Field-grown ictB tobacco transformants show no difference in photosynthetic efficiency for biomass relative to the wild type

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Abstract

In this study, four tobacco transformants overexpressing the inorganic carbon transporter B gene (ictB) were screened for photosynthetic performance relative to the wild type (WT) in field-based conditions. The WT and transgenic tobacco plants were evaluated for photosynthetic performance to determine the maximum rate of carboxylation ($V_{c,max}$), maximum rate of electron transport ($J_{max}$), the photosynthetic compensation point ($Γ^*$), quantum yield of PSII ($Φ_{PSII}$), and mesophyll conductance ($g_m$). Additionally, all plants were harvested to compare differences in above-ground biomass. Overall, transformants did not perform better than the WT on photosynthesis-, biomass-, and leaf composition-related traits. This is in contrast to previous studies that have suggested significant increases in photosynthesis and yield with the overexpression of ictB, although not widely evaluated under field conditions. These findings suggest that the benefit of ictB is not universal and may only be seen under certain growth conditions. While there is certainly still potential benefit to utilizing ictB in the future, further effort must be concentrated on understanding the underlying function of the gene and in which environmental conditions it offers the greatest benefit to crop performance. As it stands at present, it is possible that ictB overexpression may be largely favorable in controlled environments, such as greenhouses.

Keywords: Biomass production, crop production, ictB gene, photosynthesis, photosynthetic efficiency, water use efficiency.
Introduction

By the year 2050 it is projected that global food supply will need to increase by 50–85% to keep pace with a growing human population and shifting dietary preferences with greater emphasis on the consumption of animal products (Tilman et al., 2011; Ray et al., 2012, 2013; Long et al., 2015; FAO et al., 2020). As a result, yields of staple crops must increase at a considerably greater rate than today to ensure future food security. Furthermore, future crop varieties must be more sustainable and utilize water and nutrients more efficiently if they are to be environmentally sustainable (Foley et al., 2011; Tilman et al., 2011). While properties such as harvest index and light interception by the canopy have been improved close to their theoretical maxima over the past half century, little improvement has been made to photosynthetic efficiency in crop plants (Zhu et al., 2008). Not only is the current rate of improvement in yield of crops plants insufficient to meet the projected future demand, but it may be stagnating (Long and Ort, 2010; Ray et al., 2012; Long et al., 2015). Increasing photosynthetic efficiency is a little exploited approach that holds great potential promise for improving yield and resource use efficiencies in crops (Zhu et al., 2008; Long et al., 2015).

Most major crops consumed by humans utilize the C3 photosynthetic pathway. C3 crops assimilate CO2 from the atmosphere inefficiently due to the lack of a carbon-concentrating mechanism, several internal resistances to CO2 diffusion, and because Rubisco is catalytically slow with a slow catalytic rate of CO2 assimilation in current atmospheric conditions (Tcherkez et al., 2006; Price et al., 2013; Erb and Zarzycki, 2018). The C3 photosynthetic process is also inefficient in its use of water and nitrogen (Parry et al., 2011; Long et al., 2018). Engineering a carbon-concentrating mechanism in C3 crops, much like those seen in C4 and cyanobacteria, would significantly reduce these inefficiencies (McGrath and Long, 2014; Long et al., 2015). Indeed, many recent initiatives have aimed to improve C3 photosynthetic efficiency in crop plants to improve yield and productivity, such as the engineering of a C4 pathway in rice or constructing cyanobacterial carboxysomes in C3 chloroplasts (Mitchell and Sheehy, 2006; Long et al., 2018; Ernakova et al., 2020).

The inorganic carbon transporter B gene (ictB) is a highly conserved gene among cyanobacteria that was proposed to be involved in inorganic carbon accumulation in Synechococcus PCC 7942 (Bonfil et al., 1998; Lieman-Hurwitz et al., 2003; Price et al., 2013). Previously, it was thought that ictB functioned as a carbon pump which could increase CO2 concentration within the leaf and improve photosynthesis (Lieman-Hurwitz et al., 2003). Since then evidence has been presented showing that the ictB protein does not function as a HCO3- transporter (Xu et al., 2008; Price et al., 2013), and therefore its function remains unknown (Simkin et al., 2019).

Although the exact function of ictB is not yet known, several studies over the past 20 years have indicated that overexpressing ictB improves photosynthetic efficiency in C3 plants. Previously, Arabidopsis and tobacco transplants overexpressing ictB and grown in a controlled environment were found to have a significantly lower photosynthetic compensation point (F*) than the wild type (WT) (Lieman-Hurwitz et al., 2003). This result suggested that increased ictB expression increased [CO2] at Rubisco, consequently increasing the carboxylation rate while competitively inhibiting oxygenation (Lieman-Hurwitz et al., 2003; Hay et al., 2017). In greenhouse-grown tobacco, ictB expression led to an increase in the maximum rate of carboxylation (Vc,max), the maximum rate of electron transport (Jmax), leaf CO2 uptake rate (A), and stomatal conductance (gsv) (Simkin et al., 2015). Additionally, ictB expression may help boost photosynthetic performance in field conditions. Paddy-grown rice expressing ictB had significantly (10.5%) higher mesophyll conductance (gms) and 13.5% higher A compared with the WT (Gong et al., 2015). Field-grown maize also benefited with increases in A and carbohydrate production, with increases in yield of up to 9.4% (Koester et al., 2021). Replicated field trials of ictB-expressing soybean showed significant increases of 25% in gms, 14% in A, and 15% in seed yield relative to the WT (Hay et al., 2017). Other studies have also noted increases in biomass production (Lieman-Hurwitz et al., 2003; Yang et al., 2008; Simkin et al., 2015). Expression of ictB led to faster plant growth and greater accumulation of biomass under low–humidity conditions in Arabidopsis (Lieman-Hurwitz et al., 2003) and higher overall biomass in soybean under water deprivation conditions (Hay et al., 2017). Additionally, biomass increased by 71% in greenhouse-grown ictB tobacco transplants (Simkin et al., 2015).

However, these gains may not always translate when grown in field conditions where improvements to crops would be most relevant towards improving food production. Indeed, previous studies have shown that ictB expression has not resulted in increased biomass (Gong et al., 2015), except in drought conditions (Hay et al., 2017). Previously, ictB tobacco transplants were shown to have increased photosynthetic performance and biomass without affecting water use efficiency (Simkin et al., 2015). However, these transplants were only screened within the context of a controlled growth environment (Simkin et al., 2015). In the present study, the tobacco transplants developed and utilized in Simkin et al. (2015) were grown in field conditions to evaluate their performance. The main objectives of this study were to (i) evaluate the photosynthetic performance of ictB mutants relative to the WT in field conditions, and (ii) assess the potential of ictB to improve water use efficiency in rain-fed field conditions. We subsequently discuss why benefits might be seen in greenhouses and controlled environments for ictB transplants but not in field trials.

Materials and methods

Growing conditions and germplasm

Tobacco transplants (ictB1, ictB3, ictB4, and ictB6) were produced at the University of Essex where the ictB single construct was placed in the
tobacco (*Nicotiana tabacum*) cv. Samsun background (*Simkin et al., 2015*). Tobacco transformatants and WT tobacco plants were grown at the Energy Farm at the University of Illinois at Urbana-Champaign in Urbana, IL, USA. Seeds were sown into transplant trays on 9 July 2020, and transplanted into the field on 3 August 2020, in a random complete block design in which each genotype was replicated 12 times (*Supplementary Fig. S1*). Temperature (°C) and photosynthetic active radiation (PAR, µmol m⁻² s⁻¹) were measured throughout the field season (*Supplementary Fig. S2*). Once in the field, the plants were irrigated as needed to maintain soil moisture near field capacity (*Supplementary Fig. S2*). Measurements were made throughout August–September 2020. A full list of measured traits can be found in *Supplementary Table S1*.

### Gas exchange measurements

Leaf CO₂ uptake and modulated chlorophyll fluorescence were measured on the youngest fully expanded leaves using portable open gas exchange systems incorporating CO₂ and water vapor infra-red gas analyzers (*LI-6800, LI-COR Biosciences, Lincoln, NE, USA*). Light was provided through an integrated LED light source and modulated fluorimeter, incorporated into the head of the temperature- and humidity-controlled leaf measurement chamber (6 cm², *LI-6800-01A, LI-COR Biosciences*).

#### CO₂ and light response curves

The response of CO₂ uptake (A, µmol CO₂ m⁻² s⁻¹) to intercellular CO₂ concentration (Ci, µmol mol⁻¹) and to photosynthetic photon flux density (PPFD) was measured twice during the experiment. Response curves were performed 47 d after sowing (from 24 to 27 August 2020) and once again later in development at 61 d after sowing (from 7 to 10 September 2020). Response curves were measured for each genotype once per each block (n=12).

To examine the response of A to Ci (A/Ci curves), photosynthesis was measured at saturating light (2000 µmol m⁻² s⁻¹) and CO₂ concentrations in the following order: 400, 250, 150, 100, 50, 400, 550, 700, 900, 1100, 1500, and 1500 µmol mol⁻¹. Additionally, the block temperature was set at 28 °C, the average relative humidity was between 66% and 77%, and the vapor pressure deficit (VPD) at leaf temperature was between 0.79 kPa and 1.74 kPa. The gas exchange systems were matched before each curve, and steady-state fluorescence (Fᵢ) and maximal light-adapted fluorescence (Fₘ) were recorded at each measured Ci.

The apparent Vc max (µmol m⁻² s⁻¹) and apparent Jmax (µmol m⁻² s⁻¹) were calculated utilizing the equations from *von Caemmerer and Farquhar (1991)*. Due to the changes in ambient temperature throughout the day, the leaf temperature was variable (raw data in *Supplementary Fig. S3*). Accordingly, the temperature response curves from *Bernacchi et al. (2001, 2003)* were applied to obtain the apparent Vc max and apparent Jmax at 28 °C. The ‘apparent’ term is used because the parameters are based on Ci instead of CO₂ concentration inside the chloroplast (Cᵢ). The photosynthetic CO₂ compensation point (Γ *, µmol mol⁻¹), carboxylation efficiency (CE, µmol m⁻² s⁻¹ µbar⁻¹), and the maximum rate of CO₂ uptake in saturating light and CO₂ (Aₘ, µmol m⁻² s⁻¹) were calculated from the A/Ci curves that were fitted at 28 °C. CE was the initial slope of curves with Cᵢ ≥250 µmol mol⁻¹.

A non-linear analysis with the Marquardt method (*Moureau-Ngangue et al., 2017*) that uses the equations from the variable J method to calculate gₑ (µmol m⁻² s⁻¹) (*Harley et al., 1992*) and equations from *von Caemmerer and Farquhar (1981)* and *Farquhar and von Caemmerer (1982)* were then used to obtain Cᵢ (µmol mol⁻¹), Vc max and Jmax. For this analysis, the scaling constant (γ) and the enthalpies of activation (ΔHₐ) to calculate the Michaelis constant of Rubisco for CO₂ (Kc, µmol mol⁻¹), the inhibition constant (Ki, µmol mol⁻¹), and Γ * at 25 °C were taken from *Sharkey et al. (2007)*. Then, the Vc max, Jmax, and gₑ were adjusted to 28 °C using the equations in *Bernacchi et al. (2001, 2002, 2003)*.

The I * adjusted (Γ * adjusted) for gₑ was calculated as in *Furbank et al. (2009)* and *Walker and Cousins (2013)*: I * adjusted = IΓ + R/gₑ, where R is the daytime respiration rate obtained from the A/Ci curves. Light response curves (A/Q curves) were measured at ambient [CO₂] (400 µmol mol⁻¹) and the following PPFDs: 2000, 1700, 1400, 1100, 800, 600, 425, 250, 150, 100, 50, and 0 µmol m⁻² s⁻¹. The gas exchange systems were matched before each curve, and Fᵢ and Fₘ were recorded at each PPFD. The A/Q curves were fitted for quantum efficiency (Φₚ₅ᵢ), leaf CO₂ uptake in saturated light (Aₘ, µmol m⁻² s⁻¹), and light compensation point utilizing the (photosynthesis) R–package (*R Core Team, 2020, Stinzzano et al., 2021*), which uses the Marshall and Bisce (1980) non-rectangular hyperbola model.

### Diurnal measurements

Diurnal measurements were made every 2 h on 3 September 2020, from 08.00 h to 18.00 h. This on day, sunrise was at ~06.23 h, while sunset was at ~19.20 h. One plant of each genotype was measured in each of the 12 blocks per time point (n=12 genotype/time point). Within the cuvette, the flow rate was 500 µmol s⁻¹, [CO₂] was maintained at 400 µmol mol⁻¹, relative humidity was maintained at 70%, and actinic PPFD was 10% blue light. The PPFD and block temperature were changed at each time point to reflect ambient conditions throughout the day. The gas exchange systems were matched before each time point measurement, and Fᵢ and Fₘ were the parameters. The leaves of A, stomatal conductance (gₑ, µmol H₂O m⁻² s⁻¹), Cᵢ, and intrinsic water use efficiency (iWUE=A/gₑ, µmol CO₂ mol H₂O⁻¹) throughout the course of a day were obtained from these data.

### Confirmation of ictB expression

Leaf discs were collected into liquid N₂ the day following the diurnal measurements (4 September 2020) from one plant per tobacco genotype per block (n=12 per genotype). After the samples were ground, total RNA and protein were extracted from the same leaf discs using the NucleoSpin RNA/Protein Kit (*Machery-Nagel, http://www.mn-net.com*). Once the protocol was completed, the RNA concentration was diluted to 200 ng µ⁻¹.

cDNA was synthesized using 1 µg of total RNA in 20 µl using the oligo(dT) primer (*Invitrogen*) according to the protocol in the RevertAid Reverse Transcriptase kit (*Fermentas, Life Sciences, UK*). The cDNA was diluted 10 times. For semi-quantitative reverse transcription–PCR (RT–PCR), 10 µl of cDNA in a total volume of 25 µl was used with HS VeriFi Mix (*PCR Biosystems Ltd., UK*) according to the manufacturer’s recommendations. The PCR products were fractionated on 2.0% agarose gels. qPCRs were prepared with the 2X qPCR BIO SyGreen Mix *LoRoX* (*PCR Biosystems Ltd., UK*) with 1 µl of cDNA and 0.5 µM of each primer in a total volume of 10 µl. The amplification reaction included 40 cycles of 5 s at 95 °C, 10 s at 60 °C, and 15 s at 72 °C. The expression level of *ictB* was normalized with the values obtained for the housekeeping gene for protein phosphatase 2A (*PP2A; Supplementary Fig. S4*). Primers in 5’–3’ orientation used were RT-PCR-ictB-Fw, AGCCAAACCTGAC-GCTC TACC; RT-PCR-ictB-Rv, CGCGA C TGG GTAGGAG A T C; qPCR-ictB-Fw, GTTG GTTTTTTGCCTA GCCG; qPCR-ictB-Rv, TTGGT TAGGCCGTAGA C; qPCR-PP2A-Fw, GTGA AA C TGG GT AGGCC GTAGAC; and qPCR-PP2A-Rv, CATAG CCAGGCAC- CAAATTCC.

### Determination of leaf carbon and leaf carbon isotopic composition

Leaf discs were collected on 4 September 2020. Samples were freeze-dried and ground. Then, ~2 mg of each leaf sample was used to determine
the carbon content (leaf C, %) and the carbon isotopic composition (δ¹³C, ‰) using an elemental analyzer (Costech 4010, Costech Analytical Technologies, Valencia, CA, USA) in conjunction with an isotope ratio mass spectrometer (DeltaV Advantage, Thermo Fisher Scientific, Bremen, Germany) on continuous flow. The carbon ratios were then measured relative to laboratory standards and calibrated relative to the international Vienna Pee Dee Belemnite (VPDB) standard.

Destructive harvest and biomass quantification

All tobacco plants (~48 plants per genotype) were harvested on 16 September 2020 to obtain the total number of leaves, number of leaves on the main stem, total leaf area (cm²), and stem height (cm) per plant. Total leaf area was measured using a leaf area meter (LI-3100C Area Meter, LI-COR Environmental, Lincoln, NE, USA). Biomass samples were dried to a constant weight at 50 °C to determine leaf dry and stem dry weight (g per plant). The above-ground biomass was the combined sum of leaf and stem dry weight. Leaf area ratio (LAR, cm² g⁻¹) was determined by dividing the total leaf area by the total above-ground biomass.

Statistical analyses

After testing for normal distribution, homogeneity of variances by the Shapiro–Wilk test and Levine test, variables were analyzed with a mixed model ANOVA with or without repeated measurements. ‘Day’ was the repeated measurement factor when a variable was collected multiple times throughout the season. The fixed effects were the genotype (tobacco lines), day, and their interactions, while the block was the random effect. The Kenward–Roger method was used to calculate the degrees of freedom. Mean discrimination analysis was performed utilizing Tukey’s honest significant difference (HSD) with significance determined as P-value ≤0.05. Statistical analyses and model fitting for the A/Q curves and diurnal measurements were performed in R (version 4.0.1, R-Project). The rest of the analyses were done in SAS (version 9.4, SAS Institute Inc., Cary, NC, USA), by using the PROC UNIVARIATE procedure to assess for normality and for the discovery of outliers, and by using the PROC MIXED procedure for the ANOVA. Pair-wise comparisons were done by the least squares means test (t-test) with significance determined as a P-value ≤0.05.

Results

Confirmation of ictB expression in transgenic plants

The ictB transgenic lines used in this study are the same as those presented in Simkin et al. (2015). Semi–quantitative RT–PCR was used to detect the presence of the transcript in the ictB-expressing plant lines ictB1, ictB3, ictB4, and ictB6. No transcript was detected in WT control plants, and different levels of transgene expression were observed among transgenic lines, with ictB6 showing the highest transgene expression (Supplementary Fig. 6A). qPCR was performed to validate the differences in transgene expression between lines. No signal was detected in WT plants and ictB6 showed the highest transgene expression (Supplementary Fig. 6B). Both results are consistent and indicate that the ictB transgene is expressed in transgenic lines at different levels, and these results are also consistent with the data presented in Simkin et al. (2015).

Gas exchange data: CO₂ response curves, light response curves, and diurnals

A/Q curves were measured to allow for the determination of parameters related to how efficiently the plant is utilizing light. A/Q curves were measured on 12 plants per line (n=12). No significant differences were found between genotypes for A_sat, Φ_PSII, and light compensation point for any of the A/Q curve measurements throughout the season (Fig. 1). While not significant, the WT had one of the highest photosynthetic rates in the first set of A/Q curves but not in the second set (Fig. 1). However, indicated differences were small (Fig. 1).

The A/C_i curves were measured to determine parameters related to the biochemical performance and limitation.
of photosynthesis. These were also measured on 12 plants per line \( (n=12) \). The apparent \( V_{c,\text{max}} \), apparent \( J_{\text{max}} \), CE, \( A_{\text{max}} \), and \( \Gamma^* \) in the transgenic lines were not significantly higher than in the WT. The overall values of these parameters increased throughout the duration of the season, but without significant differences between lines (Figs 2, 3). Exceptions were that ictB3 had a lower apparent \( V_{c,\text{max}} \) and CE than the WT, ictB1, and ictB4 during the first set of measurements (Fig. 3). ictB3 had also a lower \( \Gamma^* \) than the WT and ictB1 at the beginning of the season (Fig. 3). By the end of the field season, ictB4 had an apparent \( V_{c,\text{max}} \) and CE that were lower than in ictB3 and ictB6 (Fig. 3). When considering the parameters calculated based on \( C_i \), \( V_{c,\text{max}}, J_{\text{max}} \), and \( \Gamma^* \_\text{adjusted} \) did not differ between the transgenic lines and the WT (Supplementary Fig. S5). ictB3 was the only transgenic with a \( g_m \) lower than the WT, although the difference was only significant on one date (Supplementary Fig. S5).

Finally, no significant differences were found between the genotypes for \( A \), \( g_{\text{sw}}, C_i \), and iWUE during the diurnal gas exchange measurement (Fig. 4). While the WT had the lowest overall iWUE, it was not significantly lower in the transgenic lines (Fig. 4).

**Leaf composition and biomass-related traits**

Leaf carbon content (leaf C) and \( \delta^{13}C \) varied significantly among the measured genotypes (Fig. 5). None of the transformants showed a leaf C content that was significantly different from that of the WT; however, ictB1 showed a significantly higher content than ictB4 (Fig. 5). The WT had the lowest value (most negative) for \( \delta^{13}C \) although it only varied significantly from the ictB4 genotype (Fig. 5). The \( \delta^{13}C \) values from all the ictB genotypes were compared (mean value of \(-27.48\%\)) against the \( \delta^{13}C \) in the WT (mean value of \(-27.88\%\)), showing a significantly more negative \( \delta^{13}C \) in the WT \( (P\text{-value}=0.040) \).

Significant differences were found among the genotypes for most measured biomass-related traits, including above-ground biomass, leaf and stem dry weights, total number of leaves, number of leaves on the main stem, total leaf area, and stem height (Fig. 6; Supplementary Fig. S6). Despite having the lowest total number of leaves, the WT had one of the highest total above-ground biomasses, total leaf area, and leaf dry weights (Fig. 6; Supplementary Fig. S6). The WT had significantly lower total number of leaves and number of leaves on the main stem than the ictB3 transformant. The WT also had higher above-ground biomass, stem dry weight, leaf dry weight, total leaf area, and stem height than both ictB3 and ictB4 transformants (Fig. 6; Supplementary Fig. S6). Finally, the pair-wise comparisons for LAR did not reveal significant differences between the lines (Fig. 6).

**Discussion**

Previous reports of plants transformed with the *ictB* gene indicated higher photosynthesis and biomass compared with the WT plants from which they were derived (Lieman-Hurwitz *et al.*, 2003, 2005; Simkin *et al.* 2015; Hay *et al.* 2017). However, most of these studies have been performed in controlled conditions and it is not clear if these promising improvements
in plant productivity can translate to the crops in the field. For this reason, in this experiment we grew *ictB* tobacco plants in the field to evaluate if these transgenic plants have a higher photosynthetic efficiency than the WT under field conditions. A total of four *ictB* transgenic lines were tested against tobacco WT plants from which they were derived, and were evaluated for >10 different photosynthetic parameters together with leaf composition and biomass traits (Supplementary Table S1).

The same transgenic lines were used previously in the greenhouse study of Simkin *et al.* (2015). In that experiment,
overall higher photosynthesis, apparent \( V_{\text{c, max}} \), apparent \( J_{\text{max}} \), and \( g_{\text{sw}} \) were found in these ictB lines, resulting in more leaves and stem biomass. In this experiment, we did not find any photosynthetic parameter that was higher in ictB tobacco compared with the WT (Figs 1–4; Supplementary Fig. S5). In contrast, ictB tobacco performed similarly to the WT, although one
transgenic line (ictB3) had a lower apparent $V_{c,\text{max}}$, $CE$, and $g_{\text{dm}}$ than the WT in at least one of the sets of measurements (Fig. 3; Supplementary Fig. S5). The lower $g_{\text{dm}}$ in ictB3 indicated a higher restriction to the diffusion of CO$_2$ inside the chloroplast than in the WT. However, ictB3 did show a lower $\Gamma^*$, which suggests an increased concentration of CO$_2$ around Rubisco. However, when $\Gamma^*$ was adjusted to consider the effect of $g_{\text{dm}}$, $\Gamma^*_\text{adjusted}$ did not indicate a higher amount of CO$_2$ around Rubisco in ictB3 or in any other ictB line compared with the WT (Supplementary Fig. S5). Previous studies of plants transformed with ictB have calculated $\Gamma^*$ from $A/C_i$ response curves, without accounting for $g_{\text{dm}}$ (Lieman-Hurwitz et al., 2003; Gong et al., 2015; Hay et al., 2017). The present study indicates the importance of calculating $\Gamma^*$ based on $C_c$ instead of $C_i$ for studies where the calculation of this parameter can allow a better understanding of any photosynthetic improvement achieved.

The values of apparent $V_{c,\text{max}}$ and apparent $J_{\text{max}}$ from this study were also obtained at 25 °C (Supplementary Table S2) to compare them with the values obtained in Simkin et al. (2015) which were calculated at that temperature. In our field experiment, the apparent $V_{c,\text{max}}$ at 25 °C was between 95 µmol m$^{-2}$ s$^{-1}$ and 145 µmol m$^{-2}$ s$^{-1}$, while the apparent $J_{\text{max}}$ was between 195 µmol m$^{-2}$ s$^{-1}$ and 290 µmol m$^{-2}$ s$^{-1}$, considering both ictB lines and the WT. These values are higher than the apparent $V_{c,\text{max}}$ (between 70 µmol m$^{-2}$ s$^{-1}$ and 90 µmol m$^{-2}$ s$^{-1}$) and the apparent $J_{\text{max}}$ (between 130 µmol m$^{-2}$ s$^{-1}$ and 170 µmol m$^{-2}$ s$^{-1}$) obtained in the greenhouse study of Simkin et al. (2015). It is possible that under the controlled growth conditions of the greenhouse, differences could be apparent that were later eliminated in the field. Similarly, in an ictB soybean study (Hay et al., 2017), the apparent $V_{c,\text{max}}$ and apparent $J_{\text{max}}$ were not different from the WT when grown in the field under ambient CO$_2$ concentrations; however, soybean instantaneous photosynthesis and biomass did increase. It is important to note that soybean as a legume can have an adequate nitrogen supply throughout the whole growing season, which might have contributed to its increase in carbon assimilation. In Ruiz-Vera et al. (2017), WT tobacco cv. Petit Havana, another tobacco cultivar with reduced sink capacity due to its determinate growth, grew at normal and high N soil fertilization conditions in the field. In that study, the values of apparent $V_{c,\text{max}}$ and apparent $J_{\text{max}}$ increased further under the high N treatment at ambient CO$_2$ conditions. It might be that under field conditions, other factors such as adequate nutrient supply and uptake or sink strength can influence the effect of the ictB gene in tobacco. Our results suggest that it is possible to maximize the photosynthetic performance of ictB plants over the WT under conditions where parameters such as the amount of light, temperature, relative humidity, photoperiod, nutrients, and water availability can be controlled (e.g. conditions in the ictB tobacco greenhouse experiment in Simkin et al., 2015), but this improvement might not always translate to field conditions.

The values for $A$ and other parameters obtained from the $A/C_i$ and $A/Q$ curves were higher at the end of the season compared with the beginning of the season (Figs 1–3; Supplementary Fig. S3). This trend corresponds to previous work in which values related to photosynthesis increase with leaf age (Bielczynski et al., 2017). This difference could have been influenced by the hotter temperatures during the days when the first set of measurements were carried out (approximately +5 °C; Supplementary Fig. S2) which could have also increased...
the water requirements of the plants. Previous studies suggest that ictB plants might have higher water use efficiency (WUE) than the WT because $A$ and $g_m$ tend to be higher while $g_w$ does not change (Lieman-Hurwitz et al., 2003; Gong et al., 2015; Hay et al., 2017). In our experiment, WUE was not significantly different between ictB tobacco and the WT (Fig. 4), which coincided with the lack of significant differences seen, in most of the cases, for $A, g_m, g_w$, and $\delta^{13}C$. However, ictB4 did show significantly less negative $\delta^{13}C$ than the WT, and a less negative $\delta^{13}C$ was indicated for ictB1 (Fig. 5). The $\delta^{13}C$ of leaf tissue provides an integrated signal of the WUE with which the carbon in that leaf was obtained. A less negative value indicates a higher WUE, provided that $g_m$ is not different between lines. These results suggest that the transformation with ictB may improve WUE under field conditions, although the scope may be limited (Fig. 5).

The results regarding the effect of ictB on crop biomass production have so far been inconclusive. For example, in some cases, ictB-overexpressing plants have produced significantly greater biomass (Lieman-Hurwitz et al., 2003; Hay et al., 2017; Koester et al., 2021), while in other studies overexpression of ictB did not significantly alter biomass production (Gong et al., 2015). This suggests that the mechanism that underlies ictB may be greatly affected by environmental factors, and that an increase in crop productivity may only happen under certain conditions, although these conditions have yet to be identified. Here, there were no increases in biomass in ictB tobacco; on the contrary, some ictB lines (ictB3 and ictB4) had lower biomass (above-ground biomass, leaf biomass, and stem biomass; Fig. 6) than the WT, probably because of the production of smaller leaves (total leaf area; Fig. 5) and shorter plants (Supplementary Fig. S6). This study did not measure root biomass; however, empirical observations in the previous greenhouse experiment indicated that ictB lines might have more root biomass than the WT (Simkin et al., 2015). Despite that, the effect of the ictB expression in plants remains unclear (Simkin et al., 2019); its effect might be enhanced when it is co-expressed with other genes such as with some Calvin–Benson cycle genes (Simkin et al., 2017). For example, higher dry biomass was observed in plants with the ictB gene together with the overexpression of sedoheptulose-1,7-bisphosphatase (SBPase) and fructose-1,6-bisphosphate aldolase (FBPase) (Simkin et al., 2017). Consequently, the impact of ictB on the improvement of photosynthesis and yield may be observed when it is part of a group of expressed genes rather than when it is expressed alone.

Future applications for ictB-overexpressing plants

Despite not finding a significant difference between the ictB transformants and the WT except possibly in WUE, this study was done in one field season so the replication of our results in multiple seasons is unknown. Moreover, there is still promise in utilizing expression of this gene for improved crop productivity, particularly in controlled environments. While we did not find the significant differences in biomass that were reported in Simkin et al. (2015), it is possible that field environmental conditions, which differ greatly from potted plants in the constant conditions of greenhouses and growth cabinets, play a key role in whether plants transformed with ictB perform better relative to their WT. This, combined with the successes seen in greenhouse-grown ictB transformants, could serve as encouragement for deploying this gene to improve plant productivity within the context of controlled environments. Indeed, as humans globally look to increase food production in more sustainable ways, greater emphasis has been placed on agriculture in greenhouses and vertical farming, both of which involve controlling the growing environment.

Other considerations that could be taken into account are the water status of the plant, the temperature, and the age of the plant, as all of these are factors that can play a role in how a transgene manifests itself in the field and affects photosynthetic performance (Azcon-Bieto et al., 1981). At present, it is difficult to know the true potential of transformation of crop plants with ictB, especially as the function of the gene remains unknown (Simkin et al., 2019).

Drought and heat conditions adversely affect photosynthetic performance in crops, including increasing the photorespiratory CO2 compensation point and decreasing Rubisco carboxylation or ribulose bisphosphate (RuBP) regeneration (Antolin and Sanchez-Diaz, 1993; Rensburg and Kruger, 1993; Flexas et al., 2009; Crous et al., 2013). Consequently, increases in temperature are associated with an increase in photorespiration in C3 plants, which can lead to yield penalties of up to 36% in important food crops (Walker et al., 2016). Previously, it was shown that expression of ictB in plants resulted in a decrease of $\Gamma^*$ (Lieman-Hurwitz et al., 2003; Hay et al., 2017). Testing ictB transformants under conditions that are known to affect the CO2 compensation point could help us to better understand the underlying function and see if improved performance is significantly associated with specific environmental factors. For example, if ictB transformants can maintain a lower $\Gamma^*$ under drought or heat stress conditions, then it could be a promising application for the future, especially as temperature and drought stress are projected to increase with global climate change. However, as mentioned, further effort would need to be put into understanding under which specific conditions ictB might be most beneficial.

Supplementary data

The following supplementary data are available at JXB online.

- Fig. S1. Layout of the field experiment.
- Fig. S2. Weather data for the growing season.
- Fig. S3. Raw data for $A/C_i$ curves.
- Fig. S4. Semi-quantitative RT–PCR and qPCR results of transgenic lines.
Fig. S5. Comparison of $V_{c,\text{max}} J_{\text{max}} g_{\text{int}}$ and $\Gamma^\ast_{\text{adjusted}}$ between transgenic lines and the wild type at 28 °C.

Fig. S6. Stem height and number of leaves on the main stem.

Table S1. Summary of traits measured, their abbreviations, and units.

Table S2. Apparent $V_{c,\text{max}}$ and $J_{\text{max}}$ at 25 °C.

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Author contributions

CR, TL, and SPL: conceptualization; AJS: developing the iitB transfor-
mants, supported by KLB; CA and HG: confirming the expression of iitB;
CR and TL: design; UMRZ and LGAS: performing the gas exchange measurements and field harvest; UMRZ and LGAS: data analysis and writing, with contributions from all other authors. CR agrees to serve as the corresponding author and respond to any relevant communication related to this project.

Conflict of interest

The authors declare no conflicts of interest.

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Data availability

Data are available upon request via the corresponding author.

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