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Original article

Enhancing soybean photosynthetic CO_2 assimilation using a cyanobacterial membrane protein, *ictB*☆

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ABSTRACT

Soybean C_3 photosynthesis can suffer a severe loss in efficiency due to photorespiration and the lack of a carbon concentrating mechanism (CCM) such as those present in other plant species or cyanobacteria. Transgenic soybean (*Glycine max* cv. Thorne) plants constitutively expressing cyanobacterial *ictB* (inorganic carbon transporter B) gene were generated using *Agrobacterium*-mediated transformation. Although more recent data suggest that *ictB* does not actively transport HCO3-/CO_2, there is nevertheless mounting evidence that transformation with this gene can increase higher plant photosynthesis. The hypothesis that expression of the *ictB* gene would improve photosynthesis, biomass production and seed yield in soybean was tested, in two independent replicated greenhouse and field trials. Results showed significant increases in photosynthetic CO_2 uptake (A_{net}) and dry mass in transgenic relative to wild type (WT) control plants in both the greenhouse and field trials. Transgenic plants also showed increased photosynthetic rates and biomass production during a drought mimic study. The findings presented herein demonstrate that *ictB*, as a single-gene, contributes to enhancement in various yield parameters in a major commodity crop and point to the significant role that biotechnological approaches to increasing photosynthetic efficiency can play in helping to meet increased global demands for food.

1. Introduction

The world population is projected to grow from the current 8 to over 9 billion by 2050. However, with rapidly growing purchasing power and changing diets in the emerging economies, actual demand for food is expected to rise ~87% by 2050 (Ray et al., 2013; Long et al., 2015; Tilman and Clark, 2015). One of the major challenges that will face agriculture in the next few decades is meeting this demand under conditions of increased climate uncertainties and dwindling natural resources, while yield improvements of recent decades appear to have stagnated for key crops (Long and Ort, 2010; Ray et al., 2012; Long, 2014). Innovations obtained through the tools of plant biotechnology hold great promise to aid in our ability to address this challenge of global stagnation in agronomic productivity of the last decade (Godfray et al., 2010; Raines, 2011; Ainsworth et al., 2012; Bihmidine et al., 2013a; Long et al., 2015).

The potential yield of a seed crop at a given location is the product of the efficiencies with which that crop can, over the growing season, intercept the available solar radiation (ε_i), convert the intercepted radiation into biomass (ε_c), and then partition the biomass energy into the seed (ε_p) (Monteith, 1977). The decades following the green revolution saw large increases in both ε_i and ε_p in the three major C_3 crops: wheat, rice and soybean (Long and Ort, 2010;

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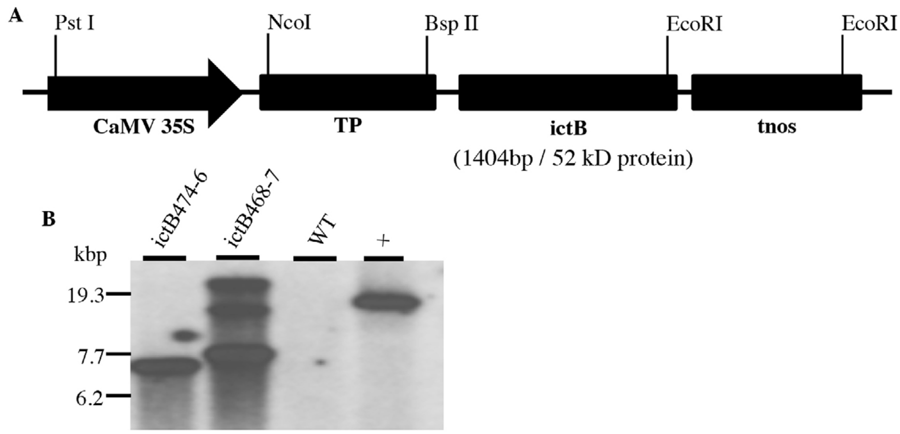

Fig. 1. Plasmid construct used in soybean transformation (A). LB and RB, T-DNA left and right borders; 35S CaMV, cauliflower mosaic virus promoter; TP, pea RbcS transit peptide; *ictB*, inorganic carbon transporter B gene; tnos, nopaline synthase gene terminator. Note: selectable marker cassette not shown, which resides proximal to LB. Southern blot analysis of total genomic DNA isolated from transgenic *ictB* soybean events and WT leaves (B). The DNA was digested with *Bgl*II and probed with *ictB* derived element. Lane A: genomic DNA from ictB474-6 plants used in this study; Lane B: genomic DNA from ictB468-7 plants; Lane C: genomic DNA from nontransformed Thorne plants; Lane D: 50 pg of a binary vector containing the *ictB* gene.

Zhu et al., 2010). Zhu et al. (2010) showed that for a modern soybean cultivar, ε_i and ε_p are 0.9 and 0.6 respectively, and very close to their theoretical maxima, leaving only ε_c as a means for further genetic improvement of yield potential, other than extending the growing season to increase the solar radiation available to the crop (Dohleman and Long, 2009; Long et al., 2006b; Zhu et al., 2010). Introducing genes from other photosynthetic organisms may be one avenue to enhance ε_c to the degree required to see improved productivity (Zhu et al., 2010; Long et al., 2015).

C_4 crops achieve a higher maximum ε_c by utilizing complex carbon concentrating mechanisms (CCMs) that requires the photosynthetic C_4 dicarboxylate cycle and compartmentation of components of both the C_4 and C_3 pathways between dimorphic photosynthetic tissues to effectively elevate CO_2 at Rubisco. This serves to accelerate RubP carboxylation and competitively inhibit oxygenation of RubP, thus minimizing photorespiratory losses. C_3 crops lack CCMs and significant losses in efficiency result from photorespiration, which rise with temperature (Long et al., 2004; von Caemmerer and Evans 2010; Price et al., 2011; Ruan et al., 2012). Cyanobacteria, the ancestors of higher plant chloroplasts, appear to have a simpler system for concentrating CO_2 at Rubisco within a single cell (Kaplan and Reinhold, 1999; Giordano et al., 2005; Zurbriggen et al., 2009; von Caemmerer and Evans, 2010; Price et al., 2011). Introduction of a single cyanobacterial membrane protein, *ictB*, from the cyanobacteria *Synechococcus* PCC 7942 into higher plants has increased photosynthetic CO_2 uptake rate in *Arabidopsis thaliana,* tobacco (*Nicotiana tabacum*) and rice (*Oryza sativa*) (Lieman-Hurwitz et al., 2003, 2005; Yang et al., 2008; Gong et al., 2015). Thus, *ictB* presents an ideal candidate gene to increase source capacity and thus improve the availability of photoassimilates to sink organs (Bihmidine et al., 2013a).

The *ictB* gene is highly conserved within cyanobacteria, indicative of its importance (Bonfil et al., 1998; Lieman-Hurwitz et al., 2005). However, its exact role is unclear and it has been shown not to be an active transporter (Price et al., 2011). Nonetheless, *ictB*-defective mutants can only survive at very high CO_2 concentrations, suggesting an essential role for the gene product in carbon uptake under ambient atmospheric conditions. If indeed *ictB* is acting to increase [CO_2] at the site of Rubisco then not only should leaf CO_2 uptake rate increase, but also the maximum quantum yield of CO_2 assimilation ($\Phi CO2_{,max}$). This is because even under strictly light-limiting conditions, if CO_2 at Rubisco is increased then less of the limiting supply of NADPH and ATP will be used in photorespiratory metabolism, making more NADPH and ATP available for CO_2

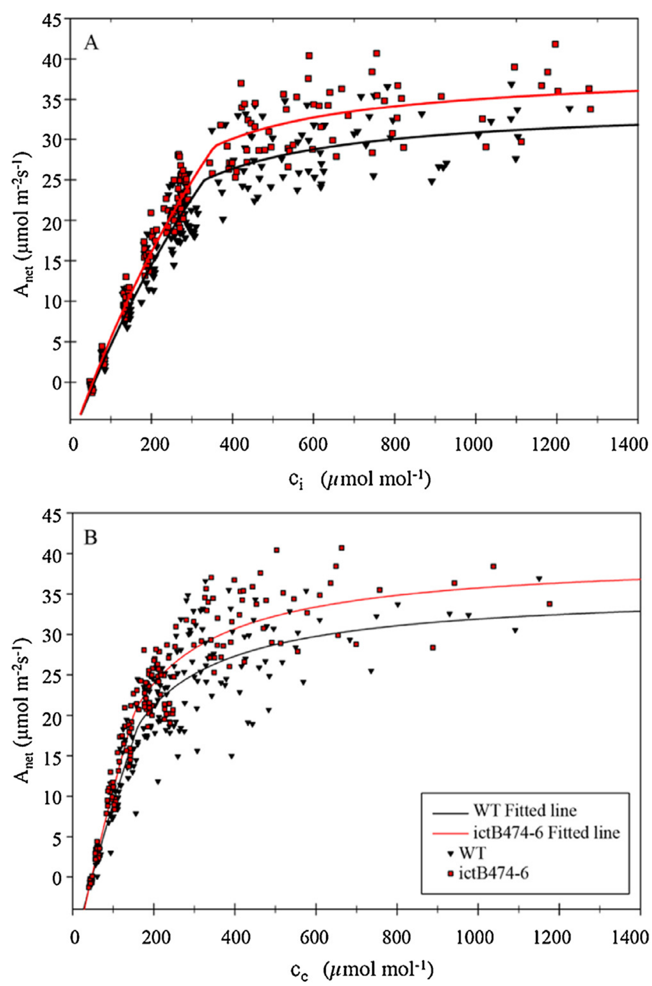

Fig. 2. Comparison of soybean wild type (WT) and transgenic ictB474-6 plants' net photosynthetic rates (A_{net}) versus plant internal [CO_2] (c_i), or versus plant chloroplast [CO_2] (c_c). Photosynthetic photon flux density (Q) was set to 1500 μmol m^{-2} s^{-1}. The fitted lines represent the calculated average net photosynthetic rates as determined by the most limiting photosynthetic process: Rubisco-carboxylation limited, RuBP-regeneration limited, or triose phosphate utilization (TPU) limited assimilation rate. The maximum rate of Rubisco carboxylation (V_{cmax}) and the maximum rate of electron transport (J_{max}) presented are calculated from the asymptotes of RuBP unsaturated assimilation and RuBP saturated assimilation respectively.

Table 1
Photosynthetic parameters under greenhouse environments.

Parameter	WT	ictB474-6	P-Value
V_{cmax} (μmol m^{-2} s^{-1})	115 ± 4	126 ± 3	0.035*
J_{max} (μmol m^{-2} s^{-1})	151 ± 5	167 ± 4	0.010*
A_{net} at Ambient [CO_2] (μmol m^{-2} s^{-1})	IL 20.43 ± 0.8	22.59 ± 0.6	0.037*
	NE 23.71 ± 0.6	26.03 ± 0.7	0.017*
Ambient Ci (μmol mol^{-1})	IL 278 ± 3	267 ± 3	0.026*
	NE 340.8 ± 3.8	327.4 ± 2.8	0.011*
Ci/Ca Ratio	IL 0.695 ± 0.008	0.668 ± 0.007	0.026*
	NE 0.784 ± 0.008	0.755 ± 0.006	0.001*
Φ_{PSII}	0.2285 ± 0.0078	0.2538 ±0 0.0066	0.019*
ΦCO_2	0.01634 ± 0.00062	0.01796 ± 0.00053	0.038*
qP	0.499 ± 0.011	0.538 ± 0.011	0.015*
g_s (mol m^{-2} s^{-1})	IL 0.336 ± 0.02	0.346 ± 0.019	0.723
	NE 0.59 ± 0.02	0.61 ± 0.02	0.5
g_m (mol m^{-2} s^{-1})	0.244 ± 0.017	0.304 ± 0.018	0.025*
Stomatal Limitation	22.72% ± 1.6	23.5% ± 1.2	0.696
Percent Carbon	45.580 ± 0.145	45.438 ± 0.141	0.485
$\delta^{13}C$	−27.5565 ± 0.1321	−27.2683 ± 0.1246	0.122

Plant photosynthetic characteristics for the wild type (WT) and ictB474-6 soybean plants monitored under greenhouse conditions in Nebraska (NE) and Illinois (IL). Photosynthetic photon flux density (Q) = 1500 μmol m^{-2} s^{-1} and ambient [CO_2] = 400 μmol mol^{-1}.
* indicate significant difference (P < 0.05) from a mixed model ANOVA with line as the main effect.

assimilation (Long et al., 2004). Increasing CO_2 at Rubisco will not only increase carbon uptake, but also increase water use efficiency, suggesting that the plant will also be more tolerant of a reduction in water supply.

Soybean is the world's fourth most important crop in regards to global quantities of seed or grain produced, and is arguably the most important source of vegetable protein for both food and feed. In 2013, 276 million metric dry tons were produced contributing $145 billion to the world's economy (FAO, 2015). Thus, improving its productivity is vital to meeting the growing demands for food and feed, and while providing a significant economic contribution to the world's economy (Ainsworth et al., 2012). The objectives of this study were to: 1) generate transgenic soybean events expressing cyanobacterial *ictB* gene, 2) test the hypothesis that photosynthetic characteristics are improved in derived transgenic events, 3) determine if this translates to increased total plant biomass and reproductive yield in the laboratory and field, and 4) examine the response of transgenic plants to water stress. The studies reported herein were performed independently at both the University of Nebraska-Lincoln and at the University of Illinois Urbana-Champaign.

2. Material and methods

2.1. Soybean transformation and selection of transgenic plants

Soybean [*Glycine max* (L.) Merrill, cultivar Thorne] was transformed using *Agrobacterium tumefaciens* (Zhang et al., 1999; Clemente et al., 2000) bearing a T-DNA construct containing the *ictB* gene fused to the coding region for a transit peptide (TP) and driven by the 35S CaMV promoter (Fig. 1A). Additional details on the selection and identification of homozygous lineages is located in the supplemental material. A number of *ictB* events were produced that showed increases in photosynthetic carbon assimilation under favorable conditions in the greenhouse (Figs. 2 and 3; Supplementary Fig. S1). The homozygous lineage of event ictB474-6 was selected as a lead event for deeper characterization relative to the control, wild type (WT, cv Thorne) for its initial strong positive performance in the greenhouse, relative to other events (Tables 1 and 2).

Table 2
Soybean plant productivity parameters under greenhouse condition for wild type (WT) and transgenic ictB474-6 event.

Parameter	WT	ictB474-6	P-Value
Seed/plant (g)	18.51 ± 0.67	21.35 ± 1.11	0.0338*
Seed (mg)	109.35 ± 2.05	120.63 ± 2.11	0.0050*
No seed	169.72 ± 7.19	176.71 ± 7.39	0.5029
No. pod	74.7 ± 2.5	76.1 ± 2.6	0.7193
Pod seed	2.26 ± 0.03	2.31 ± 0.03	0.2953
Pod mass (g)	15.07 ± 0.50	15.90 ± 0.52	0.3630
Stem (g)	37.33 ± 1.66	43.29 ± 1.59	0.0128*

Plant productivity comparisons between greenhouse grown wild type (WT) and the ictB474-6. Seed/plant refers to seed mass on a per plant basis. Seed parameter indicates mean individual seed mass. No. seed and No. pod indicate mean number of seed/pod per plant, respectively. Pod seed/Pod mass refer to mean number of seed per pod and mean pod mass, respectively. Stem indicates mean mass of stem biomass.
* indicates significant difference (P < 0.05) from a mixed model ANOVA (P < 0.05) with line as the main effect.

2.2. Gas exchange measurements under controlled greenhouse settings

A controlled growth study at the University of Nebraska-Lincoln (UNL) greenhouses, was carried out with 20 T_3 progeny from WT and homozygous selected *ictB* events. Plants were grown as described previously Bihmidine et al., 2013b. When the plants reached V3-V4 stage, corresponding to the third and fourth fully expanded young trifoliate, 10 wild type and 10 transgenic plants were selected, and used for photosynthetic phenotyping.

The greenhouse study at the University of Illinois was carried out using the same WT and *ictB* events as the University of Nebraska experiment, arranged in a completely randomized design (n = 19). The greenhouse environment at the University of Illinois was equivalent that of the UNL greenhouse study; specific conditions can be found in the supplemental material. Biomass was dried at 60 °C to constant weight and productivity parameters were determined.

Changes to carbon isotopic ratio ($\delta^{13}C$, ‰) in plant tissue can be used to detect changes to the internal conductance caused by the expression of the *ictB* gene (Farquhar et al., 1982a; Farquhar et al., 1982b; Farquhar et al., 1989). Eight to ten stems from each plant were selected at random and the mid 1 cm of the stem was removed and dried at 60 °C for 24 h, then reduced to 100 μm particles using a bead grinder (Geno/Grinder; SPEC SamplePrep, Metuchen, NJ).

Sub-samples of 2–3 mg were combusted using an elemental analyzer (ECS 4010; Costech Analytical) coupled to a Conflo IV interface (Thermo, Bremen, Germany) and an isotope ratio mass spectrometer (Delta-V advantage, Thermo) were used to determine C, H and N concentrations and $\delta^{13}C$. The average measured deviation of an in-house isotopic reference material was <0.05‰ of its known value (n = 5).

In the Nebraska greenhouse trials, gas exchange measurements were conducted at vegetative stage, 29 days after planting (DAP), with an open gas-exchange system incorporating infrared CO_2 and water vapor analyzers, with the leaf enclosed in a controlled environment cuvette and illuminated with an integrated LED light source (LI-6400-02B, LI-COR Inc. Lincoln, NE) at each growth stage. Net photosynthesis (A_{net}, $\mu mol\, m^{-2}\, s^{-1}$) and stomatal conductance (g_s, $mol\, m^{-2}\, s^{-1}$) were measured at light saturation of 1500 μmol photons $m^{-2}\, s^{-1}$ and at ambient [CO_2] of 400 $\mu mol\, mol^{-1}$. Measurements were performed between 9:00 A.M. and 1:00 P.M. on 10 plants per line, on the central leaflet of the youngest most fully expanded trifoliate.

The University of Illinois greenhouse trials conducted measurements of net photosynthetic rate (A_{net}) *vs* internal CO_2 (c_i) on three consecutive days at the V6 stage, which was, on average, 37 DAP. Leaf gas exchange measurements were measured as described for the Nebraska experiment except they included an integrated chlorophyll fluorometer in the cuvette lid (LI-6400-40 Leaf Chamber Fluorometer; LI-COR, Inc.). The procedure for measuring A_{net}/c_i was as described by Bernacchi et al. (2005). Values for A_{net} and c_i were calculated using the equations from Farquhar and von Caemmerer (1982). Fitting of the A_{net}/c_i curves and determination of the maximum rate of Rubisco carboxylation (V_{cmax}, $\mu mol\, m^{-2}\, s^{-1}$), maximum rate of electron transport (J_{max}, $\mu mol\, m^{-2}\, s^{-1}$), mitochondrial respiration (R_d, $\mu mol\, m^{-2}\, s^{-1}$) and CO_2 compensation point (Γ^*) followed the procedure from Dubois et al. (2007). Stomatal limitation was calculated from the A_{net}/c_i response curves as described by Farquhar and Sharkey, 1982. Mesophyll conductance (g_m) was estimated using the method of Bernacchi et al. (2005), and chloroplast CO_2 concentration (C_c) was estimated using the method of Bernacchi et al. (2002).

2.3. Field trials

Field trials in Nebraska were conducted at the University of Nebraska Agricultural Research and Development Center (ARDC), Mead, NE, USA. Two transgenic *ictB* events ictB474-6 and ictB468-7, were grown along with control WT, during the summers of 2009, 2010, and 2011. Seeds were planted on May 28, 26, and 10, respectively. Weather data on site were obtained from stations located at ARDC. Data from the 2010 field season are not presented in this paper due to crop damage (flood in July due to excessive rain, followed by a hailstorm in August). Plants were grown in five randomized experimental blocks, each contained one plot per line, and every plot was composed of four, 30 m long, rows where 100 seeds were planted. In the *ictB* plots, the two middle rows were composed of transgenic seeds and the wild type seeds were planted in the outer rows to serve as buffers. Measurements were taken between 9:00 AM and 1:00 PM at the vegetative (Vn), flowering (R1-R2), pod-setting to full pod (R3-R4), and seed-setting stage (R5). Phenotypic traits including yield and its components thereof were determined post harvest. Leaves of approximately the same age were randomly collected and used to measure leaf area at the different developmental stages. In addition, a near InfraRed (NIR) was used to measure the composition of the collected seeds in total protein, oil and fiber (Infratec 1241, FOSS Denmark).

Field trials in Illinois were conducted at the soybean free air gas concentration enrichment (SoyFACE) facility in Champaign, IL, USA during the 2010 and 2011 growing season. Weather data for the site was obtained from the Illinois Climate Network weather station located at Champaign, IL and within 3 miles of the experiment. The SoyFACE facility, situated on 32 ha of Illinois farmland, consisted of four blocks, each containing two 19 m diameter octagonal plots. Within each block, one plot was at current ambient [CO_2] and one plot was fumigated from sunrise to sunset to an elevated target [CO_2] of 200 $\mu mol\, mol^{-1}$ above ambient [CO_2], using the FACE design of Miglietta et al. (2001). This has been detailed previously by Morgan et al. (2005).

The Illinois field trials were arranged in a randomized complete-block design (n = 4) in the spring of 2010 and 2011. WT and ictB474-6 event were planted into four replicate blocks containing two plots each, one plot at ambient [CO_2] and one at elevated [CO_2]. Each plot, referred to hereafter as a ring plot, contained five planted rows, 3 m in length, with a row spacing of 0.38 m. The first and fifth rows in each plot were sown with the WT to serve as border rows. Each of the inner three rows were randomly assigned to be planted with either the wild type, the ictB474-6 or a transgenic event, which was a component of another study. After emergence, ten individuals in 2010 and fifteen individuals in 2011 were randomly selected and tagged from each of the treatment rows. These tagged individuals were used to track phenotypic traits.

In 2011, an additional large plot experiment was established at the SoyFACE facility to assess plant productivity over a larger plot area under ambient atmospheric [CO_2]. The large plots were planted in a completely randomized complete-block design (n = 4). Each block contained two of these large plots, one assigned to the WT and the other to ictB474-6 event. Each plot consisted of 4 rows, 4 m in length, with a row spacing of 0.45 m.

2.4. The response of leaf photosynthesis to intercellular [CO_2] (A_{net}/c_i)

In 2011 the response of leaf photosynthetic rate to intercellular [CO_2] (A_{net}/c_i) was determined, between 41 and 50 days after planting, for uppermost fully expanded leaves of four randomly selected plants per block, for both the WT and ictB474-6 event under ambient and elevated [CO_2], as described in 2.2. The leaves were excised in the field before dawn and cut under water to avoid xylem embolism. Leaf gas-exchange measurements were performed following the method described of Morgan et al., 2004.

2.5. End-of-season leaf gas exchange

Because the ictB474-6 event appeared green at the end of the growing season when the WT plants were senescing, additional gas exchange measurements were made at this stage to determine whether these leaves also remained photosynthetically active. Leaf A_{net} was measured for the WT and *ictB* lines on 16 randomly selected uppermost fully expanded soybean leaves; 99 and 105 DAP in 2010 and 2011, respectively.

2.6. Response of photosynthesis to photon flux (A_{net}/Q)

Photosynthetic light response curves (A_{net}/Q) were determined immediately following measurement of the A_{net}/c_i curves during the 2011 growing season, following the methods found in Bernacchi et al. (2005) and procedures described in 2.2; full details can be found in the supplementary. A 3-parameter non-rectangular hyperbola describing the response of A_{net} to Q was fit to the measurements made for each leaf (Long and Hällgren, 1993). The three parameters obtained from each fit were: net photosynthesis at light saturation (A_{sat}), $\Phi CO_{2,max}$ and the convexity of the transition from light-limited to light-saturated photosynthesis (θ).

2.7. Productivity parameters

At harvest, 10 individuals of each the WT and the ictB474-6 were selected at random and harvested in 2010, and 15 individuals of each in 2011. Phenotypic traits including final height, main stem circumference, pods per plant, seeds per plant, and node number were determined. The plant material was divided into stem, pod and seed and then oven dried to constant mass at 60 °C.

2.8. Drought mimic experiment under controlled greenhouse settings

Soybean plants were grown in 16 cm deep pots at the University of Nebraska-Lincoln greenhouses as described in 2.2. Drought treatment was initiated by withholding water for six days, and soil volumetric water content (SVWC, %) in pots was monitored using an ML2X ThetaProbe soil moisture sensor (Dynamax Inc., Houston, TX). Photosynthetic parameters were measured following the same procedure described above, and measurements were conducted at the V3-V4 stage of development (Fehr and Caviness 1979) as previously described by Bihmidine et al. (2013b): under no stress (NS, SVWC ≈ 40%), low stress (LS, apparent soil drying but no visible wilting of the leaves, SVWC ≈ 20-30%), medium stress (MS, leaves were wilted but still maintained visible turgidity, SVWC ≈ 10-20%), and under severe stress conditions (SS, plants were visibly stressed, SVWC < 10%). Plants were re-watered and measurements were performed at recovery (R, SVWC ≈ 40%) (Bihmidine et al., 2013b). Leaf water (Ψw, MPa) and osmotic (Ψπ, MPa) potentials were assessed using a pressure chamber instrument (PMS Instrument Co., Corvallis, OR) and a vapor pressure osmometer (Wescor, Logan, UT), respectively, on a separate set of plants at NS, SS, and R. An estimate of chlorophyll content was determined using a Minolta SPAD–502 m (Spectrum Technologies, Plainfield, IL).

Biomass related parameters, were determined by a set of plants grown under the same conditions but in soil composed of 80% sand mixed with 20% greenhouse mix to allow for root tissue recovery. Root, stem and leaf samples were oven dried at 65 °C to obtain dry weight. Total leaf area (LA_t), specific leaf area (*SLA* = total leaf area/total leaf dry weight, $cm^2\,g^{-1}$), height, and number of nodes, were also surveyed.

2.9. Statistical analysis

A complete mixed model analysis of variance (PROC MIXED, SAS System 9.2; SAS Institute, Cary, N.C.) with *G. max* line as fixed effect was conducted. Significant probability values were set, a priori, at $P < 0.05$. All measured parameters for ambient and elevated [CO_2] grown plants at the SoyFACE facility were analyzed using a complete block two-way mixed model analysis of variance (PROC MIXED) with *G. max* line, [CO_2] treatment, and *G. max* line by [CO_2] treatment interaction as fixed effect.

3. Results

3.1. Generation of transgenic soybean plants expressing ictB

To examine the contribution of the cyanobacterial *ictB* transgene to photosynthesis in soybean, an expression vector was constructed (Fig. 1A) and several transgenic T_0 soybean events were obtained; details found in supplemental section. The transgenic events were initially screened for a photosynthetic capacity boost (T_1 generation), and simplicity of the transgenic locus and expression of transgene. The selected events, based on this preliminary screen, are designated ictB468-7, ictB474-1 and ictB474-6, which Southern analysis revealed, ictB474-1 and ictB474-6 to be clones. The events, ictB468-7 ictB474-1and ictB474-6, displayed a significant boost in photosynthesis in studies conducted at both Nebraska and Illinois, while event ictB468-7 displayed a more variable response across the two locations (Figs. 2 and 3; Supplementary Fig. S1). Southern blot analysis on events ictB468-7 and ictB474-6 is shown in Fig. 1B. Expression of the *ictB* gene was confirmed using RT-PCR (Supplementary Fig. S2). No *ictB* transcript was detected in the WT plants. Homozygous T_3 or T_4 plants were used for phenotyping. The datasets gathered on the two vetted soybean *ictB* events led to the prioritization of ictB474-6 as the lead event for deeper phenotypic characterizations, given its consistency across locations, and level of photosynthetic capacity relative to WT (Fig. 2; Supplementary Fig. S1).

3.2. Photosynthetic characteristics of ictB474-6 event under favorable greenhouse conditions

The rates of net photosynthesis (A_{net}) and stomatal conductance (g_s) were investigated in the ictB474-6 event and wild type (WT, Thorne) at vegetative stage in the greenhouse at the University of Nebraska, Lincoln, under well-watered conditions. Results showed that A_{net} was significantly higher in ictB474-6 event than in WT by about 9% (Table 1). The increase in A_{net} was not the result of increase in g_s, the latter did not differ between the WT and transgenic event. These results were in agreement with those obtained in Illinois. Here, the ictB474-6 event at the V6 developmental stage showed a 10% increase ($P < 0.05$) in A_{net} at ambient [CO_2] (Fig. 2; Table 1) compared to the WT. In the ictB474-6 event this boost in A_{net} was shown to result from an increase in the rate of RuBP-limited photosynthetic CO_2 uptake (J_{max}) and RuBP-saturated (V_{cmax}) photosynthesis of 10% ($P < 0.05$) and 9% ($P < 0.01$), respectively (Fig. 2; Table 1). The quantum efficiency of photosystem II (Φ_{PSII}), the quantum yield (ΦCO_2), and photochemical quenching (*qP*) were all determined to be higher in the ictB474-6 event by 11%, 10% and 8%, respectively ($P < 0.05$; Table 1). Similar to results obtained in Nebraska, values for g_s were not significantly different between WT and the ictB474-6 event. Values of stomatal limitation were also not significantly different between WT and the ictB474-6 line. The ictB474-6 event, however, displayed a 24% ($P < 0.05$) higher mesophyll conductance (g_m) and an 11 ppm lower internal CO_2 ($P < 0.05$) at ambient [CO_2] (Table 1). The event showed no significant difference compared to WT in its percent carbon ($P = 0.4855$), and although carbon isotopic ratio ($\delta^{13}C$, ‰) was slightly reduced (i.e., more enrichment), this was not statistically significant ($P = 0.1224$) when compared to the WT (Table 1).

3.3. Productivity of ictB474-6 event under favorable greenhouse conditions

Growth rate of ictB474-6 was accelerated relative to WT, based on plant height changes monitored across vegetative growth time points (Supplementary Fig. S3). The ictB474-6 event also had an average increase in seed harvest of 15% ($P < 0.05$; Table 2). Neither the total number of seeds per plant nor the number of seeds per pod differed between WT and the ictB474-6 event. The increase in seed harvest was mainly due to an increase in the individual seed mass of 13% ($P < 0.005$; Table 2). Stem dry mass was also significantly increased in the ictB474-6 by 13% ($P < 0.05$; Table 2), but this was not accompanied by any change in the seedless pod dry mass or the total number of pods per plant (Table 2).

3.4. Evaluation of ictB474-6 event in the field

Field trials with transgenic *ictB* events and control WT plots were established to determine whether outcomes observed in the greenhouse would be translated to field conditions. In the Nebraska trials, gas exchange measurements were performed across different plant

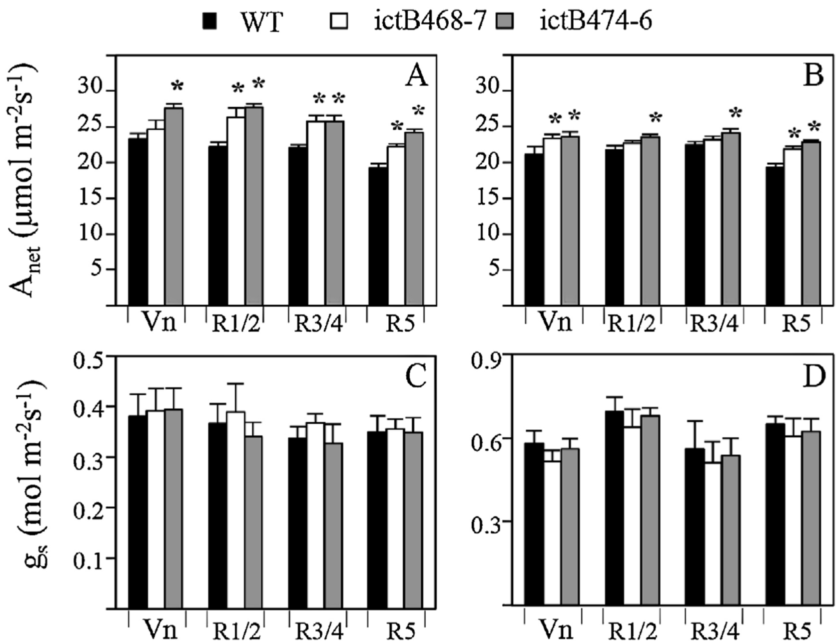

Fig. 3. Net photosynthetic rates (A_{net}, μmol m^{-2} s^{-1}) and stomatal conductance (g_s, mol m^{-2} s^{-1}) of soybean transgenic *ictB* events and wild type (WT) Thorne plants grown in the field during summer of 2009 (A & C) and 2011 (B & D). Respective bars corresponding means ± SE for data captured at Vn, vegetative stage; R1R2, flowering stage, R3R4, pod-setting to full pod stage; R5, seed-setting stage. An asterisk indicates significant difference between transgenic *ictB* and WT plants at $p \leq 0.05$.

developmental stages. The *ictB* events, ictB468-7 and ictB474-6, showed significantly higher A_{net} across all developmental stages in trials conducted in 2009 and 2011, with the latter event consistent in this phenotype in both years (Fig. 3). In the 2009 trial, the A_{net} increased in transgenic event ictB474-6 by 16%, 20%, 14% and 20% at vegetative stages R1-R2, R3-R4 and at R5 respectively. Likewise, in 2011, the A_{net} increased in this event by 10%, 8%, 7% and 16% at vegetative stages, R1-R2, R3-R4, and R5 respectively. Similar to the results obtained in the greenhouse, the rates of g_s were comparable between the *ictB* events and the WT (Fig. 3; Table 1)

In the Illinois field trials, in 2011, A_{sat} in transgenic event ictB474-6 was 15% higher at ambient [CO_2] ($P < 0.001$) and 26% higher at elevated [CO_2] relative to WT ($P < 0.0001$; Table 3). Significant increases observed in ictB474-6 event grown at ambient [CO_2] include: Φ_{PSII} ($P < 0.05$), and ΦCO_2 ($P < 0.01$). Plants grown at elevated [CO_2] also had observed increases in J_{max} ($P < 0.0001$), Φ_{PSII} ($P < 0.001$), and ΦCO_2 ($P < 0.0001$; Table 3). At ambient [CO_2], g_m for the ictB474-6 event was 24% higher than the WT, the same percentage as seen in the earlier greenhouse study; however in this case, this difference was not statistically significant ($P = 0.2150$). The maximum quantum yield of leaf CO_2 uptake ($\Phi CO_{2,max}$) was significantly higher by 6% for the ictB474-6 event in ambient [CO_2] ($P < 0.05$; Table 3), and was 5% higher (0.0715 ± 0.0011) compared to WT (0.0679 ± 0.0011) across both elevated and ambient [CO_2] ($P < 0.05$).

Gas exchange measurements were taken when senescence had begun in the ambient [CO_2] plots, at 99 days after planting (DAP) in 2010 and at 105 DAP in 2011. The ictB474-6 event in ambient [CO_2] had a higher A_{net} of 13.77 ± 1.44 ($P < 0.05$), when compared to the WT 6.72 ± 1.75 in 2010 and 6.11 ± 2.01 compared to 1.04 ± 1.67 ($P < 0.05$) in 2011.

3.5. Productivity of ictB474-6 event in the field

The ictB474-6 event showed significant increases in productivity in elevated [CO_2] on a per plant basis during the 2010 and 2011 field trials at the SoyFACE facility. The total mass per seed increased by 6% ($P < 0.05$) for the ictB474-6 event. The height of plants at the time of harvest was also 12% greater for the *ictB* event ($P < 0.0001$; Table 4).

The ictB-474-6 event at elevated [CO_2] showed a 35% increase ($P < 0.0001$) in total mass per plant, which was a result of increases in stem, pod and seed mass. The stem mass per plant increased by 47% ($P < 0.0001$), pod mass per plant increased by 32% ($P < 0.0005$), and seed mass per plant increased by 31% ($P < 0.0001$; Table 4). These increases for the ictB474-6 event were also reflected in the other measures of productivity. Plant height increased by 9% ($P < 0.0001$) and stem circumference increased by 12% ($P < 0.0005$). The transgenic event also exhibited an increase of nearly two nodes per plant ($P < 0.0005$) at the time of harvest. Unlike the ictB474-6 event grown at ambient [CO_2] there was no significant difference in the mass of individual seeds, relative to WT; however, the transgenic event grown at elevated [CO_2] had 46.6 more seeds per plant compared to wild type, corresponding to an increase of 29% ($P < 0.0005$; Table 4).

In the ambient [CO_2] ring plots the transgenic plots showed a 30% increase ($P < 0.001$) in total mass per plant, which was a result of increases in stem, pod and seed mass. The stem mass per plant increased by 28% ($P < 0.01$), pod mass per plant increased by 31% ($P < 0.001$; Table 5), and seed mass per plant increased by 30% ($P < 0.001$; Table 5). There was no significant increase in the individual mass per seed, indicating that the 30% increase in seed yield resulted from increased seed number. Importantly, the *ictB* event was observed to have 9% more nodes per plant when compared with the wild type ($P < 0.0001$). As in 2010, the average plant height showed no significant difference between wild type and *ictB* event, but stem circumference was increased ($P < 0.05$). The number of pods per plant increased by 29% ($P < 0.001$), and the total number of seeds per plant increased by 27% ($P < 0.01$; Table 5).

At elevated [CO_2], the *ictB* event showed a 28% increase ($P < 0.001$) in total mass per plant, as was seen in the ambient [CO_2]; this was a result of increases in stem, pod and seed mass. The stem mass per plant increased by 34% ($P < 0.0001$), pod mass per plant increased by 29% ($P < 0.001$; Table 4), and seed mass per plant increased by 23% ($P < 0.01$; Table 5). The *ictB* event was observed to have a significant increase of almost two nodes per plant when

Table 3
Photosynthetic parameters in wild type (WT) and transgenic ictB474-6 soybean plants grown in ambient (400 μmol mol^{-1}), and elevated (600 μmol mol^{-1}) [CO_2] during the 2011 field season in.

	WT	ictB474-6	P-Value
Ambient [CO_2]			
V_{cmax}(μmol m^{-2} s^{-1})	129.97 ± 3.80	134.42 ± 4.18	0.4140
J_{max}(μmol m^{-2} s^{-1})	153.84 ± 4.02	163.33 ± 4.36	0.0767
Φ_{PSII}	0.1911 ± 0.007	0.2141 ± 0.0074	0.0267*
ΦCO_2	0.01498 ± 0.00051	0.01694 ± 0.00052	0.0028*
$\Phi CO_{2,max}$	0.06247 ± 0.00120	0.06648 ± 0.00138	0.0361*
qP	0.4813 ± 0.0114	0.5090 ± 0.0119	0.0943
A_{sat}(μmol m^{-2} s^{-1})	30.63 ± 1.28	36.14 ± 1.33	0.0010*
c_i(μmol mol^{-1})	261.41 ± 10.13	277.12 ± 10.83	0.2919
g_s (mol m^{-2} s^{-1})	0.5337 ± 0.0296	0.5424 ± 0.0274	0.8282
g_m (mol m^{-2} s^{-1})	0.314 ± 0.042	0.391 ± 0.044	0.2150
R_d(μmol m^{-2} s^{-1})	1.63 ± 0.09	2.08 ± 0.11	0.0008*
Elevated [CO_2]			
V_{cmax}(μmol m^{-2} s^{-1})	104.08 ± 6.18	117.98 ± 6.31	0.0523*
J_{max}(μmol m^{-2} s^{-1})	131.32 ± 5.61	152.02 ± 5.70	<0.0001*
Φ_{PSII}	0.1708 ± 0.0081	0.2102 ± 0.0078	0.0007*
ΦCO_2	0.01595 ± 0.00056	0.01915 ± 0.00055	<0.0001*
$\Phi CO_{2,max}$	0.07335 ± 0.00129	0.07651 ± 0.00167	0.1444
qP	0.4198 ± 0.01300	0.5013 ± 0.0126	<0.0001*
A_{sat}(μmol m^{-2} s^{-1})	33.83 ± 1.46	42.13 ± 1.43	<0.0001*
c_i(μmol mol^{-1})	430.63 ± 14.00	413.41 ± 15.81	0.2322
g_s (mol m^{-2} s^{-1})	0.4772 ± 0.0290	0.4976 ± 0.0254	0.5960
g_m (mol m^{-2} s^{-1})	0.221 ± 0.370	0.230 ± 0.384	0.8664
R_d(μmol m^{-2} s^{-1})	1.77 ± 0.09	1.90 ± 0.12	0.3860

Photosynthetic photon flux density Q = 2000 μmol m^{-2} s^{-1}. Estimates of V_{cmax} and J_{max} were determined from fitted (A_{net}/ci) response curves by the most limiting photosynthetic process: Rubisco-carboxylation limited, RuBP-regeneration limited, or triose phosphate utilization (TPU) limited assimilation rate. The estimate of A_{sat} and R_d were determined from (A/Q) response curves using a 3-parameter non-rectangular hyperbola describing the response of A_{net} *to Q.*
* indicates significant difference (P < 0.05) determined from a complete block two-way mixed model ANOVA with [CO_2] and line as fixed effects.

Table 4
Soybean harvest productivity parameters in wild type (WT) and transgenic ictB474-6 soybean plants grown in ambient (400 μmol mol^{-1}), and elevated (600 μmol mol^{-1}) [CO_2] during the 2010 field season in.

	WT	ictB474-6	P-Value
Ambient [CO_2]			
Height (cm)	65.59 ± 1.34	73.64 ± 1.31	<0.0001*
Circumference (cm)	2.64 ± 0.07	2.66 ± 0.05	0.866
No. of nodes	14.76 ± 0.42	15.18 ± 0.36	0.4717
Total number of seeds per plant	128.6 ± 8.7	141.8 ± 7.0	0.2415
Individual seed mass (mg)	140.2 ± 2.7	148.4 ± 2.2	0.0223*
Seed mass (g)	18.12 ± 1.26	20.86 ± 1.01	0.0960
Stem mass (g)	7.45 ± 1.56	8.33 ± 1.5	0.3228
Pod mass (g)	7.91 ± 0.56	8.05 ± 0.66	0.6576
Total mass (g)	32.33 ± 2.19	36.43 ± 3.56	0.3750
Harvest Index	0.56 ± 0.01	0.56 ± 0.01	0.2582
Ratio of stem mass to total mass	0.21 ± 0.02	0.24 ± 0.01	0.0010*
Elevated [CO_2]			
Height (cm)	75.75 ± 1.14	82.37 ± 0.70	<0.0001*
Stem circumference (cm)	2.87 ± 0.09	3.21 ± 0.09	0.0003*
No. of nodes per plant	15.45 ± 0.46	17.38 ± 0.41	0.0002*
Total number of seeds per plant	158.4 ± 12.5	205.0 ± 13.8	0.0004*
Individual seed mass (mg)	149.3 ± 2.4	152.2 ± 1.9	0.199
Seed mass (g)	24.16 ± 2.06	31.58 ± 2.38	0.0050*
Stem mass (g)	10.06 ± 0.84	14.81 ± 0.95	<0.0001*
Pod mass (g)	8.69 ± 0.69	11.51 ± 0.83	0.0002*
Total mass (g)	42.92 ± 2.50	57.91 ± 4.13	<0.0001*
Harvest Index	0.56 ± 0.00	0.54 ± 0.00	0.0208*
Ratio of stem mass to total mass	0.24 ± 0.00	0.26 ± 0.00	<0.0001*

Productivity parameters from the 2010 SoyFACE experiment (IL). Data within the respective WT (wild type) and ictB474-6 columns are means corresponding to the parameter.
* indicates significant difference (P < 0.05) determined from a complete block two-way mixed model ANOVA with [CO_2] and line as fixed effects.

compared to the wild type (P < 0.0001). At elevated [CO_2] the total number of nodes per plant, as well as the average increase observed in the *ictB* event, was consistent across the 2010 and 2011 years. When grown at elevated [CO_2], the *ictB* event had an increase in average plant height of 5% (P < 0.001). There was also an increase observed in the stem circumference (P < 0.01), as well as a 28% (P < 0.0001) increase in the number of pods per plant and a 21% (P < 0.01) increase in the total number of seeds per plant (Table 5). Despite the large increases observed in individual plant productivity the end-of-season harvest of the large plots only showed a significant increase of 6.75% in yield per unit land area for the *ictB* event (4.09 t/ha^{-1}) compared to the wild type (3.83 t/ha^{-1}; P < 0.05; Supplementary Fig. S6).

In the 2009 and 2011 Nebraska field trials yield and its components were also determined to evaluate whether the increase in A_{net} had any impact on overall yield and the amount of seed

Table 5
Soybean harvest productivity parameters in wild type (WT) and transgenic ictB474-6 soybean plants under ambient (400 μmol mol^{-1}), and elevated (600 μmol mol^{-1}) [CO_2] during the 2011 field season in IL.

	WT	ictB474-6	P-Value
Ambient [CO_2]			
Height (cm)	80.08 ± 0.78	81.7 ± 0.74	0.1661
Circumference (cm)	1.79 ± 0.04	1.90 ± 0.04	0.0397*
No. of nodes per plant	14.45 ± 0.22	15.77 ± 0.24	<0.0001*
Pods per plant	17.13 ± 0.76	22.08 ± 0.96	0.0006*
No. of seeds per plant	41.4 ± 1.9	52.4 ± 2.1	0.0018*
Individual seed mass (mg)	139.8 ± 1.7	142.7 ± 1.5	0.2047
Seed mass (g)	5.77 ± 0.26	7.51 ± 0.33	0.0006*
Stem mass (g)	3.83 ± 0.17	4.90 ± 0.19	0.0018*
Pod mass (g)	2.63 ± 0.12	3.45 ± 0.14	0.0006*
Total mass (g)	12.24 ± 0.55	15.87 ± 0.65	0.0006*
Harvest Index	0.47 ± 0.00	0.47 ± 0.00	0.7969
Ratio of stem mass to total mass	0.31 ± 0.00	0.31 ± 0.003	0.6339
Elevated [CO_2]			
Height (cm)	83.87 ± 1.31	87.80 ± 1.10	0.0009*
Circumference (cm)	1.88 ± 0.03	2.04 ± 0.04	0.003*
No. of nodes per plant	15.98 ± 0.25	17.58 ± 0.24	<0.0001*
Pods per plant	20.17 ± 1.12	25.83 ± 1.25	<0.0001*
No. of seeds per plant	48.6 ± 2.8	58.6 ± 3.1	0.004*
Individual seed mass (mg)	134.5 ± 1.5	135.6 ± 1.5	0.6193
Seed mass (g)	6.55 ± 0.39	8.06 ± 0.47	0.003*
Stem mass (g)	4.73 ± 0.23	6.35 ± 0.29	<0.0001*
Pod mass (g)	3.26 ± 0.19	4.19 ± 0.21	0.0001*
Total mass (g)	14.55 ± 0.80	18.60 ± 0.94	0.0001*
Harvest Index	0.45 ± 0.00	0.43 ± 0.01	0.0003*
Ratio of stem mass to total mass	0.33 ± 0.01	0.35 ± 0.01	0.0034*

Productivity parameters from the 2011 SoyFACE experiment (IL). Data within the respective WT (wild type) and ictB474-6 columns are means corresponding to the parameter.
* indicates significant difference ($P < 0.05$) determined from a complete block two-way mixed model ANOVA with [CO_2] and line as fixed effects.

components (Supplemental Table S1). In contrast to the Illinois SoyFACE findings, no significant differences were found between the transgenic and WT plants in terms of total seeds dry weight, total number of seeds, and yield. Similarly, analysis of total content of seeds in proteins, oil, and fiber showed no statistical differences between ictB474-6 event and WT (Supplementary Table S2).

3.6. Physiological response of ictB474-6 event to drought mimic

The enhanced leaf A_{net} of the *ictB* events prompted us to test whether their performance will change in response to water deprivation. In this study, plants were subjected to a dry-down water deprivation period followed by re-watering recovery phase. Transgenic ictB474-6 plants displayed A_{net} rates that were 19%, 26%, 30%, 77%, and 27% higher than the WT plants at the NS, LS, MS, SS, and R measurement dates, respectively (Fig. 4). All the increases in A_{net} in the transgenic plants were statistically significant at $p \leq 0.05$ except under SS conditions.

A separate set of plants was grown to determine leaf water relations and biomass related parameters, and measurements were taken at NS, SS, and R dates (Table 6). Compared to the WT plants, transgenic ictB474-6 plants responded better and maintained higher biomass throughout the study period (Table 6). While no statistical differences were observed in leaf water (Ψw, MPa) and osmotic ($\Psi \pi$, MPa) potentials, estimated leaf chlorophyll content (SPAD value), total root dry weight (DWR, g), total leaf area (LA_t, cm^2) and node numbers throughout the study period, ictB474-6 line displayed significantly higher stem dry weight (DWS; g) by 25%, 23%, and 21%, higher leaves dry weight (*DWL*, g) by 14%, 13%, 17%, and higher total dry weight (*DWT*, g) by 15%, 13%, and 16%, during NS, SS, and R period, respectively, compared to WT plants. Moreover, the ictB474-6 event showed an 11% and 8% increase in height at NS and SS, respectively, but the difference was not statistically significant at recovery (R). In addition, ictB474-6 showed a decrease of 20% in specific leaf area (SLA, $cm^2\ g^{-1}$) after drought compared to the WT (Table 6).

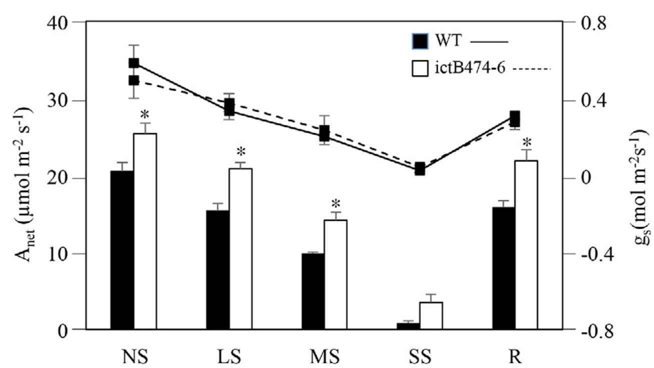

Fig. 4. Photosynthetic rates (A_{net}, μmol $m^{-2}\ s^{-1}$) and stomatal conductance (g_s, mol $m^{-2}\ s^{-1}$) for wild type (WT) and transgenic ictB474-6 soybean plants, under gradual soil dehydration followed by a period of recovery. Respective bars (A_{net}) and lines (g_s) are corresponding means ± SE for treatments, NS, no stress conditions; LS, low stress; MS, medium stress; SS, severe stress; R, recovery. An asterisk (above bar) indicates significant differences between transgenic ictB474-6 and WT plants at $p \leq 0.05$.

4. Discussion

Phenotyping of a soybean event expressing the putative cyanobacterial inorganic carbon transporter, *ictB,* at two independent locations in both controlled greenhouse and in replicated field environments over three years revealed significant increases in both light-saturated and light-limited photosynthesis, and in various components of yield.

Under greenhouse conditions, transgenic plants showed significantly higher A_{net} compared to WT, while g_s did not differ between the two, indicating that the increase in photosynthesis was not due to higher stomatal conductance of CO_2 in the transgenic plants. These results are in agreement with Simkin et al. (2015) and Lieman-Hurwitz et al. (2003) who also found that transgenic tobacco and Arabidopsis plants that express the *ictB* gene,

Table 6
Soybean parameters monitored during the drought mimic study in NE greenhouses, for wild type (WT) and transgenic ictB474-6 event.

Parameter	Pre-Stress		Stress		Post-Stress	
	WT	ictB474-6	WT	ictB474-6	WT	ictB474-6
SVWC, %	40.8 ± 1.5	39.6 ± 3.5	6.5 ± 1.3	6.6 ± 2.2	39.2 ± 5.5	37.5 ± 5.5
Ψ_w, MPa	−0.27 ± 0.06	−0.27 ± 0.06	−1.53 ± 0.06	−1.57 ± 0.21	−0.23 ± 0.06	−0.18 ± 0.1
Ψ_π, MPa	−1.08 ± 0.06	−1.12 ± 0.08	−1.9 ± 0.45	−1.81 ± 0.31	−1.17 ± 0.32	−1.03 ± 0.12
SPAD	39.8 ± 2.0	38.7 ± 2.6	33.4 ± 4.9	34.3 ± 2.9	36.8 ± 4.7	37.2 ± 3.3
DW_R, g	1 ± 0.1	1.07 ± 0.06	1.2 ± 0.06	1.2 ± 0.1	1.3 ± 0.10	1.3 ± 0.15
DW_S, g	1 ± 0.2	1.3 ± 0.06*	1.3 ± 0.06	1.7 ± 0.15*	1.4 ± 0.12	1.8 ± 0.15*
DW_L, g	1.83 ± 0.21	2.13 ± 0.15	2.03 ± 0.15	2.33 ± 0.06*	2.23 ± 0.21	2.70 ± 0.10*
DW_T, g	3.8 ± 0.06	4.5 ± 0.15*	4.6 ± 0.1	5.2 ± 0.21*	5 ± 0.20	5.9 ± 0.15*
LA_t, cm^2	483 ± 37	452 ± 69	645 ± 20	629 ± 45	716 ± 61	703 ± 41
SLA, $cm^2\ g^{-1}$	268 ± 53	214 ± 47	318 ± 16	270 ± 18*	323 ± 45	260 ± 12
Height, cm	21.3 ± 0.58	24 ± 0.0*	26 ± 1.0	28.2 ± 0.76*	27.8 ± 0.29	29 ± 1.0
Node number	7 ± 0	8 ± 1	7.67 ± 0.58	8.67 ± 0.58	8.33 ± 0.58	9.33 ± 0.58

Data within each column refer to wild type (WT) and ictB474-6 event prior to (Pre-stress), end of dry-down (Stress) and following re-water (Post-Stress). Soil volumetric water content (%), leaf water potential (Ψ_w, MPa), leaf osmotic potential (Ψ_π, MPa), SPAD chlorophyll value, root dry weight (DW_R, g), stem dry weight (DW_S, g), leaf dry weight (DW_L, g), total dry weight (DW_T, g), total leaf area (LA_t, cm^2), specific leaf area (SLA, $cm^2\ g^{-1}$), height (cm), and number of nodes. Values are means ± SD.
* indicates significant differences between transgenic and WT plants within the same period at $P \leq 0.05$.

exhibited higher rates of photosynthesis without a change in stomatal conductance compared to the WT.

The *ictB* event showed a significant increase of 24% in its g_m, as well as a significant decrease of c_i by 11 ppm and 13.4 ppm, which similar outcomes were observed in rice (Gong et al., 2015). It has been shown that mesophyll resistance (the inverse of g_m) imposes a limitation of 0.1–0.2 on photosynthesis; therefore, if g_m were infinite, photosynthetic CO_2 uptake would be increased by 10–20% (Bernacchi et al., 2002). The significant decrease in the c_i/c_a was consistent throughout the greenhouse experiments, as was the significant decrease of c_i. However, increases in g_m alone could not account for the improvements in A_{net}, as demonstrated by higher CO_2 uptake for the *ictB* event, even at equivalent chloroplast concentrations of CO_2 (c_c; Fig. 2). An additional advantage of the increase in g_m, without an increase in g_s, is the improvement in the rate of CO_2 uptake without a negative effect on plant water use efficiency (*WUE*). It is important to note that while the variable J method provides reliable estimates of g_m, it is more sensitive to errors than the isotopic method that was not possible here (Pons et al., 2009); nevertheless Bernacchi et al. (2002) showed good agreement between our method and the isotopic method.

Tobacco and *Arabidopsis thaliana* expressing *ictB* were reported to have higher rates A_{net} at low [CO_2], and a higher V_{cmax} when grown in a low relative humidity environment, but there was no observed increase in RuBP-limited photosynthetic CO_2 uptake (J_{max}) (Lieman-Hurwitz et al., 2003; Simkin et al., 2015). However, in this study the ictB474-6 event was observed to have a significant increase in both V_{cmax} and J_{max}. The increase in RuBP-limited leaf photosynthetic CO_2 uptake is consistent with a reduction in photorespiration due to improved CO_2 availability at the site of Rubisco; RuBP limited photosynthesis increases in elevated [CO_2] environments due to decreased Rubisco oxygenation reactions, thus diverting ATP and NADPH to photosynthetic assimilation instead (Long et al., 2004). Consistent with these changes, the Φ_{PSII} and ΦCO_2 displayed increases in the transgenic *ictB* event compared to the WT.

Most significant with respect to the effect of *ictB* is the observed increase in the maximum quantum yield of CO_2 assimilation ($\Phi CO_{2,max}$), i.e. the initial slope of the response of A_{net} to absorbed photons (I_{abs}). This could only be increased by three possible routes: 1) The Rubisco in the *ictB* plants has a higher specificity for CO_2; 2) the oxygen concentration at Rubisco is decreased; or 3) CO_2 at Rubisco is increased. Since Rubisco is highly conserved within a species and intercellular O_2 will reflect the atmospheric level, increased CO_2 appears the only plausible explanation. Since photosynthesis is improved under both light-saturating and light-limiting conditions, it follows that net plant CO_2 assimilation, A_{net}, should be improved, along with productivity.

Field trials confirmed the higher rate of leaf photosynthesis in the ictB474-6 event compared to WT initially observed in the greenhouse, with even greater differences under field conditions. This could be explained by the environmental differences between greenhouse and field conditions. Indeed, a previous study showed that under high relative humidity, *ictB* expression did not affect Rubisco properties or growth, but under low humidity, its expression led to an increase in the enzyme activity and accelerated growth of the transgenic plants compared to the wild type (Lieman-Hurwitz et al., 2003). Similar to the results from the greenhouse experiments, in the SoyFACE field experiments the *ictB* event showed significant increases in several measurements of photosynthetic performance. The rate of light-saturated photosynthetic CO_2 uptake (A_{sat}) increased relative to WT in both ambient [CO_2] and elevated [CO_2]. The effect of elevated [CO_2] on light-saturated CO_2 uptake was an increase of 7% and 12% in the WT and the *ictB* event, respectively. This was slightly less than the 13% average increase in light-saturated C_3 photosynthesis for 45 species measured at 11 different FACE studies (Leakey et al., 2009). This further suggests that the expression of the *ictB* gene produces similar increases in light-saturated CO_2 uptake as elevating [CO_2] for C_3 crops.

Estimates of A_{sat} were 18% and 25% higher for the *ictB* event than the WT in ambient [CO_2] and elevated [CO_2], respectively (Table 3; Supplementary Fig. S5). The reported effect of elevated (567 μmol mol^{-1}) [CO_2] on A_{sat} in legumes was nearly a 20% increase, but showed a lower impact on plants that were RuBP-limited (Ainsworth and Rogers, 2007). The effect of elevated [CO_2] on estimates of A_{sat} from A_{net}/Q curves was 10.5% and 17% for the WT and ictB474-6 event, respectively. However, the interaction term for genotype with [CO_2] treatment was not significant ($P = 0.3203$), i.e. there was no statistical evidence of an altered effect of transformation with *ictB* at elevated [CO_2] of 600 μmol mol^{-1}, indicating that the benefit would not be diminished with rising [CO_2]. It is important to note that during the 2011 growing season, at the SoyFACE facility, there was no reported stimulation of A_{sat} by elevated [CO_2] in the soybean cultivar Pioneer 93B15 due to drought stress (Carl Bernacchi, personal communication). In the growing season months of July through September, the SoyFACE facility received 46% less cumulative precipitation (154.1 mm) than the 30 year average (287.5 mm). Plant drought stress is consistent with the small increases in A_{sat} shown in the WT grown at elevated [CO_2].

The ictB474-6 event showed increases in most measures of photosynthetic capacity: *qP*, V_{cmax}, J_{max}, Φ_{PSII}, and ΦCO_2 measured in saturating light. Increase in V_{cmax} was not due to a change in Rubisco content, since protein immunoblot blots analysis provided no evidence of any difference in Rubisco content between the *ictB* event and wild type in either ambient or elevated CO_2 treatments (Supplementary Fig. S4). The observed decreases in V_{cmax} in elevated $[CO_2]$ were also found by Bernacchi et al. (2005) and assumed to be due to reductions in Rubisco activity.

As noted for the controlled greenhouse grown plants, the maximum quantum yield of CO_2 assimilation ($\Phi CO_{2,max}$) was significantly increased by 5% in the ictB474-6 event relative to WT. This finding is particularly significant since it provides strong evidence that *ictB* is likely acting to increase $[CO_2]$ at Rubisco site. While a multitude of pleiotropic effects could increase A_{sat}, for example by increases in a wide range of proteins involved in the light-reactions and carbon metabolism (Long et al., 2006a,b; Zhu et al., 2007), an increase in $\Phi CO_{2,max}$ is only possible if $[CO_2]$ at Rubisco is increased, $[O_2]$ is decreased, or the kinetic properties of Rubisco are changed. It seems very unlikely that transformation with the *ictB* gene could alter the latter two.

The mechanism of action of the *ictB* product is not fully understood (Shibata et al., 2002; Price et al., 2011). While this study is unable to determine the molecular mechanism of the *ictB* gene, significant decreases in c_i, along with large increases in g_m, seem to suggest some form of internal membrane alteration that allows for easier diffusion of CO_2 from the intercellular air space to Rubisco. Interestingly, in rice, *ictB*, fused to a transit peptide, appears to remain in the cytosol (Gong et al., 2015).

Greenhouse seed harvest and mass per seed in the ictB474-6 event were increased by 15% and 13%, respectively, compared to WT (Table 2). The increases in seed harvest and mass per seed are similar to the average observed increase in yield of 13% for C_3 crops grown in elevated (550 ppm) $[CO_2]$, which is also attributed to an increase in photosynthesis (Long et al., 2006a,b). The ictB474-6 event showed increases in height, stem dry weight, leaf dry weight, and total dry weight from the drought mimic study (Table 6). The increases in seed mass and overall plant biomass are consistent with higher rates of CO_2 uptake, and previously observed increased growth rate as well as mass accumulation in *Arabidopsis thaliana* and Tobacco expressing the *ictB* transgene in a low humidity environment (Lieman-Hurwitz et al., 2003). Biomass comparison of plants before exposing them to water deprivation showed that transgenic ictB474-6 event exhibited higher values compared to WT (Table 6). These results are in agreement with findings previously observed in transgenic rice expressing the *ictB* gene that displayed both enhanced growth and yield parameters as a result of increased photosynthesis (Yang et al., 2008; Gong et al., 2015).

Water availability is one of the major environmental factors that affect CO_2 fixation and limit photosynthesis (Ruan et al., 2012). Plants respond to drought stress by first closing their stomata to minimize water loss. However, stomatal closure also leads to limitation in CO_2 supply to Rubisco, resulting in a decrease in photosynthesis. Because expression of the *ictB* gene in soybean did not have any effect on g_s under favorable conditions, the transgenic plants displayed A_{net}, which led to better biomass in terms of stem, leaf and total dry weight. This could be explained by the fact that *ictB* allows more CO_2 availability at the site of Rubisco without transpiring any more water, as inferred by the lack of effect on g_s.

Consistent with results using individual plants in greenhouse experiments, the ictB474-6 event displayed significant increases in productivity compared to WT plants in response to both ambient and elevated $[CO_2]$ treatments during the SoyFACE 2010 and 2011 field trials. This is consistent with the observed increases in leaf CO_2 uptake and the maintenance of photosynthetic capacity into late reproduction stages, and increase in node number (Table 4 and 5; Fig. 3). The increase in A_{net} observed in ambient $[CO_2]$ due to *ictB* was almost identical to the increase observed in WT plants grown in elevated $[CO_2]$ (Morgan et al., 2005).

Additionally, the boost in yield observed in one year (Supplementary Fig. S6) may be the result of mitigation of reduction in photosynthetic capacity under stress as suggested by results from the drought mimic study. Plants expressing *ictB* were observed to maintain photosynthetic activity and biomass accumulation under drought stress and, as previously stated, the SoyFACE facility had received 46% less precipitation (154.1 mm) than the 30 year average (287.5 mm). The Nebraska field trials received slightly greater cumulative precipitation (245.9 mm) than their historic average (241.8 mm), and ultimately 60% more precipitation than the trials at the SoyFACE facility in July through September of 2011. Therefore, it is possible that the 7% increase in productivity with the ictB474-6 event compared to WT plots may be due to its protection of yield under stress. The *ictB* event displayed improved photosynthetic rates compared to wild type plants in all but the most severe drought mimic conditions (Fig. 4).

There is always a possibility that a somaclonal mutation could explain a phenotype observed in a transgenic plant. However, given the increase in photosynthesis and productivity, observed across multiple *ictB* events (Fig. 3, Supplementary Fig. S1), the likelihood of such a phenomenon accounting for the results communicated herein is remote.

The results of this study translate the findings observed in rice (Yang et al., 2008; Gong et al., 2015) and tobacco (Lieman-Hurwitz et al., 2003) to a major oilseed, *Glycine max*, wherein the cyanobacterial *ictB* transgene significantly increases photosynthesis and components of yield. The observed increase in photosynthetic CO_2 assimilation induced by *ictB* may have a positive effect on the plant's *WUE* due to the observed increase in g_m without a corresponding of increase in g_s. Likely because of an improved maximum ε_c, the *ictB* event was able to produce significantly higher outcomes in various yield parameters. The final location of the *ictB* gene product is uncertain; the use of the RbcS chloroplast transit peptide has been previously demonstrated to fail to successfully target the chloroplast envelope (Gong et al., 2015; Rolland et al., 2016). The expression of the *ictB* gene results in significant improvements in photosynthesis but the current study is unable to determine the mode of action of *ictB* or the definitive cause of the photosynthetic improvement. The work presented here aids in further elucidating the underlying mode of action of *ictB in planta*. Whether the transit peptide is processed or successfully plastid targeted in soybean was not determined in this study. However, confirmatory subcellular localization of *ictB* is additional information that will aid in further gaining insight into the underlying mechanism, which in turn will help guide next generation synthetic design approaches to further boost carbon capture and flux in higher plants as a means break the predicted yield barriers of crops.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2017.02.003.

References

Ainsworth, E.A., Rogers, A., 2007. The response of photosynthesis and stomatal conductance to rising (CO2): mechanisms and environmental interactions. Plant. Cell Environ. 30, 258–270.

Ainsworth, E.A., Yendrek, C.R., Skoneczka, J.A., Long, S.P., 2012. Accelerating yield potential in soybean: potential targets for biotechnological improvement. Plant Cell Environ. 35, 38–52.

Bernacchi, C.J., Portis, A.R., Nakano, H., von Caemmerer, S., Long, S.P., 2002. Temperature response of mesophyll conductance: implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis in vivo. Plant Physiol. 130, 1992–1998.

Bernacchi, C.J., Morgan, P.B., Ort, D.R., Long, S.P., 2005. The growth of soybean under free air [CO_2] enrichment (FACE) stimulates photosynthesis while decreasing in vivo Rubisco capacity. Planta 220, 434–446.

Bihmidine, S., Hunter III, C.T., Johns, C.E., Koch, K.E., Braun, D.M., 2013a. Regulation of assimilate import into sink organs: update on molecular drivers of sink strength. Front. Plant Sci. 4, 177, http://dx.doi.org/10.3389/fpls.2013.00177.

Bihmidine, S., Lin, J., Stone, J.M., Awada, T., Specht, J.E., Clemente, T.E., 2013b. Activity of the Arabidopsis RD29A and RD29B promoter elements in soybean under water stress. Planta 237, 55–64.

Bonfil, D.J., Ronen-Tarazi, M., Sültemeyer, D., Lieman-Hurwitz, J., Schatz, D., Kaplan, A., 1998. A putative HCO3- transporter in the cyanobacterium *Synechococcus* sp. strain PCC 7942. FEBS Lett. 430, 236–240.

Clemente, T., La Vallee, B.J., Howe, A.R., Conner-Ward, D., Rozman, R.J., Hunter, P.E., Broyles, D.L., Kasten, D.S., Hinchee, M.A., 2000. Progeny analysis of glyphosate selected transgenic soybeans derived from *Agrobacterium*-mediated transformation. Crop Sci. 40, 797–803.

Dohleman, F.G., Long, S.P., 2009. More productive than maize in the midwest: how does miscanthus do it? Plant Physiol. 150, 2104–2115.

Dubois, J.-J.B., Fiscus, E.L., Booker, F.L., Flowers, M.D., Reid, C.D., 2007. Optimizing the statistical estimation of the parameters of the Farquhar-von Caemmerer-Berry model of photosynthesis. New Phytol. 176, 402–414.

FAO, 2015. FAOSTAT. Food and Agriculture Organization of the United Nations, Rome, Italy.

Farquhar, G.D., Sharkey, T., 1982. Stomatal conductance and photosynthesis. Ann. Rev. Plant Physiol. 33, 317–345.

Farquhar, G.D., von Caemmerer, S., 1982. Modeling of photosynthetic response to environmental conditions. Encycl. Plant Physiol. 12, 549–587.

Farquhar, G.D., Ball, M., von Caemmerer, S., Roksandic, Z., 1982a. Effect of salinity and humidity on delta 13C value of halophytes—Evidence for diffusional isotope fractionation determined by the ratio of intercellular/atmospheric partial pressure of CO_2 under different environmental conditions. Oecologia 52, 121–124.

Farquhar, G.D., O'Leary, M.H., Berry, J.A., 1982b. On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. Funct. Plant Biol. 9, 121–137.

Farquhar, G.D., Ehleringer, J.R., Hubick, K.T., 1989. Carbon isotope discrimination and photosynthesis. Ann. Rev. Plant Physiol. Plant Mol. Biol. 40, 503–537.

Fehr, W.R., Caviness, C.E., 1979. Stages of Soybean Development. Cooperation Extension Service, vol. 80. Iowa State University, pp. 1–12.

Giordano, M., Beardall, J., Ravan, J.A., 2005. CO_2 concentrating mechanism in algae: mechanisms, environmental modulation and evolution. Ann. Rev. Plant Biol. 56, 99–131.

Godfray, H.C., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F., Pretty, J., Robinson, S., Thomas, S.M., Toulmin, C., 2010. Food security: the challenge of feeding 9 billion people. Science 327, 812–818.

Gong, H.Y., Li, Y., Fang, G., Hu, D.H., Jin, W.B., Wang, Z.H., Li, Y.S., 2015. Transgenic rice expressing IctB and FBP/Sbpase derived from cyanobacteria exhibits enhanced photosynthesis and mesophyll conductance to CO_2. PLoS One 10 (10), e0140928.

Kaplan, A., Reinhold, L., 1999. CO_2 concentrating mechanisms in photosynthetic microorganisms. Ann. Rev. Plant Physiol. Plant Mol. Biol. 50, 539–570.

Lieman-Hurwitz, J., Rachmilevitch, S., Mittler, R., Marcus, Y., Kaplan, A., 2003. Enhanced photosynthesis and growth of transgenic plants that express ictB: a gene involved in HCO3- accumulation in cyanobacteria. Plant Biotechnol. J. 1, 43–50.

Lieman-Hurwitz, J., Asipov, L., Rachmilevitch, S., Marcus, Y., Kaplan, A., 2005. Expression of cyanobacterial ictB in higher plants enhanced photosynthesis and growth. In: Plant Responses to Air Pollution and Global Change., pp. 133–139.

Long, S., Hällgren, J.-E., 1993. Measurement of CO_2 assimilation by plants in the field and the laboratory. In: Photosynthesis and Production in a Changing Environment. Springer, 129-167.

Long, S.P., Ort, D.R., 2010. More than taking the heat: crops and global change. Curr. Opin. Plant Biol. 13, 241–248.

Long, S.P., Ainsworth, E.A., Rogers, A., Ort, D.R., 2004. Rising atmospheric carbon dioxide: plants face the future. Ann. Rev. Plant Biol. 55, 591–628.

Long, S.P., Ainsworth, E.A., Leakey, A.D.B., Nösberger, J., Ort, D.R., 2006a. Food for thought: lower-than-expected crop yield stimulation with rising CO2 concentrations. Science 312, 1918–1921.

Long, S.P., Zhu, X.G., Naidu, S.L., Ort, D.R., 2006b. Can improvement in photosynthesis increase crop yields? Plant Cell Environ. 29, 315–330.

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

Long, S.P., 2014. We need winners in the race to increase photosynthesis in rice, whether from conventional breeding, biotechnology or both. Plant. Cell Environ. 37, 19–21.

Monteith, J., 1977. Climate and the efficiency of crop production in Britain. Philos. Trans. R. Soc. Lond. 281, 277–294.

Morgan, P.B., Bernacchi, C.J., Ort, D.R., Long, S.P., 2004. An *in vivo* analysis of the effect of season-long open-air elevation of ozone to anticipated 2050 levels on photosynthesis in soybean. Plant Physiol. 135, 2348–2357.

Morgan, P.B., Bollero, G.A., Nelson, R.L., Dohleman, F.G., Long, S.P., 2005. Smaller than predicted increase in aboveground net primary production and yield of field-grown soybean under fully open-air [CO2] elevation. Glob. Change Biol. 11, 1856–1865.

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. J. Exp. Bot. 60, 2217–2234.

Price, G.D., Badger, M.R., von Caemmerer, S., 2011. The prospect of using cyanobacterial bicarbonate transporters to improve leaf photosynthesis in C3 crop plants. Plant Physiol. 155, 20–26.

Raines, C.A., 2011. Increasing photosynthetic carbon assimilation in C3 plants to improve crop yield: current and future strategies. Plant Physiol. 155, 36–42.

Ray, D.K., Ramankutty, N., Mueller, N.D., West, P.C., Foley, J.A., 2012. Recent patterns of crop yield growth and stagnation. Nat. Commun. 3, 1293, http://dx.doi.org/10.1038/ncomms2296.

Ray, D.K., Mueller, N.D., West, P.C., Foley, J.A., 2013. Yield trends are insufficient to double global crop production by 2050. PLoS One 8, e66428.

Rolland, V., Badger, M.R., Price, G.D., 2016. Redirecting the cyanobacterial bicarbonate transporters BicA and SbtA to the chloroplast envelope: soluble and membrane cargos need different chloroplast targeting signals in plants. Front. Plant Sci. 7, 185, 10.3389/fpls.2016.00185.

Ruan, C.J., Shao, H.B., Teixeira da Silva, J.A., 2012. A critical review on the improvement of photosynthetic carbon assimilation in C3 plants using genetic engineering. Crit. Rev. Biotechnol. 32, 1–21.

Simkin, A.J., McAusland, L., Headland, L.R., Lawson, T., Raines, C.A., 2015. Multigene manipulation of photosynthetic carbon assimilation increases CO2 fixation and biomass yield in tobacco. J. Exp Bot 66, 4075–4090.

Tilman, D., Clark, M., 2015. Food, agriculture & the environment: can we feed the world & save the earth? Daedalus 144, 8–23.

Yang, S., Chang, C., Yanagisawa, M., Park, I., Tseng, T., Ku, M.S.B., 2008. Transgenic rice expressing cyanobacterial bicarbonate transporter exhibited enhanced photosynthesis, growth and grain yield. Photosynthesis. Energy from the Sun: 14th International Congress on Photosynthesis, 1243–1246.

Zhu, X.G., de Sturler, E., Long, S.P., 2007. Optimizing the distribution of resources between enzymes of carbon metabolism can dramatically increase photosynthetic rate: a numerical simulation using an evolutionary algorithm. Plant Physiol. 145, 513–526.

Zhu, X.G., Long, S.P., Ort, D.R., 2010. Improving photosynthetic efficiency for greater yield. Ann. Rev. Plant Biol. 61, 235–261.

Zurbriggen, M.D., Carrillo, N., Hajirezaei, M., 2009. Use of cyanobacterial proteins to engineer new crops. In: Kirakosyan, A., Kaufman, P.B. (Eds.), Recent. Advances in Plant Biotechnology. Springer, New York, NY, pp. 65–88.

Supplementary: Material and methods

S.1 Soybean transformation and selection of transgenic plants

The T-DNA region contained a *bar* gene cassette that confers tolerance to treatment with the herbicide, glufosinate, which was utilized as a selectable marker during the transformation process. The primary transformants were first tested for glufosinate tolerance in the greenhouse, allowed to self to T_3 and T_4 generations, from which homozygous lineages were then used for photosynthetic phenotyping and field evaluations. PCR analysis was performed to monitor segregation of the transgene using the *ictB*F/5'-ATGGATCTACGGCGTTGAAG-3' forward and *ictB*R/5'-AAGGCCGATCTTGAATCAT-3' reverse primers, and to identify homozygous lines. To examine the complexity of the T-DNA insertions in the soybean genome, total DNA was extracted following the protocol of Dellaporta et al. (1983), which was used for Southern blot hybridization according to Eckert et al. (2006).

S.2 Monitoring ictB transcript accumulation in transgenic soybean events

Total RNA was isolated from top most leaf at V5 stage of development using TRIzol® LS reagent (Life Technologies). RT-PCR reactions were conducted using primer set pICTB-1: CTTCTTGCTTGCTGTCGTCTAC and pICTB-2: ATAGAGGGGATAAACCAGGTTAAAG. Two controls soybean genotypes (NE3001 and Thorne), along with binary plasmid used for transformation were used as negative and positive controls, respectively. Amplification of 18S ribosomal RNA subunit was used as a internal reaction control for the RT-PCR assay.

S.3 Gas exchange measurements under controlled greenhouse settings

A controlled growth study at the University of Nebraska-Lincoln (UNL) greenhouses, was carried out with 20 T_3 progeny from WT and homozygous selected *ictB* events. Plants were grown in 16 cm diameter pots in a greenhouse soil mix (40% Canadian peat, 40% coarse vermiculite, 15% masonry sand, and 5% screened topsoil), at 14 h photoperiod, day/night temperatures of 25/22°C, RH of 45±5%, and were well watered throughout the whole study period.

The greenhouse study at the University of Illinois was carried out using the same WT and *ictB* events as the University of Nebraska experiment, arranged in a completely randomized design (n=19). The greenhouse environment at the University of Illinois was also a 14 h photoperiod, day/night temperatures of 25/20°C, RH of 50±5%. Natural daylight was supplemented with 600 μmol photons $m^{-2}s^{-1}$ from high pressure mercury lamps. Plants were grown in 25 cm diameter pots containing soilless potting media (Sunshine Mix LC1; Sun Gro Horticulture, Bellevue, WA). Plants were well watered throughout their growth cycle, and their position in the greenhouse was randomly rotated biweekly. Plant height was measured at 11 time points spread across the growing season. Plants were allowed to dry down prior to harvest, post R8 stage, and productivity parameters were determined. Biomass was dried at 60°C to constant weight and productivity parameters were determined.

The procedure for measuring A_{net}/c_i was as described by Bernacchi et al. (2005). The leaf temperature was maintained at 25-27ºC and photon flux at 1500 μmol $m^{-2}s^{-1}$ using the cuvette integrated red-blue LED light source. Following the procedure described by Long and Bernacchi (2003), each leaf was allowed to reach steady-state CO_2 and water vapor exchange at the set ambient CO_2 of 400 μmol mol^{-1}. Measurements of A_{net} were made starting at 400 μmol mol^{-1} [CO_2] surrounding the leaf, and [CO_2] was decreased stepwise from 300, 200, 100 and finally to 50 μmol mol^{-1} [CO_2]. The [CO_2] was then returned to 400 μmol mol^{-1} for two consecutive measurements before being increased stepwise from 600, 800, 1000 and finally to 1500 μmol mol^{-1}. In total, eleven measurements were taken per plant at varying [CO_2].

Stomatal limitation was calculated from the A_{net}/c_i response curves following the equation (Farquhar and Sharkey, 1982)

$$l = \frac{(A_{ca} - A_{ci})}{A_{ca}} \qquad \text{Eq. 1}$$

where A_{ca} is the value of A_{net} as determined from the A_{net}/c_i where the c_i was 400 µmol mol^{-1}, i.e. current ambient [CO_2], and the value that would be obtained if stomatal conductance (g_s) was infinite. A_{ci} is the A_{net} achieved with the actual g_s when c_a is 400 µmol mol^{-1}.

Mesophyll conductance was calculated by using A_{net}, J measured from gas exchange and chlorophyll fluorescence at ambient [CO_2], Γ*, and R_d from the fitted A_{net}/c_i curves to determine g_m:

$$g_m = \frac{A_{net}}{c_i - \dfrac{\Gamma^*(J + 8(A_{net} + R_d)}{J - 4(A_{net} + R_d)}} \qquad \text{Eq. 2}$$

The chlorophyll fluorescence terms and definitions follow those of Baker and Oxborough (2004). Photochemical efficiency of photosystem II (Φ_{PSII}) for a light adapted leaf was determined by measuring the steady state-fluorescence (F_s) and the maximum fluorescence during a light saturating pulse of >7 mmol m^{-2} s^{-1} (F_m'; Equation 3; Genty et al.,1989).

Quantum yield of CO_2 assimilation (ΦCO_2) was determined by equation 4, where A_{net} is CO_2 assimilation or net photosynthetic rate (µmol m^{-2} s^{-1}), A_{dark} is dark CO_2 assimilation rate (µmol m^{-2} s^{-1}), I is incident photon flux density (µmol m^{-2} s^{-1}), and α_{leaf} is leaf absorptance, which was calculated as described previously (Bernacchi et al., 2002). Photochemical quenching (qP) was determined by using equation 5: where F_o is the minimum fluorescence.

$$\Phi_{PSII} = F_m' - \frac{F_s}{F_m'} \qquad \text{Eq. 3}$$

$$\Phi_{CO_2} = \frac{A_{net} - A_{dark}}{I\alpha_{leaf}} \quad \text{Eq. 4}$$

$$qP = \frac{F_m^{'} - F_s}{F_m^{'} - F_o} \quad \text{Eq. 5}$$

S.4. The response of leaf photosynthesis to intercellular [CO_2] (A_{net}/c_i)

Excised leaves were kept in low light (<10 μmol m^{-2} s^{-1}) until 15 min prior to measurements, after which, they were light-acclimated (approximately 1,000 μmol m^{-2} s^{-1} white light) outside the gas exchange chamber. Leaf gas-exchange measurements were coupled with measurements of chlorophyll fluorescence using an open gas-exchange system and fluorometer (LI-6400 and LI-6400-40; LI-COR), and V_{cmax} and J_{max} determined, as described in 2.2

S.5. End-of-season leaf gas exchange

Because the ictB474-6 event appeared green at the end of the growing season when the WT plants were senescing, additional gas exchange measurements were made at this stage to determine whether these leaves also remained photosynthetically active. Leaf A_{net} was measured for the WT and *ictB* lines on 16 randomly selected uppermost fully expanded soybean leaves; 99 and 105 DAP in 2010 and 2011, respectively.

S.6. Response of photosynthesis to photon flux (A_{net}/Q)

Leaves were acclimated to 2,000 μmol m^{-2} s^{-1} light, and the [CO_2] surrounding the leaf was set to the [CO_2] of the ambient and or the elevated SoyFACE treatment. Leaves were allowed to achieve steady-state, i.e. A_{net} did not vary by more than ±3% over 2 minutes, before measurements commenced. Once steady-state had been obtained at 2,000 μmol m^{-2} s^{-1} Q, the associated gas exchange and fluorescence parameters were recorded and photon flux was then decreased stepwise finishing in complete darkness (Q=2000, 1500, 1000, 500, 200, 100, 75, 50, 25, 0 μmol m^{-2} s^{-1}), repeating the steady-state measurements at each photon flux. All light response curves were completed within 6 h of leaf collection

S.7. Productivity parameters

At 75 days after planting, three plants per treatment were randomly selected and all above ground biomass harvested from the SoyFACE ring plots in 2011. Three leaves were randomly selected per plant and a circular punch, 3 cm^2, was used to cut leaf discs to determine specific leaf area (m^2 gram^{-1}). All material was dried to constant weight at 60 C^o. Total leaf area was estimated as the product of the specific leaf area by total leaf mass of each plant. Leaf area index (one-sided leaf area per unit ground area; Beadle 1993), was determined by dividing the estimated plant leaf area by the ground area occupied by one plant, i.e. the inverse of the number of plants per square meter of plot.

S.8. Protein immunoblot analysis for Rubisco

In 2011, at 45 days after emergence, protein was extracted from 1.27 cm^2 (four 0.316 cm^2 subsamples) of leaf tissue from the WT and ictB474-6 event for each biological replicate (n=4), following the procedure of Spence et al. (2014). Samples were cut with a circular punch from the center trifoliate of each leaf using the same leaves for which A_{net}/c_i and A_{net}/Q response curves were determined. Rubisco polypeptides were detected with rabbit polyclonal antibodies to the spinach Rubisco large subunit (Agrisera Antibodies, Vännäs, Sweden). After primary antibody incubation the membranes were washed and then incubated with anti-rabbit IgG alkaline phosphatase antibody produced in goat, affinity isolated (A7539, Sigma-Aldrich). The secondary antibody was detected with a mixture of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) (Western Blue® Stabilized Substrate, Promega, Madison, WI, USA). After air drying, each membrane was photographed using a single-lens reflex digital camera (D80 and AF Micro Nikkor 60mm lens, Nikon, Chiyoda-ku, Tokyo, Japan). The relative volume and intensity for each band was quantified by 2-D image analysis, and reported as ImageQuant volume (IQV; ImageQuant TL 7.0, GE Healthcare Biosciences, Little Chalfont, U.K.). In quantifying, amounts were diluted to ensure that the relationship of relative volume to amount of Rubisco remained linear.

Supplementary Results:

SR.3. Productivity of ictB474-6 event under favorable greenhouse conditions

The growth rate of ictB474-6 event was significantly greater than WT, as determined by increases in plant height ($P<0.01$) on 10 of the 11 height measurement days throughout vegetative growth (Supplementary Fig. S2).

SR.2 Productivity of ictB474-6 event in the field

In the 2011 field season total mass, leaf mass and LAI in the SoyFACE ring plots were larger in the ictB474-6 event in both ambient and elevated [CO_2] plots, 75 days after planting, but the increases were only significant for total mass and leaf mass in ambient [CO_2] ($P<0.10$; Table S2).

SR.3 The relative amount of Rubisco in the ictB474-6 event

There were no significant differences in Rubisco protein content between the WT and the ictB474-6 event at either [CO_2] treatments at SoyFACE plots (Supplementary Fig. S3). The protein immunoblot blot analysis ImageQuant volume for the wild type line at ambient [CO_2] was 692 ±63 AI (arbitrary units) compared to *ictB* at 641 ±105 ($P = 0.5816$). The volume for the WT line at elevated [CO_2] was 551 ±37 compared to *ictB* 542 ±47 ($P = 0.9207$).

SR.4. Development of ictB474-6 event in the field

The transgenic event ictB474-6 displayed significant differences in the timing of development, when compared to WT, in the SoyFACE ring plots in Illinois. In both years and under both treatments, though, the time between emergence and completion of seed fill and dry-down was significantly extended by an average of 1.3 days in the transgenic plots. In the 2010 growing season it took 1.7 more days for the ictB474-6 event to complete its life cycle, when grown in ambient [CO_2] relative to the WT (ictB474-6 103.4 ±0.5 days *vs* WT 101. 8 ±0.6 days; $P<0.01$). In the 2011 growing season the ictB474-6 event again showed an increase in days required to complete its life cycle, by 1.4 days (ictB474-6 116.6 ±0.1 days *vs* WT 115.3 ±0.3 days; $P<0.0001$), In the large plots a similar increase in total growing days was also observed in the *ictB* event (111.3 days ±0.2) compared with the wild type line (110.5 days ±0.2, $P<0.01$).

Supplemental References:

Baker N.R. and Oxborough K. (2004). Chlorophyll fluorescence as a probe of photosynthetic productivity. In G Papageorgiou, Govindjee, eds, Chlorophyll fluorescence: a signature of photosynthesis. Kluwer Academic Publishers, Dordrecht.

Beadle C.L. (1993). Growth analysis. Photosynthesis and Production in a Changing Environment. A Field and Laboratory Manual, eds Hall D.O., Scurlock J.M.O., Bolhar-Nordenkampf H.R., Leegood R.C., and Long S.P., pp. 36–46. Chapman & Hall, London.

Bernacchi C.J., Morgan P.B., Ort D.R. and Long S.P. (2005). The growth of soybean under free air [CO2] enrichment (FACE) stimulates photosynthesis while decreasing in vivo Rubisco capacity. Planta, 220: 434-446.

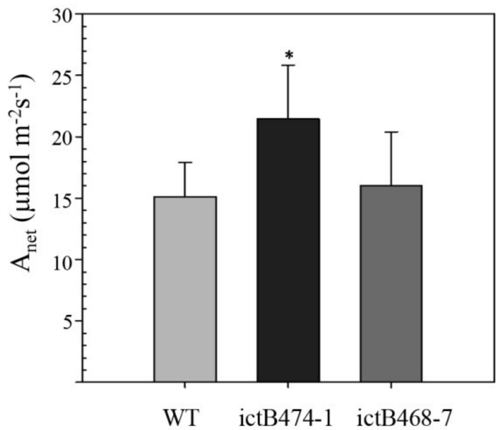
Dellaporta S., Wood J., Hicks J. (1983). A plant DNA minipreparation: Version II. Plant Molecular Biology Reporter 1: 19-21.

Eckert H., La Vallee B., Schweiger B.J., Kinney A.J., Cahoon E.B., and Clemente T. (2006). Co-expression of the borage Delta 6 desaturase and the Arabidopsis Delta 15 desaturase results in high accumulation of stearidonic acid in the seeds of transgenic soybean. Planta, 224: 1050-1057.

Genty B., Briantais J.M., and Baker N.R. (1989). The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta (BBA)-General Subjects, 990(1), 87-92.

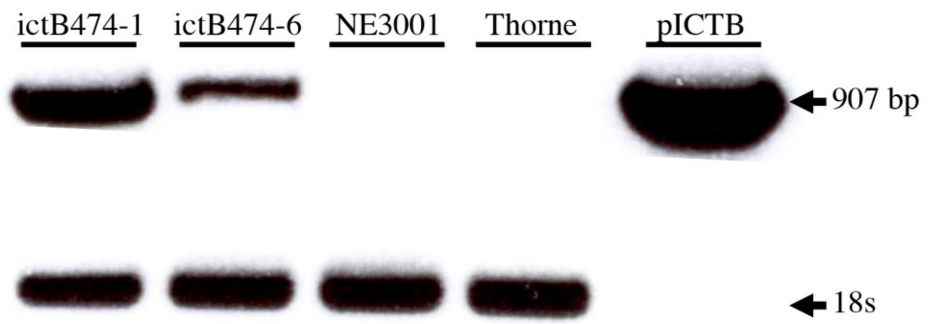
Long S.P. and Bernacchi C.J. (2003). Gas exchange measurements, what can they tell us about the underlying limitations to photosynthesis? Procedures and sources of error. Journal of Experimental Botany, 54: 2393-2401.

Supplemental figures and tables:

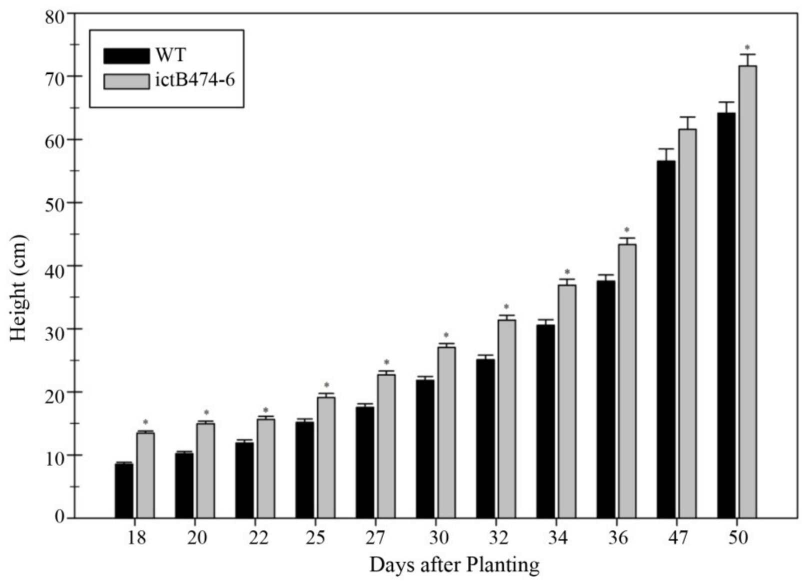


Supplementary Fig S1: Photosynthetic rates (A_{net}, µmol m^{-2} s^{-1}) of greenhouse grown transgenic *ictB*474-1, *ictB*468-7 and wild type (WT) control Thorne soybean plants. Plants grown at the University of Illinois greenhouses in conditions as discussed previously in section 2.2. An asterisk indicates significantly different means between transgenic lines and WT at $p \leq 0.05$.

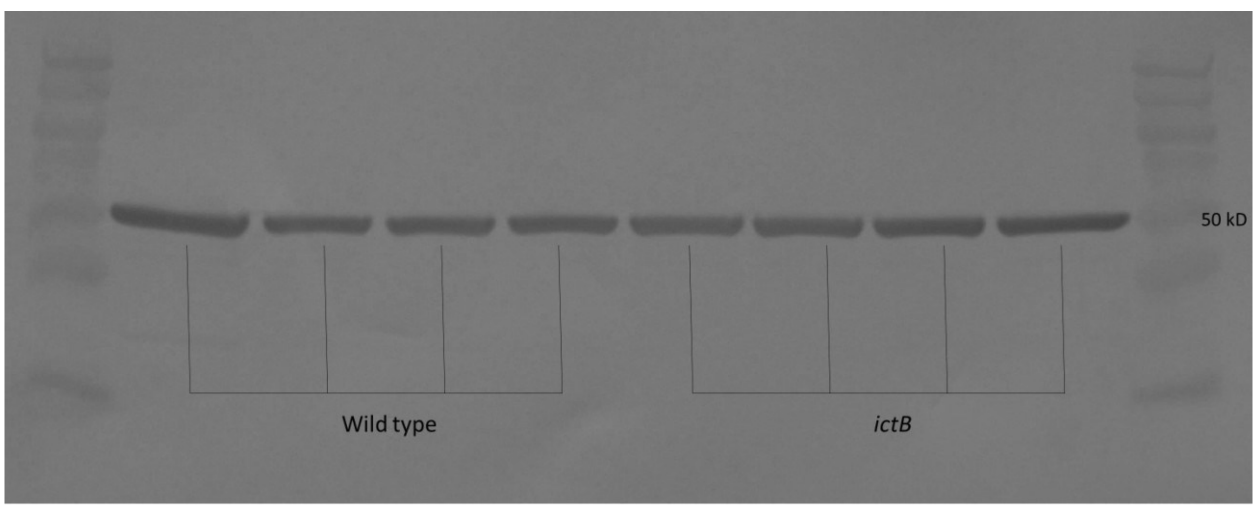
Supplemental Fig S2: RT-PCR analysis for *ictB* expression in soybean



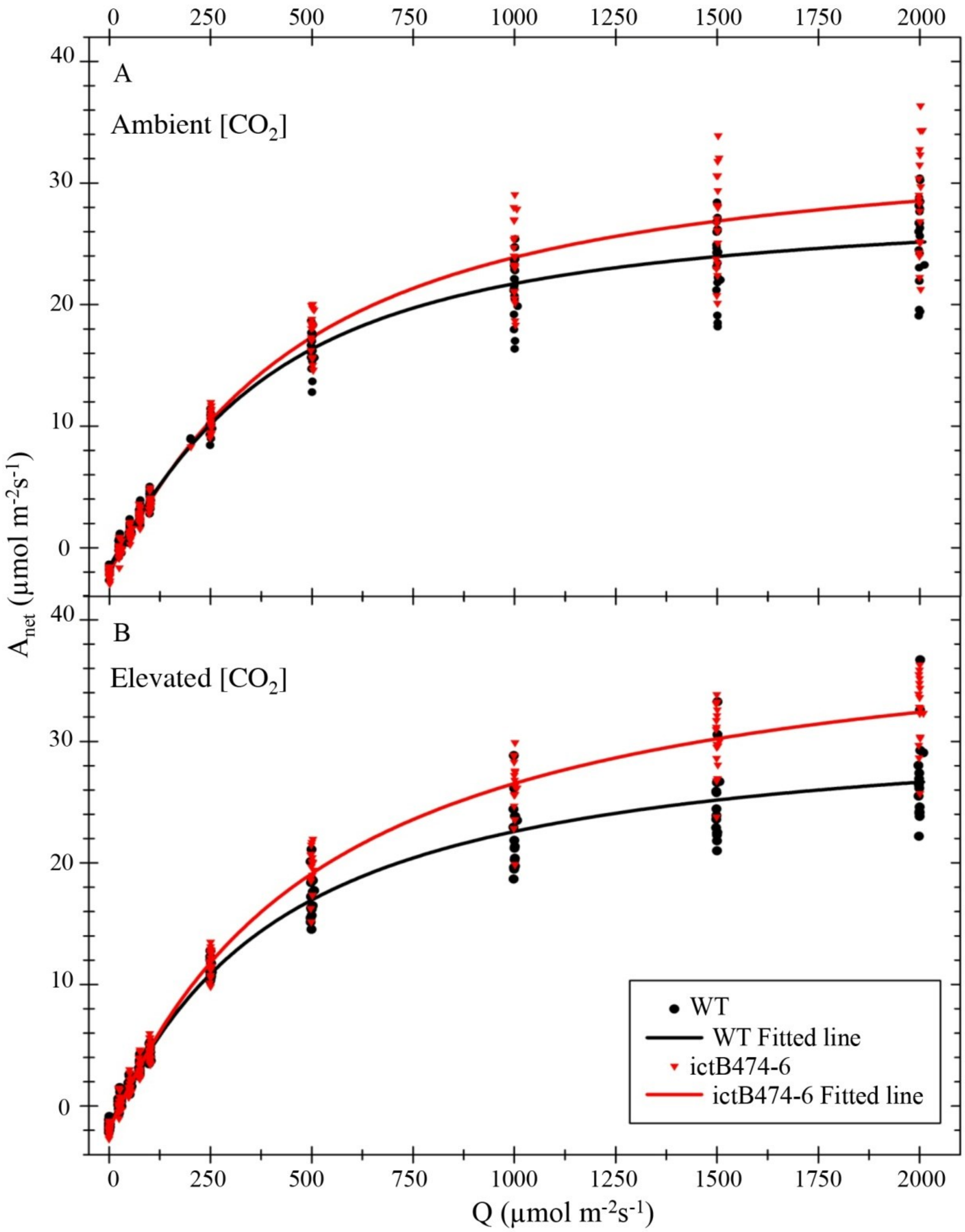
RT-PCR analysis conducted on transgenic soybean events, ictB474-1 and ictB474-6, along with two wild type controls, NE3001 and Thorne. Total RNA extracted from top fully expanded leaf at V5 stage of development. pICTB lane refers to PCR amplification of ictB region from binary vector used for transformation. Bottom panel shows RT-PCR of 18S ribosomal RNA as an internal control.



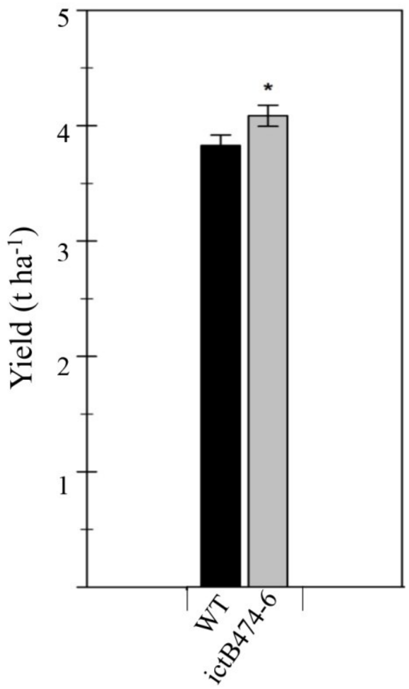
Supplementary Fig S3: Plant height, measured on 11 days basis throughout the vegetative growing season (January-February 2009) for greenhouse grown soybean transgenic ictB474-6 and wild type (WT) plants. Bars represent arithmetic means ± SE n=18. An asterisk indicates a significant difference between lines at P<0.01 from.



Supplementary Fig S4: Quantitative Rubisco protein immunoblot blot of the soybean wild type (WT) and *ictB* transgenic line 45 days after planting (DAP), grown at elevated [CO2] of 600 μmol mol^{-1}. Lane 1 started on the left side of the image. Lanes 1 and 10 were molecular weight protein standards, lanes 2-5 were biological replicates of the WT, lanes 6-9 were biological replicates of the *ictB* line



Supplementary Fig S5. Net photosynthetic rates (A_{net}) versus photosynthetic photon flux (*Q*) curves at ambient (400 µmol mol^{-1}) and elevated [CO_2] (600 µmol mol^{-1}) and leaf temperature of 25 ºC, in wild type (WT) and transgenic ictB474-6 soybean plants. Measurements were taken 41-50 DAP in the 2011 field season. The fitted lines are a 3-parameter non-rectangular hyperbola describing the response of A_{net} to Q fit to the measurements made for each leaf (n=19 at ambient [CO_2]; n=16 for elevated [CO_2]).



Supplementary Fig S6. 2011 SoyFACE ambient [CO_2] large soybean field plot, seed yield per hectare for wild type (WT) and transgenic ictB474-6 plants. Bars represent the arithmetic mean yield per hectare^{-1} ±SE. An asterisk indicates a significant difference $P<0.05$ from a complete block mixed model ANOVA with line as the fixed effect (n=3).

Table S1: Soybean agronomic traits obtained from field trials conducted in NE, for wild type (WT) and transgenic ictB474-6 event during the 2009 and 2011 growing seasons.

Year	Plant	Pod weight	No. of seed/kg	Yield (tonnes/ha)	Protein%	Oil%
2009	WT	1.96±0.08	5.10±0.02	4.21±0.17	37.57±0.13	17.70±0.15
2009	ictB474-6	1.85±0.14	5.11±0.05	3.99±0.03	37.37±0.09	17.53±0.09
2011	WT	1.87±0.05	6.3±0.12	4.02±0.10	35.9±0.21	19.08±0.08
2011	ictB474-6	1.87±0.05	6.28±0.11	4.03±0.11	36.1±0.17	19.06±0.10

Yield and some of its components taken in field trials during 2009 and 2011seasons in NE. Values are means ± SE. Plot weight in kg. No. of seed/kg (x10^3).

Table S2: Biomass of soybean wild type (WT) plants and transgenic ictB474-6 event under 2011 field conditions in IL.

Ambient [CO_2]	**WT**	**ictB474-6**	**P-Value**
Total biomass (g)	15.18±1.51	19.73±1.48	0.06*
Leaf biomass (g)	4.12±0.39	5.39±0.50	0.06*
LAI	7.32±0.70	8.27±0.66	0.32

Elevated [CO_2]	**WT**	**ictB474-6**	**P-Value**
Total biomass (g)	17.62±2.62	20.45±1.78	0.24
Leaf biomass (g)	4.47±0.69	5.04±0.52	0.39
LAI	6.81±0.88	7.32±0.65	0.60

Means of biomass parameters and leaf area index (LAI) taken at late V5 stage of development under elevated and ambient CO_2 levels from 2011 field plots in IL. P-values with an asterisk indicates significant difference ($p<0.1$).