Overexpression of cytoplasmic C4 Flaveria bidentis carbonic anhydrase in C3 Arabidopsis thaliana increases photosynthetic potential and biomass

Deepika Kandoi[1], Kamal Ruhil[1], Govindjee Govindjee1[2], and Baishnab Tripathy[1]

[1]Jawaharlal Nehru University
[2]University of Illinois at Urbana-Champaign

March 30, 2022

Abstract

One of the important ways to improve photosynthetic capacity in C3 crops, such as rice and wheat, is to transfer efficient C4 characters to them. Here, cytosolic carbonic anhydrase (βCA3) of the C4 Flaveria bidentis (Fb), having low Km for CO2, was overexpressed under the control of 35S promoter in Arabidopsis thaliana, a C3 plant, to enhance its photosynthetic efficiency. Overexpression of CA resulted in higher [HCO3-] in the cytosol of the overexpressors, and increased endogenous phospho-enolpyruvate carboxylase activity generating oxaloacetate that feeds into the tricarboxylic acid cycle. This provided more carbon skeleton for increased synthesis of amino acids and proteins. Further, transgenic expression of FbβCA3 in Arabidopsis led to pleiotropic expression of several genes/proteins involved in chlorophyll biosynthesis and photosynthesis leading to higher chlorophyll content and photosynthetic capacity in the transformants. Due to the presence of higher CO2 in the chloroplast, pleiotropic effect overexpressors had enhanced CO2 assimilation, starch content, and plant dry weight. In addition, transgenic plants had lower stomatal conductance, reduced transpiration rate and higher water use efficiency. These results, taken together, show that expression of C4 CA in the cytosol of a C3 plant can indeed improve its photosynthetic capacity with enhanced water use efficiency.

Regular paper

Overexpression of cytoplasmic C_4 *Flaveria bidentis* carbonic anhydrase in C_3 *Arabidopsis thaliana* increases photosynthetic potential and Biomass

Deepika Kandoi[1]· Kamal Ruhil[1]·Govindjee Govindjee[2,#]·Baishnab C. Tripathy[1, 3*]

[1]School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India

[2]Department of Plant Biology, Department of Biochemistry, and Center of Biophysics & Quantitative Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

[3]Department of Biotechnology, Sharda University, Greater Noida 201306, UP, India

[#]Since 2019, Govindjee's legal name is Govindjee Govindjee

*Corresponding author's email is: baishnabtripathy@yahoo.com

Running title: Boosting photosynthesis by C4 carbonic anhydrase

Address for correspondence

Baishnab C. Tripathy

Department of Biotechnology, Sharda University, Greater Noida 201310, UP, India

This work was supported by the National Agriculture Innovation Project (grant number: PAC-SLS-BCT-NAIP-09080312-371 to BCT) from the Indian Council Agriculture Research (ICAR) and BSR Faculty Fellowship to BCT, and by the National Post-Doctoral Fellowship (PDF-2017-000632) to DK from the Science and Engineering Research Board, Government of India.

Acknowledgments

Govindjee thanks the School of Life Sciences, Jawaharlal Nehru University for their hospitality during his stay when the experiments were being done and when the paper was written and finalized.

Summary statement (max. 300 characters with spaces).

Expression of C_4 carbonic anhydrase from *Flaveria bidentis* in the cytoplasm of C_3 *Arabidopsis thaliana* significantly improves its photosynthetic capacity and biomass, with enhanced water use efficiency.

Author Contributions

BCT planned and designed the experiments; DK performed the experiments, and analysed the data; BCT, DK, GG and KR wrote the paper.

Conflict of interest statement

There is no known conflict of interest

Abbreviations

APT Adenosyl phosphoryl transferase;

CaMV Cauliflower mosaic virus;

CA Carbonic anhydrase;

C_c Chloroplast CO_2 concentration;

Chl Chlorophyll;

ETR Electron transport rate;

ETRI Electron transport rate of PSI, see text for details;

ETRII Electron transport rate of PSII, see text for details;

F_m Maximum Chl fluorescence;

F_o Minimum Chl fluorescence;

F_v Variable Chl fluorescence,F_m -Fo;

g_s Stomatal conductance;

g_m Mesophyll conductance;

LED Light-emitting diode;

nptII Neomycin phosphotransferase (kanamycin resistance gene);

PAR Photosynthetically active radiation;

PBGS Porphobilinogen synthase;

PEPC Phosphoenolpyruvate carboxylase;

PFD Photon flux density;

PORC Protochlorophyllide oxidoreductase;

PPOX Protoporphyrinogen oxidase;

PSI Photosystem I;

PSII Photosystem II;

P700 Reaction centre chlorophyll of PSI, with absorption maximum at 700 nm;

rubisco Ribulose 1, 5-bisphosphate carboxylase/oxygenase;

TCA Tricarboxylic acid cycle;

UROD Uroporphyrinogen decarboxylase;

VC Vector control;

WUE Water use efficiency.

Key words: *Arabidopsis thaliana* , Carbonic anhydrase, C4 photosynthesis, Photosystem I, Photosystem II, CO_2assimilation, Water Use Efficiency

Abstract

One of the important ways to improve photosynthetic capacity in C_3 crops, such as rice and wheat, is to transfer efficient C_4 characters to them. Here, cytosolic carbonic anhydrase (*βCA3*) of the C_4*Flaveria bidentis* (*Fb*), having low Km for CO_2, was overexpressed under the control of 35S promoter in *Arabidopsis thaliana* , a C_3 plant, to enhance its photosynthetic efficiency. Overexpression of *CA* resulted in higher [HCO_3^-] in the cytosol of the overexpressors, and increased endogenous phosphoenolpyruvate carboxylase activity generating oxaloacetate that feeds into the tricarboxylic acid cycle. This provided more carbon skeleton for increased synthesis of amino acids and proteins. Further, transgenic expression of *ΦββCA3* in Arabidopsis led to pleiotropic expression of several genes/proteins involved in chlorophyll biosynthesis and photosynthesis leading to higher chlorophyll content and photosynthetic capacity in the transformants. Due to the presence of higher CO_2 in the chloroplast, pleiotropic effect overexpressors had enhanced CO_2 assimilation, starch content, and plant dry weight. In addition, transgenic plants had lower stomatal conductance, reduced transpiration rate and higher water use efficiency. These results, taken together, show that expression of C_4 *CA* in the cytosol of a C_3plant can indeed improve its photosynthetic capacity with enhanced water use efficiency.

Introduction

The current global research efforts are actively focusing on increasing crop yield for sustainable food and fuel production (Long et al., 2015). C_3 photosynthesis is limited by available CO_2. Therefore, it is important to pump more CO_2 into the leaf cells to alleviate carbon limitation in C_3 plants. We consider it to be an important option to enhance crop productivity by manipulating carbonic anhydrase (CA, EC 4.2.1.1), which is mostly a zinc-containing metalloenzyme that catalyses the inter-conversion of CO_2 and HCO_3^- and is widely distributed in eukaryotes and prokaryotes (Hewett-Emmett and Tashian, 1996; Liljas and Laurberg, 2000; Moroney et al., 2001; Bonacci et al., 2012). *CAs* belong to 6 independent gene families (Moroney et al., 2011): *α-CAs* , *β-CAs* , *γ-CAs* , *δ-CAs* ,*ϵ-CA* and *ζ-CA* . In higher plants, only *α-CA* ,*β-CA* , and *γ-CA* exist. In spite of their structural differences, these 3 isoforms share the same general catalytic mechanism (Lindskog, 1997). In C_3 plants, up to 2% of the total leaf protein is CA and 95% of the total CA activity is in chloroplast stroma (Okabe et al., 1984; Tsuzuki et al., 1985). Further, in C_3 plants, chloroplastic *CA* is responsible for maintaining CO_2 around **r** ib**u** lose**bis** phosphate **c** arb**o** xylase/**o** xygenase (*rubisco*) by facilitating its diffusion across the chloroplast envelope, after its formation from bicarbonate (Giordano et al., 2005). Without the *CA* , the hydration reaction of CO_2 is usually very slow (Raven, 1997). *CA* also participates in respiration, pH regulation, inorganic carbon transport, ion transport, and water and electrolyte balance (Tashian, 1989; Henry, 1996; Smith and Ferry, 2000; Badger and Price, 2003). In *Arabidopsis thaliana* ,*βCAs*

are responsible for the uptake of HCO_3^- from soil by the roots. Bicarbonate absorbed by roots is transported to stem and leaves and is assimilated by *rubisco* or phosphoenolpyruvate carboxylase,*PEPC* (Dabrowska-Bronk et al., 2016). High light and long photoperiods favor rubisco activation leading to higher biomass. Conversely, low light and short photoperiods activate PEPC in C_4 plants (Bailey et al., 2007). Further, it has been suggested that a thylakoid CA may be involved in Photosystem II reactions (Stemler, 1997; Shevela et al., 2012). In the cytoplasm of C_4 mesophyll cells, *CA* is known to catalyse the very first reaction in its carbon fixation pathway (Hatch and Burnell, 1990), which is the hydration of CO_2 producing HCO_3^- , the latter being the substrate for *PEPC* (also see Ludwig, 2012). Plants with C_4 photosynthesis are efficient in carbon hydration and assimilation and have an advantage over C_3photosynthesis. In the recent past, there have been several attempts to overexpress or regulate C_4 pathway genes into C_3 plants (for a review, see Miyao et al., 2011; Schuler et al., 2016; for attempts from our Lab, see: Kandoi et al., 2016, 2018; Borba et al., 2018; Lin et al., 2020). To enhance CO_2uptake by C_3 plants, carbonic anhydrase content needs to be substantially increased in the cytosol. Most studies have attempted to overexpress C_3 *CA* in the cytosol or chloroplasts of C_3 plants (Hu et al., 2010; Pal et al., 2015); however, this has not resulted in enhancing their rates of photosynthesis. Further, *Arabidopsis* double knockout mutants of the cytosolic *β-ϡA2* and *β-ϡA4* had even reduced growth rates and chlorosis of the younger leaves relative to that in the wild-type plants, when grown at low concentration of CO_2(DiMario et al., 2016). Amino acid sequence alignment of cytoplasmic*Flaveria bidentis* (C_4 plant) *βϡA3*(*ΦββϡA3*), with different isoforms of *Arabidopsis thaliana* (C_3 plant) *βϡAς* (*AτβϡAς*), demonstrates that all the sequences have similar amino acid binding sites for Zn^{2+} as well as for the substrates (see**Fig. S1**).

A pertinent question that remains to be answered is whether overexpression of C_4 carbonic anhydrase, having low Km for CO_2 (Hatch and Burnell, 1990; Ignatova et al., 1998) and ability to efficiently hydrate CO_2, in the cytosol of C_3 plants, improves photosynthetic efficiency. To explore this question, we have, in this study, overexpressed carbonic anhydrase *βϡA3* from a dicot C_4 plant*Flaveria bidentis* in the C_3 plant*Arabidopsis thaliana* (**Fig. 1**). Our results show that overexpression of *ΦββϡA3* in the C_3 plant results in improved photosynthesis, water use efficiency, higher starch content and biomass.

Typesetter to place Fig. 1 here

Materials and methods

Sequence analysis

Homology search was made with **b** asic **l** ocal**a** lignment **s** earch **t** ool (BLAST) by using Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalo/). Signal sequence predictions were made by Target P (http://www.cbs.dtu.dk/services/TargetP/). Nucleotide sequence data were taken from the database at the following website: <http://www.ncbi.nlm.nih.gov/>.

Generation of transgenic and growth conditions

The full length *ΦββϡA3* (Accession no-AY167113) was cloned in pGEM-T Easy vector and subsequently, in modified pCAMBIA1304 (Pattanayak and Tripathy, 2011), and then transformed in *Arabidopsis* using agrobacterium-mediated floral dip method, as described by Kandoi et al. (2016) (see **Table S1** for details of the primer). Vector control (VC) plants containing the null vector, pCAMBIA1304 (binary vector without *ΦββϡA3* cDNA) were also generated for our research. Seeds of the transformed plants were screened on half-strength Murashige and Skoog (MS) agar medium containing 50 μg/ml kanamycin; these were grown up to T4 generation. Stratified *Arabidopsis* seeds were sown in agropeat: vermiculite (1:4) mixture in pots and grown under cool-white-fluorescent light (100 μmol photons $m^{-2}s^{-1}$), under 14 h light/10 h dark photoperiods, at 21±1ºC.

Confirmation of transgenic lines by polymerase chain reaction (PCR) and Southern blot analysis

Genomic DNA was isolated by **c** etyl **t** rimethyl**a** mmonium **b** romide (CTAB) method (Nickrent, 1994) from 4-week old plants of the T1 generation. The presence of trans-gene in the plants was confirmed by PCR using 35S forward internal primer and*ΦββˆA3* reverse primer to ensure the incorporation of the whole cassette in sense orientation. The presence of *ΦββˆA3* transgene was analysed by Southern blot analysis, as described by Kandoi et al. (2016).

qRT-PCR

Total RNA was extracted from leaves of 4-week old transgenic and VC plants, using the trizole method (Sigma-Aldrich, USA). RNA samples were reverse transcribed into cDNA, using the first strand cDNA synthesis kit from Thermo Fisher Scientific, according to manufacturer's instructions.

Relative expression of different genes was studied using qRT-PCR on ABI Prism 7500 Sequence Detection System (Applied Biosystems, USA), and the design of the primers was based on sequence details (**Table S2**). Adenine phosphoribosyl transferase (APT1) was used as a housekeeping gene. The relative gene expression data were analysed using the $2^{-\Delta\Delta^{\wedge}\tau}$ quantitation method (Livak and Schmittgen, 2001).

Western blot analysis

For immunoblot analysis, total protein was extracted from 4-week-old leaves of plants (Jilani et al., 1996). Protein concentration of the supernatants was measured, as described by Bradford (1976). Electrophoresis was carried out on a 15% (w/v) SDS-PAGE gel using a total of 25 µg of soluble plant protein per lane. Separated polypeptides were blotted on nitrocellulose membranes (Jilani et al., 1996). Proteins were probed with a primary antibody from Imgenex (India). 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-**b** romo-4-**c** hloro-3-**i** ndolyl**p** hosphate (BCIP) and **n** itro **b** lue**t** etrazolium (NBT) and quantified using an Alpha Imager 3400.

CO_2 hydration activity

CO_2 hydration activity was determined, using an electrometric method, in 5ml of 20 mM Tris (pH 8.3) at 0^{o}C. This reaction was started by the addition of 1.5 ml of ice-cold CO_2 saturated water to the enzyme solution. The activity was calculated as µmol CO_2hydrated min^{-1} (mg Chl)$^{-1}$ mg Chl^{-1} using the following equation:

CA activity = $[2\times(T_0\text{-}T)]/[$Tx amount of Chl in reaction mixture (mg)$]$,

where, T_0 and T are the times for pH drop from 8.3 to 6.8 for the non-enzymatic and the enzymatic reactions, respectively (Wilbur and Anderson, 1948).

To eliminate the non-enzymatic CA reaction, the enzyme extract was inactivated at 95oC and the CA activity was determined. The heat-denatured CA had negligible activity.

Estimation of Chlorophyll, protein and free amino acids

Chl was extracted in 80% acetone and estimated as described by Porra et al. (1989). The leaf soluble protein was measured according to Bradford (1976). Free amino acids were analysed by the ninhydrin colorimetric method, using leucine as a standard (Misra et al. 1975).

PEPC activity

PEPC activity was measured in leaves of VC and FbβCA3 overexpressor plants, as described by Kandoi et al. (2016). Enzyme specific activity was expressed as µmol of NADH oxidised per mg of protein per hr.

Whole-chain (WC), PSII, and PSI reactions

Thylakoid membranes were isolated in isolation buffer containing 0.01M Tris and 1 mM EDTA (pH 7.5). The homogenate was centrifuged at 12,000xg for 5 min and the pellet containing thylakoid membrane was suspended in suspension buffer containing 0.4 M sorbitol, 0.05 M Tris (pH 7.5), 1 mM MgCl2 and 1 mM

EDTA (see Sood et al., 2005; Gupta and Tripathy, 2010). Using thylakoid membranes isolated from VC and CAx plants, we monitored (a) the light-saturated whole chain (WC) electron transport, from water to methyl viologen [MV]); (b) the partial reaction of PSII from water to phenylenediamine [PD], and (c) that of PSI from ascorbate/ dichlorophenol-indophenol to MV, using a Clark type oxygen electrode.

Pulse amplitude modulation (PAM) measurements

Chl *a* fluorescence (for PSII activity) and transmission changes for PSI activity (at 830nm) were measured simultaneously by the Dual-PAM-100 measurement system using the automated "Light Curve" program provided by a software from Walz (For details see Yuan et al., 2014).

Handy PEA

Chl *a* fluorescence induction was measured using Handy PEA (**P** lant **E** fficiency **A** nalyzer), Hansatech Instruments, UK. Arabidopsis seedlings were pre-darkened for 20 min at room temperature (for details, see Kandoi et al. (2016), and references therein) before fluorescence measurements.

Light response curve

Light response curves of photosynthesis of six-week old VC and transgenic plants, grown under 10h L/14h D, in soil, were measured using**i** nfra**r** ed **g** as **a** nalyser, IRGA (LiCor 6400XT), with 6400-18A RGB (Red, Green, Blue) light source at different light intensities. Sample chamber CO_2 concentration was maintained at 400 µmol mol^{-1}. The air temperature in the sample chamber was maintained at 25°C, and the relative humidity at 60%.

A/C_i curve (net CO_2 assimilation rate, A, versus calculated internal CO_2 concentrations, C_i)

A/C_i curves of VC and transgenic plants, grown in soil, were measured using IRGA, as described above. Light intensity in the chamber was maintained at 400 µmol photons $m^{-2}s^{-1}$, using 6400-18A RGB (Red, Green, Blue) light source, whereas, the temperature was maintained at 25°C. To obtainA/C_i curves, we measured CO_2assimilation, at different CO_2 concentrations. For the measurements at 2% O_2, a gas cylinder containing a mixture of 2% O_2 and 98% N_2 was used. The gas from the cylinder was humidified and supplied to the LiCor 6400XT, where CO_2 was mixed with the above gas mixture. The *CE* was calculated from the slope of theA/C_i curve (Li et al., 2009).

Estimation of mesophyll conductance (g_m) and CO_2 concentration in the chloroplast (C_c)

Estimation of g_m was made by the method of Harley et al. (1992), using the following equation:

$$J = 4\,(A + R_L)\,(C_i - A_n/g_m) + 2\,\Gamma^*/\,(C_i - A/g_m) - \Gamma^*,$$

where, J is photosynthetic electron transport, A - net CO_2 assimilation, C_i- intercellular [CO_2], R_L- the rate of mitochondrial respiration in the light, Γ^*- the CO_2 compensation point in the absence of R_L (Evans & Loreto, 2000), and the factor 4 is for the minimum electron requirement for carboxylation. Values for J were derived from Chl fluorescence. The CO_2 concentration in the chloroplast (C_c) was measured by using$C_c = C_i - A_n/g_m$ (Pons et al., 2009).

Starch content

Leaf samples were collected immediately prior to the end of light period for the measurement of starch content. Samples were digested with perchloric acid, and then the starch was assayed spectrophotometrically, using anthrone, a color reagent (Rose et al., 1991).

Growth parameters

VC and *ΦββΫA3* overexpressor plants were grown vertically for three weeks in MS medium in petri-dishes, and then used for measuring root length, shoot length and rosette diameter. For dry weight measurements, whole plants were first kept in an oven at 80^{o}C for 72 h.

Statistical Analysis

Statistical analysis was performed by using Microsoft Excel. After the calculation of averages, standard deviations and standard errors for each of the growth parameters were determined. A 't' test was used to assess the difference between VC and transgenic plants for all the parameters.

Results

Σεχυενχε αλιγνμεντ οφ *Φλαερια βιδεντις βˇA3* ωιτη*Αραβιδοπσις τηαλιανα βˇA* ισοφορμς

A comparison of the amino acid sequence of *Αραβιδοπσις τηαλιανα βˇA* (*ΑτβˇA)* isoforms with *βˇA3* of *Flaveria bidentis* demonstrates that six isoforms of *βˇA* are present in*Arabidopsis thaliana,* out of which three are cytosolic, two chloroplastic, and one mitochondrial having 57%-72%, 36%-59% and 33% homology with *ΦββˇA3* **(see Table S3**). The catalytic mechanism of the enzyme is highly conserved in the *ΑτβˇA´ζ*sequences and *ΦββˇA3* (**Fig. S1**).

Transformation

In order to overexpress cytoplasmic C_4 carbonic anhydrase into C_3 plants, we cloned *ΦββˇA3* into a transformation vector pCAMBIA1304 (**Fig. 2a**). Recombinant pCAMBIA1304:: *ΦββˇA3* was then used for *Agrobacterium*-mediated *Arabidopsis* transformation.

Typesetter to place Fig. 2 here

Genomic DNA analysis of transgenic plants

Genomic DNA was isolated from different transgenic *Arabidopsis*lines overexpressing *ΦββˇA3* , and then the *ΦββˇA3* was amplified by PCR using 35S forward and *ΦββˇA3* specific reverse primers that yielded a fragment of ˜0.8 kb of*ΦββˇA3* suggesting that the transgene had been integrated into to the host genome **(Fig. S2a)** . In order to confirm the insertion of the binary vector, without the gene in VC plants, kanamycin (*nptII*) gene was amplified by PCR, using *nptII* specific forward and reverse primers. Our PCR results showed that a fragment of ˜0.8 kb from the transformants contained *nptII*(**Fig. S2b**). These confirmed individual transgenic lines were then grown to harvest seeds. Seeds collected from these plants were grown again in kanamycin plates to select T2 transgenic lines. Transgenic seeds were then grown to get T4 generations in order to obtain homozygous transgenic plants for further use.

Southern blot analysis

The number of integrations of the T-DNA cassettes containing*ΦββˇA3* cDNA in the *Arabidopsis thaliana* host genome was checked by southern blot analysis, using the *nptII* probe. The transgenic lines CAx2, CAx3 and CAx5 showed single bands confirming single integration of the transgene into the *Arabidopsis*genome. The CAx6 line showed a double band, which means that here, there was a two-time integration of the transgene into the host genome (**Fig. S2c**). VC showed the presence of a single copy of T-DNA cassette and, thus, there was no band of *nptII* in the wild type (WT).

Phenotype of plants

Figure 2b shows a photograph of a four-week old VC as well as that of CA overexpressed plants. Visually, plants of CAx3 and CAx5 transgenic lines show better growth than those of VC.

Gene expression

The qRT-PCR analysis, using gene-specific primers, showed that the expression of the *ΦββˇA3* varied among the different transgenic lines. The transcript abundance in CAx1, CAx2, CAx3, and CAx5 was 1.5, 1.2, 2 and 2.7 fold higher, respectively than that in the VC (**Fig. 2c**).

Immunoblot analysis

To check if the transgenic plants had the expected changes in their protein expression, we did western blot analysis. Total protein was isolated from 4-week-old *ΦββˇA3ξ* lines. Equal loading of the protein (25 μg) was checked by running 15% SDS-PAGE (**Fig. 2d).**Polyclonal antibodies, raised against *ΦββˇA3* protein, were

used to immuno-detect the CA protein in different *ΦββΫA3ξ* lines**(Fig. 2e).** In the transgenic lines, the CA protein (˜28 kD) expression was 1.3 to 2.1 fold higher than in the VC **(Fig. 2f)** . The CA protein was also present in the VC and showed the expected band in the blot, which was due to the high homology of *Aτβ Ϋ΄ς* with *ΦββΫA3* .

Enzyme activity of carbonic anhydrase

CA activity in VC was 276 µmol CO_2 hydrated (mg Chl)$^{-1}$ min^{-1}; this activity, in different transgenic lines, ranged from 348 to 550 µmol CO_2 hydrated (mg Chl)$^{-1}$min^{-1}. Thus, the CA activity of the transgenic lines was 1.3 to 2.0 fold higher than in the VC **(Fig. 2g).**

Based on their higher transgene expression, protein abundance and enzymatic activity, CAx3, and CAx5 transgenic lines were selected for further studies.

Typesetter to place Table 1 here

Chlorophyll

The CAx3 and CAx5 transgenic lines had 11% to 15% higher total Chl than the VC plants **(Table 1).** However, the Chl a/b ratio remained similar both in the VC and in the transgenics **(Table 1)** .

Protein

The total free amino acids increased by 12% in CAx3 and 16% in CAx5 lines. Further, the protein content of CAx3 and CAx5 was 9% -12% higher than in the VC plants **(Table 1)** .

Enzyme activity of phosphoenolpyruvate carboxylase (PEPC)

For the in-vitro assay of PEPC, 10 mM sodium bicarbonate was exogenously added to the reaction mixture. Consequently, the PEPC activity remained almost similar in the VC and transgenic plants (˜3.45 µmol (mg protein)$^{-1}$ hr^{-1})

(Fig. 3a).

Typesetter to place Fig. 3 here

PSII, Whole-chain (WC), and PSI reactions

Under saturating light, and on Chl basis, thylakoids from the *CA*overexpressor plants, as compared to that from the VC plants, we observed higher electron transfer rates for PSII (21%-26%), WC (20% - 28%), and PSI (23% - 32%) (**Figs. 3b,c,d**).

Photosynthetic efficiency

To obtain information on the effect of overexpression of *ΦββΫA* on photosynthetic efficiency of *Arabidopsis* , Chl *a*fluorescence was monitored as a non-invasive signature of photosynthesis, especially of PSII (see e.g., Govindjee, 1995; Govindjee, 2004). Various Chl *a* fluorescence parameters were measured as described under *Materials and Methods.* Our results on different parameters of photosynthesis are described below.

Electron transport rate (ETR II and ETR I): At the saturating light intensity (540 µmol photons m^{-2}s^{-1}), the calculated values of ETR II and ETR I were higher by 13% to 19% and 17% to 25% in the transgenics than in the VC plants **(Fig. 3e,f).**

Typesetter to place Figure 4 here

Chl *a* fluorescence induction transient: For these measurements, OJIP curves were recorded up to 2 s, after excitation with 650 nm light of high intensity (3500 µmol photons m^{-2}s^{-1}), as provided by an array of 3 LEDs.**Figure 4a** shows Chl *a* fluorescence transients of dark-adapted leaves of *Arabidopsis thaliana* , plotted on a logarithmic time scale from 20 µs to 2 s; here, all the curves were normalized at Fo. Fluorescence transients of both VC and transgenic plants show typical OJIP curves. The transgenic CAx3 and CAx5

have a similar fluorescence rise from O to J, but a faster rise from J to I and I to P as compared to those in the VC.

To study further differences between the transgenics and the controls, the IP rise was normalized, both at the I level (30 ms) as well as at the P level (290 ms) (see **Fig. 4b**). The normalized I-P curves clearly reveal that the transgenic plants had faster IP rise than the VC. Further, when the OJIP curves were normalized at I only, we observe a faster IP rise in the transgenics than in the vector controls (**Fig. 4c**).

Typesetter to place Table 2 here

F_o : The F_o was measured in dark adapted leaves as the initial minimum fluorescence. As compared to the VC, CAx3 and CAx5 plants had ˜8% and ˜11% higher F_o values, but this must be simply due to higher [Chl] in the transgenics **(Table 2);** there was no difference in the values ofF_o/Chl.

F_m : Maximal fluorescence was measured during the first saturation pulse, after dark adaptation (see Material and Methods). The F_m values of CAx3 and CAx5 plants are observed to be higher by ˜18% and ˜25% **(Table 2);** theF_m/Chl values are 6%-9% higher in the transgenics than in the VC.

F_v/F_m : This ratio is an estimate of the maximum potential quantum efficiency of Photosystem II, if all the reaction centers are open.; this is usually the case when dark-adapted samples are used, as was the case in our experiments; this ratio increased only slightly (˜4%) in the transgenic plants **(Table 2).**

*Area over the OJIP curve***:** This area is betweenF_o and F_m, and is proportional to the size of the pool of the electron acceptors in PSII, mainly the plastoquinone molecules (Malkin and Kok, 1966); we observe it to be higher (19%-22%) in CAx3 and CAx5 than in the VC **(Table 2).**

F_v/F_o*:* This 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 (Burke, 1990), was higher (11%- 16%) in the transgenics than in the VC **(Table 2).**

*PI, the performance index***:** The calculated PI (Tsimilli-Michael et al., 2000) was higher (10%- 20%) in the transgenics than in the VC **(Table 2).**

Non Photochemical quenching (NPQ): NPQ of the excited state of Chl increased with light intensity. At high light intensity (540 µmol photons m^{-2} s^{-1}), the NPQ in CAx3 and CAx5 transgenic lines is slightly lower (5% - 9%) than in the VC

(Fig. 4d).

Typesetter to place Figure 5 here

Gene expression

*Expression of chlorophyll biosynthesis genes***:** To understand the reasons for increased Chl biosynthesis (see above) in*ΦββˆA* overexpressor plants, the expression of a few selected genes involved in Chl biosynthesis pathway was monitored. In comparison with the VC, the message abundance of *PBGS*(porphobilinogen synthase), involved in the synthesis of the pyrrole ring porphobilinogen, was found to be 1.7 and 2.2 fold higher in CAx3 and CAx5, respectively, than in the VC **(Fig. 5a**). The transcript expression of *UROD* (uroporphyrinogen decarboxylase), responsible for the synthesis of coproporphyrinogen, was 2.0 and 2.2 fold higher in CAx3 and CAx5 as compared to VC **(Fig. 5a)** . The gene expression of *PPOX* (protoporphyrinogen oxidase), responsible for protoporphyrin IX synthesis, increased 1.4-3 fold in the transgenics (**Fig. 5a**). The message abundance of *CHLI*(protoporphyrin-IX Mg-chelatase), one of the 3 genes responsible for Mg insertion to protoporphyrin IX moiety, increased by 1.4 and 1.5 fold in CAx3 and CAx5, respectively, than in the VC (**Fig. 5a**). The expression of light-inducible *PORC* (protochlorophyllide oxidoreductase) was 1.8-2.5 fold higher in transgenics than in the VC plants **(Fig 5a)** .

***Expression of photosynthesis-related genes* :** The gene expression of *Lhcb1* and *Lhcb2.1*encoding components, of the light harvesting complex associated with PSII, was 2.1-2.5 and 1.9-2.5 fold higher in

the transgenics**(Fig 5b)** . The gene expression of *Lhca1* and *Lhca2*(encoding components of the light harvesting complex associated with PSI) increased by 2- 3.45 and 1.2 –1.4 fold in the overexpressors**(Fig. 5b)** . Expression of two core proteins PsbA, and, PsbD, encoding photosystem II D1 and D2 proteins, was 3.2-3.6 and 1.7-2.3 fold higher in the transgenics **(Fig. 5b)** . *PsbO* (encoding for OEC33, the oxygen-evolving complex) increased, as compared to that in the VC plants, by 1.4 and 2.4 fold in CAx3 and CAx5 as compared to VC

(Fig. 5b).

Western blots of electron transport chain components

To understand the reasons why PSI and PSII dependent electron transfer rates increased at limiting as well as saturating light intensities in*CAx* plants **(Fig. 3e,f)** , certain components of the Chl biosynthesis, light-harvesting complex, photosynthetic electron transport chain and rubisco were analysed by the western blot. Equal loading of the protein (25 µg) was checked by running 12% SDS-PAGE (**Fig. 5c**). The abundance of Chl biosynthetic enzyme UROD increased by 15% -25% in CAx3 and CAx5 plants (**Figs. 5c,d**). As compared with VC plants, the abundance of LHCII, light-harvesting Chl-binding proteins, associated with PSII, increased by 12%-17% in CAx plants (**Figs. 5c,d**). The inter-system electron transport components between PSI and PSII, i.e., Cytb6f complex, and Cyt f in it, increased by 25%-35% and 16%-20% in CAx3 and CAx5, respectively (**Figs. 5c,d**). Similarly, the oxygen evolving complex protein OEC33 (PsbO) had increased by 22% and 27% in CAx3 and CAx5 (**Figs. 5c,d**). One of the electron transport components of PSI, PsaE (PSIIV) increased by 20%-25% in the overexpressors (**Figs. 5c,d**). The protein abundance of the large subunit (LSU) as well as the small subunit (SSU) of rubisco had also increased by 11%-14% and 8%-11%, respectively, in the overexpressors (**Figs. 5c,d).**

Typesetter to place Figure 6 here

Light response curves for photosynthesis

The photosynthetic light response curves were measured by IRGA (LiCOR-6400/XT), using 6400-18A RGB (red, green, blue) light source, in the attached leaves of VC as well as in transgenic plants, under ambient CO_2 (400 µmol mol^{-1}) and ambient O_2 (21%). At saturating light intensity (800 µmol photons m^{-2} s^{-1}), leaves of CAx3 and CAx5 transgenic plants had 17% and 23% higher net CO_2assimilation rate, than in the VC plants **(Fig. 6a)** . The rate of respiration in the leaves of transgenics, measured in the dark, as compared to the vector control was also higher (˜8% -12%) (**Figs. 6a,b**). The quantum yield of CO_2fixation, as measured at limiting-light intensities (up to 80 µmol photons m^{-2} s^{-1}) was 0.0407 mol mol^{-1} in VC plants, but it increased by 17%- 21% in CAx3 and CAx5, respectively (**Fig. 6b**).

Stomatal conductance (g_s) and water use efficiency (WUE)

The CO_2 assimilation rate, measured at 400 µmol photons m^{-2} s^{-1}, was 14% and 19% higher in CAx3 and CAx5 than in the VC (**Fig. 6c**). It is important to note that increases in photosynthetic rates were associated with decreases in stomatal conductance and transpiration rates in the transgenic lines. Stomatal conductance decreased by 7% - 13% in the transgenics, as compared to VC plants (**Fig. 6d**). Consequently, the transpiration rate decreased by 7% -10% in *ΦββΤA* overexpressor plants (**Fig. 6e**). Therefore, the water use efficiency, which is the ratio of the CO_2 assimilation rate to the transpiration rate (µmol CO_2 $m^{-2}s^{-1}$/mmol H_2O $m^{-2}s^{-1}$) was higher by 22% and 26% in CAx3 and CAx5, respectively, than that in VC (**Fig. 6f**).

Typesetter to place Figure 7 here

A/C_i curve

In order to evaluate the contribution of photorespiration, carbon assimilation rates were measured as a function of the intercellular CO_2 (A/C_i curve) under 21% and 2% oxygen **(Fig. 7a).** In 21% O_2, the transgenic plants had higher photosynthetic capacity (the maximum rate of photosynthesis under CO_2 saturation at saturating light) than the VC plants. At saturating C_i(˜1000 ppm), CO_2 assimilation rate increased by

˜19% in CAx3 and ˜22% in CAx5 transgenic plants than in the VC (**Fig. 7a**). The carboxylation efficiency (*CE*), as calculated from the initial slope of A/C_i curve (i.e. between 50 and 200 μmol mol^{-1} CO_2), in VC was 0.038 mol m^{-2} s^{-1}. It increased (by ˜17%) to 0.044 mol $m^{-2}s^{-1}$ in the transgenics under 21% O_2. Further, there was no significant difference in the CO_2compensation point of the VC and the transgenic plants. Similarly, the V_{cmax} (Bernacchi et al., 2001) was 34 μmol m^{-2} s^{-1} for VC and the same increased to 40 μmol m^{-2} s^{-1} i.e., by ˜18% in transgenics. The electron transport rate J_{max}, derived from the A/C_i curve, was 67 μmol $m^{-2}s^{-1}$ in VC and it increased to 84 μmol m^{-2} s^{-1} i.e., by ˜18% in the overexpressors.

In 2% oxygen, the maximum rate of CO_2 assimilation at saturating CO_2 was similar to that under 21% O_2 in all plants. However, the net CO_2 assimilation was 22% –25% higher in the transgenics than in the VC. The *CE* significantly increased with the decrease in the O_2 level, from 21% to 2%. The*CE* in VC was 0.045 mol $m^{-2}s^{-1}$ and it increased to 0.054-0.056 mol m^{-2} s^{-1} (19%-23%) in the transgenics under 2% O_2.

Mesophyll conductance (g_m) and chloroplast CO_2 concentrations (Cc)

Leaf g_m to CO_2 and C c were calculated from the combined gas exchange and other parameters (Harley et al., 1992). The g_m in VC was 0.05 mol CO_2 m^{-2} $s^{-1}bar^{-1}$ and C_c was substantially lower than C_i (173 μmol CO_2mol^{-1} at 319 μmol CO_2mol^{-1}). In the two transgenics,g_m increased to 0.069 or 0.073 mol CO_2 m^{-2} $s^{-1}bar^{-1}$ which led to significantly higherC_c (8% - 10%) than in the VC. Although we had problems in accurately measuring g_m andC_c because of the small size of*Arabidopsis* leaves, yet, we consistently observed small increases in C_c for *ΦββˇA3* overexpressors over the VC.

Typesetter to place Table 3 here

Growth, weight and starch content

The up-regulation of any functionally indispensable gene product(s) usually results in alterations in the development of the plant. We compared the overall growth of VC, CAx3 and CAx5 plants, all for 3-week old plants. The CAx3 and CAx5 plants had longer (˜14%) roots than that of VC **(Table 3).** However, their shoot lengths were almost similar **(Table 3)** . The CAx3 and CAx5 plants had bigger (11%-15%) rosette diameter than the VC plants **(Table 3)** . After 3 weeks of growth, the dry weight of CAx3 and CAx5 plants increased by 14%, and 20%, respectively than that in VC **(Fig. 7b)** . Furthermore, increased photosynthetic carbon assimilation in the transgenics resulted in higher starch content (10-19%), estimated before the end of the day **(Fig. 7c)** .

Discussion

In our study, we have generated several lines of *Arabidopsis thaliana* overexpressing C_4 *Φλαεριαβιδεντις βˇA3* that, in general, has a low Km for CO_2. Overexpression of C_4 *ΦββˇA3* under the control of constitutive 35S promoter resulted in higher gene expression, higher protein abundance and higher enzymatic activity of *CA* , in the transgenic lines than in the VC (**Fig. 2f, g**). In the *CA*overexpressors, higher activity of *CA* resulted in hydration of extra CO_2 and its conversion into bicarbonate, the substrate for *PEPC* (Hatch and Burnell, 1990) (**see Fig. 1**). However, in vitro activity of *PEPC* was similar in the transgenics and in the VC plants, as expressed on equal protein basis, in the presence of equal concentration of the substrate (**Fig. 3a)** . This implies that the protein abundance of PEPC did not substantially increase in response to *CA* overexpression.*PEPC* has a much higher (25 mM) Km of HCO_3^- (Bauwe 1986) than the concentration of HCO_3^- in the cytoplasm of C_3 plants (Mukerji and Yang, 1974; Sato et al., 1988). Due to the higher concentration of HCO_3^- in the cytosol of *CA*overexpressors, their endogenous *PEPC* activity must have increased generating more oxaloacetic acid (OAA) (**see Fig. 1**). The OAA generated by the increased carboxylation activity of endogenous cytoplasmic *PEPC* may lead to an increase in the carboxylic acid pool of the tricarboxylic acid (TCA) cycle. The anaplerotic role played by *ΦββˇA* in the cytoplasm by providing the pool of carboxylic acids as carbon skeleton to TCA cycle for amino acid and protein synthesis must have, we suggest, increased their content in the transgenics (**see Fig. 1**).

Although Chl and proteins are synthesized via two different metabolic processes, their biosynthetic pathways may be co-regulated by the nitrogen status of the system (Garai and Tripathy, 2018). Higher protein content

in the transgenics would have resulted in increased Chl synthesis that is regulated by several genes. We propose that higher Chl content must have been due to increased expression of several Chl biosynthetic genes, such as *PBGS* , *UROD* , *PPOX* ,*CHLI* and *PORC* (**Fig. 5a**). Similarly, overexpression of one of the Chl biosynthesis genes is known to up-regulate the abundance of message of several other genes involved in the greening process, suggested to be due to the presence of regulatory network among Chl biosynthesis genes (Alawady and Grimm, 2005; Pattanayak and Tripathy, 2011; Biswal et al., 2012). Our results demonstrate the pleiotropic effect of *ΦββA3* overexpression on transcript abundance of genes involved in Chl biosynthesis.

Chlorophyll *a* fluorescence and photosystem activities in the trangenics

Chl *a* fluorescence has been used as a non-invasive signature of photosynthesis, particularly of PSII (Krause and Weis, 1991; Strasser and Srivastava, 1995; Papageorgiou and Govindjee (eds), 2004; Baker, 2008; Stirbet et al., 2018). When a dark-adapted photosynthetic organism is exposed to blue light (λ- 460 mm), Chl *a* fluorescence rises from a low initial minimum level (F_o) to a high level (F_m) (Munday and Govindjee, 1969a; Munday and Govindjee, 1969b). The *CA* over-expressers had higher F_o (**Table 2**) partly due to higher Chl content. However, there could be several other reasons to have higher F_o in the transgenics, e.g., higher concentration of Q_B^- in dark-adapted samples (Schreiber and Armond, 1978; Cao and Govindjee, 1990; Yamane et al., 1997). However, the maximum primary photochemical efficiency of PSII, as inferred from F_v/F_m in dark-adapted leaves, was ~5% higher in the transgenic plants demonstrating higher efficiency of PSII (Genty et al., 1992; Baker et al., 2007).

Higher photosynthetic efficiency in the transgenics

The increased ETR of PSII and PSI at lower light intensities (50-125 μmol photons m^{-2} s^{-1}) in intact leaves of *CAx* plants is suggested to be mostly due to efficient energy capture by the larger antenna, which must have been due to increased gene/protein expression of the light-harvesting components of PSII and PSI i.e. *Lhcb2.1* , LHCII, *Lhcb1* , *Lhca1* and*Lhca2* (**Figs. 5b,c,d**). ETR measured at higher light intensity (~550 μmol photons $m^{-2}s^{-1}$) was also higher (13%-19% for PSII; 17%-25% for PSI) in *CAx* plants than in the VC (**Fig. 3e,f**). These values of PSI and PSII ETR of intact leaves, derived from Chl *a*fluorescence parameters, were confirmed by actual measurements of light-saturated PSII, whole-chain and PSI in isolated thylakoid membranes (**Figs. 3b,c,d**). We suggest that higher light-saturated PSII- and PSI-dependent ETR in the intact leaves and PSII and PSI activities of thylakoid membranes, isolated from transgenics, are due to the increased gene expression/ protein abundance of essential components of PSII (*PsbA* , *PsbD*), oxygen evolving complex (*PsbO*), PSI subunit IV (PsaE), as well as a few other thylakoid membrane-bound photosynthetic proteins. On the basis of all the results, presented in this paper, we conclude that the*CAx* plants had (**i**) a higher light-harvesting capacity under limiting light intensities due to larger light-harvesting apparatus and (**ii**) a greater ability to use light at higher light intensities due to more and efficient PSII, PSI and intersystem electron transport components. It is known that OEC33, which is on the electron donor (oxidising) side of PSII, binds calcium as well as GTP (Suorsa and Aro, 2007); furthermore, this side has a carbonic anhydrase activity (Lu et al., 2005). Increased protein abundance of OEC33 in the transgenics may have been responsible for higher CA activity on the oxidising side of PSII resulting in enhanced PSII-dependent ETR especially under high light. The presence of *CA* in thylakoid membranes (Stemler, 1997), generating HCO_3^-, could have contributed to increased PSII activity by binding at the reducing side of PSII (for a review on the "bicarbonate" effect, see Shevela et al., 2012). Coupled with higher PSI and PSII reactions, increased protein abundance of intersystem electron transport components, i.e., cytochrome b6/f (Cyt b6/f) complex, which includes cytochrome f, must have contributed to higher whole chain electron transport in the transgenic lines. These conclusions are consistent with earlier work, from our laboratory, where higher PSI, PSII and whole chain electron transport activities were observed in chlorophyllide a oxygenase (*CAO*) overexpressor lines of tobacco, which was also due to an increase in photosynthetic proteins (Biswal et al., 2012); this finding is in complete agreement with the data of Simkin et al. (2017), on Arabidopsis, overexpressing Rieske FeS protein of the Cyt b6/f complex resulting in increase of components of PSI, PSII, and the ATPase. The Cyt b6/f is known to mediate electron transfer from the

plastoquinol to plastocyanin, and is involved in noncyclic electron flow (Wood and Bendall, 1976; Hurt and Hauska, 1981), as well as in the cyclic electron flow around PSI (Lam and Malkin, 1982). In addition, PsaE protein on the electron- acceptor side of PSI is involved in cyclic electron transport, and is responsible for preventing electron leakage to molecular oxygen (Mehler reaction) (Jeanjean et al., 2008). We speculate that the increased protein abundance of PsaE (**Fig. 5d**) in CAx plants may have minimized the photoreduction of reactive oxygen species by PSI.

Chl fluorescence transient (or induction) curve includes what is known as the OJIP rise, where "O" is the first measured point when a dark-adapted plant is exposed to continuous light; then it rises to a peak P with two inflection points J and I. In the OJIP curve, normalized at the "O" level (F_o), we observe, in the transgenics, a faster JI rise as well as a faster IP rise. We interpret this to suggest that there a faster efficiency of electron transport in the transgenics (**Fig. 4a**) (Kandoi et al., 2016; Jiménez-Francisco et al., 2020). The higher amount of bicarbonate in the transgenics might have possibly helped in keeping bicarbonate bound to the non-heme iron on the reducing side of PSII (as mentioned above), probably affecting OJIP kinetics indirectly. Further research is needed to understand this phenomenon in terms of the various available models (see e.g. Stirbet et al., 2020). However, the faster IP rise in the transgenics suggests that they may be more efficient in photosynthesis, as empirically found in other systems (Hamdani et al., 2015; Soda et al., 2018). The "performance index" in the CAx plants was observed to be better than in the VC plants; clearly indicating higher efficiency of the photosynthetic machinery in the transgenics. 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 plastoquinone molecules (Malkin and Kok, 1966) was higher (19%-22%) in the CAx3 and CAx5 lines than in VC, once again confirming the advantage of the transgenics, over the controls

(Table 2).

Carbon fixation and water use efficiency (WUE)

Our data clearly demonstrate that the constitutive overexpression of*ΦββˇA3* in the cytoplasm of the transgenics resulted in higher rates of photosynthetic carbon assimilation, per unit leaf area, than in the VC plants (**Fig. 6a**); this corresponds to the higher electron transport rates of PSI and PSII that provided sufficient NADPH and ATP for increased CO_2 assimilation. Higher protein content must have been the reason not only for the elevation of the rates of photosynthesis, but for the increased metabolic processes including Chl biosynthesis (**Table1**), and photosynthesis. In the transgenics, as compared to VC, the quantum yield of CO_2assimilation measured in limiting light intensities increased by 17% to 20%. These observations suggest that the transgenics had inherently higher photosynthetic efficiency and that they utilized the absorbed light energy much more efficiently.

The *ΦββˇA3* overexpressors had lower stomatal conductance, decreased transpiration rate per unit leaf area, and a better WUE (**Figs. 6d,e,f**). Overexpression of *βˇA1* or *βˇA4* in guard cells of *Arabidopsis thaliana* has shown a reduced stomatal conductance and a substantial increase in the WUE (cf. Hu et al., 2010); however, there was no increase in the rate of photosynthesis in that work.

Owing to the overexpression of *ΦββˇA3* , having a higher affinity for CO_2 than that of endogenous *CA* in the cytosol, the production of HCO_3^- is definitely expected to be higher diminishing the cytoplasmic [CO_2]. It would provide a higher concentration gradient for a better diffusion of CO_2 from the intercellular space to the cytoplasm of the mesophyll cells to keep a balance between CO_2 uptake and HCO_3^- assimilation by cytoplasmic endogenous PEP carboxylase of *Arabidopsis thaliana* . The overexpression of *ΦββˇA3* could, indeed, produce enough HCO_3^- under ambient CO_2 to partially close the stomata to reduce the stomatal conductance and transpiration rate and increase the WUE (**Figs. 6d,e,f**). Conversely, Kolbe et al. (2018) found that the*βςa1βςa2* maize mutants, with 3% of wild type CA activity, had higher stomatal conductance, a slower stomatal closure in response to an increase in CO_2 concentration. Further, in transgenic lines of *Setaria viridis* (C_4 grass), with less than 13% CA activity, Osborn et al. (2017) observed that the photosynthetic rates decreased at lower CO_2concentrations. Further, in CA-deficient (less than 20% of the wild type) *F. bidentis* little effect was found on carbon assimilation, and on g_m (Cousins et al., 2006;

Ogee et al., 2018). However, a knockout of chloroplastic βCA1 in *A. thaliana*has been shown to have poor seedling establishment due to decreased photosynthetic performance under ambient CO_2 (Ferreira et al., 2008). For efficient photosynthetic performance by plants, relatively high CA activity is one of the important requirements in*A. thaliana* . On the basis of the above results, we conclude that the CAx plants had higher photosynthetic efficiency with higher water use efficiency.

CO_2 response curves

In general, response of the light-saturated CO_2assimilation rate (A) to leaf intercellular CO_2mole fraction (C i) consists of three phases**: (i)** the first phase is when assimilation is limited by the amount of active*rubisco* (slope of the initial phase); **(ii)** the second phase is an inflection to a slower rise, where$A_{\max}$ is reached due to limitation by the supply of substrate (ribulose 1,5-bisphosphate, RuBP); and **(iii)** the third phase is where photosynthesis does not respond to increasing CO_2, nor it is inhibited by increasing oxygen concentration; further, it is often limited by triose-phosphate utilization (von Caemmerer and Farquhar 1981; Sharkey, 1985;; Ainsworth and Long, 2005; Sharkey et al., 2007; Bernacchi et al., 2013). In 21% O_2 the transgenics had higher photosynthetic capacity,CE and V_{cmax} than VC. This was partly due to increased g_m and higherC_{c} in the transgenics, albeit to a smaller extent. Higher g_{m} has potential to simultaneously improve photosynthetic efficiency and intrinsic water use efficiency (Flexas et al., 2013). The CO_2 must diffuse to the cytoplasm and the chloroplasts either through the lipid bilayer or probably through the pores of certain aquaporins (Tyerman et al., 2002; Flexas et al., 2008; Groszmann et al., 2017). However, the diifusion is slow and dependent upon the concentration gradient and inversely proportional to the distance. Therefore, diffusion of CO_2 has to be high enough to match the rate of carbon assimilation. The cytoplasmic reserve of HCO_3^- generated by overexpressed*ΦββΤA* , when required, could be converted back to CO_2 by the reverse reaction of CA, and the CO_2 thus produced could diffuse through the envelope membrane due to high CO_2 gradient created by consumption of CO_2 by *rubisco* in the stroma during the day time (**Fig. 1**). This would have resulted in a partially higher [CO_2] in the stroma (C_{c}) to enhance photosynthetic carbon assimilation (**Fig. 7a**). In higher plants, no bicarbonate transporter has been found in their chloroplast envelope membranes (Poschenrieder et al., 2018). However, bicarbonate functions as a small molecule activator of SLAC1 anion channels in the guard cells and may partly contribute to bicarbonate transport into the stroma (Xue et al., 2011). Nonetheless, the possibility of HCO_3^- directly diffusing from the cytosol to stroma to act as a reservoir for CO_2 to increased plastidic [CO_2], is rather small.

Increase in CE and V_{cmax} in the transgenics suggest that at the atmospheric [CO_2], the higher rates of photosynthesis is due to enhancedC_{c} although the impact of pleiotropic effect on the overexpressors is not ruled out. We have used theA/C_{i} curves to obtain information on theCE and V_{cmax} ; we found it to be clearly higher in the transgenics. We suggest that this was, in part, due to an increase in the *rubisco* content and in chloroplastic CO_2 (C_{c}). In several earlier studies, the V_{cmax}/CE has been shown to be strongly correlated with the content of *rubisco* (von Caemmerer and Farquhar, 1981; Makino et al., 1994; Jacob et al., 1995, Manter et al., 2004). The transgenics, in our work, had 8%-10% higher concentration of C_{c} than the VC, and, we suggest that it must have partially contributed to the enhanced CE . As the CO_2 compensation point remained almost similar both in the VC and in the transgenic plants, the rate of photorespiration, probably, did not decrease in the CA overexpressors. Increase in photosynthetic capacity could be attributed to an increase in the rates of ETRII and ETRI. The RuBP regeneration was better in the transgenics probably due to a general increase in the protein content. Rubisco activation state and RuBP levels were shown to be higher in other transgenic plants (Miyagawa et al., 2001; also see von Caemmerer & Evans, 2010). It has been demonstrated in C_3 plants that facilitating electron transport by overexpressing the components of electron transfer chain can result in higher assimilation rates (Simkin et al., 2017). The higher carbon fixation by transgenics could be partly due to recapture of photorespiratory CO_2 by the efficient *ΦββΤA*present in the cytoplasm. The increase in the rate of carbon assimilation in transgenics when photosynthesis becomes oxygen insensitive cannot be the result of better CO_2 supply. It may be due to better RuBP regeneration capacity in the transgenics. The A/C_{i} curves further suggest that transgenics may have limitation in triose phosphate utilization (Sharkey et al., 2007). The higher photosynthesis rate in transgenics is also due to higher g_{m}. The relationship betweeng_{m} and CA activity on photosynthesis

rate is dependent on the environment and/or the photosynthetic pathway (e.g. Cano et al., 2019; also see a review by Momayyezi et al., 2020).

Under 2% O_2, the rate of photorespiration is expected to have been very minimal. The backward extrapolation of theA/C_i curve in 2% O_2 shows that the CO_2 compensation point was close to 7 μmol mol^{-1} in these experiments. Under 2% O_2 the A/C_i curves for the VC as well as the transgenics saturated almost at ambient CO_2. In 2% O_2, the *CE* of transgenics was higher than that in the VC plants which suggests that the increases in *CE* and in the maximum rates of photosynthesis, under ambient CO_2, were not due to a reduction in photorespiration but due to an increase in the inherent efficiency of photosynthesis of the transgenics possibly due to pleiotropic effect of *CA*overexpression. In 2% O_2, the maximum rate of CO_2 assimilation is the same whether measured in 20% or 2% oxygen; this indicates that starch and sucrose synthesis set a ceiling on the rate of photosynthesis. This ceiling is higher in the plants with extra CA.

Due to their higher photosynthetic efficiency, the starch content of the transgenic plants was substantially higher than in the VC plants. The additional starch produced in *CA* overexpressors was partly used in the dark period by respiration, most likely for increased growth of plants that had higher biomass. The increase in dark respiration rate in the transgenics supported the energy demand for higher plant growth. This finding is in complete agreement with data from several earlier reports (Lefebvre et al., 2005; Biswal et al., 2012; Kandoi et al., 2016; Ermakova et al., 2019), where enzymes, such as sedoheptulose-bisphosphatase, chlorophyllide *a* oxygenase, *PEPC* and Rieske FeS protein of the Cytochrome b6f complex, were overexpressed one at a time. In these experiments, PSII photosynthetic efficiency, carbon assimilation, starch content, and dry matter accumulation were shown to have consistently increased. It is obvious to us that the increases in overall biomass is very important towards the goal of obtaining high yielding bioenergy crops (see Ort et al., 2015). Our results, reported here, on overexpressing cytoplasmic C_4 carbonic anhydrase from*Flaveria bidentis* in *Arabidopsis thaliana* clearly demonstrates highly significant increases in photosynthesis which is in the right direction to meet the global needs ahead of us.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

Ainsworth, E.A., & Long, S.P. (2005). What have we learned from 15 years of free air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. *New Phytologist,* 165, 351-372.

Alawady, A.E., & Grimm, B. (2005). Tobacco Mg protoporphyrin IX methyltransferase is involved in inverse activation of Mg porphyrin and protoheme synthesis. *Plant Journal,* 41(2), 282-290.

Badger, M.R., & Price, G.D. (2003). CO_2 concentrating mechanisms in cyanobacteria: molecular components, their diversity and evolution. *J Exp Bot,* 54, 609–622.

Bailey, K.J., Gray, J.E., Walker, R.P., & Leegood, R.C. (2007). Coordinate regulation of phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase by light and CO_2during C4 photosynthesis. *Plant Physiol,* 144, 479-486.

Baker, N.R. (2008). Chlorophyll fluorescence: a probe of photosynthesis in vivo. *Annu Rev Plant Biol,* 59, 89–113.

Baker, N.R., Harbinson, J., & Kramer, D.M. (2007). Determining the limitations and regulation of photosynthetic energy transduction in leaves. *Plant, Cell & Environment,* 30(9), 1107-25.

Bauwe, H. (1986). An efficient method for the determination of Km values for $HCO3^-$ of phosphoenolpyruvate carboxylase.*Planta,* 169, 356–360.

Bernacchi, C. J., Singsaas, E.L., Pimentel C., Portis, A.R. Jr, & Long, S.P. (2001). Improved temperature response functions for models of Rubisco-limited photosynthesis. *Plant, Cell and Environ* , 24(2), 253-260.

Bernacchi, C.J., Bagley, J.E., Serbin, S.P., RuizVera, U.M., Rosenthal, D.M., & Vanloocke, A. (2013). Modelling C_3 photosynthesis from the chloroplast to the ecosystem**.** *Plant Cell Environ* , 36 (9), 1641-1657.

Biswal, A.K., Pattanayak, G.K., Pandey, S.S., Leelavathi, S., Reddy, V.S., Govindjee, & Tripathy, B.C. (2012). Light intensity-dependent modulation of chlorophyll b biosynthesis and photosynthesis by overexpression of chlorophyllide a oxygenase in tobacco. *Plant Physiol,* 159(1),433-449.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem,* 72, 248–254.

Bonacci, W., Teng, P.K., Afonso, B., Niederholtmeyer, H., Grob, P., Silver, P.A., & Savage, D.F. (2012). Modularity of a carbon-fixing protein organelle. *Proc Natl Acad Sci U.S.,* 109, 478–483.

Borba, A.R., Serra, T.S., Gorska, A., Gouveia, P., Cordeiro, A.M., Reyna-Llorens, I., Knerova, J., Barros P.M., Abreu, I.A., Oliveira, M.M., Hibberd, J.M., & Saibo, N.J.M. (2018). Synergistic binding of bHLH transcription factors to the promoter of the maize NADP-ME gene used in C4 photosynthesis is based on an ancient code found in the ancestral C3 state. *Mol. Biol. Evol,*35, 1690–1705.

Burke, J.J. (1990). High temperature stress and adaptation in crops. In: Stress Response in Plants: Adaptation and Acclimation Mechanisms. (eds R.G. Alscher & J.R. Cummings.pp. 295-309. Wiley-Liss, New York.

Cano, F.J., Sharwood, R.E., Cousins, A.B., & Ghannoum, O. (2019). The role of leaf width and conductances to CO2 in determining water use efficiency in C4 grasses. *New Phytol,* 223, 1280–1295.

Cao, J. & Govindjee. (1990). Chlorophyll a fluorescence transient as an indicator of active and inactive Photosystem II in thylakoid membranes.*Biochim Biophys Acta,* 1015, 180–188.

Cousins, A.B., Badger, M.R. & von Caemmerer, S. (2006). A transgenic approach to understanding the influence of carbonic anhydrase on C18OO discrimination during C4 photosynthesis. *Plant Physiology,* 142, 662–672.

Dabrowska-Bronk, J., Komar, D.N., Rusaczonek, A., Kozłowska-Makulska, A., Szechyńska-Hebda, M., & Karpiński, S. (2016) β-carbonic anhydrases and carbonic ions uptake positively influence Arabidopsis photosynthesis, oxidative stress tolerance and growth in light dependent manner. *J Plant Physiol* ., 203,44-54.

DiMario, R.J., Quebedeaux, J.C., Longstreth, D.J., Dassanayake, M., Hartman, M.M., & Moroney, J.V. (2016). The cytoplasmic carbonic anhydrases bCA2 and bCA4 are required for optimal plant growth at low CO2. *Plant Physiol,* 171, 280–293.

Ermakova, M., Lopez-Calcagno, P.E., Raines, C.A., Furbank, R.T., & von Caemmerer, S. (2019). Overexpression of the Rieske FeS protein of the Cytochrome b6f complex increases C4 photosynthesis in Setaria viridis.*Commun. Biol,* 2, 1 –12

Evans, J.R., & Loreto, F. (2000). Acquisition and diffusion of CO_2 in higher plant leaves. In: Leegood, R.C., Sharkey, T.D., Caemmerer, S., eds. *Photosynthesis: physiology and metabolism* . Dordrecht, the Netherlands: Springer, 321–351.

Ferreira, F.J., Guo, C., & Coleman, J.R. (2008). Reduction of plastid-localized carbonic anhydrase activity results in reduced Arabidopsis seedling survivorship. *Plant Physiol,* 147, 585–594.

Flexas, J., Bota, J., Loreto, F., Cornic, G., & Sharkey, T.D. (2008). Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. *Plant Biol,* 6, 269–279.

Flexas, J., Niinemets, U, Gallé, A, Barbour, M.M., Centritto, M., Diaz-Espejo, A., Douthe, C., Galmés, J., Ribas-Carbo, M., Rodriguez, P.L., Rosselló, F., Soolanayakanahally, R., Tomas, M., Wright, I.J., Farquhar,

G.D., & Medrano, H. (2013). Diffusional conductances to CO_2 as a target for increasing photosynthesis and photosynthetic water-use efficiency.*Photosynthesis Research* 117, 45–59.

Garai, S., & Tripathy, B.C. (2018). Alleviation of Nitrogen and Sulfur Deficiency and Enhancement of Photosynthesis in Arabidopsis thaliana by Overexpression of Uroporphyrinogen III Methyltransferase (UPM1).*Front Plant Sci,* 9, 1365.

Genty, B., Goulas, Y., Dimon, B., Peltier, G., Briantais, J.M., & Moya, I. (1992). Modulation of efficiency of primary conversion in leaves.*Photosynthesis Research,* 34, 106.

Giordano, M., Beardall, J., & Raven, J.A. (2005). CO_2concentrating mechanisms in algae: mechanisms, environmental modulation, and evolution. *Annu Rev Plant Biol,* 56, 99–131. Govindjee (1995). Sixty-three years since Kautsky: chlorophyll a fluorescence.*Aust J Plant Physiol,* 22, 131–160.

Govindjee (2004). Chlorophyll a fluorescence: a bit of basics and history. In: (eds Papageorgiou G.C. & Govindjee) Chlorophyll a fluorescence: a probe of photosynthesis, advances in photosynthesis and respiration, vol 19, pp. 2–42, Springer, Dordrecht.

Groszmann, M., Osborn, H.L., & Evans, J.R. (2017). Carbon dioxide and water transport through plant aquaporins. *Plant, Cell & Environment,* 40, 938-961

Hamdani, S., Qu, M., Xin, C.P., Li, Ming., Chu, C., Govindjee, & Zhu, X.G. (2015) Variations between the photosynthetic properties of elite and landrace Chinese rice cultivars revealed by simultaneous measurements of 820 nm transmission signal and chlorophyll a fluorescence induction. *J Plant Physio* l, 177,128–138.

Gupta, V., & Tripathy, B.C., (2010). Effect of light quality on chlorophyll accumulation and protein expression in wheat (Triticum aestivum L.) seedlings. *International Journal of Biotechnology & Biochemistry,* 6(4), 521-537.

Harley, P.C., Loreto, F., Marco, G.D., & Sharkey, T. D. (1992) Theoretical Considerations when Estimating the Mesophyll Conductance to CO_2 Flux by Analysis of the Response of Photosynthesis to CO_2 *Plant Physiol* , 98, 1429-1436.

Hatch, M.D. & Burnell, J.N. (1990). Carbonic anhydrase activity in leaves and its role in the first step of c(4) photosynthesis.*Plant Physiol,* 93, 825–828.

Henry, R.P. (1996). Multiple roles of carbonic anhydrase in cellular transport and metabolism. *Annu Rev Physiol,* 58, 523–538.

Hewett-Emmett, D., & Tashian, R.E. (1996). Functional diversity, conservation, and convergence in the evolution of the α-, β-, and γ-carbonic anhydrase gene families. *Mol Phylogenet Evol,* 5, 50–77.

Hu, H., Boisson-Dernier, A., Israelsson-Nordström, M., Böhmer, M., Xue, S., Ries, A., Godoski, J., Kuhn, J.M., & Schroeder, J.I. (2010). Carbonic anhydrases are upstream regulators of CO_2-controlled stomatal movements in guard cells.*Nat Cell Biol,* 12, 87–93.

Hurt, E., & Hauska, G. (1981). A cytochrome f/b6 complex of five polypeptides with plastoquinol-plastocyanin-oxidoreductase activity from spinach chloroplasts. *Eur J Biochem.,* 117(3), 591-5.

Ignatova, L.K., Moskvin, O.V., Romanova, A.K., & Ivanov, B.N. (1998). Carbonic anhydrases in the C3 -plant leaf cell Aust. *J. Plant Physiol,* 25, 673-677.

Jacob, J., Greitner, C., & Drake, B.G. (1995). Acclimation of photosynthesis in relation to RuBisco and non-structural carbohydrate contents and in situ carboxylase activity in *Scirpus olneyi* grown at elevated CO, in the field. *Plant Cell Environ,* 18, 875-884.

Jeanjean, R., Latifi, A., Matthijs, H.C., & Havaux, M. (2008). The PsaE subunit of photosystem I prevents light-induced formation of reduced oxygen species in the cyanobacterium Synechocystis sp. PCC 6803.*Biochim Biophys Acta,* 1777(3), 308-16.

Jilani, A., Kar, S., Bose, S., & Tripathy, B.C. (1996). Regulation of the carotenoid content and chloroplast development by levulinic acid.*Physiol Plant,* 96, 139–145.

Jiménez-Francisco, B., Stirbet, A., Aguado-Santacruz, G.A., Campos, H., Conde-Martínez, F.V., Padilla-Chacón, D., Trejo, C., Bernacchi, C.J., & Govindjee, G. (2020). A comparative chlorophyll a fluorescence study on isolated cells and intact leaves of Bouteloua gracilis (blue grama grass) *Photosynthetica,* 58, 262-274.

Kandoi, D., Mohanty, S., & Tripathy, B.C. (2018). Overexpression of plastidic maize NADP-malate dehydrogenase (ZmNADP-MDH) in Arabidopsis thaliana confers tolerance to salt stress. *Protoplasma,* 255(2), 547-563.

Kandoi, D., Mohanty, S., Govindjee, & Tripathy, B.C. (2016). Towards efficient photosynthesis: overexpression of Zea mays phosphoenolpyruvate carboxylase in *Arabidopsis thaliana* . *Photosynth Res,*130(1-3), 47-72.

Krause, G.H., & Weis, E. (1991). Chlorophyll Fluorescence and Photosynthesis: The Basics. *Annu Rev Plant Physiol Plant Mol Biol,* 42, 313–349.

Kolbe, A.R., Brutnell, T.P., Cousins, A.B., & Studer, A.J. (2018). Carbonic anhydrase mutants in *Zea mays* have altered stomatal responses to environmental signals. Plant Physiol., 177(3),980–9.

Lam, E., & Malkin, R. (1982). Reconstruction of the chloroplast noncyclic electron transport pathway from water to NADP with three integral protein complexes. *Proc Natl Acad Sci U S A,*79(18),5494-8.

Lefebvre, S., Lawson, T., Fryer, M., Zakhleniuk, O.V., Lloyd, J.C., & Raines, C.A. (2005). Increased sedoheptulose-1,7-bisphosphatase activity in transgenic tobacco plants stimulates photosynthesis and growth from an early stage in development. *Plant Physiol,* 138, 451–460.

Li, Y., Gao, Y., Xu, X., Shen, Q., & Guo S. (2009) Light saturated photosynthetic rate in high-nitrogen rice (Oryza sativa L.) leaves is related to chloroplast CO2 concentration. *J Exp Bot* , 60, 2531–2360.

Liljas, A., & Laurberg, M. (2000). A Wheel Invented Three Times: The Molecular Structures of the Three Carbonic Anhydrases. *EMBO,* 1, 16–17.

Lin, H.C., Arrivault, S., Coe, R. A., Karki, S., Covshoff, S., Bagunu, E., Lunn, J.E., Stitt, M., Furbank, R.T., Hibberd, & J.M., Quick, W.P. (2020). A Partial C_4 Photosynthetic Biochemical Pathway in Rice. *Frontiers in Plant Science* , 11, 1581.

Lindskog, S. (1997). Structure and mechanism of carbonic anhydrase. Pharmacol Ther, 74, 1–20.

Livak, K.J., Thomas, D., & Schmittgen, T.D. (2001). Analysis of Relative Gene Expression DataUsing RealTime Quantitative PCR and the 22DDCT Method. METHODS, 25, 402–408.

Long S.P., Marshall-Colon A., & Zhu X.G. (2015) Meeting the global food demand of the future by engineering crop photosynthesis and yield potential. *Cell,* 161, 56–66.

Lu, Y.K., Theg, S.M., & Stemler, A.J. (2005). Carbonic anhydrase activity of the photosystem II OEC33 protein from pea. Plant Cell Physiol,46(12), 1944-53.

Ludwig, M. (2012) Carbonic anhydrase and the molecular evolution of C4 photosynthesis. Plant Cell Environ, 35, 22-37.

Malkin, S., & Kok, B. (1966). Fluorescence induction studies in isolated chloroplasts. I. Number of components involved in the reaction and quantum yields. *Biochim. Biophys. Acta,* 126, 413-432.

Makino, A., Nakano, H., & Mae, T. (1994). Responses of ribulose-1,5- bisphosphate carboxylase, cytochrome *J* and sucrose synthesis enzymes in rice leaves to leaf nitrogen and their relationships to photosynthesis. *Plant Physiol* , 105, 173-179.

Manter, D.K., & Kerrigan, J. (2004). A/C(i) curve analysis across a range of woody plant species: influence of regression analysis parameters and mesophyll conductance. *J Exp Bot,* 55(408), 2581-8.

Misra, P.S., Mertz, E.T., & Glover, D.V. (1975). Studies on corn proteins: VIII. Free amino acid content of opaque-2 and double mutants.*Cereal Chem,* 52, 844–848.

Miyao, M., Masumoto, C., Miyazawa, S., & Fukayama, H. (2011). Lessons from engineering a single-cell C(4) photosynthetic pathway into rice.*J Exp Bot,* 62, 3021–3029.

Miyagawa, Y., Tamoi, M., & Shigeoka, S. (2001) Overexpression of a cyanobacterial fructose-1,6-/sedoheptulose-1,7-bisphosphatase in tobacco enhances photosynthesis and growth. *Nat Biotechnol* , 19, 965–9.

Momayyezi, M., McKown, A.D., Bell, S.C.S., & Guy R.D. (2020). Emerging roles for carbonic anhydrase in mesophyll conductance and photosynthesis. *Plant J,* 101(4), 831-844.

Moroney, J.V., Bartlett, S.G., & Samuelsson, G. (2001). Carbonic anhydrases in plants and algae: Invited review. *Plant, Cell Environ,* 24, 141–153.

Moroney, J.V., Ma, Y., Frey, W.D., Fusilier, K.A., Pham, T.T., Simms, T.A., DiMario, R.J., Yang, J., & Mukherjee, B. (2011). The carbonic anhydrase isoforms of Chlamydomonas reinhardtii: intracellular location, expression, and physiological roles. *Photosynth Res,* 109, 133–49.

Munday, J.C.M. Jr., & Govindjee. (1969a). Light-Induced Changes in the Fluorescence Yield of Chlorophyll a Fluorescence in Vivo. III. The Dip and the Peak in Fluorescence Transient of Chlorella Pyrenoidosa.*Biophysic. J.,* 9, 1–21.

Munday, J.C.M. Jr., & Govindjee. (1969b). Light-Induced Changes in the Fluorescence Yield of Chlorophyll a Fluorescence in Vivo. IV. The Effect of Preillumination on the Fluorescence Transient of Chlorella Pyrenoidosa. *Biophysic. J.,* 9, 22–35

Mukerji, S., & Yang, S. (1974). Phosphoenolpyruvate Carboxylase from Spinach Leaf Tissue Inhibition by Sulfite Ion. *Plant Physiol,* 53, 829-834.

Nickrent, D.L. (1994). From field to film: Rapid sequencing methods for field-collected plant species. *Biotechniques,* 16, 470–5.

Ogée, J., Wingate, L., & Genty, B. (2018). Estimating mesophyll conductance from measurements of C18OO photosynthetic discrimination and carbonic anhydrase activity. *Plant Physiol,* 178, 728–752.

Okabe, K., Yang, S-Y., Tsuzuki, M., & Miyachi, S. (1984). Carbonic anhydrase: Its content in spinach leaves and its taxonomic diversity studied with anti-spinach leaf carbonic anhydrase antibody. *Plant Sci Lett,* 33, 145–153.

Ort, D.R., Merchant, S.S., Alric, J., Barkan, A., Blankenship, R.E., Bock, R., Croce, R., Hanson, M.R., Hibberd, J.M., Long, S.P., Moore, T.A., Moroney, J., Niyogi, K.K., Parry, M.A., Peralta-Yahya, P.P., Prince, R.C., Redding, K.E., Spalding, M.H., van Wijk, K.J., Vermaas, W.F., von Caemmerer, S., Weber, A.P., Yeates, T.O., Yuan, J.S., & Zhu, X.G. (2015). Redesigning photosynthesis to sustainably meet global food and bioenergy demand. *Proc Natl Acad Sci USA,* 112, 8529–8536.

Osborn, H.L., Alonso-Cantabrana, H., Sharwood, R.E., Covshoff, S., Evans, J.R., Furbank, R.T., & von Caemmerer, S. (2017). Effects of Reduced Carbonic Anhydrase Activity on CO2 Assimilation Rates in*Setaria Viridis* : A Transgenic Analysis. *Journal of Experimental Botany,* 68, 299–310.

Pal, A., & Borthakur, D. (2015). Transgenic overexpression of Leucaena β-carbonic anhydrases in tobacco does not affect carbon assimilation and overall biomass. *Plant Biosystems.* 150(5), 1-10.

Papageorgiou, G.C., & Govindjee. (2004). Chlorophyll a fluorescence: a signature of photosynthesis. Springer Netherlands ISBN: 978-1-4020-3217-2. doi: 10.1007/978-1-4020-3218-9.

Pattanayak, G.K., & Tripathy, B.C. (2011). Overexpression of Protochlorophyllide Oxidoreductase C Regulates Oxidative Stress in Arabidopsis. *PLoS One,* 6(10), e26532.

Pons, T.L., Flexas, J., von Caemmerer, S., Evans, J.R., Genty, B., Ribas-Carbo, M., & Brugnoli E. (2009). Estimating mesophyll conductance to CO_2: methodology, potential errors, and recommendations. *Journal of Experimental Botany* , 60 (8),2217–2234.

Porra, R.J., Thompson, W.A., & Kriedemann, P.E. (1989). Determination of Accurate Extinction Coefficients and Simultaneous-Equations for Assaying Chlorophyll-a and Chlorophyll-B Extracted with 4 Different Solvents - Verification of the Concentration of Chlorophyll Standards by Atomic-Absorption Spectroscopy. *Biochim Biophys Acta,* 975, 384–394.

Poschenrieder, C., Fernández, J.A., Rubio, L., Pérez, L., Terés, J., & Barceló, J. (2018). Transport and Use of Bicarbonate in Plants: Current Knowledge and Challenges Ahead. *Int J Mol Sci.,* 3, 19(5).

Raven, J.A. (1997). Inorganic carbon acquisition by marine autotrophs.*Adv Bot Res,* 27, 85–209.

Rose, R., Rose, C.L., Omi, S.K., Forry, K.R., Durall, D.M., & Bigg, W.L. (1991). Starch determination by perchloric acid vs enzymes: evaluating the accuracy and precision of six colorimetric methods.*J. Agric. Food Chem* , 39, 2–11.

Sato, F., Koizumi, N., & Yamada, Y. (1988). Purification and characterization of phosphoenol pyruvate carboxylase of photomixotrophically cultured green tobacco cells. *Plant Cell Physiol,* 29, 329-337.

Schreiber, U., & Armond, P. (1978). Heat-induced changes of chlorophyll fluorescence in isolated chloroplasts and related heat-damage at the pigment level. *Biochim Biophys Acta,* 502, 138–151.

Schuler, M.L., Mantegazza, O., & Weber, A.P. (2016). Engineering C4 photosynthesis into C3 chassis in the synthetic biology age. *Plant J.* 87, 51-65.

Sharkey, T.D. (1985) O_2-Insensitive Photosynthesis in C_3 Plants. Its occurrence and a possible explanation.*Plant Physiol.* , 78(1), 71–75.

Sharkey, T.D., Bernacchi, C.J., Farquhar, G.D., & Singsaas, E.L. (2007) Fitting photosynthetic carbon dioxide response curves for C3 leaves. *Plant Cell & Environment* , 30 (9), 1035-1040.

Simkin, A.J., McAusland, L., Lawson, T., & Raines, C.A. (2017) Overexpression of the RieskeFeS protein increases electron transport rates and biomass yield. *Plant Physiol.* **175** , 134–145.

Smith, K.S., & Ferry, J.G. (2000). Prokaryotic carbonic anhydrases. FEMS Microbiol Rev, 24, 335-366.

Soda, N., Gupta, B.K., Anwar, K., Sharan, A., Govindjee, Singla-Pareek, S.L., & Pareek, A. (2018). Rice intermediate filament, OsIF, stabilizes photosynthetic machinery and yield under salinity and heat stress.*Sci Rep,* 9(1), 11015.

Stemler, A.J. (1997). The case for chloroplast thylakoid carbonic anhydrase. *Physiol Plant,* 99, 348–353.

Shevela, D., Eaton-Rye, J.J., Shen, J.R., & Govindjee. (2012). Photosystem II and the unique role of bicarbonate: a historical perspective. *Biochim Biophys Acta.,* 1817, 1134-1151.

Strasser, R.J. & Srivastava, A. (1995). Polyphasic chlorophyll a fluorescence transient in plants and cyanobacteria. *Photochem Photobiol,* 61, 32–42.

Stirbet, A., Lazár, D., Kromdijk, J., & Govindjee (2018). Chlorophyll a fluorescence induction: Can just a one-second measurement be used to quantify abiotic stress responses? *Photosynthetica,* 56, 86-104.

Stirbet, A., Lazár, D., Guo, Y., & Govindjee, G. (2020) Photosynthesis: basics, history and modelling *Annals of Botany* , 126, 4, 511–537.

Sood, S., Gupta, V., & Tripathy, B.C. (2005). Photoregulation of the Greening Process of Wheat Seedlings Grown in Red Light. *Plant Molecular Biology* 59(2), 269-287.

Suorsa, M., & Aro, E.M. (2007). Expression, assembly and auxiliary functions of photosystem II oxygen-evolving proteins in higher plants.*Photosynth. Res,* 93, 89–100.

Tashian, R.E. (1989). The carbonic anhydrases: widening perspectives on their evolution, expression and function. *Bioessays,* 10, 186–92.

Tsimilli-Michael, M., Eggenberg, P., Biro, B., Köves-Pechy, K., Vörös, I., & Strasser, R.J. (2000). Synergistic and antagonistic effects of arbuscular mycorrhizal fungi and Azospirillum and Rhizobium nitrogen-fixers on the photosynthetic activity of alfalfa, probed by the polyphasic chlorophyll a fluorescence transient O-J-I-P. *Appl Soil Ecol.,* 15, 169–182.

Tsuzuki, M., Miyachi, S., & Edwards, G. (1985). Localization of carbonic anhydrase in mesophyll cells of terrestrial C3 plants in relation to CO_2 assimilation. *Plant Cell Physiol,*26, 881 – 91.

Tyerman, S.D., Niemietz, C.M., & Bramley, H. (2002). Plant aquaporins: multifunctional water and solute channels with expanding roles.*Plant Cell Environ,* 25(2), 173-194.

von Caemmerer, S., & Farquhar, G.D. (1981). Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves.*Planta* , 153, 376-387.

von Caemmerer, S., & Evans, J.R. (2010). Enhancing C3 photosynthesis. *Plant Physiol,* 154(2), 589-92.

Wilbur, K.M., & Anderson, N.G. (1948). Electrometric and Colorimetric Determination of Carbonic Anhydrase. *J Biol Chem,* 176, 147–154.

Wood, P.M., & Bendall, D.S. (1976). The reduction of plastocyanin by plastoquinol-1 in the presence of chloroplasts. A dark electron transfer reaction involving components between the two photosystems. *Eur J Biochem,* 61(2), 337-44.

Xue, S.W., Hu, H.H., Ries, A., Merilo, E., Kollist, H., & Schroeder, J.I. (2011). Central functions of bicarbonate in S-type anion channel activation and OST1 protein kinase in CO_2 signal transduction in guard cell. *EMBO Journal,* 30, 1645–1658.

Yamane, Y., Kashino, Y., Koioke, H., & Satoh, K. (1997). Increase of the fluorescence F0 level and reversible inhibition of photosystem II reaction center by high-temperature treatments in higher plants.*Photosynth Res,* 52, 57–64.

Yuan, X., Zhang, L., Ning, N., Wen, Y., Dong, S., Yin, M., Guo, M., Wang, B., Feng, L., & Guo, P. (2014). Photosynthetic physiological response of Radix Isatidis (Isatis indigotica Fort.) seedlings to nicosulfuron. *PLoS One,* 28:9(8), e105310.

Table 1 Total chlorophyll (Chl), Chl a/b ratio, free amino acids and total protein of vector control (VC) and CAx plants grown in soil. Each data point is an average of six replicates. The error bars represent standard error (+-SE). Asterisks indicate significant differences determined by t test (*P<0.05).

	WT	**WT**	**VC**	**CAx3**	**CAx5**
Total Chl, mg (g FW)$^{-1}$	2.0±0.3	2.1±0.4	2.1±0.4	2.5±0.2	2.7±0.3*
Chl a/b	2.3±0.2	2.3±0.3	2.3±0.3	2.4±0.3	2.4±0.2
Φρεε αμινο αςιδς, μμολ (γ ΦΩ)$^{-1}$	5.5±0.6	5.5±0.4	5.5±0.4	6.2±0.5	6.4±0.3*
Total protein, mg (g FW)$^{-1}$	10.1±0.8	10.1±0.6	10.1±0.6	11.1±0.5	12±0.5*

Table 2 Chlorophyll fluorescence measurements of VC and CAx plants grown in soil. VC and *CAx Arabidopsis thaliana* plants were cultivated for 6 weeks in soil and watered with half-strength MS medium two times in a week. Minimal chlorophyll (Chl) *a*fluorescence (F_o), maximal Chl fluorescence (F_m), maximal variable fluorescence (F_v), and F_v/F_m ratio, F_v/F_o and performance index (PI) where$F_v = F_m - F_o$ (WT- wild type, VC-vector control; CAx3 and CAx5- two different transgenic lines). Each data point is the average of six replicates with standard error, ±SE. Asterisks indicate significant differences determined by t test (*P<0.05, **P<0.001).

	VC	**CAx3**	**CAx5**
F_o	184± 8	199±11	204±9*
F_m	1028±59	1211±67*	1290±56**
F_v / F_m	0.81±0.03	0.84±0.01	0.84±0.02
Area	15240±176	18074±141*	18583±187**
F_v / F_o	4.6±0.7	5.1±0.9*	5.3±0.8*
PI	1.51±0.05	1.66±0.06*	1.82±0.09**

Table 3 Morphological parameters of the transgenics and the vector control plants. *Arabidopsis* vector control, CAx3 and CAx5, grown at 21^0C under 14h L/ 10h D photoperiod in cool-white-fluorescent light (100µmol photons $m^{-2}s^{-1}$) for 3 weeks in petri-dishes. Each data point is an average of 6 replicates and error bars represent SD. Asterisks indicate significant differences determined by t test (*P<0.05).

	VC	**CAx3**	**CAx5**
Root length (cm)	6.4±0.5	7.9±0.8*	8.0±0.9*
Shoot length (mm)	4.5±0.8	4.7±0.6	4.7±0.9
Rosette diameter (mm)	22±1	24.5±1*	25.5±1.2*

Figure legends

Fig. 1 A proposed model of photosynthetic carbon flow in*Arabidopsis thaliana* overexpressing *ΦββΆA3* . The cytosolic*ΦββΆA3* having low Km for CO_2 increase the hydration of CO_2. The dashed arrows indicate the diffusion of CO_2 and HCO_3^- within the cytosol and the chloroplast. The relatively higher amounts of CO_2 and HCO_3^- present in the mesophyll cells of overexpressor likely to increase the CO_2 diffusion gradient into the chloroplast during day time. Utilization of diffused CO2 by *rubisco* to make 3-phosphoglycerate would accelerate the carbon reduction cycle favoring the carboxylation activity.*ΦββΆA3* overexpression increased the flux of the carboxylic acid to the tricarboxylic acid cycle (TCA) in mitochondria to play an anaplerotic role to synthesize higher amounts of total amino acids and proteins that contribute to increase photosynthetic efficiency and biomass.

Fig. 2 A schematic representation of the transgene used for*Arabidopsis* transformation, photographs and conformation of*Flaveria bidentis CA* overexpressed *in Arabidopsis.***(a)** : *Φλαεριια βιδεντις βΆA3* cloned to pCAMBIA1304 vector having CaMV35S-Ω-poly A promoter cassette; CaMV35S-npt, coding region of neomycin phosphotransferase gene with CaMV35S promoter; CaMV35SΩ, CaMV 35S promoter with omega (Ω) enhancer; *ΦββΆA3* cDNA, coding region of *Fb* CA gene; Poly A, Poly A tail; (**b):***Arabidopsis* vector control (VC) and CAx (CAx3 & CAx5) plants grown at 21^0C under 14h L / 10h D photoperiod in cool-white-fluorescent light (100 µmol photons $m^{-2}s^{-1}$) for 4 weeks in pots**; (c):** qRT-PCR of*ΦββΆA3* —relative gene expression of *CA* in VC and transgenic lines; (**d):** 15% SDS-PAGE- twenty five µg protein was loaded in each lane and SDS-PAGE was run to check equal loading; (**e):** Western blot- protein samples from the gel were transferred to nitrocellulose membrane and immunoblot analysis of CA protein was made using *Flaveria bidentis* CA antibodies; (**f):** Quantification of CA blot- relative expression of CA in transgenic lines; (**g):** CA enzymatic activity- the activity of CA in VC was 276 µmol CO_2 hydrated (mg Chl)$^{-1}$ min^{-1}. CA activity ranged from 348 to 550 µmol CO_2 hydrated (mg Chl)$^{-1}$ min^{-1} in different transgenic lines (CAx1, CAx2, CAx3 and CAx5); VC- vector control plants containing the null vector pCAMBIA1304). Each data point is the average of five replicates, and error bars represents ±SD; asterisks indicate significant differences determined by t test (*P < 0.05)

Fig. 3 PEPC enzymatic activity in-vitro, electron transport reactions in isolated thylakoid membrane and electron transport rates (ETRII & ETRI) in the intact leaves. (**a**): PEPC enzymatic activity- the activity of PEPC in transgenics was similar to vector control plants (~3 µmol/mg protein/hr); (**b):**Electron transport

through PSII (oxygen evolution; water to PD); **(c)** : whole chain (water to MV; oxygen uptake); **(d):**PSI (ascorbate to MV; oxygen uptake) was measured polarographically, as described under "Materials and Methods"; **(e):** ETRII; **(f):** ETRI, VC- vector control; and 2 different transgenic lines, CAx3 and CAx5. Each data point is the average of five replicates and the error bars represent ±SE; asterisks indicate significant differences determined by t test (*P < 0.05)

Fig. 4 The OJIP curve of chlorophyll *a* fluorescence and the non-photochemical quenching (NPQ) of the excited state of chlorophyll *a* of the vector control and *ΦββˆA3ξ* plants grown in soil. **(a):** Chl *a* fluorescence transients, the OJIP curves normalized at the O level; **(b):** Variable fluorescence transients from the I to the P—double normalized between I (F_I) and P (F_P): $V_{IP} = (F_t - F_I)/(F_P - F_I)$; **(c):** Variable fluorescence transients from the I –single normalization; F, in the diagram, stands for fluorescence at time t (F_t), and F_o is for fluorescence at the O level;**(d):** NPQ of the excited state of chlorophyll at different light intensities. Each data point is an average of 8 replicates and error bars represent ±SE; asterisks indicate significant differences determined by t test (*P < 0.05)

Fig. 5 Relative gene expression and immunoblot analysis. Relative expression of genes related to **(a):** chlorophyll biosynthesis; **(b):** photosynthesis; **(c):** SDS-PAGE (12%) of protein (20 µg) isolated from VC and transgenic plants to check equal loading and the immunoblot to check the abundance of electron transport chain components; **(d):** Quantification of western blot by densitometry analysis using Alpha Ease FC software,;*PBGS* - porphobilinogen synthase; *UROD*- encoding uroporphyrinogen decarboxylase;*PPOX1* - encoding protoporphyrinogen oxidase; *CHLI* - encoding protoporphyrin-IX Mg-chelatase; *PORC* - encoding the light-inducible protochlorophyllide oxidoreductase; *Lhcb1* and*Lhcb2,* encoding components of the light harvesting complex associated with PSII; *Lhca1* and *Lhca2,* encoding components of the light harvesting complex associated with PSI; PsbA and PsbD, Core proteins, encoding photosystem II D1 and D2 proteins; *PsbO* , encoding for OEC33, the oxygen-evolving complex; LHCII- light-harvesting chlorophyll-binding proteins; Cytb6f and Cytf, psaE- PSIIV; rubisco LSU and SSU- rubisco large and small subunit. Western blot data is an average of three independent replicates. qRT-PCR data are expressed as the mean ± SE of three independent experiments performed in triplicate. Asterisks indicate significant differences determined by Student's t-test compared to control (*P < 0.05, **P < 0.01)

Fig. 6 Photosynthesis (net CO_2 assimilation rate) light response curve. **(a)** : Net CO_2assimilation rates of vector control and CAx plants were monitored by IRGA (LiCor-6400/XT) in ambient CO_2 at 400 µmol photons m^{-2} s^{-1} at 21ºC; **(b)** : Net CO_2 assimilation rates upto 80 µmol photons m^{-2} s^{-1}; **(c):** Net CO_2 assimilation rates; **(d):** Stomatal conductance (gs); **(e):** Transpiration rate; **(f):** Water use efficiency (WUE) of vector control and transgenic *Arabidopsis*plants. Each data point is an average of five replicates and error bars represent SE. Asterisks indicate significant differences determined by t test (*P<0.05).

Fig. 7 Photosynthetic carbon fixation rate as a function of increasing intercellular [CO_2] at 21% O_2 and 2% O_2. **(a):**A/C_i curve; dashed lines represents to at 2% O_2; **(b):** Dry weight; **(c):** Starch content, per g FW. Each data point is an average of five replicates and error bars represent SE. Asterisks indicate significant differences determined by t test (*P<0.05).

Hosted file

`Kandoi et al Figures PCE.pdf` available at https://authorea.com/users/470790/articles/562690-overexpression-of-cytoplasmic-c4-flaveria-bidentis-carbonic-anhydrase-in-c3-arabidopsis-thaliana-increases-photosynthetic-potential-and-biomass