCO, which exclusively operates in the bundle sheath cells in C4 plants (see O'Leary 1982; Hatch 1987; Chollet et al. 1996; Izui et al. 2004; Raghavendra and Sage 2011). Higher concentration of CO_2 in bundle sheath cells outcompetes O_2 to suppress photorespiration and increases carboxylation efficiency in C4 plants.

In C3 plants, PEPC plays an anaplerotic role of replenishing the citric acid cycle intermediates, oxaloacetate and malate, which are required for nitrogen assimilation and amino acid biosynthesis (Fukayama et al. 2003; Miyao and Fukayama 2003; Masumoto et al. 2010; O'Leary et al. 2011). OAA is the precursor for the synthesis of several amino acids including aspartic acid, arginine, threonine, methionine, and lysine. Furthermore, malate is involved in many other physiological functions (see e.g., Lance and Rustin 1984). PEPC is also involved in the regulation of pH and electroneutrality in the system (Latzko and Kelly 1983). The PEPC gene family of *Arabidopsis thaliana* includes four PEPC genes: AtPEPC1, AtPEPC2, AtPEPC3, and AtPEPC4; these have 84–91 % sequence identity including a conserved phosphorylation domain at the *N*-terminal end (Vidal and Chollet 1997). However, AtPEPC4 encodes a polypeptide without any phosphorylation domain (Sanchez et al. 2006).

Further, we note that C4, as compared to C3, plants have higher solar radiation utilization efficiency (Zhu et al. 2008), higher water use efficiency, and, are tolerant to both

water stress, and salt stress (Osmond et al. 1982; Long 1999).

A number of attempts have been made to introduce single cell C4-like pathway into the mesophyll cells of C3 plants (for a review, see Miyao et al. 2011). Further, cDNAs or the gene coding for PEPC from various sources have been introduced into tobacco (Hudspeth et al. 1992), rice (Ku et al. 1999; Agarie et al. 2002; Fukayama et al. 2003; Bandyopadhyay et al. 2007; Ding et al. 2007), *Arabidopsis* (Wang et al. 2012), and potato (Gehlen et al. 1996; Häusler et al. 1999; Beaujean et al. 2001; Rademacher et al. 2002). In addition, the gene for pyruvate orthophosphate dikinase (PPDK) has been introduced into *Arabidopsis* (Ishimaru et al. 1997) and potato (Ishimaru et al. 1998), and cDNA for NADP-malic enzyme (NADP-ME), from maize, has been introduced into rice (Takeuchi et al. 2000; Tsuchida et al. 2001). Transgenic rice plants expressing C4-specific PEPC at a remarkably high level in their leaves showed reduced sensitivity to inhibition of photosynthesis by oxygen, but no change in the photosynthetic efficiency of the overexpressors (Ku et al. 1999; Fukayama et al. 2003). Transgenic potato expressing both PEPC of *Corynebacterium* (C.) *glutamicum* in cytosol, and NADP-ME of *Flaveria pringlei* in chloroplasts, showed a reduced requirement of electrons for CO_2 fixation under strong light and at high temperature (Lipka et al. 1999). Similarly, transgenic rice plants overexpressing C4-type PEP carboxykinase and/or PEP carboxylase in chloroplasts showed a C4-like photosynthetic carbon flow (Suzuki et al. 2000, 2006). For a discussion on the conversion of C3 into C4 machinery, as well as problems and issues related to it, see Gowik and Westhoff (2011) and Raines (2011).

Salt stress causes ionic imbalance and hyperosmotic stress in plants, and this leads to stomatal closure. It hampers CO_2 influx into the mesophyll cells during the daytime, reducing photosynthesis; further, there is increased oxidative stress, membrane disorganization, and reduction of cell division and expansion (Hasegawa et al. 2000; Munns 2002). Further, Ding et al. (2007) and Fang et al. (2008) found that under drought or high temperature stress, PEPC transgenic rice had higher photosynthetic efficiency than the controls. However, impact of salt stress has not been addressed thus far with this and other transgenic plants. Here, we present our results on molecular characterization, pigment and protein content, chlorophyll *a* fluorescence, photosynthesis (electron transport, CO_2 fixation), and non-photochemical quenching in the PEPC overexpressing transgenic plants; our data clearly show improved photosynthetic efficiency and tolerance to salt stress in the transgenics.

Materials and methods

Plasmid constructs, plant material, transformation

The cDNA of *Zea mays* PEPC (accession No. NM_001111948) (2913 bp) was amplified by polymerase chain reaction (PCR) using a pair of primers 5′-GGT ACC ATG GCG TCG ACC AAG GCT CCC g-3′ and 5′-G GGT ACC CTA GCC AGT GTT CTG CAT GCC GG-3′. In both the primer(s), KpnI restriction sites were introduced, as underlined above. The amplified cDNA fragment having KpnI restriction sites was ligated to pGEMT-Easy, then KpnI-digested PEPC cDNA fragment was taken out from the cloned pGEMT-Easy and ligated with modified pCAMBIA1304 plant transformation vector under the control of CaMV (Cauliflower Mosaic Virus) ^{35}S promoter with omega (Ω) enhancer (Pattanayak et al. 2005). In pCAMBIA1304, hygromycin (hpt) marker gene was replaced with kanamycin (nptII) gene. The recombinant plasmid (pCAMBIA1304::PEPC) was transformed into *Agrobacterium tumefaciens* strain (GV1301) and introduced into 6-week-old *A. thaliana* plants (cv. columbia) via agrobacterium-mediated floral dip method (Clough and Bent 1998). Vector control (VC) plants containing the null vector, pCAMBIA1304 (binary vector without PEPC cDNA), were also generated.

Selection of transgenic lines and plant growth conditions

Seeds collected from both the transformed and the vector control plants were plated on half-strength MS (Murashige and Skoog) agar medium (Sigma-Aldrich, USA) containing kanamycin (50 mg/L). Seedlings resistant to kanamycin were transferred to pots and then grown in a controlled chamber under a day (14 h light) night (10 h dark) cycle at a temperature of 22 ± 1 °C, and an irradiance of 75 µmol photons m^{-2} s^{-1}.

Genomic DNA isolation and PCR analysis

Genomic DNA was isolated by CTAB (Cetyl Trimethyl Ammonium Bromide) method (Nickrent 1994) from one month old plants of the T1 generation. Overexpression of maize PEPC gene in the plants was confirmed by PCR using ^{35}S forward internal primer (5′-CCC ACT ATC CTT CGC AAG AC-3′) and maize PEPC reverse primer (5′-G GGT ACC CTA GCC AGT GTT CTG CAT GCC GG-3′) to ensure incorporation of the whole cassette in a proper (sense) orientation.

Southern blot

The presence of PEPC transgene was checked by Southern blot analysis. The genomic DNA from the leaves of the T3 generation of the vector control and transgenic plants (PEPCx7, PEPCx11, and PEPCx43) was digested with the restriction enzyme XbaI. Thirty µg of DNA was loaded and resolved on 1 % agarose gel and blotted onto Nylon 66 membrane (MDI) (Sambrook and Russell 2001). The nptII coding sequence amplified from plasmid was used for probe preparation and labeled with (α^{32}P) dCTP, using a radioactive random primer labeling kit (Amersham-GE, UK). Southern blot was developed as described by Sambrook and Russell (2001).

Total RNA isolation, reverse transcription polymerase chain reaction (RT-PCR), and qRT-PCR

Total RNA was isolated from the leaves of 4-week-old vector control and transgenic plants, using the trizole method (Sigma-Aldrich, USA); cDNA was prepared using "First strand cDNA synthesis kit" from Thermo Fisher Scientific, following manufacturer's protocol. Reverse transcription polymerase chain reactions were performed using the following gene-specific internal primers: PEPC F, 5′-GTA CCG CGA GTG GCC CGA GG-3′ and PEPC R, 5′-CGT CCA TGA GCT TGC GCC AC-3′. Actin was used as an endogenous control (Actin F, 5′-ATG GCT GAT GGT GAA GAC ATT-3′ and Actin R, 5′-TCA GAA GCA CTT CCT GTG AAC A-3′). The PCR products were resolved on 0.8 % TAE-agarose gel.

Relative expression of different genes was studied by performing qRT-PCR on ABI Prism 7500 Sequence Detection System (Applied Biosystems, USA) using maize PEPC (PEPC F, 5′-AGA ACT CAA GCC CTT TGG GAA GC-3′ and PEPC R, 5′-GTC GGC GAA CTC CTT GGA CAG C-3′) and *A. thaliana* adenine phosphoribosyl transferase (APT1) (F, 5′-TTC TCG ACA CTG AGG CCT TT-3′ and R, 5′-TAG CTT CTT GGG CTT CCT CA-3′) primers. APT was used as a housekeeping gene (Wang et al. 2012). The relative gene expression data were analyzed using the $2^{-\Delta\Delta C_t}$ quantitation method (Livak and Schmittgen 2001). Increase in the expression of ZmPEPC was calculated using, as a reference, a transgenic line (PEPCx6) that has a low expression of ZmPEPC.

Western blot

Leaves harvested from 4-week-old vector control and transgenic plants were frozen in liquid nitrogen and pulverized. Extracted proteins (22 µg) were separated on 12 % (w/v) SDS-PAGE. Separated polypeptides were blotted on nitrocellulose membranes (Towbin et al. 1979). Proteins were probed with anti-maize PEPC antibody. The rabbit anti-mouse IgG (1:25,000) was used as a secondary antibody, conjugated to alkaline phosphatase. Blots were stained for alkaline phosphatase, using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT), and quantified using an Alpha Imager 3400.

PEPC enzyme assay

One hundred mg of leaf discs were quickly ground in 1 ml of extraction buffer containing 100 mM TRIS–HCl (pH 7.3), 2 mM K_2HPO_4, 10 mM $MgCl_2$, 1 mM acid EDTA, 10 % (v/v) glycerol, 10 mM β-mercaptoethanol, 10 mM NaF, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg ml^{-1} chymostatin, and 2 % (w/v) insoluble polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 14,000×g for 5 min and the supernatant was used immediately for spectrophotometric assay of PEPC at room temperature. The assay buffer contained 50 mM TRIS–HCl (pH 7.3), 5 mM $MgCl_2$, 0.2 mM NADH, 2 units of NAD-MDH, 10 mM $NaHCO_3$, 2.5 mM phosphoenolpyruvate (PEP), and 20 µl crude extract in 1 ml reaction (Avasthi et al. 2011). This reaction was initiated by adding PEP, and measured, for 3 min, as a decrease in absorbance at 340 nm. Protein concentration in the enzyme extracts was determined by the method of Bradford (1976); enzyme specific activity was expressed in terms of µmols of NADH oxidized per mg of protein per hr.

Spectrophotometric assays for chlorophyll, total protein, and free amino acids

Chlorophyll content was estimated in 80 % acetone (Porra et al. 1989), and the total protein content of leaves was measured according to Bradford (1976). Free amino acids were analyzed by the ninhydrin colorimetric method, using leucine as a standard (Misra et al. 1975).

Chlorophyll *a* fluorescence measurements and analysis

For a background on the basics and use of chlorophyll *a* fluorescence for measuring different reactions in photosynthesis, see Govindjee et al. (1986) and Papageorgiou and Govindjee (2004). The instruments used and the details of the experiments are described below.

PAM-2100

Chlorophyll *a* (Chl *a*) fluorescence from the leaves of 6-week-old vector control and transgenic plants was measured with a PAM-2100 fluorometer (Walz, Germany), as described by Dutta et al. (2009). Before each measurement,

the sample leaf was dark-adapted for 20 min (Demmig et al. 1987). Optimum quantum efficiency (φPSII, also referred to as Y) of Photosystem II (PSII) was inferred from $F_v/F_m = (F_m - F_o)/F_m$ (Schreiber and Armond 1978), where F_o is the minimum fluorescence, F_m is the maximum fluorescence, and F_v is the variable fluorescence ($F_m - F_o$). Electron transport rate (ETR) was estimated as described by Schreiber et al. (1995): ETR = $Y \times$ PAR $\times$ 0.5 $\times$ 0.84, where Y is the overall photochemical quantum yield (inferred from $(F'_m - F_t)/F'_m = \Delta F/F'_m$, with F_t being the measured fluorescence yield at any given time (t) and F'_m the maximal fluorescence yield in a pulse of saturating light when the sample is pre-illuminated), PAR, the photosynthetically active radiation, is flux density of incident light (μmol photons m^{-2} s^{-1}), the factor 0.5 indicates that the PSII: PSI ratio is 1:1, and 0.84 is the assumed fractional absorption of light by the leaf. In addition, non-photochemical quenching (NPQ) of the excited state of Chl *a* was calculated from $(F_m - F'_m)/F'_m$ (Schreiber 2004).

IMAGING-PAM

Images, taken by IMAGING-PAM chlorophyll fluorometer, were analyzed with the *Imaging-Win software* (Walz, Germany), as reviewed by Krause and Weis (1991), Oxborough (2004) and Baker (2008). The relaxation kinetics of NPQ was also measured by IMAGING-PAM (Walz, Germany). For quenching analyses, 6-week-old plants were dark-adapted for 20 min (Demmig et al. 1987), and subsequently exposed to 360 s by actinic light at two different intensities, 116 μmol photons m^{-2} s^{-1} and 336 μmol photons m^{-2} s^{-1}, provided by light-emitting diodes (LEDs). Relaxation of NPQ was followed by turning the light off and keeping the samples in dark up to 840 s. Saturating light pulses (1500 μmol photons m^{-2} s^{-1}) were given periodically to assess the maximal fluorescence level. For a complete description and understanding of NPQ, see Demmig-Adams et al. (2014).

Handy PEA

Chlorophyll *a* fluorescence induction was measured using Handy PEA (Plant Efficiency Analyzer), Hansatech Instruments, UK. *Arabidopsis* seedlings were pre-darkened for 20 min at room temperature. Chlorophyll *a* fluorescence induction transients were measured, up to 2 s, by excitation with 650 nm light of high intensity (3500 μmol photons m^{-2} s^{-1}), as provided by an array of 3 LEDs. These data were then analyzed by the so-called OJIP-test (Strasser et al. 2004); here, O (origin) is the (measured) initial minimum fluorescence, which is followed by a rise to a J level (2 ms), an inflection I (30 ms), and then finally the peak P (260 ms) (see Strasser et al. 1995). For an early discussion of the meaning of the "O" to "P" rise, see Munday and Govindjee (1969a, 1969b) and for a current understanding and assumptions involved, see Stirbet and Govindjee (2011, 2012), and Stirbet et al. (2014).

Parameters labeled as PI (performance index), RC/ABS (estimated ratio of reaction center to PSII antenna absorption), F_v/F_o (variable to minimal fluorescence), and an area over the fluorescence induction curve were obtained by using "*PEA plus software*" (Strasser et al. 2004). The PI represents a combination of three independent functional steps of photosynthesis, the density of RCs in the chlorophyll bed, excitation energy trapping, and conversion of excitation energy to electron transport, into a single multi-parametric expression (Strasser et al. 1999; Tsimilli-Michael et al. 2000); it was calculated as PI_{ABS}: RC/ABS * $\phi P_O/(1 - \phi P_O)$ * $\psi_0/(1 - \psi_0)$, where RC/ABS is the inferred density of reaction centers per PSII antenna chlorophyll, ϕP_O is the number of excitons trapped per photon absorbed, and ψ_0 is the probability that an electron can move all the way to PSI (Strasser et al. 2000, 2004). In addition, we measured 2 other parameters: (i) *variable to minimum fluorescence* (F_v/F_o), which is considered to be proportional to the activity of the water-splitting complex on the donor side of the PSII; and (ii) *Area*, the area above the chlorophyll fluorescence curve between F_o and F_m, which estimates the size of the plastoquinone pool.

To examine the OJIP data from different samples, fluorescence transients were normalized at the F_o (the O level). To assure ourselves the reproducibility of this F_o, we varied the dark time before the measurements, and we observed that it did not change above 15 min pre-dark period, and was linearly proportional to light intensity.

Further, fluorescence transients were normalized in the O-J region to provide relative variable fluorescence (general symbol V): $V_{OJ} = (F_t - F_o)/(F_J - F_o)$, and $\Delta V_{OJ} = V_{OJ}$ (treated) $- V_{OJ}$ (untreated); a plot of ΔV_{OJ} revealed another inflection, called the K-band (at about 300 μs) (Strasser et al. 2004, 2007; Tsimilli-Michael and Strasser 2008). Further, normalization at the "O" (20 μs) and the "K" (300 μs) levels gave us information on the O to K rise: $V_{OK} = (F_t - F_o)/(F_K - F_o)$; and, $\Delta V_{OK} = V_{OK}$(treated) $- V_{OK}$(untreated) revealed another band, the L-band (at about 150 μs). In addition, we also calculated $V_{OP} = (F_t - F_o)/(F_P - F_o)$ and $V_{IP} = (F_t - F_I)/(F_P - F_I)$.

Carbon assimilation (photosynthesis) at different light intensities

Photosynthetic light response curves of 6-week-old vector controls and transgenic plants, grown under short-day condition (8 h L/16 h D) in soil, were measured using an Infrared gas analyzer (Portable Gas Exchange Fluorescence

System GFS3000, Walz) using a standard head, for known leaf areas. The CO_2 concentration in the sample chamber was maintained at 400 ppm, air temperature at 22 °C, and relative humidity at 50 %. To obtain light response curves, we measured CO_2 assimilation, at different light intensities up to 500 µmol photons m^{-2} s^{-1}. For measurements of stomatal conductance (gs), transpiration rate (E), and water use efficiency (WUE), we used a light intensity of 400 µmol photons m^{-2} s^{-1} (von Caemmerer and Farquhar 1981).

Size and weight of plants

For the measurement of root length, vector control, and ZmPEPCx transgenic plants were grown vertically for 3 weeks in the MS medium in petri dishes. For the measurement of fresh weight, plants were taken from the petri dishes, and their weight was measured. For dry weight, whole plants were kept in an oven at 80 °C for 72 h, before the measurement.

Starch content

For the estimation of starch, samples were digested with an acid, and then assayed spectrophotometrically, using anthrone, a color reagent (Rose et al. 1991).

Salt treatment (salt stress)

To measure the effect of salt stress on photosynthetic efficiency of plants, vector control, and transgenic plants were plated on the MS medium for 2 weeks, and then transferred to the same medium with or without 150 mM NaCl for 8 days. Growth was measured during this period to evaluate salt tolerance.

Proline content

Free proline was estimated from leaf samples using ninhydrin, as described by Bates et al. (1973).

Anti-oxidative enzymes

Leaf samples (0.1–0.2 g) were homogenized in ice-cold extraction buffer (50 mM HEPES, pH 7.5), 0.4 mM EDTA, 5 mM $MgCl_2$, 10 % glycerol, 1 % PVP, 2 mM dithiothreitol (DTT), and 1 mM PMSF (Gegenheimer 1990). The homogenate was centrifuged (14,000×g) at 4 °C for 20 min. The supernatant was used as the crude extract for the assay of enzyme activities; it was then stored at −80 °C for further processing. Total protein was determined as described by Bradford (1976).

The catalase activity was measured as described by Cakmak and Marschner (1992). Assay mixtures contained 25 mM phosphate buffer (pH 7.0), 10 mM H_2O_2, and crude extract. The decomposition of H_2O_2 was measured at 240 nm (E = 39.4 mM cm^{-1}), and the specific activity of the enzyme was expressed as µmoles of H_2O_2 oxidized per min per mg protein.

The peroxidase activity was assayed spectrophotometrically at 436 nm, using Guaiacol, a hydrogen donor (Putter 1974).

Lipid peroxidation

For monitoring malondialdehyde (MDA), an indicator of lipid peroxidation, we used the method of Hodges et al. (1999).

Transmission electron microscopy (TEM)

A. thaliana leaves were vacuum infiltrated with 2.5 % glutaraldehyde solution for 30 min and kept overnight in the same solution (Karnovsky 1965). This solution was then replaced by 0.1 M sodium-phosphate buffer (pH 7.0); after this, we followed the procedure of Jiang et al. (2011). Sections of samples were then viewed in a transmission electron microscope (JEOL 2100F) at the Advanced Instrumentation Research Facility of Jawaharlal Nehru University, New Delhi, India.

Statistical analysis

Excel was used for statistical analyses. After the calculation of averages, standard deviations and standard errors for each of the parameters were determined. A *t* test was used to assess the differences between the vector control and transgenic plants in each parameter (see Ruhil et al. 2015, for details).

Results

Figure 1a shows a photograph of the vector control and the PEPC overexpressed plants (PEPCx), used in our measurements. They were grown for 5 weeks in a controlled chamber under a day (14 h light) night (10 h dark) cycle at 22 ± 1 °C, and at a light intensity of 75 µmol photons m^{-2} s^{-1}. Visually, plants of transgenic lines (PEPCx11 and PEPCx43) had somewhat better growth (in terms of number of leaves per plant) than the vector control (see Fig. 1a); also see below under "Plant morphology".

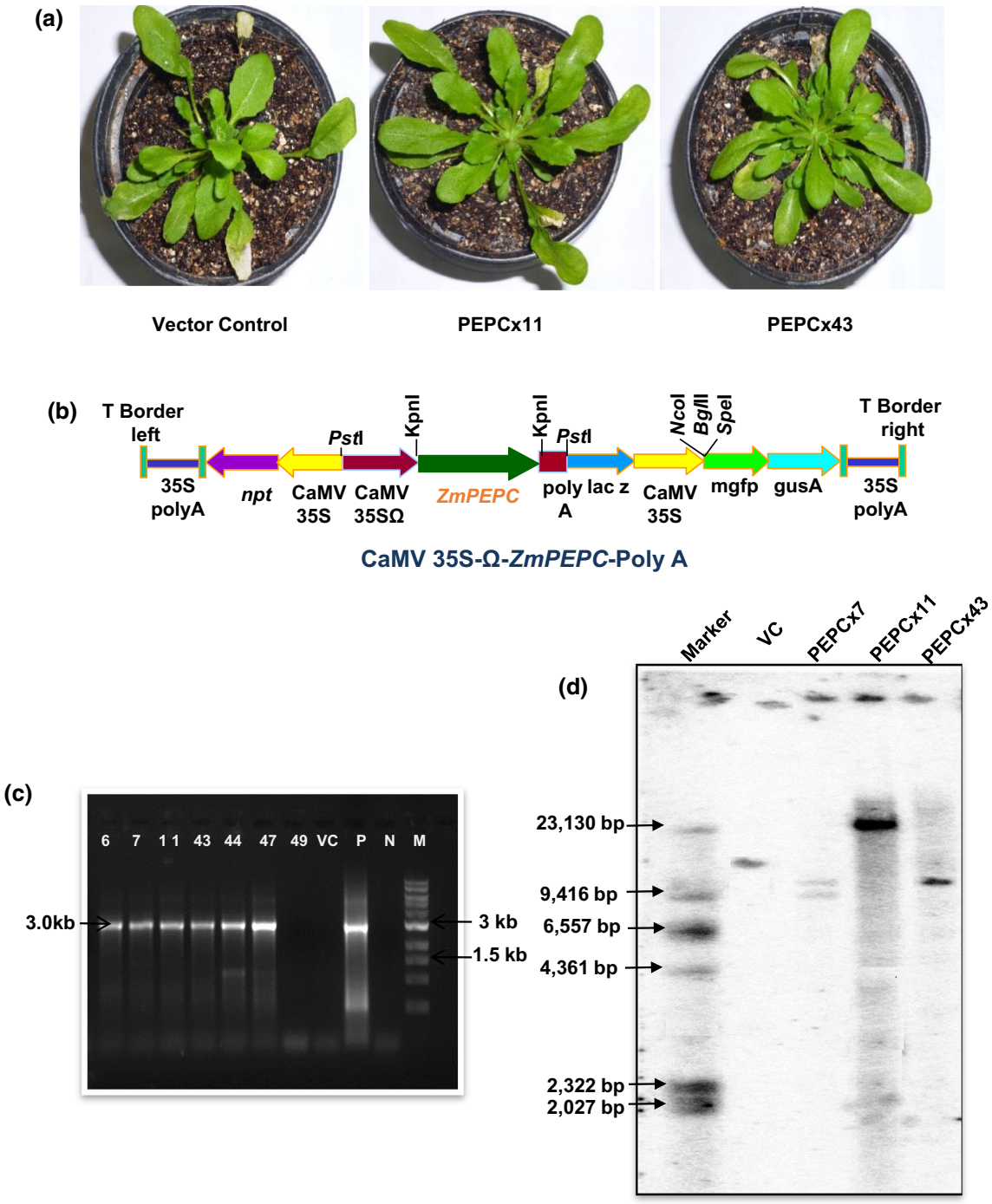


Fig. 1 Transgenic plants and vector control, cloning strategy, and confirmation of *Zea mays* phosphoenolpyruvate carboxylase (PEPC)-overexpressed *Arabidopsis thaliana*. **a** A photograph of vector control and ZmPEPCx plants—*Arabidopsis* vector control and ZmPEPCx (PEPCx11 and PEPCx43) were grown at 22 °C under 8 h L/16 h D photoperiod in cool-white-fluorescent light (75 µmol photons m^{-2} s^{-1}) for 5-weeks in soil. **b** Schematic representation of the construct used to overexpress *Zm*PEPC into *Arabidopsis*—CaMV35S-npt, coding region of neomycin phosphotransferase gene with Cauliflower Mosaic Virus ^{35}S promoter; CaMV35SΩ, CaMV 35S promoter with omega (Ω) enhancer; ZmPEPC cDNA, coding region of ZmPEPC gene; Poly A, Poly A tail; LacZ, β-galactosidase; mGFP, green fluorescent protein; gusA, β-glucuronidases; **c** PCR amplification of the genomic DNA—PCR reaction was performed with genomic DNA, using ^{35}S internal forward primer and gene-specific reverse primer, which yielded a fragment size of 3.0 kb in the transgenics confirming the integration of the T-DNA cassette with the host *Arabidopsis* genome in "sense orientation"; the numbers 6, 7, 11, 43, 44, 47, and 49 represent different transgenic lines, line 49 being a non-transformed escape; VC-vector control plants containing the null vector pCAMBIA1304 (binary vector without ZmPEPC cDNA); P-positive control (plasmid containing PEPC gene); N-negative control; M-marker (1-kb DNA ladder); **d** Southern blot, obtained after using npt II as a probe—Marker-λ DNA-HindIII Digest; VC—null vector control; PEPCx7, PEPCx11, and PEPCx43-transgenic lines

Characterization of the transgenics versus vector controls

Molecular characterization of transgenic plants

Using an *Agrobacterium tumefaciens*-mediated gene transfer system, we have transformed *Arabidopsis* (*A.*) *thaliana*, with cDNA of *Z. mays* (Zm) into C4-specific PEPC under the control of CaMV ^{35}S promoter and Ω enhancer (Fig. 1b). Several transgenic (T1) *Arabidopsis* plants were obtained from this transformation. Integration of the PEPC gene into the genome of *A. thaliana* was confirmed by PCR, using ^{35}S internal forward primer and PEPC gene-specific reverse primer (Fig. 1c; see figure legend for details); the presence of a 3-kb fragment proved that the transformation was successful (in vector control, the gene was not amplified). All these plants exhibited normal phenotype with their usual life cycle, grew to maturity, flowered, and set seeds.

Southern blot analysis, using nptII probe to estimate the copy number of the transgene, revealed that PEPCx11 and PEPCx43 lines had a single copy of the transgene nptII, but PEPCx7 had two copies (Fig. 1d; in VC—15 kb, in PEPCx7—9 kb and 11 kb, in PEPCx11—24 kb, in PEPCx43—11 kb). Plants were confirmed to be homozygous by looking at the T3 generation. Vector control had a single copy of nptII. Two homozygous lines, PEPCx11 and PEPCx43 from the T3 generation, each of which had a single copy of the transgene, were used for further experiments.

Based on isolated RNA, RT-PCR revealed a substantial increase in the ZmPEPC expression in PEPCx11, PEPCx7, PEPCx6, and PEPCx43 lines over the vector control (Fig. 2a); *β*-actin was used as an internal control. Quantitative reverse transcription (qRT)-PCR analysis, using gene-specific primers, showed that the expression of the ZmPEPC gene varied among the different transgenic lines. The transcript abundance in PEPCx7, PEPCx11, and PEPCx43 was 1.9-, 2.3-, and 2.6-fold higher, respectively, than that in PEPCx6 (Fig. 2b).

Figure 2c shows the SDS-PAGE data confirming that we had equal loading of proteins in different samples. Further, Fig. 2d shows that increased gene expression of ZmPEPC resulted in increased protein abundance (determined from the Western blot using a maize PEPC antibody). A 109 kD protein was detected in the vector control, in all the 4 transgenic lines, as well as in the maize (Fig 2d). As compared to the vector control *Arabidopsis*, PEPC protein abundance was ∼18-fold higher in maize; further, it was ∼7–10-fold higher in the transgenic lines (Fig 2e).

As compared to PEPC abundance in maize, the same in *Arabidopsis* transgenic lines was ∼35 to ∼56 %; further, their enzymatic activities were almost half of it, i.e., ∼19 to ∼29 % of maize suggesting a role of post-translational modification (including phosphorylation) to realize the full potential of the C4 enzyme (Fig. 2f).

Pigments and proteins

Based on their higher transgene expression, protein abundance and enzymatic activities, PEPCx11, and PEPCx43 transgenic lines were selected for further studies. Total Chl per fresh weight was higher in PEPCx11 and PEPCx43 by ∼16 and ∼19 %, as compared to the vector control (Table 1). However, no significant change in Chl a/b ratio was observed (Table 1). On the other hand, the carotenoid content increased by 10–13 % in the two transgenic lines (Table 1).

The protein content, on fresh weight basis, of transgenic plants was also higher by ∼7 and ∼16 % in PEPCx11 and PEPCx43, respectively (Table 1). Table 1 shows that the total free amino acids, also on fresh weight basis, increased in these transgenic lines by ∼6 % (in PEPCx11) and ∼11 % (in PEPCx43).

Chlorophyll a fluorescence

When a photosynthetic organism, kept in dark, is exposed to light, Chl fluorescence rises from a low minimum level ("O" level or F_o) to a higher maximum level ("P" level or F_m). Table 2 shows our results. The maximum primary photochemical efficiency of PSII, estimated from F_v/F_m was identical in the transgenics to that in the vector control (Table 2).

Our results on Chl *a* fluorescence, using pulse amplitude modulated (PAM 2100, Walz, Germany) fluorometer, revealed that the calculated electron transport rate (ETR) (μmol electrons m^{-2} s^{-1}) increased in response to photon flux density (PFD). The light response curves (see Fig. 3a) show, as expected, that ETR (see "Materials and methods" section for details) increases in response to light intensity. The ETR of PEPCx11 and PEPCx43 at 430 μmol photons m^{-2} s^{-1} was ∼10 to ∼19 % higher than in the vector controls (Fig. 3a); for results at 30–60 μmol photons m^{-2} s^{-1}, see Supplementary Material (Fig. S1).

Figure 3b shows a plot of NPQ of Chl *a* fluorescence. As expected, NPQ is shown to increase with increasing light intensity (see e.g., Demmig-Adams et al. 2014); at 430 μmol photons m^{-2} s^{-1}, the NPQ in PEPCx43 and PEPCx11 transgenic lines was lower than in vector control by 10–15 %.

Figures 3c, and 3d show time-dependent changes in NPQ, measured by IMAGING-PAM, in the upper leaves of vector control, PEPCx11, and PEPCx43, at two different light intensities (116 and 336 μmol photons m^{-2} s^{-1}). Our

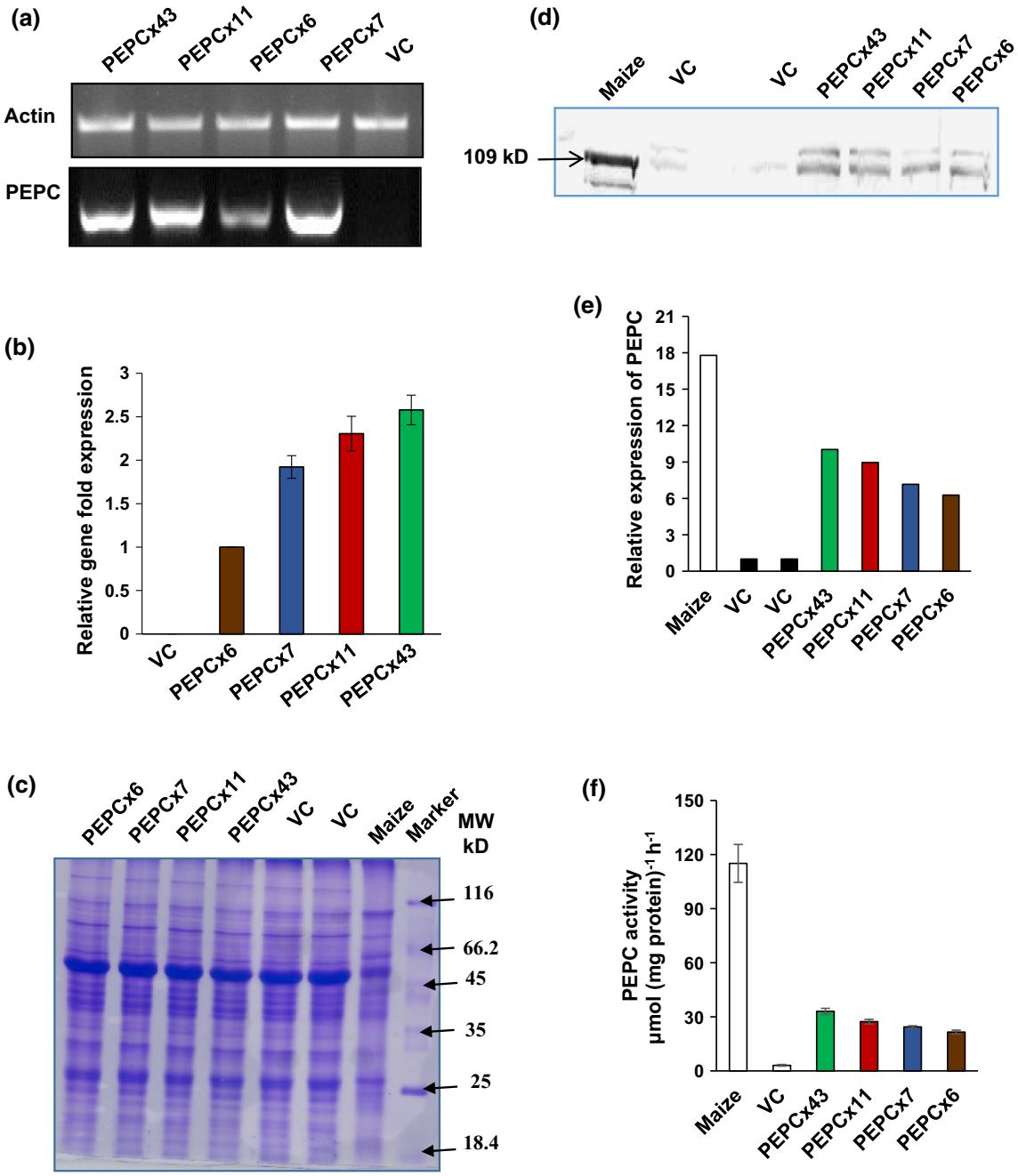


Fig. 2 Gene expression, protein abundance, and PEPC enzymatic activity of vector control and transgenic plants. **a** Semi-quantitative RT-PCR—*Zm*PEPC gene-specific primers were used to check PEPC expression. Actin was used as an internal control; **b** qRT-PCR of PEPC—relative gene expression of PEPC in VC and transgenic lines; **c** SDS-PAGE—protein profile of total proteins isolated from vector control, *Zm*PEPC-overexpressed *Arabidopsis* and field-grown maize. Twenty-two µg protein was loaded in each lane and 12 % SDS-PAGE was run to check for equal loading; **d** Western blot—protein samples from the gel were transferred to nitrocellulose membrane, and immunoblot analysis of PEPC protein was done, using PEPC antibody; **e** Relative expression of PEPC—Data are shown for transgenic lines, vector control, and maize; **f** PEPC enzymatic activity—the activity of PEPC ranged from ~22 to 33 µmol/mg protein/hr in transgenic *Arabidopsis*, which was ~7–11-fold higher than that in vector control plants (~3 µmol/mg protein/h). Note that PEPC activity of maize leaves was ~37-fold higher (115 µmol/mg protein/h) than that in vector control *Arabidopsis* plants, when assayed under the same conditions. VC stands for vector control; PEPCx6, PEPCx7, PEPCx11, and PEPCx43 for the transgenic lines. Each data point is the average of three replicates, and *error bars* represent ±SD

Table 1 Total chlorophylls (Chl), carotenoids, proteins, and free amino acids of vector control and ZmPEPCx transgenic plants

	VC	PEPCx11	PEPCx43
Total chlorophyll, mg (g FW)$^{-1}$	1.36 ± 0.09	1.57 ± 0.12*	1.61 ± 0.12*
Chl a/b ratio	3.32 ± 0.08	3.36 ± 0.17	3.38 ± 0.11
Carotenoids, mg (g FW)$^{-1}$	0.29 ± 0.01	0.32 ± 0.02*	0.33 ± 0.02
Total proteins, mg (g FW)$^{-1}$	11.5 ± 1.4	12.3 ± 1.4	13.3 ± 1.4
Free amino acids, μmol (g FW)$^{-1}$	5.90 ± 0.12	6.30 ± 0.22	6.60 ± 0.4

MS grown 11-days-old seedlings of *Arabidopsis* vector control and ZmPEPCx (PEPCx11 and PEPCx43) plants were transferred into pots, grown at 22 °C under 14 h L/10 h D photoperiod in cool-white-fluorescent light (75 μmol photons m^{-2} s^{-1}) for 4 weeks. FW stands for fresh weight, VC stands for vector control; and PEPCx11 and PEPCx43 for two transgenic lines. Each data point is the average of five replicates, and error bars represent ±SD; asterisks indicate significant differences determined by *t* test (*$P < 0.05$)

Table 2 Chl *a* fluorescence measurements of *Arabidopsis thaliana*

	VC	PEPCx11	PEPCx43
F_o	296.14 ± 6.0	302.30 ± 6.3	300.42 ± 4.0
F_m	1974.5 ± 14.7	2010.5 ± 35.2	2107.8 ± 37.6
F_v	1678.18 ± 17.5	1708.2 ± 29.5	1807.23 ± 32.6
F_v/F_m	0.85 ± 0.05	0.85 ± 0.03	0.86 ± 0.04

Plants were grown in pots at 22 °C under 14 h L/10 h D photoperiod under cool-white-fluorescent light (75 μmol photons m^{-2} s^{-1}) for 4 weeks. Minimal fluorescence (F_o), maximal fluorescence (F_m), maximal variable fluorescence (F_v), and F_v/F_m ratio, where $F_v = F_m - F_o$ (VC-vector control; PEPCx11 and PEPCx43- two different transgenic lines). Each data point is the average of six replicates with standard error, ±SE

results show that (1) NPQ was higher at the higher light intensity than at the lower light intensity, as expected; (2) the value of NPQ was higher in the vector control than in the transgenics at the higher light intensity, Fig. 3d; (3) when the continuous light was turned off, NPQ relaxed faster (within a minute) in the vector control than in the transgenics (several minutes), see Fig. 3d. The NPQ in the transgenics, as compared to the vector control (taken as 100 %), showed first a relative decrease with time, in the 50–70 s range, and then an increase reaching the same level as that in the vector control. When the light was turned off, it increased further going above the vector control, more at the higher, than at the lower, light intensity (Fig. 3e, f).

The relaxation of NPQ in the dark is due to a minimum of three components: qE (high energy quenching), qT (state change quenching), and qI (photoinhibition) (Allen and Mullineaux 2004; Müller et al. 2001). Since we did not wait for qI to decay, we could only make an estimate of NPQ relaxation for qE, and qT + qI, after the light was turned off; it led to the following estimate for the higher light intensity (Fig. 3d): (1) the amplitude of qE was higher (∼50 % of the total quenching) in the vector control than in the transgenics (∼10–15 %), whereas the half-time of decay was slightly shorter (∼45 s) than in the transgenics (∼60–90 s); (2) qT + qI was ∼50 % in the vector control as compared to ∼85–90 % in the transgenics, whereas the half-time of the decay was very slow in all cases (∼13 min in the vector control; and ∼15–18 min in the transgenics).

Light response curves of net CO_2 assimilation

Figure 4a shows data, measured by IRGA, on photosynthetic CO_2 assimilation in attached leaves of pot-grown vector control and ZmPEPCx *Arabidopsis* plants. As compared to the vector controls, transgenics (PEPCx11 and PEPCx43) had higher (∼14–18 %) net, and higher (∼13–21 %) gross photosynthetic rates at high-light intensities (400–500 μmol photons m^{-2} s^{-1}), (Fig. 4a). However, the quantum yield of CO_2 fixation, at low-light intensities (up to 80 μmol photons m^{-2} s^{-1}), was essentially the same in the vector control as in the transgenics (Fig. 4b). Further, the rate of respiration measured in the dark was higher (25–63 %) in the transgenics than in the vector control. The light compensation point in the vector control was at ∼5 μmol photons m^{-2} s^{-1}, which increased to ∼7 to ∼9 μmol photons m^{-2} s^{-1} in the transgenics (Fig. 4b)

Data shown in Table 3 confirm that the CO_2 assimilation rate was higher at 400 μmol photons m^{-2} s^{-1} in PEPCx11 (by 13 %) and PEPCx43 (by 18 %) than in the vector controls. We note that the above-mentioned increases in the photosynthetic rates were associated with increases in the stomatal conductance by 34 and 53 % in PEPCx11 and PEPCx43 (Table 3), and in the transpiration rate by 15 and 20 % (Table 3). However, the water use efficiency (WUE) was almost similar in the vector control and the transgenics because of almost similar increases in CO_2 assimilation and transpiration rates (Table 3).

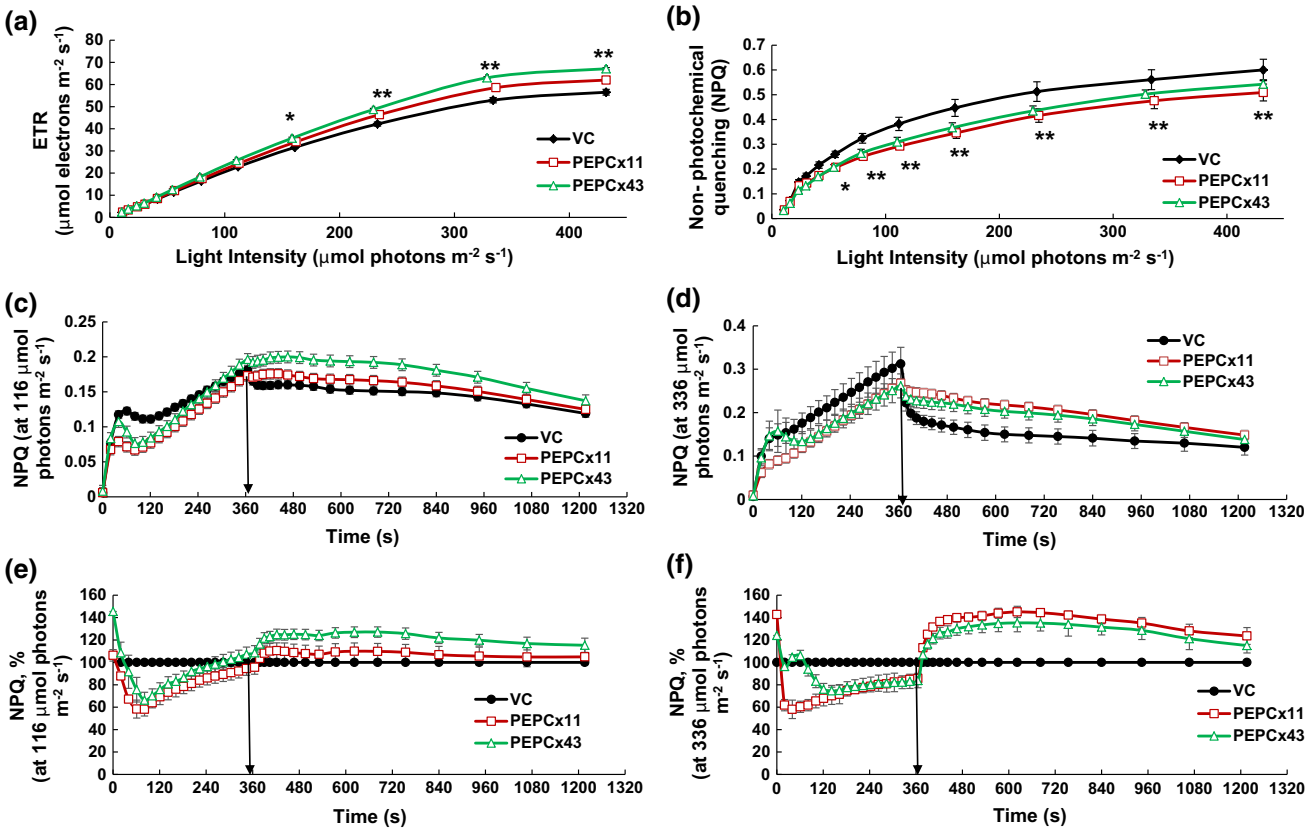


Fig. 3 Chlorophyll *a* fluorescence measurements on leaves of *Arabidopsis thaliana*. The vector control and ZmPEPCx (PEPCx11 and PEPCx43) plants were grown for 11 days in MS medium. Then, they were transferred into pots and grown for 4 weeks at 22 °C under 14 h L/10 h D in cool-white-fluorescent light (75 μmol photons m^{-2} s^{-1}). Plants were then dark-adapted for 20 min before making the following measurement. **a** Electron transport rates—ETR = Yield × PAR × 0.5 × 0.84; see text for details; **b** Non-photochemical quenching (NPQ) of excited state of chlorophyll at different light intensities—NPQ = $F_m - F'_m/F'_m$, where F_m is maximum fluorescence in dark, and F'_m is maximum fluorescence in light; **c**, **d** NPQ in light followed by relaxation in dark—during and after 116 μmol photons m^{-2} s^{-1} (**c**), or 336 μmol photons m^{-2} s^{-1} (**d**) (VC-vector control; and the transgenic lines PEPCx11 and PEPCx43); **e**, **f** NPQ, in arbitrary units both in light and in the following dark period, taking values for vector control as 100—during and after 116 μmol photons m^{-2} s^{-1} (**e**) or 336 μmol photons m^{-2} s^{-1} (**f**). Each data point is the average of five replicates, and *error bars* represent ±SE. *Asterisks* indicate significant differences determined by *t* test (*$P < 0.05$, **$P < 0.001$)

Plant morphology: fresh weight, dry weight, and root length

After 3-weeks of growth, both the fresh weight and the dry weight were higher in PEPCx11 (by 7 and 6.5 %) and in PEPCx43 (by 15 and 16 %) (Table 4).

Transgenic plants showed little change in root morphology; the root length was slightly reduced in the vector controls and in the transgenics (Table 4). However, the number of lateral roots was greater in the transgenics compared to the vector controls (data not shown).

Starch content

Increased CO_2 assimilation often results in increased stored carbohydrates, i.e., starch. Our data show that the starch content in PEPCx11 and PEPCx43, was higher by 10 and 18 % than in the vector controls (Table 4).

Comparison of the effect of salt stress on the vector control with that on the transgenics

As compared to the controls, the transgenics, constructed in our work, were tolerant to salt stress; thus, we focus below on this advantage by presenting results both on their composition and the functional characteristics.

Pigments and proteins

As compared to that of the vector control, the Chl content was higher in the transgenics. In salt-treated plants, the Chl content declined by 39 % in the vector controls, but by

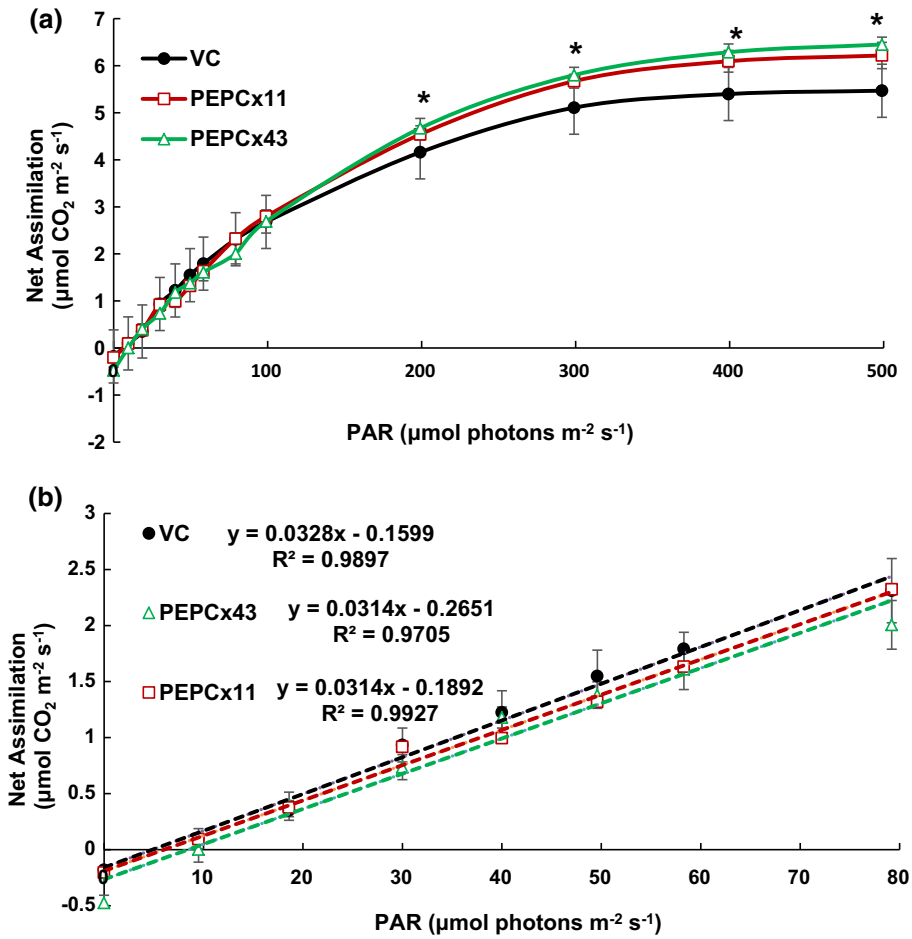


Fig. 4 Photosynthesis (net CO_2 assimilation rate) light response curves from attached leaves of vector control and PEPCx plants. Measurements, at 22 °C, were made by IRGA (GFS-3000, Walz) in ambient CO_2 at different light intensities, up to 500 µmol photons m^{-2} s^{-1} **(a)** and on an expanded X-axis up to 80 µmol photons m^{-2} s^{-1} **(b)** (VC—null vector control; PEPCx11 and PEPCx43—transgenic lines). The top curve **a** shows that the transgenic had higher saturating rate of CO_2 fixation and the bottom curve **b** shows the quantum yield (slope of the curve) is essentially the same in vector control and in the transgenic lines. Each data point is the average of five replicates, and *error bars* represent ±SE. *Asterisks* indicate significant differences determined by *t* test (*$P < 0.05$)

Table 3 Measurements of photosynthesis by IRGA (GFS-3000, Walz) in ambient CO_2 at 400 µmol photons m^{-2} s^{-1}

	VC	PEPCx11	PEPCX43
Net assimilation (µmol CO_2 m^{-2} s^{-1})	5.40 ± 0.41	6.09 ± 0.23*	6.285 ± 0.17*
Stomatal conductance (mmol H_2O m^{-2} s^{-1})	62.24 ± 2.8	83.39 ± 3.81*	95.01 ± 4.57*
Transpiration rate (mmol H_2O m^{-2} s^{-1})	0.57 ± 0.08	0.65 ± 0.04*	0.68 ± 0.06*
WUE (µmol CO_2 m^{-2} s^{-1}/mmol H_2O m^{-2} s^{-1})	9.10 ± 1.38	9.10 ± 0.53	9.03 ± 1.3

Attached leaves of vector control and PEPCx plants were monitored at 22 °C for net CO_2 assimilation rate, Stomatal conductance (gs), Transpiration rate, and Water Use Efficiency (WUE). VC stands for vector control; and PEPCx11 and PEPCx43 for two transgenic lines. Each data point is the average of five replicates and error bars represent ±SE. Asterisks indicate significant differences determined by t test (*$P < 0.05$)

Table 4 Fresh weight, dry weight, root length, and starch content of vector control and ZmPEPCx plants

	VC	PEPCx11	PEPCX43
Fresh weight (mg)	17.86 ± 0.62	19.10 ± 0.70*	20.6 ± 0.57*
Dry weight (mg)	1.60 ± 0.06	1.70 ± 0.04*	1.86 ± 0.048*
Root length (cm)	10.03 ± 0.15	9.29 ± 0.24	9.4 ± 0.16
mg starch (g FW)$^{-1}$	1.90 ± 0.14	2.10 ± 0.19	2.25 ± 0.27*

Arabidopsis vector control and ZmPEPCx (PEPCx11 and PEPCx43) were grown at 22 °C under 8 h L/16 h D photoperiod in cool-white-fluorescent light (75 µmol photons m^{-2} s^{-1}) for 3-weeks in petri dishes. FW stands for fresh weight, VC stands for vector control, and PEPCx11 and PEPCx43 for two transgenic lines. Each data point is the average of six replicates, and error bars represent ±SE. Asterisks indicate significant differences determined by *t* test (*$P < 0.05$)

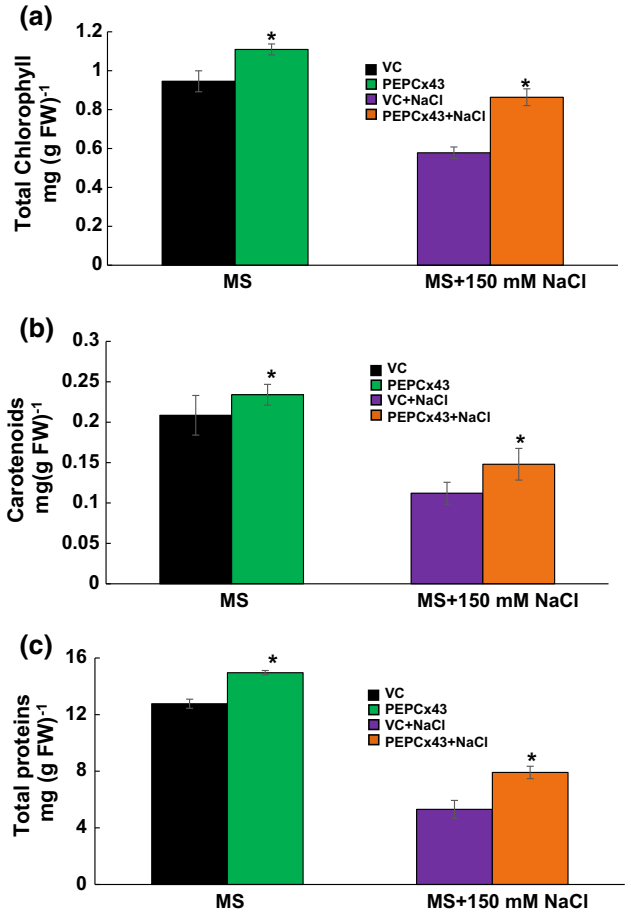


Fig. 5 Total chlorophylls (**a**), carotenoids (**b**), and proteins (**c**) of vector control and ZmPEPCx plants grown under salt stress. *Arabidopsis* vector control and ZmPEPCx43 plants were grown for 15 days in MS medium. Then, they were transferred on the MS medium with or without 150 mM NaCl, and grown for 8 days at 22 °C under 14 h L/10 h D in cool-white-fluorescent light (75 µmol photons m^{-2} s^{-1}). VC stands for vector control and PEPCx43 for a transgenic line. Each data point is the average of five replicates, and *error bars* represent ±SE. *Asterisks* indicate significant differences determined by *t* test (*$P < 0.05$)

only 22 % in PEPCx43 (Fig. 5a); on the other hand, the carotenoid content declined by 46 % in the vector control, but only by 37 % in PEPCx43 (Fig. 5b).

The protein content was higher in the transgenics (Table 1; Fig. 5c). However, in salt-stressed plants, decreases in protein were 58 and 47 % in the vector controls and the transgenic PEPCx43, respectively (Fig. 5c).

Chlorophyll a fluorescence and photosynthetic efficiency

Figure 6a shows false color images of F_v/F_m, using IMAGING-PAM, from vector control and transgenic *Arabidopsis* plants, grown with and without 150 mM NaCl. We note that in response to NaCl treatment, F_v/F_m and the diameter of the rosette leaves declined to a very large extent in the vector control (left panels), whereas in the PEPCx plants, this decrease was much less (right panels; also see Table 5). This suggests that, under salt stress, the maximum primary photochemical efficiency of PSII was highly reduced in the vector control; however, it was only slightly lowered in the transgenics.

The OJIP curves Chl *a* fluorescence transients of the dark-adapted leaves of *A. thaliana* plants are shown, on a logarithmic time scale from 10 µs up to 2 s, in Fig. 6b. The transgenic PEPCx43 had a slightly slower fluorescence rise from O to J, but a slightly faster rise from J to I, and almost similar I to P rise, as compared to the vector control (Fig. 6b). A faster IP rise has been observed in rice plants that have higher photosynthesis and growth (Hamdani et al. 2015). Further experiments are needed to examine the implications of these results.

In salt-treated plants, both F_o and F_m decreased (Table 5; see below for detailed discussion) and the O-J, J-I, and I-P phases had lowered amplitudes, compared to those without salt treatment. To reveal changes in the fluorescence kinetics, we normalized the OJIP curves at F_o, and present them as relative variable fluorescence versus time (on logarithmic time scale) (Fig. 6b). To further elucidate differences in the fluorescence kinetics, subsequent normalization (and subtraction) was carried out. The relative variable fluorescence, from the O to the J level, normalized between 20 µs and 2 ms (V_{OJ}) is shown in Fig. 7a and in Supplementary Material (Fig S2); it shows changes in V_{OJ} due to salt stress. The inset of Fig. 7a, ΔV_{OJ}, shows the K-band at 300 µs in salt-treated samples (see Srivastava et al. 1997; and Strasser et al. 2004). This K-band has a higher amplitude in salt-treated vector control plants than in the salt-treated transgenic PEPCx43 (Fig. 7a). The difference, observed in Chl *a* fluorescence transient between the normalized curves of plants with and without salt treatment, during 20–300 µs, is shown in Fig. 7b. Here, we see the so-called L-band, which appears as a peak around 150 µs, and is higher in salt-treated vector control than in the transgenic samples (Fig. 7b).

When the OJIP curves are doubly normalized at the O level (20 µs) and at the P level (260 ms), the transgenic plant has a relatively lower "J" level (Fig. 7c). Upon salt treatment, there is a decrease in the J level in both the vector control and the transgenic line (6.3 % in VC; 5.7 % in PEPCx43). When the IP curves are double normalized at the I (30 ms) and at the P levels, we do observe differences. At 150 ms, there is no difference between VC and PEPCx43, but there is a larger decrease in the VC by salt treatment (Fig. 7d). Further analysis is needed to understand the differences observed here.

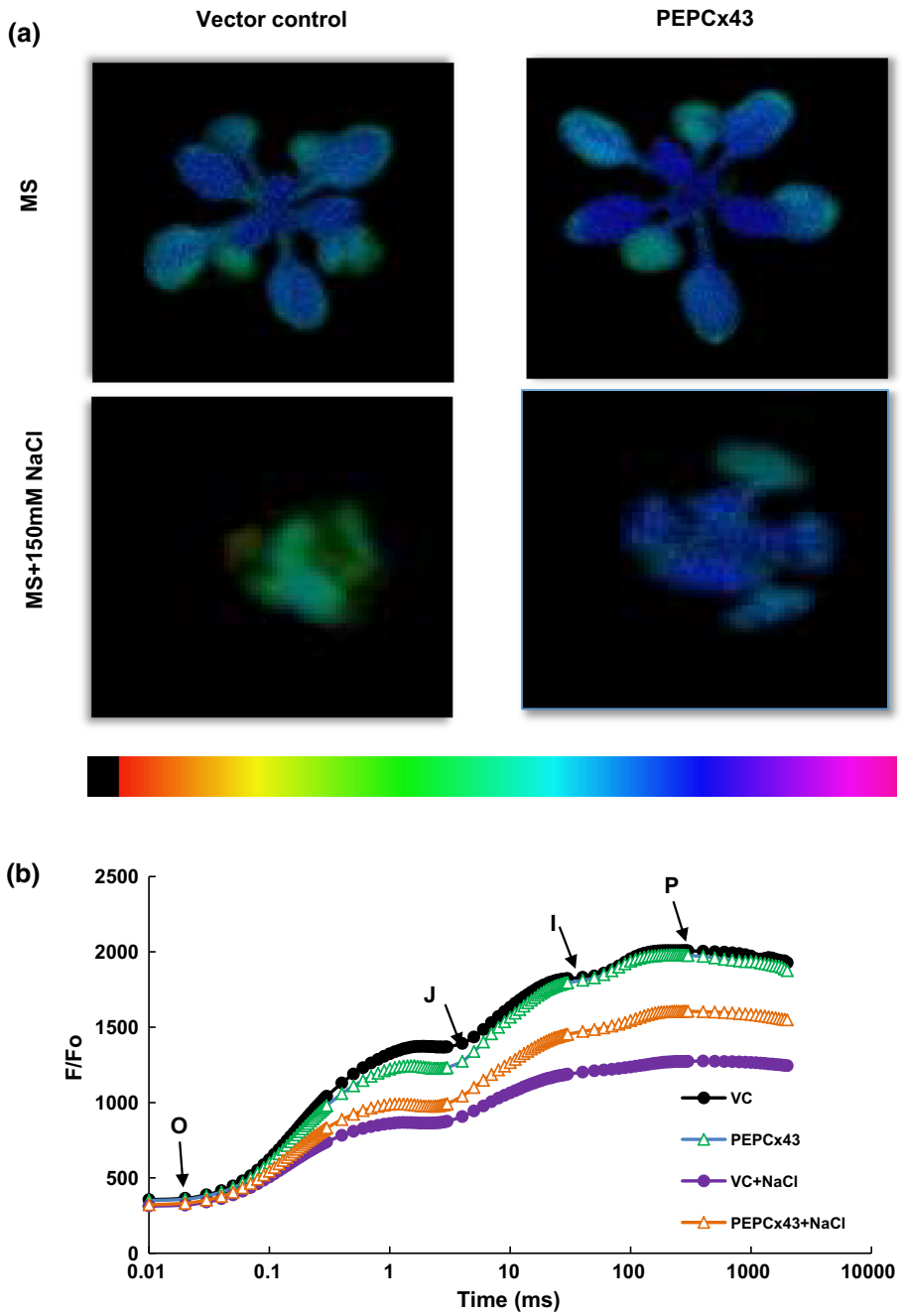


Fig. 6 Chlorophyll *a* fluorescence measurements: effects of salt. See the legend of Fig. 5 for details of plant growth and salt treatment. **a** False *color images* of F_v/F_m of untreated and salt-treated *Arabidopsis* vector control and ZmPEPCx43 plants—images by the IMAGING-PAM. The *colored scale bar* shows the F_v/F_m increasing in value from *left* (*green*) to *right* (*blue*); **b** Chl *a* fluorescence transients, the OJIP curves normalized at the O level. *F*, in the diagram, stands for fluorescence at time t (F_t), and F_o for fluorescence at the O level. VC is for vector control, and PEPCx43 for a transgenic line

Parameters from fluorescence curves, their meaning and analysis Salt stress significantly affected chlorophyll *a* fluorescence parameters, as shown in the fluorescence transient, the OJIP curves (Fig. 6b) (Strasser et al. 1995, 1999). In our experiments, the parameters most affected, by salt treatment, were minimal Chl fluorescence, F_o, maximum Chl fluorescence, F_m, ratio of variable to maximum fluorescence, F_v/F_m, the so-called Performance Index, PI, ratio of the inferred reaction center to the antenna, RC/ABS, variable to minimum Chl fluorescence, F_v/F_o, and the area over the OJIP curve (Table 5) (for further detailed definition of these parameters, see "Materials and methods" section).

We list below the information obtained on the various fluorescence parameters:

- Under salt stress (150 mM NaCl), the F_o of the vector control and PEPCx43 decreased by 20 and 18 % due mainly to equivalent reduction in the Chl content (Table 5).
- The F_m of the vector control and PEPCx43 decreased, in 150 mM NaCl samples, by 49 and 33 % (Table 5),

Table 5 Chlorophyll *a* fluorescence measurements on *Arabidopsis thaliana*

	VC	VC + NaCl	PEPCx43	PEPCx43 + NaCl
F_o	285 ± 8	229 ± 18	288 ± 7	235 ± 11
F_m	1900 ± 51	1020.4 ± 92	1987.4 ± 42	1320 ± 42*
F_v/F_m	0.85 ± 0.001	0.77 ± 0.015	0.85 ± 0.004	0.82 ± 0.007*
F_v/F_o	6.0 ± 0.07	3.5 ± 0.32	5.9 ± 0.18	4.7 ± 0.2*
PI	1.11 ± 0.09	0.64 ± 0.11	1.37 ± 0.07*	1.1 ± 0.05*
RC/ABS	0.49 ± 0.02	0.43 ± .02	0.52 ± .01	0.50 ± 0.01
Area	21,104 ± 537	11,161 ± 2141	21,258 ± 1370	15,740 ± 1344*

MS grown 2 weeks old seedlings of *Arabidopsis* control and ZmPEPCx43 plants, grown in MS medium, were transferred to MS or MS + 150 mM NaCl and grown at 22 °C under 14 h L/10 h D photoperiod in cool-white-fluorescent light (75 µmol photons m^{-2} s^{-1}) for 8 days. The data points are average of 8 replicates and error bars represent ±SE. Asterisks indicate significant differences determined by *t* test (*$P < 0.05$). See list of abbreviation for the definition of fluorescence parameter

also due mainly to decreased Chl content. Since this decrease was much larger than on the F_o, it reflects differences in the ability of the two to withstand salt stress, the transgenics being more resistant than the vector controls.

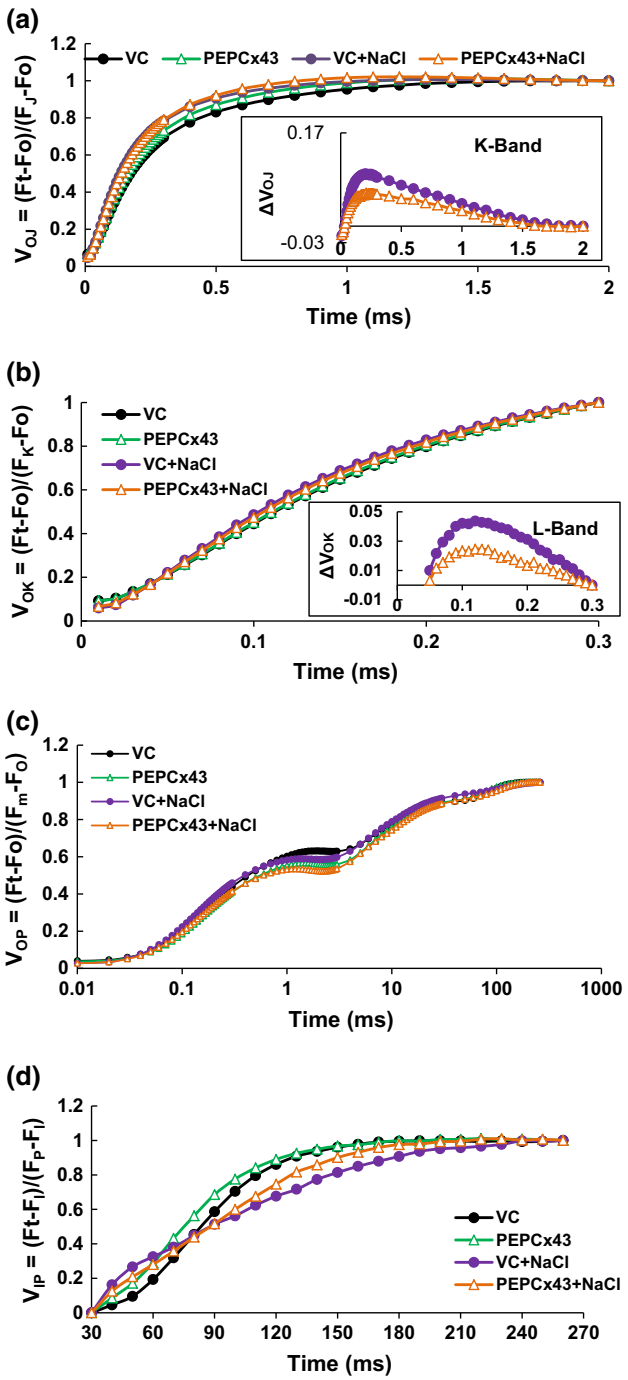
- Under salt stress (150 mM), the F_v/F_m ratio was higher (by 6.5 %) in the transgenics than in the vector controls (Table 5). However, the F_v/F_m ratio in the vector control decreased by 9.4 % upon salt stress, but in PEPCx43, it decreased by only 3.5 %, showing better salt tolerance in this transgenic plant (Table 5). The F_v/F_m ratio, which reflects the quantum efficiency of photosystem II, is indeed very high (0.85) in the leaves in our study, as observed by others for healthy higher plants (0.85; see Table 5; see Björkman and Demmig 1987, and Govindjee 2004); it is shown to be proportional to the efficiency of photosynthesis (see e.g., Basu et al. 1998).
- The F_v/F_o ratio, which is known to reflect the efficiency of the (electron) donor side of PSII, i.e., the activity of the oxygen-evolving (or the water-splitting) complex, is a highly sensitive parameter (Burke 1990). A decrease in this ratio after salt treatment, both in the vector control and the transgenics, may be due to impairment of photosynthetic electron transport on the electron donor side of PSII (Pereira et al. 2000). However, a decrease of F_v/F_o in the transgenics, by salt treatment, was much smaller than in the vector control (see Table 5), showing its advantage.
- The calculated performance index (PI) was higher (23 %) in the transgenics than in the vector controls, in agreement with other data showing their advantage. Salt (150 mM) treatment of vector control plants led to a decrease of PI by 42 %, but in the transgenics, this decrease was only about half as much, ~20 % (Table 5)—again showing their advantage over the vector controls.
- The ratio RC/ABS, which reflects the density of reaction centers per PSII antenna chlorophyll (its inverse being the antenna size of chlorophyll molecules per PSII reaction center), was slightly higher in the transgenics (by ~6 %) than in the vector controls, but it decreased less (4.6 %) under salt stress in PEPCx43 plants than in the vector controls (13.3 %) in agreement with transgenics bring more resistant to salt stress (see Table 5).
- The area over the OJIP curve, between F_o and F_m, which is proportional to the size of the pool of the electron acceptors in PSII, mainly the plastoquinones (Malkin and Kok 1966), decreased, upon 150 mM NaCl treatment, by 47 % in the vector controls, but only by 26 % in the transgenic PEPCx43 (Fig. 6b, Table 5), once again confirming the advantage of the transgenics, over the control, during salt stress.

Electron transport rate and non-photochemical quenching

The ETR (see "Materials and methods" section for details) increased in response to light intensity (Fig. 8a); it is significantly higher (19 %) in the transgenic plants, as compared to that in the vector control. Salt treatment led to decreases in ETR in both the vector control and in the PEPCx43, especially at higher light intensities. However, at saturating light intensities, the extent of decline of the ETR in the PEPCx43 plants was less (36 %) than that in the vector controls (57 %) (Fig. 8a). We further note that salt treatment increased NPQ in the vector control by ~23 %, but in PEPCx43 by only 11 % (Fig. 8b).

Proline content

Proline is known to play a critical role in protecting plants under stress, particularly under saline conditions (Madan et al. 1995; Petrusa and Winicov 1997). Although the proline content of both the vector control and the



◀**Fig. 7** Photosynthetic parameters, deduced from the analysis of chlorophyll *a* fluorescence transient. See the legend of Fig. 5 for details of plant growth and salt treatment. **a** Variable fluorescence transients from the O to the J—double normalized between O (F_o) and J (F_J): $V_{OJ} = (F_t - F_o)/(F_J - F_o)$, F_t stands for fluorescence at time t. The *inset* shows kinetic difference of V_{OJ}, $\Delta V = V_{OJ\,(treated)} - V_{OJ(untreated)}$, which shows the K-band; **b** Variable fluorescence transients from the O to the K—double normalized between O and K (F_K): $V_{OK} = (F_t - F_o)/(F_K - F_o)$. The *inset* is the kinetic difference of V_{OK}, $\Delta V = V_{OK(treated)} - V_{OK(untrated)}$, showing the L-band; **c** Variable fluorescence transients from the O to the P—double normalized between O and P (F_P) phases: $V_{OP} = (F_t - F_o)/(F_P - F_o)$; **d** Variable fluorescence transients from the I to the P—double normalized between I (F_I) and P phases $V_{IP} = (F_t - F_I)/(F_P - F_I)$. VC stands for vector control; and PEPCx43 for a transgenic line

transgenic plants was similar, salt stress increased proline content by 100 % in the vector control, but by 390 % in the transgenics (Fig. 9a).

Antioxidant enzymes and oxidative damage

Catalase is one of the most potent catalysts in nature; it catalyzes the conversion of H_2O_2, a powerful and potentially harmful oxidizing agent, to water and molecular O_2. In the control seedlings (without salt treatment), the catalase activity was almost similar in the vector control and in the transgenic plants (Fig. 9b). Although in response to salt treatment, catalase activity increased in both vector control and transgenic plants, the relative increase in catalase activity was higher (113 %) in the transgenics than in the vector control (71 %) (Fig. 9b).

Further, in response to salt treatment, guaiacol peroxidase increased by 76 % in the vector controls, but much more, by 106 %, in PEPCx43 (Fig. 9c).

Malondialdehyde (*MDA*) level, an index for lipid peroxidation, was quite similar in the vector control and the transgenic control plants; however, upon salt treatment, there was an increase in the MDA content by ~250 % in the vector controls, but much less (~120 %) in the transgenics, suggesting lesser lipid peroxidation in salt-treated transgenic plants (Fig. 9d).

Transmission electron microscopy (TEM)

TEM of the chloroplasts of the control vector control and of the PEPCx43 plants showed typical well-developed grana and stroma thylakoids (Fig. 9e). We are unable to see any striking difference between the two samples. The vector control plants showed salt-induced structural distortion, such as swelling of thylakoids (see left bottom panel, Fig. 9e). However, consistent with our observations about the advantages of the transgenic plants, 150 mM salt treatment did not produce swollen thylakoids; instead, they had typical well-developed grana and stroma lamellae (Fig 9e).

Discussion

The PEPC and its implications

PEPC is an essential enzyme that catalyzes biosynthesis of OAA from PEP and is crucial for the primary metabolism

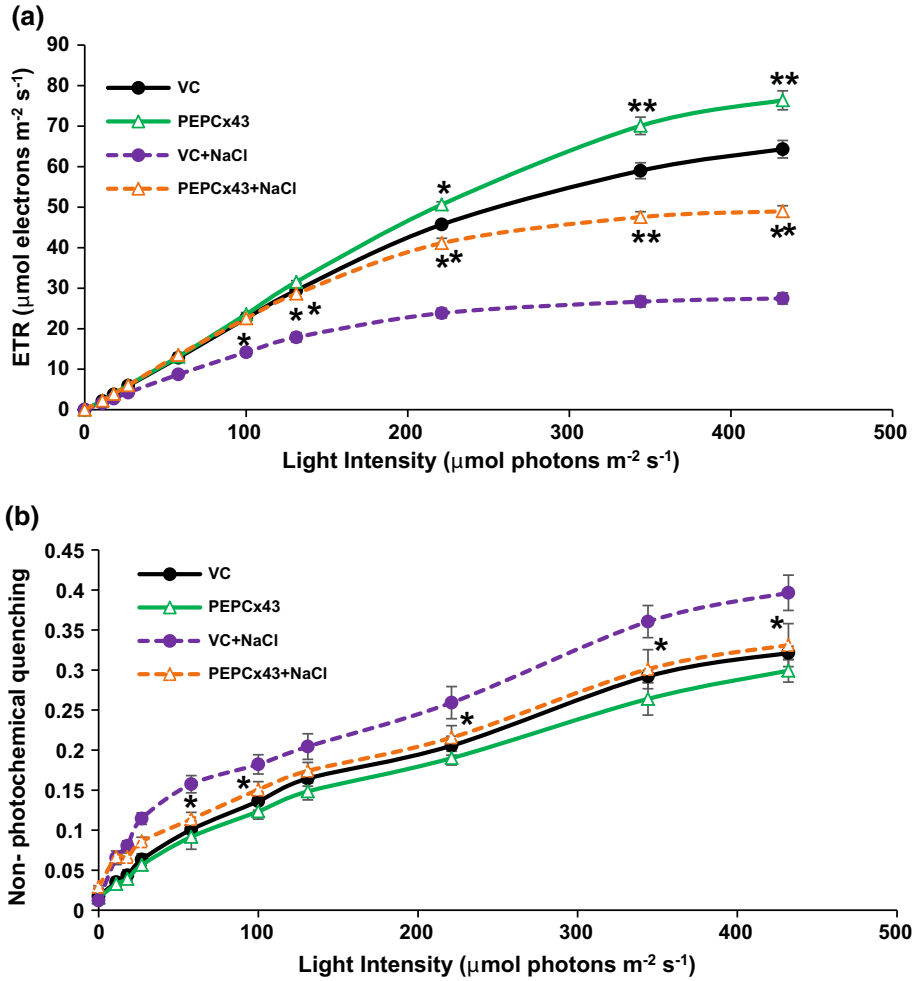


Fig. 8 Calculated electron transport rates (**a**) and non-photochemical quenching (NPQ) of Chl *a* fluorescence (**b**) of *Arabidopsis* vector control and ZmPEPCx43 plants. See legend of Fig. 5 for details of plant growth and salt treatment. ETR = Yield × PAR × 0.5 × 0.84; NPQ = $F_m - F'_m/F_m$; see "Materials and methods" section for details. VC stands for vector control; and PEPCx43 for a transgenic line. Each data point is the average of five replicates, and *error bars* represent ±SE. *Asterisks* indicate significant differences determined by *t* test (*$P < 0.05$, **$P < 0.001$)

of all C4 plants (O'Leary 1982; Chollet et al. 1996; Izui et al. 2004). Therefore, introduction of PEPC to C3 plants is considered essential for achieving C4 photosynthesis and, thus, higher productivity in these plants. In our work, presented here, we have produced and characterized several lines of *A. thaliana* overexpressing ZmPEPC (Fig. 1c). Quantitative real-time PCR analysis showed that the expression level of ZmPEPC was ~2.3- and ~2.6-fold higher in the PEPCx11 and PEPCx43, respectively compared to that in PEPCx6 (Fig. 2b). This is consistent with the observation of Wang et al. (2012); also see Hudspeth et al. (1992). The observed differences in the level of expression among transgenic lines may be related to the diversity of the available insertion sites. Similarly, the protein abundance of ZmPEPC in our transgenic lines was 7–10-fold higher than in the vector controls, which is also consistent with earlier reports (Hudspeth et al. 1992; Ku et al. 1999; Bandyopadhyay et al. 2007).

A closer analysis of protein abundance and enzymatic activity of PEPC in the transgenics and in maize reveals that, on equal protein basis, abundance of ZmPEPC in the overexpressors is 44–65 % lower than in the maize. The PEPC activity, measured per mg protein, in the transgenics is 72–82 % lower than in the maize. However, when the PEPC activity is corrected for its reduced protein abundance in the *Arabidopsis* transgenics as compared to maize, the enzymatic activity is found to be almost half of that in the C4 plants. This difference could be attributed to endogenous post-translational phosphorylation of C4 PEPC, by PEPC kinase in C3 plants. (For details on PEPC

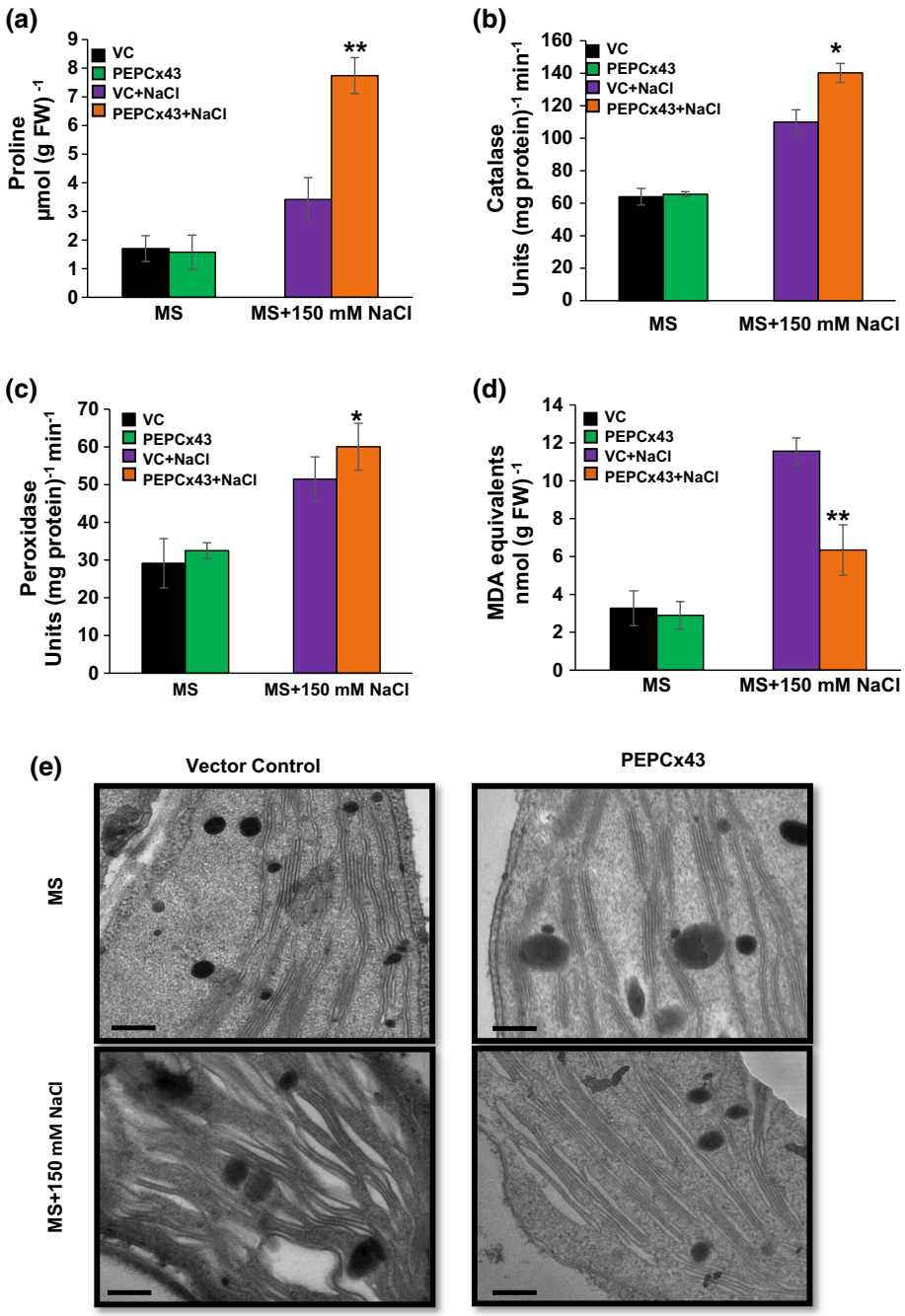


Fig. 9 *Top* Bar graphs for **a** Proline; **b** Catalase activity; **c** Peroxidase activity; **d** MDA content. *Bottom* **e** Ultrastructure of chloroplasts; magnification ×15,000 (*scale bar* 100 nm). See legend of Fig. 5 for details of plant growth and salt treatment. Each data point is the average of five replicates, and *error bars* represent ±SD. *Asterisks* indicate significant differences determined by *t* test (*$P < 0.05$, **$P < 0.001$)

kinase, see Vidal and Chollet 1997; Nimmo 2000; Meimoun et al. 2009.)

Results, reported in this paper, demonstrate that different transgenic *Arabidopsis* lines used here had 7–10 fold higher PEPC activity than the vector controls. This was substantially higher (2–5.5 times) than in other transgenic plants (say, e.g., tobacco, and potato) that had overexpressed maize or bacterial PEPC cDNA (Hudspeth et al. 1992; Kogami et al. 1994; Gehlen et al. 1996). Wang et al. (2012) reported a maximum increase of 5.8-fold in the PEPC activity in the transgenics. Overexpression of intact maize C4-specific PEPC gene, with its own promoter and terminator sequences, in rice has indeed led to much higher level expression of PEPC protein (Ku et al. 1999; Fukayama et al. 2003; Bandyopadhyay et al. 2007); further, its enzyme activity has been shown to be 14–110-fold higher than in the non-transformants (Ku et al. 1999; Jiao et al. 2002; Fukayama et al. 2003; Suzuki et al. 2006; Taniguchi et al. 2008).

Although Chls and proteins are synthesized by two different metabolic processes, their biosyntheses are co-

regulated especially those related to the status of the nitrogen in the system. Higher increase in the total protein content is expected to result in higher Chl synthesis (Table 1) due to an overall increase in the enzymes involved in Chl biosynthesis. PEPC plays a central metabolic role in interaction between carbon and nitrogen in the plants; nitrogen is an important regulator not only of carbon flow but also of gene expression of some proteins, including Chl a/b apoprotein, in higher plants (Sugiharto and Sugiyama 1992; Plumley and Schmidt 1989). As mentioned in the *Introduction*, PEPC plays an anaplerotic role of replenishing the tricarboxylic acid cycle with intermediates to meet the demand of carbon skeletons for the synthesis of organic acids and amino acids in C3 plants (Miyao and Fukayama 2003). Our results demonstrate that overexpression of ZmPEPC may have led to increased amino acid content in the transgenics because of the increased production of OAA, which is the precursor for the synthesis of several amino acids. We consider it likely that the OAA could have migrated from the cytoplasm to mitochondria, and may have contributed to the increased carboxylic acid pool. Further, increased amino acids in the transgenics may have contributed to higher protein content of plants that led to increases in metabolic processes including photosynthesis and respiration. Gehlen et al. (1996), Häusler et al. (1999), and Rademacher et al. (2002) have demonstrated 20–90 % increases in amino acid content in PEPC-overexpressed plants. Our results suggest that increased amino acid content in transgenics must have resulted in higher protein content (Table 1); there are no other previous reports of increase in total protein content of PEPCx plants.

Exploiting chlorophyll *a* fluorescence

Chl *a* fluorescence, a non-invasive probe for measurement of photosynthetic functions (for reviews, see Krause and Weiss 1991; Govindjee 1995, 2004; Baker 2008), has provided us the following results.

The light response curves clearly demonstrate that ETR at high-light intensities is higher in the transgenic plants (Fig. 3a). We note that Gehlen et al. (1996) did not observe any significant change in the quantum yield of PSII between WT and PEPC overexpressing potato plants (cf. Biswal et al. 2012).

Non-photochemical processes that dissipate excitation energy by quenching the excited state of Chl *a* are lower in the transgenic *Arabidopsis* than in the vector control demonstrating that in the transgenics, light energy is more efficiently utilized by PSII reactions (Fig. 3b). The three components of NPQ (Horton and Hague 1988; Krause and Weis 1991; Horton et al. 1996; Müller et al. 2001; Nilkens et al. 2010; Murchie and Niyogi 2011) are: (1) the faster (seconds) qE due to pH changes, which lead to conformational reorganization in the system involving a PsbS protein, and a xanthophyll cycle (Demmig-Adams et al. 2014); (2) the slower (minutes) qT due to "state transition 1 to 2"; excess light in PSII initiates a set of reactions (see Allen and Mullineaux 2004) that lead to movement of a mobile light harvesting complex from the highly fluorescent PSII to the weakly fluorescent PSI, and the reverse when there is too much excess light in PSI; and (3) the slowest (minutes to hours) qI due to photoinhibition of photosynthesis (Niyogi 1999). At moderate light intensities, the contribution of both qT and qI is usually negligible in higher plants, whereas qE accounts for most of the NPQ (Baker 2008; Nilkens et al. 2010). Both at 110 µmol photons m^{-2} s^{-1} and 336 µmol photons m^{-2} s^{-1}, used in our work, the increase in NPQ after the onset of actinic illumination was lower in the transgenics as compared to that in the vector controls (Fig. 3c, d); this clearly indicates that the transgenic plants dissipate lower amount of the absorbed energy by NPQ (see Murchie and Niyogi 2011). Further, the NPQ relaxed at much slower rate in PEPC overexpressors; here, the total carotenoid content was higher than in the vector controls. A larger xanthophyll cycle pool is known to retard both the kinetics of formation and relaxation of qE (Johnson et al. 2008). In our experiments, we have observed that the estimated ratio of qE to qT + qI was 1:1 in the vector control, but 1:9 in the transgenics. We do not yet understand the reasons for this difference; further experiments are needed to answer this question.

Chlorophyll fluorescence induction curves normalized at F_o reveal that the PEPCx43 has a relatively lower J value (in the OJIP transient) than the vector control (Fig. 6b). This decrease in O-J rise in the transgenic plant is likely to be due to increased Q_A^- re-oxidation by Q_B (Stirbet and Govindjee 2011). However, the faster rise from J to I may be due to a decrease in electrons being transferred to Photosystem I by the intersystem electron acceptor components (Fig. 6b) (cf. Yusuf et al. 2010).

Carbon assimilation

Our data clearly show that, in response to PEPC overexpression, the light-saturated photosynthetic carbon assimilation, on a leaf area basis, increased significantly in PEPCx plants. As shown in Fig. 4a, transgenic plants had higher assimilation rates than vector control plants. The CO_2 assimilation of vector control and transgenic plants grown under 75 µmol photons m^{-2} s^{-1} saturated at 400 µmol photons m^{-2} s^{-1}. These results clearly show and confirm earlier observations (Jiao et al. 2002) that whole-plant photosynthesis can be increased via PEPC overexpression in C3 plants. For increased CO_2 assimilation, NADPH and ATP must have been provided by higher

light-dependent electron transport in the transgenics (Fig. 3a). Higher total protein content in PEPCx plants, as shown in Table 1, is expected to have contributed to RuBP regeneration in the carbon reduction cycle. PEPC is involved in organic acid metabolism in guard cells and that it remains phosphorylated during stomatal opening to regulate stomatal conductance (Outlaw et al. 2002; Vavasseur and Raghavendra 2005). Further, higher stomatal conductance in the transgenics might have contributed to their enhanced photosynthesis efficiency.

The quantum yield of photosynthetic CO_2 assimilation, measured in limiting light intensities, was almost similar in both the vector controls and the transgenics (Fig. 4b). Further, when grown in low-light intensities (75 µmol photons m^{-2} s^{-1}), both the vector control and the transgenic plants had low-light compensation points. Saturation of photosynthetic carbon reduction at lower light intensity has been observed in shade plants (Nobel 1976; Powles and Critchley 1980; Ellsworth and Reich 1993). Slightly higher light compensation point in PEPCx43 is because it has higher rate of respiration (Fig. 4b).

Our study reveals that photosynthetic carbon assimilation is significantly higher (14–18 %) in the transgenic plants than in the vector controls (Fig. 4a). Almost similar results have been obtained in PEPC transgenic rice (Bandyopadhyay et al. 2007). Our present results demonstrate that although *Arabidopsis* transformants had 10-fold increased PEPC activity, they had only 18 % higher CO_2 assimilation rate than the vector controls. These observations are apparently similar to those of Ku et al. (1999), where overexpression of native PEPC in rice had demonstrated a 100-fold increase in PEPC activity, but only a 20 % enhancement of carbon assimilation. However, a detailed analysis of the above PEPCx rice plants by the same authors indicated that the native maize PEPC actually did not contribute to higher photosynthetic CO_2 fixation of the transgenic plants (Fukayama et al. 2003). This suggests that very high activity (100 fold increase over WT) of PEPC alone in PEPCx plants does not necessarily lead to higher CO_2 assimilation. Similarly, overexpression of maize PEPC cDNA in tobacco was shown to result in a 2-fold increase in PEPC activity but without any change in photosynthetic carbon assimilation (Hudspeth et al. 1992). Further, although overproduction of PEPC in rice resulted in 2–30-fold higher PEPC activity than that of non-transformants, it had a lower capacity of CO_2 assimilation (Agarie et al. 2002). These differences could be due to differential integration of the transgene into the host genome in addition to other physiological conditions. Furthermore, higher carbon assimilation efficiency, and apparently higher respiration rates, seem to have contributed to increased plant growth, fresh weight, starch content, and dry matter accumulation in the transgenics in the studies presented in this paper.

Generally, C4 and CAM plants are better adapted to arid environments since they have higher water use efficiency (WUE) than the C3 plants (Long 1999). C4 plants have higher photosynthetic efficiency than C3 plants, in arid, hot, and under high-light conditions, since they possess an additional carbon fixation pathway and characteristic anatomy to limit photorespiration (Long 1999; Sage et al. 1999). Although carbon assimilation rate had increased, no change in WUE was observed in this study. This demonstrates that simply the overexpression of PEPC may not convert a C3 plant into a C4 plant. We believe that our results demonstrating improvement of photosynthesis is in the right direction to meet the global needs (Kirst et al. 2014; Ort et al. 2015).

Effects of salt treatment

PEPCx plants are known to be tolerant to water stress (Ding et al. 2012b). The PEPCx plants not only had higher photosynthesis and plant productivity, but they were relatively resistant to salt stress. In this study, we have compared the impact of ionic imbalance caused by salinity over and above the osmotic stress. In salt-treated plants, photosynthetic pigments, i.e., Chl content declined in the vector control plants, whereas they were substantially higher in the transgenics. Turan and Tripathy (2015) have shown that salt stress causes down-regulation of Chl biosynthesis due to decreased synthesis of 5-aminolevulinic acid (ALA) and its subsequent metabolism to Chl by impairing activities of 5-aminolevulinic acid (ALA) dehydratase, porphobilinogen deaminase, coproporphyrinogen III oxidase, protoporphyrinogen IX oxidase, Mg-protoporphyrin IX chelatase, and protochlorophyllide oxidoreductase. S. Turan and B.C. Tripathy (unpublished, 2015; personal communication) have shown that salinity-induced suppression of Chl biosynthesis is lower in salt-tolerant and higher in salt-sensitive rice cultivars. Experiments with sunflower callus and rice plants (dos Santos and Caldeira 1999; Santos 2004; Akram and Ashraf 2011; Turan and Tripathy 2015) have shown that salt stress markedly affects Chl biosynthesis over Chl breakdown; our results show promise of improvement in the right direction.

Addition of 150 mM NaCl to both the vector controls and the transgenics led to a large decrease in the amplitude of the fluorescence intensity during the O-P transient, although to a smaller extent in the PEPCx43 (Fig. 6b). The O-J rise represents the conversion of absorbed light energy to the trapping (TP) flux, i.e., the energy flux leading to the reduction of the electron acceptors of PS II (Yusuf et al. 2010). Figure 7a shows a doubly normalized curve between the "O" and the "J" levels; the shape of this curve is sigmoidal in both the vector control and the transgenics

(see Fig. S2 in the Supplementary Material), suggesting that the cooperativity between the PSII units remained essentially unchanged (Stirbet 2013). The decline in O-J was higher in salt-treated vector control plants than in PEPCx43 plants (Fig. 6b) suggesting a better trapping efficiency in salt-treated transgenics. In salt-treated samples, the J-I rise was higher in PEPCx43 plants than vector control suggesting a better efficiency of the electron transport (ET) beyond Q_A (Fig. 6b). However, the faster IP rise has been associated, in a complex manner, with better photosynthetic potential, and, thus, higher productivity in rice (see Hamdani et al. 2015). In our experimental conditions, transgenics that have higher carbon assimilation efficiency and increased biomass did not show any significant change in the IP rise than in the vector controls. This needs further investigation.

The term PI (Abs) denotes the photosynthetic performance on the basis of light absorption; it has been considered to be one of the most sensitive parameters of the OJIP curve as it includes in its calculation—besides maximum quantum yield of primary photochemistry, the probability to move an electron further than Q_A^-, the ability to reduce an electron acceptor at the end of PS I, and RC/ABS ratio, i.e., Q_A^- reducing RCs per PSII antenna Chl (Strasser et al. 2000, 2004). In control samples, the PI in transgenics was higher than in the vector control (Table 5) suggesting better overall higher potential for energy conservation due to higher energy transduction process in the overexpressors. In salt-treated transgenics, as compared to the vector controls, a higher PI (Table 5) suggests improved stability of their PSIIs. Compared to the vector controls, the relative degradation of Chls in salt-treated samples was lower in the transgenics (Fig. 5a). The calculated RC/ABS ratio was higher in salt-treated transgenics due, perhaps, to comparatively higher stability of reaction centers. This might have contributed to higher PI in the transgenics subjected to salt stress. In spite of these results, the meaning and interpretation of calculated PI remains to be proven with future experiments.

Strasser et al. (2004) have suggested that the K-band (at 300 μs), in the difference fluorescence kinetics, when it is positive, reflects, an inactivation of the oxygen-evolving complex (OEC), especially of the Mn-protein complex. We have observed here that the salt treatment led to an increase in the K-band both in the vector controls as well as in the transgenics, although the increase in the transgenics was smaller than in the vector controls (Fig. 7a). This is, in all likelihood, due to salt-induced inactivation of the Mn-oxygen-evolving complex, which seems to be less in the transgenics than in the vector controls.

The difference in fluorescence transients from the O to the K level between salt-treated and control samples of the vector control and the transgenics revealed a positive band, called L, at ~150 μs (Fig. 7b, inset). This suggests, according to Strasser et al. (2004), a higher positive connectivity of PSII units in salt-treated transgenic plants than in the vector controls; a higher connectivity results in a better utilization of the excitation energy, and a higher stability of the system (see Stirbet 2013). However, our data, presented in the Supplementary Fig. S2, during the O to P transient, do not reveal any significant differences in connectivity between the PSII units.

Salt-stress-induced inhibition in plants is often ascribed to their reduced photosynthetic performance (Wu et al. 2010; Akram and Ashraf 2011; Silva et al. 2011). Reduced Chl content in vector controls resulted in decreased F_o in salt-treated samples. The value of F_m declined drastically in salt-treated samples, indicating that salt stress caused damage on the electron donor side of PSII (see e.g., Mohanty et al. 1974). The F_v/F_m ratio, a measure of the quantum efficiency of PSII, in dark-adapted leaves, declined in vector controls and transgenic plants when grown in 150 mM NaCl; however, the decrease in transgenics was less than in the vector controls (Table 5). In fact the estimated ETR was higher in the transgenic plants, especially under salt-stress conditions.

The PSII-dependent photosynthetic ETR (μmol electrons m^{-2} s^{-1}) increased with increasing light intensity. However, salinity stress decreased ETR at high light (200–420 μmol photons m^{-2} s^{-1}) both in the vector controls as well as in the transgenics (Fig. 8a); this decrease, which may be due to the damage of the reaction centers, was smaller in the transgenics than in the vector controls.

The NPQ, which measures the dissipation of absorbed light energy as heat, was lower in 150 mM salt-stressed transgenics than in the vector controls (Fig. 8b). These results suggest that the absorbed light energy was better utilized in photochemical reactions in the transgenics, than in the vector controls, under salt stress conditions. From the data that could be related to the overall structure (Fig. 9e; lower panels), we suggest that better photosynthetic function of the overexpressors, under salt stress conditions, may have been due to better stacking of thylakoids in grana, and preserved membrane architecture. Under identical conditions, organization and architecture of thylakoid membranes were grossly affected in the vector control plants, which limited their photosynthesis potential (Fig. 9e).

We consider it highly likely that in the transgenics, overexpression of ZmPEPC led to higher fixation of CO_2 to produce oxaloacetate, which eventually provided carbon skeletons for the synthesis of an important osmolyte proline that, perhaps, increased the capacity of osmotic adjustment in salt-stressed plants (Delauney and Verma 1993). Proline is an osmoprotecting molecule that is known

to accumulate in response to environmental stress (Delauney and Verma 1993; Khedr et al. 2003; Claussen 2005; Gleeson et al. 2005; Younis et al. 2009; Gururani et al. 2015). When treated with salts, proline content in both the transgenics and the vector controls increased. However, proline content in the transgenic plants increased much more than in the vector controls, and was significantly higher than that in salt-treated vector controls (Fig. 9a). Although proline can be synthesized from either glutamate or ornithine, glutamate is the primary precursor in osmotically stressed cells (Boggess et al. 1976; Rhodes et al. 1986), and less than 15 % of glutamate, which is converted to proline derives from pre-existing protein (Venekamp and Koot 1988). Formation of glutamate requires an anaplerotic reaction that is catalyzed by phosphoenolpyruvate carboxylase (Melzer and O'Leary 1987). Therefore, synthesis of excess proline is a key step involved in tolerance of PEPCx plants to environmental stress.

Antioxidant content and the activities of ROS scavenging enzymes have been correlated with tolerance to environmental stress, while the MDA content could reflect the peroxidation level of plant cell membrane (Ding et al. 2012a). Finally, our results (shown in Fig. 9b–d) suggest that higher activity of ROS scavenging enzymes, i.e., of catalase and peroxidase, in the transgenics may lead to efficient detoxification of active oxygen species and reduced membrane lipid peroxidation and MDA content under stressful environment.

Concluding remarks

Our paper deals with the beneficial effect of overexpressing a C4-type PEPC from maize on the components and the function of *A. thaliana*, a C3 plant. In the transformants, especially in PEPCx43, we observed a significant increase in the PEPC activity, increased electron transfer rate, increased CO_2 assimilation, increased stomatal conductance, and even increased size and fresh weight, but importantly, increased tolerance to salt stress. Further, we observed (i) a small but significant increase in the total amino acids, total chlorophyll and, total protein content; (ii) unchanged variable to maximum fluorescence (F_v/F_m), suggesting no effect on the quantum yield of photosystem II; (iii) higher stability of the system, as reflected in the ultrastructure of the grana; (iv) a decrease in the NPQ of the excited state of Chl; (v) higher proline level; (vi) higher levels of catalase and peroxidase in salt-stressed transgenic line; and (vii) lower lipid peroxidation. We cannot and do not claim that we have converted a C-3 plant into a C-4 plant, but we have certainly produced transformants that grow better, and have increased tolerance to salts, such as NaCl. Since the overall problem is highly complex, we refrain from making speculations concerning the role of mitochondria and respiration in this study (see e.g., Fukayama et al. 2003). Further research is needed to exploit the full potential of our research.

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