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

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Summary
An important method to improve photosynthesis in C₃ crops, such as rice and wheat, is to transfer efficient C₄ characters to them. Here, cytosolic carbonic anhydrase (CA: pCA3) of the C₄ Flaveria bidentis (FB) was overexpressed under the control of 35S promoter in Arabidopsis thaliana, a C₃ plant, to enhance its photosynthetic efficiency. Overexpression of CA resulted in a better supply of the substrate HCO₃⁻ for the endogenous phosphoenolpyruvate carboxylase in the cytosol of the overexpressers, and increased its activity for generating malate that feeds into the tricarboxylic acid cycle. This provided additional carbon skeleton for increased synthesis of amino acids aspartate, asparagine, glutamate, and glutamine. Increased amino acids contributed to higher protein content in the transgenics. Furthermore, expression of FbβCA3 in Arabidopsis led to a better growth due to expression of several genes leading to higher chlorophyll content, electron transport, and photosynthetic carbon assimilation in the transgenics. Enhanced CO₂ assimilation resulted in increased sugar and 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.

Introduction
The current global research efforts are focusing on increasing crop yield for food and fuel production (Long et al., 2015). C₃ photosynthesis is often limited by available CO₂ for ribulose bis phosphate carboxylase/oxygenase (rubisco). Therefore, it is important to modulate C₃ photosynthesis to enhance plant productivity. Carboxic anhydrase (CA, EC 4.2.1.1) is mostly a zinc-containing metalloenzyme that catalyses the interconversion of CO₂ and HCO₃⁻ and is widely distributed in both euukaryotes and prokaryotes (Badger and Price, 1994; Bonacci et al., 2012; Hewett-Emmett and Tashian, 1996; Liljas and Laurberg, 2000; Momayyzei et al., 2020; Moroney et al., 2001). CAs belong to six 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, they share the same general catalytic mechanism (Lindskog, 1997). Amino acid sequence alignment of cytoplasmic C₄ Flaveria bidentis pCA3 (FbβCA3), with different isoforms of C₃ Arabidopsis thaliana pCAs (AtPCAs), demonstrates that these sequences have similar binding sites for Zn²⁺ as well as for the substrates (see Figure S1). In addition, great deal is now known about the structure and varied functions of carbonic anhydrases in plants (DiMario et al., 2017).

In C₃ plants, up to 2% of the total leaf protein is CA, and 95% of its activity is in chloroplast stroma (Okabe et al., 1984; Tsuzuki et al., 1985). Without the CA, the hydration of CO₂ is very slow (Raven, 1997). The role of various CA components in terrestrial C₃ plants in photosynthesis is less understood seemingly due to the compensatory effect of multiple CA isoforms (DiMario et al., 2018). The C₃ isoforms of CA are involved in seed germination, lipid biosynthesis, morphogenesis, nodule of legumes, and responses to abiotic stress (Flemetakis et al., 2003; Floryszak-Wieczorek and Arasimowicz-Jelonek, 2017; Hoang and Chapman, 2002; Kavroulakis et al., 2000). The CO₂ usually diffuses from the cytoplasm to the chloroplasts either through the lipid bilayer or through the pores of aquaporins (Flexas et al., 2008; Groszmann et al., 2017; Tyerman et al., 2002). For research on the transport of bicarbonate in plants and algae, see Poschenrieder et al. (2018). Furthermore, thylakoid CAs are involved in converting CO₂ to HCO₃⁻ in Photosystem II (DiMario et al., 2016), where the latter is needed not only for binding on the non-heme iron for the functioning of electron flow for the reduction of plastoquinone but also for binding near manganese on the ‘oxygen evolving complex’ for water oxidisation (Shevela et al., 2012; Shitov et al., 2018; Stemler, 1997). Without bicarbonate bound in Photosystem II, there is no photosynthesis.

In C₄ mesophyll cells, CA is known to catalyse the hydration of CO₂ producing HCO₃⁻ (Hatch and Burnell, 1990), the latter being the substrate for phosphoenolpyruvate carboxylase (PEPC, Ludwig, 2012). C₄ plants are efficient in carbon assimilation and have an advantage over C₃ plants under several ecological conditions. In the past, there have been attempts to overexpress
(or regulate) C₄ pathway genes into C₃ plants (Borba et al., 2018; Kandoi et al., 2016, 2018; Lin et al., 2020; Miyao et al., 2011; Schuler et al., 2016; Sen et al., 2017). Although Hu et al. (2010) and Pal and Borthakur (2015) have succeeded in overexpressing C₃ CA in the cytosol, in chloroplasts, and in the guard cells of C₂ plants, no increase in the rates of photosynthesis was observed. Furthermore, Majeau et al. (1994) showed that the chloroplastic CA antisense plants compensate by increasing their stomatal conductance leading to an increase in water loss. In tobacco, antisense/mutant of chloroplastic CA that had substantial loss of CA produced no significant decrease in the rate of photosynthesis (Hines et al., 2021; Price et al., 1994).

The seedling establishment was affected in chloroplastic βCA1-deficient Arabidopsis (Ferreira et al., 2008). The knockout of cytosolic βca2 and βca4 reduced Arabidopsis growth rates and caused chlorosis of the younger leaves, when grown at low [CO₂] due to reduction in amino acid biosynthesis (DiMario et al., 2016); further, βCAs gene expression and enzymatic activities are important for optimal plant growth (Daßrowska-Bronk et al., 2016). A pertinent question is whether overexpression of cytoplasmic C₂ CA, having higher affinity for CO₂ (Kₘ = 0.8–2.8 mM) than that of C₃ CA (Kₘ = 15–42 mM) (Hatch and Burnell, 1990; Ignatova et al., 1998; Pocker and Ng, 1973; Tobin, 1970), and its higher ability to hydrate CO₂, in the cytosol of C₂ plants, can increase amino acid content and photosynthetic efficiency.

To explore this question, we have overexpressed carbonic anhydrase βCA3 from Flaveria bidentis into Arabidopsis thaliana (Figure 1); we show here that this indeed leads to increased amino acid (aspartate, asparagine, glutamate and glutamine) content, improved photosynthesis (16%–22%), better water-use efficiency (22%–26%), higher starch content (10%–19%), and even higher biomass (14%–20%).

### Results

#### Sequence alignment of Flaveria bidentis βCA3 with Arabidopsis thaliana βCA isoforms

Six isoforms of βCA are present in Arabidopsis thaliana: three are cytosolic, two chloroplastic, and one mitochondrial. A comparison of the amino acid sequence of Arabidopsis with Flaveria cytosolic βCA3 demonstrates homology of 57%–72% among cytosolic isoforms, 36%–58% in chloroplastic isoforms, and 33% in mitochondrial isoforms (Table S1); furthermore, the catalytic sites of the enzyme are highly conserved in the AtβCAs and in FbjCA3 (Figure S1).

#### Transformation, genomic DNA analysis of transgenic plants

To overexpress cytoplasmic C₄ CA into C₃ plants, we cloned FbjCA3 into a transformation vector pCAMBIA1304 (Figure 2a). Recombinant pCAMBIA1304: FbjCA3 was then used for Agrobacterium-mediated Arabidopsis transformation to raise transgenic plants.

Genomic DNA was isolated from different transgenic Arabidopsis lines overexpressing FbjCA3, and then the FbjCA3 was amplified by PCR using 5’S and 3’S specific forward and reverse primers that yielded a fragment of ~0.8 kb of FbjCA3, suggesting that the transgene had been integrated into the host genome (Figure S2a). To confirm the insertion of the binary vector, without the gene in vector control (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 (Figure S2b). Plants of these confirmed individual transgenic lines were then grown to harvest seeds; these seeds were grown again in kanamycin plates to select T₂ transgenic lines. Transgenic seeds were then grown to get T₄ generations to obtain homozygous transgenic plants for further use.

#### Southern blot analysis

The number of integrations of the T-DNA cassettes containing FbjCA3 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. However, the CAx₆ line showed a double band, which implies that there was a two-time integration of the transgene into the host genome (Figure S2c). VC showed the presence of a single copy of T-DNA cassette and, 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 the VCs (see the last section of ‘Results’ for differences in growth, and weight of these plants).

#### Gene expression

The qRT-PCR analysis, using gene-specific primers, showed that the expression of the FbjCA3 varied among the transgenic lines. The transcript abundance in CAx₁, CAx₃, and CAx₅ was 1.3-, 1.8-, and 2.3-fold higher, respectively, compared with that in the CAx₂ (Figure 2c).

#### Immunoblot analysis

To check changes in protein expression in the transgenics, we performed Western blot for proteins isolated from 4-week-old VC and FbjCA3x lines. Polyclonal antibodies raised against FbjCA3 protein, as described in Experimental Procedures, were used to immuno-detect the CA protein in different FbjCA3x lines. In the transgenic lines, the abundance of CA protein (~28 kD) was higher than in the VCs (Figure 2d,e). The VC showed the expected band due to the high homology of AtβCAs with FbjCA3.

#### Enzyme activity of carbonic anhydrase

CA activity in VC was 276 μmol CO₂ hydrated (mg Chl)⁻¹ min⁻¹, however, this activity, in different transgenic lines, ranging from 348 to 550 μmol CO₂ hydrated (mg Chl)⁻¹ min⁻¹, was 1.3- to 2.0-fold higher than in the VCs (Figure 2f). Based on their higher transgene expression, protein abundance and enzymatic activity, CAx₃, and CAx₅ transgenic lines were selected for further studies.

#### Chlorophyll, free amino acids, and protein content

There was no significant difference in the Chl content, Chl a/b ratio, free amino acids and the total protein content of WT and VC plants (Figure 3a-d). The CAx₃ and CAx₅ transgensics had 11% to 15% higher total Chl than the WT and the VC plants.
Figure 1 A proposed model of photosynthetic carbon flow in Arabidopsis thaliana overexpressing FbβCA3. The overexpressed cytosolic FbβCA3, having low Km for CO₂, increases the hydration of CO₂. The dashed arrows indicate the diffusion of CO₂ within the cytosol and the organelles. FbβCA3 overexpression increases the flux of the carboxylic acid to the tricarboxylic acid (TCA) cycle in mitochondria and plays an anaplerotic role in synthesizing higher amounts of total amino acids and proteins that contribute to increases in photosynthetic efficiency and biomass (OAA- oxaloacetic acid; PEP- phosphoenolpyruvate; PEPC- phosphoenol pyruvate carboxylase; TCA cycle- tri carboxylic acid cycle).

Enzyme activity of phosphoenolpyruvate carboxylase (PEPC)

In vitro assay of PEPC, where 10 mM sodium bicarbonate was exogenously added to the reaction mixture, showed its activity to be similar in the VC and transgenic plants (~3.45 µmol (mg protein)⁻¹ h⁻¹) (Figure S3).

Whole-chain, PSII, and PSI reactions

Under saturating light, and on Chl basis, thylakoids isolated from the CA overexpresser plants, as compared to that from the VC plants, showed higher (20%–28%) electron transfer rates for the whole chain (WC) reaction (Figure 3g). The partial reactions of PSII as well as of PSI increased by 21%–26% and 23%–32%, respectively, in the transgenics (Figure 3h,i).

Chlorophyll a fluorescence

To obtain further information on WC, particularly on PSII, we used Chl a fluorescence as its non-invasive signature. Various Chl a fluorescence parameters were measured as described under Experimental Procedures; these results are described below.

Rates of electron transport (ETR II and ETR I)

At saturating light intensity (540 µmol photons m⁻² s⁻¹), the calculated values of ETR II and ETR I were higher by 13%–19%
and 17%–25%, respectively, in the transgenics than in the VC plants (Figure 4a,b).

Chlorophyll a fluorescence transient

For these measurements, OJIP curves were recorded up to 1 s, after excitation with 650 nm light of high intensity (3500 µmol photons m⁻² s⁻¹), provided by an array of 3 LEDs. Different curves in Figure 4c are for Chl a fluorescence transients of dark-adapted leaves of Arabidopsis thaliana (Figure 4c) plotted on a logarithmic time scale from 20 µs to 1 s; here, all the curves were normalized at 20 µs (taken to be Fo). Fluorescence transients of both VC and transgenic lines show typical OJIP curves; CAx3 and CAx5 show 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.

When we double-normalize the IP rise, that is, both at the I level (30 ms) as well as at the P level (290 ms) (see Figure 4d), we observe that the transgenic plants have a faster IP rise than the VC. Furthermore, when the OJIP curves are normalized at ‘I’ only, we observe a faster IP rise in the transgenics than in the vector controls (Figure 4e).

We now describe observations on quantitative differences between the VC and the transgenics on several key Chl a fluorescence parameters.

1. \( F_o \), the initial minimum fluorescence: As compared to the VC, CAx3 and CAx5 plants have ~8% and ~11% higher values (Table S2), but this is simply due to higher [Chl] in the transgenics (Figure 3a) since there was no difference in the values of \( F_d/\text{Chl} \).

2. \( F_m \), the maximal fluorescence (see Experimental Procedure): The \( F_m \) values of CAx3 and CAx5 plants are higher by ~18% and ~25% (Table S2); furthermore, the \( F_m/\text{Chl} \) values are 6%–9% higher in the transgenics than in the VC.

3. \( F_o/F_n \), an estimate of the maximum potential quantum efficiency of PS II: when all the reaction centres are open (i.e. when dark-adapted samples are used), this ratio increased only slightly (~5%) in the transgenic plants (Table S2).

4. Area over the OJIP curve, an area between \( F_o \) and \( F_m \), which is proportional mainly to the size of the plastocyanine pool (Malkin and Kok, 1966): It was clearly higher (19%–22%) in CAx3 and CAx5 than in the VCs (Table S2).

5. \( F_d/F_o \), reflecting the efficiency of the (electron) donor side of PSII (Burke, 1990): It was higher (11%–16%) in the transgenics than in the VCs (Table S2).

6. \( P_l \), the performance index (see Tsimilli-Michael et al., 2000): It was higher (10%–20%) in the transgenics than in the VCs (Table S2).
7. Non-Photochemical Quenching (NPQ) of the excited state of Chl: This parameter, which increases with light intensity, was lower (5%–9%) in CAx3 and CAx5 than in the VCs at 540 μmol photons m⁻² s⁻¹ (Figure 4f).

Gene expression
1. Expression of chlorophyll biosynthesis genes: (i) In comparison with the VC, PBGS (porphobilinogen synthase) was 1.7- and 2.2-fold higher in CAx3 and CAx5 (Figure 5a). (ii) 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 than in the VCs (Figure 5a). (iii) The gene expression of PPOX (protoporphyrinogen oxidase), responsible for protoporphyrin IX synthesis, increased 1.4–3-fold in the transgenics (Figure 5a). (iv) Furthermore, protoporphyrin-IX Mg-chelatase (CHL1), 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, compared with that in the VCs (Figure 5a). (v) The expression of light-inducible protochlorophyllide oxidoreductase (PORC) was 1.8–2.5-fold higher in the transgenics than in the VC plants (Figure 5a).

2. Expression of photosynthesis-related genes: (i) The gene expression of Lhcb1 and Lhcb2.1, encoding components of LHCII, was 2.1–2.5 and 1.9–2.5-fold higher in the transgenics (Figure 5b). (ii) The gene expression of Lhca1 and Lhca2, encoding components of LHClI, increased by 2–3.45- and 1.2–1.4-fold in the overexpressers (Figure 5b). (iii) Expression of PsbA and PsbD, encoding PSI’s D1 and D2 proteins, was 3.2–3.6- and 1.7–2.3-fold higher in the transgenics (Figure 5b). (iv) 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 (Figure 5b).

Western blots of electron transport chain components
To ascertain if increased gene expression resulted in higher protein abundance in the transgenics, proteins of certain gene products were analysed by immunoblot. Besides, other photosynthetic proteins, that is, those involved in Chl biosynthesis, light harvesting, photosynthetic electron transport chain, and rubisco were analysed by Western blot (Figure 5c).

The images of Western blots revealed the abundance of UROD; LHClI, light-harvesting Chl-binding proteins, that is, Cytb6f complex, and Cyt f; electron transport proteins of PSI, PsAE (PSI subunit IV); both the rubisco subunits (SU), the large (LSU) as
well as the small (SSU), increased in the CA overexpressers (Figure 5c). Light response curves for photosynthesis Figure 6 shows the photosynthetic light response curves of the attached leaves, of both VC and the transgenics, as measured by IRGA (LiCOR-6400/XT), using 6400-18A RGB (red, green, blue) light source under ambient CO₂ (400 μmol/mol) and ambient O₂ (21%). At saturating light intensity (800 μmol photons m⁻² s⁻¹), leaves of CAx3 and CAx5 had 17% and 23% higher net CO₂ assimilation rate, than in the VC plants (Figure 6a). The rate of respiration in the leaves of transgenics, as compared to the vector control, was also higher (~12%–16%) (Figure 6a, b). The quantum yield of CO₂ fixation, as measured at limiting-light intensities (up to 80 μmol photons m⁻² s⁻¹) was 0.0423 in VC plants; it was higher by ~18% in CAx3 and CAx5 (Figure 6b). [We note that while calculating the quantum yield of CO₂ fixation, data at very low light intensity (10 μmol photons m⁻² s⁻¹) were excluded from the regression analysis to avoid mixing it with the Kok effect, that is, inhibition of respiration by light (Sharp et al., 1984).]

Stomatal conductance (gₛ) and water-use efficiency (WUE) The CO₂ assimilation rate, measured at 400 μmol photons m⁻² s⁻¹, was 16% and 22% higher in CAx3 and CAx5 than in the VCs (Figure 6c). We note that the increased photosynthetic rate was associated with decreased stomatal conductance and transpiration rates in the transgenics. Stomatal conductance decreased by 10%–16% in the transgenics, as compared to that in the VC plants (Figure 6d); in addition, the transpiration rate decreased by ~7% in the FbβCA overexpressers (Figure 6e). Therefore, the water-use efficiency, the ratio of the CO₂

Figure 4 Electron transport reactions, the OJIP curves of chlorophyll a fluorescence and the non-photochemical quenching (NPQ) of the excited state of chlorophyll a of the vector control and FβCA3x plants grown in soil. (a): ETRII; (b): ETRI; (c): Chl a fluorescence transients, with the OJIP curves normalized at the O level; (d): Variable fluorescence transients from the I to the P—for double normalization between I (F_I) and P (F_P); V_IIP = (F_t−F_I)/(F_P−F_I); (e): 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; (f): NPQ of the excited state of chlorophyll at different light intensities. Each data point is an average of five replicates for 4a, b, and f and average of 8 replicates for 4c, d, and e; error bars represent ± SE.
assimilation rate to the transpiration rate (μmol CO₂ m⁻² s⁻¹/ mmol H₂O m⁻² s⁻¹), was higher by 22% and 26% in CAx3 and CAx5 than that in VC (Figure 6f).

A/Cᵢ, Carbon assimilation rates and intercellular CO₂

In order to evaluate the contribution of photosynthesis, A/Cᵢ curves, under 21% and 2% O₂ were measured (Figure 7a). In 21% O₂, the transgenic plants had higher photosynthetic capacity (the maximum rate of photosynthesis under CO₂ saturation at saturating light) than the VC plants. At saturating Cᵢ (~1000 ppm), CO₂ assimilation rate increased by ~19% in CAx3 and ~22% in CAx5 transgenics as compared to the VC curves (Figure 7a). Under 21% O₂, the carboxylation efficiency (CE), the initial slope of A/Cᵢ curve (i.e. between 50 and 200 μmol mol⁻¹ CO₂), in VC was 0.038 mol m⁻² s⁻¹, which increased (by ~17%) to 0.044 mol m⁻² s⁻¹ in the transgenics. Further, there was no significant difference in the CO₂ compensation point of the VC and the transgenics. Similarly, the Vᵢmax (cf. Bernacchi et al., 2001) was 34 μmol m⁻² s⁻¹ for VC, which increased to 40 μmol m⁻² s⁻¹, that is, by ~18% in transgenics. The electron transport rate Jᵢmax was 67 μmol m⁻² s⁻¹ in the VC, and it was higher by ~18%, that is, 84 μmol m⁻² s⁻¹ in the overexpressers. At higher CO₂ concentration, the A/Cᵢ curve was parallel to the X-axis suggesting that the VCs and the transgenics may have limitation in triose phosphate utilization (Sharkey et al., 2007).

In 2% oxygen, the maximum rate of CO₂ assimilation, at saturating CO₂, was similar to that under 21% O₂ in all plants. However, the net CO₂ assimilation was 22%–25% higher in the transgenics than in the VCs. The carboxylation efficiency (CE) significantly increased with the decrease in the O₂ level, from 21% to 2%. The CE in VC was 0.045 mol m⁻² s⁻¹ but, it was 0.054-0.056 mol m⁻² s⁻¹ (higher by 19%–23%) in the transgenics under 2% O₂.

Mesophyll conductance (gᵢ) and chloroplast CO₂ concentrations (Cc)

In 6-week-old VC plants, leaf gᵢ, estimated from the combined gas exchange and other parameters, was 0.064 mol CO₂ m⁻² s⁻¹ bar⁻¹. In the two transgenics, gᵢ increased to 0.073 or 0.084 mol CO₂ m⁻² s⁻¹ bar⁻¹. However, these gᵢ values were comparatively lower in the VC as well as in the transgenics since the experiments were done on six-week-old plants (Flexas et al., 2007). Although there are problems in accurately measuring gᵢ in Arabidopsis leaves, because of their small size, we consistently observed increases in gᵢ in the transgenics. Similarly, using Thomas Sharkey’s A/Cᵢ curve fitting model (Sharkey et al., 2007), the gᵢ values had a similar pattern (15%–22% increase) in the overexpressers. However, this model did not predict a significant increase in the Cs of the transgenics.

Growth, weight, sugar 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 3-week-old VC, CAx3, and

Figure 5  Relative gene expression and immunoblot analysis. Relative expression of genes related to (a): Chlorophyll biosynthesis; (b): Photosynthesis; (c): Abbreviations: PBGS: porphobilinogen synthase; UROD: encoding uroporphyrinogen decarboxylase; PPOX: 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 PSI; Lhca1 and Lhca2, encoding components of the light harvesting complex associated with PSII; 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; Cytb₅₆ and CytI, psaE- PSIV; rubisco LSU and SSU: rubisco large and small subunits. qRT-PCR data are expressed as the mean ± SE of three independent experiments performed in triplicate. Asterisks indicate significant differences determined by ANOVA along with Dunnett’s post hoc test compared to control (*P < 0.05, **P < 0.01).
CAX5 plants: both the overexpressers had longer (~14%) roots than in the VC plants (Table S3). Although their shoot lengths were almost similar (Table S3), they had larger (11%–15%) rosette diameter than in the VC plants (Table S3). After 3 weeks of growth, the dry weight of CAX3 and CAX5 plants increased by 14%, and 20%, respectively, as compared to VC plants (Figure 7b). Furthermore, increased photosynthetic carbon assimilation in the transgenics resulted in higher sugar and starch content. The content of glucose, fructose, and sucrose increased by 100%, 34%, and 19%, respectively, as compared to VC plants (Figure 7c). Similarly, the starch content of the leaves, harvested before dusk, were almost similar (Table S3), they had larger (11%–15%) roots than in the VC plants (Table S3). Although their shoot lengths were almost similar (Table S3), they had larger (11%–15%) rosette diameter than in the VC plants (Table S3). After 3 weeks of growth, the dry weight of CAX3 and CAX5 plants increased by 14%, and 20%, respectively, as compared to VC plants (Figure 7b). Furthermore, increased photosynthetic carbon assimilation in the transgenics resulted in higher sugar and starch content. The content of glucose, fructose, and sucrose increased by 100%, 34%, and 19%, respectively, as compared to VC plants (Figure 7c). Similarly, the starch content of the leaves, harvested before dusk, increased by 10%–19% (Figure 7d).

All the above results clearly show that the transgenics had higher carboxylic acids, amino-acids, proteins, photosynthesis, carbohydrate, and biomass content than the VCs.

**Discussion**

We have generated *Arabidopsis thaliana* lines overexpressing C₄ *Flaveria bidentis* pCA3 with higher affinity for CO₂. Overexpression of C₄ FbB1CA3 under the control of 3SS promoter resulted in higher gene expression, protein abundance, and enzymatic activity of CA, in the transgenic lines (Figure 2c,f,g). The expression of a transgene in the chloroplast usually results in higher level of transgene expression (Daniell et al., 2001, and Lee et al., 2003).

Silencing of cytosolic pCA2 and pCA4 that decreased the carbon skeleton pool substantially reduced the aspartate, glutamate, and glutamine content in *Arabidopsis thaliana* (DiMario et al., 2016). One of the reasons is that PEPC in the cytoplasm uses HCO₃⁻ to generate 50% of the aspartate in leaves (Melzer and O’Leary, 1987). The aspartate content was reduced to about two-thirds by the knockdown of PEPC (Osppc4) in rice (Masumoto et al., 2010). Conversely, our data demonstrate that increased CA activity resulted in higher aspartate in the transgenics. The aspartate and its derivative asparagine increased by 70%–80% (Figure 3f). Similarly, glutamate and glutamine synthesized from the enriched supply of TCA cycle intermediate oxoglutarate also increased (10%–17%) in the transgenics (Figure 3d). Increased amino acid synthesis was due to the increased supply of carbon skeleton by TCA cycle intermediates, that is, OAA and oxoglutarate. We observed 40% increase of malate (Figure 3e), generated by the reduction of OAA by malate dehydrogenase (MDH). Furthermore, fumarate, another TCA cycle intermediate, increased by 30% (Figure 3e).

The increased availability of amino acids resulted in higher protein content in the transgensics (see Figure 1). Although Chl and proteins are synthesized via two different metabolic processes, their biosynthetic pathways are co-regulated by the nitrogen status of the plant (Garai and Tripathy, 2018). We propose that higher Chl content in the transgensics must have been due to increased expression of Chl biosynthetic genes.
Higher photosynthetic efficiency in the transgenics

The increased ETR of PSII and PSI at low light (50–125 mol photons m$^{-2}$ s$^{-1}$) in CA overexpressor plants may be due to the availability of more photons absorbed by the larger antenna, and the latter may be due to increased gene/protein expression of light harvesting components of PSII and PSI: Lhcb2.1, Lhcb1, Lhca1, and Lhca2 (Figures 5b,c and S5). ETR at high light (~550 mol photons m$^{-2}$ s$^{-1}$) was also higher (13%–19% for PSII, 17%–25% for PSI) in CAx plants than in the VC (Figure 4a, b), consistent with light-saturated PSI, whole-chain and PSI rates in isolated thylakoid membranes (Figure 3g–i). We suggest that higher light-saturated PSI- and PSI-dependent ETR in the leaves, and higher PSII and PSI activities of thylakoid membranes, from the transgenics, are due to the increased gene expression/protein abundance of PsbA, PsbD, PsbO, PsaE, Cytb$\alpha$f complex, and other proteins. We conclude that the CAx plants have (i) a higher light-harvesting capacity under limiting light due to larger antenna and (ii) a greater ability to use high light due to more and efficient PSII, PSI, and intersystem ETCs. Also, OEC33, on the electron donor side of PSI, has CA activity (Lu et al., 2005). Thus, higher OEC33 in the transgenics may have led to enhanced PSI-dependent ETR, under high light. Coupled with higher PSI and PSII reactions, increased cytochrome b$\alpha$f complex may have contributed to higher WC activity in the transgenics. For other systems, see Biswal et al. (2012) and Simkin et al. (2017). Further, PsaE (PSIIV, Figure 5c), involved in PSI cyclic electron transport, which prevents electron leakage to O$_2$ (see Jeanjean et al., 2008), was increased in CAx plants; thus, this may have minimized the photoreduction of ROS by PSI.

In the Chl a fluorescence induction (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; this may be due to higher efficiency of electron transport in the transgenics (Figure 4c; cf. Kandoi et al., 2016; Jiménez-Francisco et al., 2020). The faster IP rise in the transgenics suggests higher photosynthesis efficiency (cf. Hamdani et al., 2015; Soda et al., 2018), consistent with their higher ‘performance index’. The area over the OJIP curve, between $F_o$ and $F_m$, proportional to the size of the pool of the electron acceptors in PSII (Malkin and Kok, 1966), was higher (19%–22%) in the CAx3 and CAx5 than in the VC plants (Table S2), confirming the advantage of the transgenics over the controls.
Carbon fixation and water-use efficiency (WUE)

The rate of CO₂ fixation measures the performance of plants. We demonstrate here that overexpression of FbjCA3 resulted in higher rates of photosynthetic carbon assimilation, per unit leaf area, than in the VC plants (Figure 6a). In the transgenics, the quantum yield of CO₂ assimilation, measured in limiting light intensities, other than at 10 μmol m⁻² s⁻¹ (to minimize Kok effect; Sharp et al., 1984; Figure 6b), increased by ~18%. Thus, the transgenics had higher photosynthetic efficiency and they utilized the absorbed light energy much more efficiently. Leaf is critical for transpiration, and vascular water transport sets limits on growth and drought tolerance. Our results demonstrate that the overexpression of FbjCA3 in A. thaliana led not only to higher photosynthesis but also to lower stomatal conductance, decreased transpiration, and a better WUE (Figure 6c). Although the overexpression of jCA1 or jCA4 in the guard cells resulted in better WUE, there was no increase in photosynthesis rates (Hu et al., 2010), showing the key role of CA in mesophyll cells. The overexpression of FbjCA3 could, indeed, produce enough HCO₃⁻ under ambient CO₂ to partially close stomata to reduce the stomatal conductance and transpiration rate and, thus, increase the WUE. Our results further demonstrate that for efficient photosynthetic performance, relatively high CA activity is one of the important requirements in A. thaliana. For results on other systems where reduced CA activity leads to reduced photosynthesis and higher stomatal conductance, see von Caemmerer et al. (2004), Cousins et al. (2006); Osborn et al. (2017), Kolbe et al. (2018) and Ogé et al. (2018).

CO₂ response curves

CO₂ response curves provide crucial information on the photosynthetic capacity of plants at varying levels of CO₂. A typical response of light-saturated CO₂ assimilation rate (A) to leaf intercellular CO₂ mole fraction (C) has three phases: (i) when assimilation is limited by active rubisco (slope of the initial phase); (ii) when Amax is reached due to limitation by the supply of RuBP; and (iii) when there is no increase in carbon assimilation with increasing [CO₂], or to increasing [O₂], or when the system is limited by triose-phosphate utilization (Ainsworth and Long, 2005; Bernacchi et al., 2013; von Caemmerer and Farquhar, 1981; Sharkey, 1985; Sharkey et al., 2007). In 21% O₂, the transgenics had higher A, CE, V_cmax and q_m than the vector control. Higher q_m may improve photosynthetic efficiency and intrinsic water-use efficiency (Flexas et al., 2013). Increase in CE and V_cmax in the transgenics suggests that at the atmospheric [CO₂], the higher rates of photosynthesis is predominantly due to the anaplerotic role played by overexpressed CA that enhanced carboxylic acids, amino acids and protein content of plants (Figure 3d–f). The protein content of the leaves increased in the FbjCA3 overexpressers. Since nearly half of the leaf protein is in rubisco, it is highly likely that transgenics had relatively higher rubisco content (Majeau et al., 1994, and Majeau and Coleman, 1994), resulting in increased CE. Furthermore, V_cmax/CE has been shown to correlate with the amount of rubisco (von Caemmerer and Farquhar, 1981; Jacob et al., 1995; Makino et al., 1994; Manter and Kerrigan, 2004).

The CO₂ compensation point was almost similar both in the VC and in the transgenic plants and hence the rate of photorespiration did not decrease in the CA overexpressers. The increase in V_cmax and RuBP regeneration, in the transgenics, probably was partly due to a general increase in the protein content of leaves. The transgenics could recapture photorespiratory CO₂ by the efficient FbjCA3 present in their cytoplasm. Under 2% O₂, photorespiration is expected to be minimal. Extrapolation of the A/CO₂ curve at 2% O₂ shows the CO₂ compensation point to be at ~7 μmol mol⁻¹ (Figure 7a). Furthermore, the A/CO₂ curve in both the VC and the transgenics saturated at ambient CO₂; however, the CE of the transgenics was higher than that of the VC plants. The increases in CE and in the maximum rates of photosynthesis, under ambient CO₂, in the transgenics was clearly due to an increase in the inherent efficiency of photosynthesis. Under 2% O₂, the maximum rate of CO₂ assimilation is the same as under 20% oxygen, indicating 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 content of carbohydrates (glucose, fructose, sucrose and starch) was substantially higher in the transgenics (Figure 7c,d). The dark respiration is an important part of the plant carbon budget. The increased dark respiration, in the transgenics, is commensurate with higher carboxylic acid flux to the TCA cycle in these plants. The additional starch produced in CA overexpressers was only partly used in the dark by respiration, perhaps for increased growth with higher biomass, in agreement with the data of others (Biswal et al., 2012; Ermakova et al., 2019; Kandoi et al., 2016; Lefebvre et al., 2005).

Increases in overall biomass is important for obtaining high-yielding bioenergy crops (Ort et al., 2015), where PSII photosynthetic efficiency, carbon assimilation, starch content, and dry matter accumulation have been shown to be high. Our results on transferring carbonic anhydrase from Flaveria bidentis to Arabidopsis thaliana have demonstrated a significant increase in carboxylic acids, amino acids, protein content, electron transport, carbon assimilation, starch content, and dry matter, which is in the right direction to meet the global needs ahead of us.

Experimental procedures

Sequence analysis

Homology search was made with basic local alignment search tool (BLAST), using Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalo/), signal sequence predictions by Target P (http://www.cbs.dtu.dk/services/TargetP/), and nucleotide sequence data were from a web site: http://www.ncbi.nlm.nih.gov/.

Generation of transgenic and growth conditions

The full-length FbjCA3 (accession no-AY167113) was cloned in pGEM-T Easy vector and subsequently in modified pCAMBIA1304 (Pattanyak and Tripathy, 2011), and then transformed in Arabidopsis using agrobacterium-mediated floral dip method (see Kandoi et al., 2016, and Table S4). VC plants containing the null vector pCAMBIA1304 (i.e. without FbjCA3 cDNA) were also generated and used for comparison with the transgenics since no significant differences in growth parameters were seen between the wild type (WT) and the VC plants.

Seeds of the transformed plants were screened on half-strength Murashige and Skoog (MS) agar medium containing 50 μg/mL kanamycin. Stratified Arabidopsis seeds were sown in agroplant: 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 ± 1 °C. Plants' position was randomized, and the position of the trays rotated daily under the light.
Confirmation of transgenic lines by polymerase chain reaction (PCR) and Southern blot analysis

Genomic DNA was isolated by cetyl trimethyl ammonium bromide (CTAB) method (Nickrent, 1994) from four-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 FbjCa3 reverse primer to ensure the incorporation of the whole cassette in sense orientation. The presence of FbjCa3 transgene was analysed by Southern blot analysis. The genomic DNA from the leaves of the T3 generation of the vector control and the transgenic plants was digested with the restriction enzyme XbaI. The nptII coding sequence, amplified from the plasmid, was used for probe preparation, and labelled with (32P) dCTP, using a radioactive random primer labelling kit (Amersham-GE, UK). Southern blot was developed and used, as described by Sambrook and Russell (2001).

qRT-PCR

Relative expression of different genes was checked by 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 S5). Following the minimum information for publication of quantitative real-time PCR experiment (MIQE) guidelines (Bustin et al., 2009), all the details are provided in the supplementary material of our paper. The relative gene expression data were analysed using 2^(-ΔΔCt) quantification methods (Livak and Schmittgen, 2001; Taylor et al., 2019). Details of the procedure are also described in the supplementary material.

Western blot analysis

For immunoblot analysis, electrophoresis was carried out using 25 μg of plant protein, extracted from four-week-old leaves. Proteins were quantified by spectrophotometry, and Coomassie brilliant blue R250 methods were used for the visualization of proteins separated by SDS-PAGE. Separated proteins were blotted on nitrocellulose membranes (Towbin et al., 1979). The blots were probed using antibodies raised against the specific protein. To track the transfer efficiency of proteins in the blot lanes, reversible Ponceau staining was used because of its cost effectiveness (Sander et al., 2019). The rabbit anti-mouse IgG (1:20 000) conjugated to alkaline phosphatase (Sigma-Aldrich) was used as a secondary antibody. Blots were stained for alkaline phosphatase using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. Additionally, polyclonal antibodies were used (for details, see Supplementary Table S6) for some major enzymes of Chl biosynthetic pathway. CA polyclonal antibody was raised in rabbits against epitope sequence TAQLTDSTKPGFDPVER and commercially prepared by Imgenx. Enzyme-specific activity was expressed as μmol of NADH oxidized per mg of protein per hour. These experiments were performed using five biological replicates.

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

Thylakoid membranes were isolated in a buffer containing 0.01 M Tris and 1 mM EDTA (pH 7.5). The homogenate was centrifuged at 12 000 g for 5 min and the pellet, containing thylakoid membranes, was suspended in a buffer containing 0.4 M sorbitol, 0.05 M Tris (pH 7.5), 1 mM MgCl2, and 1 mM EDTA (Gupta and Tripathy, 2010; ilani et al., 1996; Mohapatra and Tripathy, 2003). Using thylakoid membranes isolated from both WC and CAx plants, we monitored electron transport: (i) from water to methyl viologen [MV], involving both PSII and PSI; (ii) from water to phenylenediamine [PD], which measures only PSII; and (iii) from ascorbate/dichlorophenol-indophenol to MV, which measures only PSI. All the above measurements used a Clark-type oxygen electrode, as described by Chakraborty and Tripathy (1992). These experiments were performed using five biological replicates.

Determination of metabolite levels by GC-MS

For gas chromatography-mass spectrometry (GC-MS) analysis, polar metabolites were extracted with isopropanol: acetonitrile: water (3:3:2) mixture from 100 mg of complete rosettes ground previously to a fine powder. Metabolite samples were derivatized by methoxyamination, using a 20 mg/mL solution of methoxyamine hydrochloride in pyridine, and subsequent trimethylsilylation with N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA). An aliquot of the derivate was injected into a GC-MS system (GCMS QP2010 Plus, Shimadzu). Signals were normalized to an internal standard molecule introduced into the samples (ribitol), allowing a relative quantification of the metabolites. Data sets presented were normalized to the wild-type control of each measured batch as a reference. These experiments were performed using three biological replicates.

Pulse-amplitude modulation (PAM) and Handy PEA measurements

Chl a fluorescence (for PSI activity) and transmission changes at 830 nm (for PSI activity) were measured simultaneously by the
Dual-PAM-100 system, using an automated “Light Curve” program from Walz (for details, see Yuan et al., 2014). These experiments were performed using five biological replicates. In addition, Chl a fluorescence induction was measured using Handy PEA (Plant Efficiency Analyzer), Hansatech Instruments, UK. For these measurements, Arabidopsis seedlings were pre-darkened for 20 min at room temperature (for details, see Kandoi et al., 2016, and references therein). These experiments were performed using eight biological replicates.

**Carbon dioxide assimilation**

Rates of CO₂ assimilation, at different light intensities (constant [CO₂] 400 μmol mol⁻¹) as well as at constant light intensity (400 μmol photons m⁻² s⁻¹), different [CO₂] were measured using infrared gas analyzer, IRGA (LiCor 6400XT), with 6400-18A RGB (Red, Green, Blue) light source. During the experiments, the temperature of the chamber was maintained at 25 °C, and a relative humidity of 60%–70%. These experiments were performed using five biological replicates. For the measurements of A/C curve (net CO₂ assimilation rate, A, versus calculated internal CO₂ concentration, C), humidified 2% O₂ and 98% N₂ was used; the carboxylation efficiency was obtained from the slope of the A/C curve (Li et al., 2009). Mesophyll conductance and chloroplastic CO₂ (Cc) were calculated using Thomas Sharkey’s curve fitting model (Sharkey et al., 2007).

**Starch content**

For measuring the starch content, leaf samples were collected from three-week-old plants in the plant growth chamber immediately prior to the end of the light period. Samples were digested with percolic acid, and then the starch was assayed spectrophotometrically, using anthrone, a colour reagent (Rose et al., 1991). These experiments were performed using five biological replicates.

**Statistical analysis**

All the details for this analysis are described in the Supplementary Material.

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**Conflict of interest**

There is no known conflict of interest.

**Author contributions**

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

Data availability statement

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

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.
**Figure S1** Comparison of the amino acid sequences of *At*βCAs with *Fb*βCA3 (cytoplasmic carbonic anhydrase, CA).

**Figure S2** Confirmation of *Flaveria bidentis* CA overexpressed plants (a): PCR reaction was performed with genomic DNA isolated from different transgenic lines of T1 generation plants by using 35S internal forward primer and gene specific reverse primer; (b): Genomic DNA of vector control and T1 generation of CAx plants, having resistance to nptII, were used as template and PCR of nptII was done by using nptII primers; (c): Southern blotting using nptII as a probe.

**Figure S3** PEPC enzymatic activity in vitro; the activity of PEPC in the transgensics was similar to that in the vector control plants (~3 μmol/mg protein/h).

**Figure S4** RNA denaturing agarose gel electrophoresis of VC and several transgensics.

**Figure S5** Immunoblot analysis of LHCII and LSU of rubisco in the vector control and the two transgensics CAx3 and CAx5 (a) LHCII; (b) rubisco large subunit (LSU).

**Table S1** Score of pairwise alignment of different sequences of *Arabidopsis thaliana* βCA with *Flaveria bidentis* βCA3 (258 amino acids) and localization of these isoforms.

**Table S2** Chlorophyll a fluorescence measurements of VC and CAx plants grown in soil.

**Table S3** Morphological parameters of the transgensics and the vector control plants.

**Table S4** Primers used for cloning.

**Table S5** Primers used for RT-qPCR analysis.

**Table S6** Antibodies used in this study.