Multigene manipulation of photosynthetic carbon assimilation increases CO₂ fixation and biomass yield in tobacco

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Abstract

Over the next 40 years it has been estimated that a 50% increase in the yield of grain crops such as wheat and rice will be required to meet the food and fuel demands of the increasing world population. Transgenic tobacco plants have been generated with altered combinations of sedoheptulose-1,7-bisphosphatase, fructose-1,6-bisphosphate aldolase, and the cyanobacterial putative-inorganic carbon transporter B, ictB, of which have all been identified as targets to improve photosynthesis based on empirical studies. It is shown here that increasing the levels of the three proteins individually significantly increases the rate of photosynthetic carbon assimilation, leaf area, and biomass yield. Furthermore, the daily integrated measurements of photosynthesis showed that mature plants fixed between 12–19% more CO₂ than the equivalent wild-type plants. Further enhancement of photosynthesis and yield was observed when sedoheptulose-1,7-bisphosphatase, fructose-1,6-bisphosphate aldolase, and ictB were over-expressed together in the same plant. These results demonstrate the potential for the manipulation of photosynthesis, using multigene-stacking approaches, to increase crop yields.

Key words: Biomass, Calvin–Benson cycle, chlorophyll fluorescence imaging, gas exchange, gene stacking.

Introduction

Increasing demands of the growing world population for food and fuel are putting ever greater pressure on the need to develop higher-yielding crop varieties. It has been estimated that increases of 50% will be required in the yield of grain crops such as wheat and rice if food supply is to meet the demands of the increasing world population (Fischer and Edmeades, 2010). The maximum yield of a crop is determined by the yield potential, which is the biomass produced per unit area of land over the growing season under optimal conditions and is influenced by genetic factors and agronomic practice. The primary determinant of crop yield is the cumulative rate of photosynthesis over the growing season which is the result of the crop’s ability to capture light, the efficiency by which this light is converted to biomass, and how much biomass is converted into the usable product, for example, grain in the case of wheat and rice. Traditional breeding and agronomic approaches have maximized light capture and the conversion of biomass to end-products and, therefore, in order to increase yield, the efficiency of energy conversion will have to be improved (Zhu et al., 2010). In plants that fix atmospheric CO₂ using the Calvin–Benson (C3) cycle enzyme, ribulose-1,5-bisphosphate carboxylase, the theoretical maximum energy conversion efficiency attainable is 4.6%, but, in the field, efficiencies of less than 50% of
this are realized. Kinetic models based on ordinary differential equations (ODEs) have been developed to describe the responses of photosynthetic carbon assimilation (Peterson and Ryde-Peterson, 1988; Laisk et al., 1989; Poolman et al., 2000). Further development of these models to include not only the reactions in the Calvin cycle but, importantly, those in the pathways of sucrose and starch biosynthesis and photorespiration has led to the construction of a dynamic model of carbon metabolism (Zhu et al., 2007). The outputs of this modelling work suggested that an increase in the Calvin cycle enzymes sedoheptulose-1,7-bisphosphatase (SBPase: EC.3.1.3.37) and fructose-1,6-bisphosphate aldolase (FBPA: EC 4.1.2.13) and the starch biosynthesis enzyme ADP-glucose pyrophosphorylase (AGPase), together with a decrease in the photorespiratory enzyme glycine decarboxylase (GDC), could increase photosynthetic carbon assimilation.

In addition to these theoretical predictions there is compelling evidence from transgenic studies that manipulation of the C3 cycle will contribute to closing this gap in efficiency and that this could increase yield in the absence of significant stress (Raines, 2006, 2011; Zhu et al., 2010). In the 1990s, analysis of transgenic plants, in which the levels of individual proteins or enzymes were manipulated, changed the view that there was a single limiting step in photosynthetic carbon assimilation (Stitt and Schulze, 1994; Raines, 2003). These studies demonstrated that small reductions in either SBPase or FBPA in the C3 cycle impacted negatively on photosynthesis, indicating that these enzymes had significant control over the rate of carbon assimilation and growth (Haake et al., 1998, 1999; Harrison et al., 1998; Raines et al., 1999; Lawson et al., 2006; Raines and Paul, 2006). These experiments suggested that improvements in photosynthetic carbon fixation may be achieved by increasing the activity of these enzymes individually. Evidence supporting this hypothesis came from transgenic tobacco plants in which the levels of the cyanobacterial SBPase/FBPA (Miyagawa et al., 2001) or the enzyme SBPase (Lefebvre et al., 2005; Rosenthal et al., 2011) were increased. The single manipulation of SBPase resulted in an increase in photosynthesis, leaf area and total biomass was up by as much as 30% in plants grown in high light (Lefebvre et al., 2005). However, growth of SBPase over-expressing plants in greenhouse conditions in the winter, when the day length was shorter and light levels lower, resulted in only minimal increases in growth. Furthermore, growth of these transgenic plants in the field in elevated CO2 conditions also enhanced photosynthesis and growth due to a higher internal CO2 concentration around ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) resulting in higher enzyme activity (Lieman-Hurwitz et al., 2003).

The work in this paper aims to test the hypothesis that gene-stacking of components of the C3 cycle with the putative-inorganic carbon transporter B can have a synergistic effect on photosynthesis and yield. To test this, several sets of transgenic tobacco plants co-expressing SBPase and ictB, either alone or in combination, and plants co-expressing SBPase, FBPA, and ictB were generated. It has been shown that the simultaneous manipulation of multiple targets leads to a cumulative impact on photosynthesis and biomass yield which will benefit substantially the biomass requirements of both the biofuel and food industries.

**Materials and methods**

**Construct generation**

Constructs were generated using Gateway cloning technology and vectors pGWB2 (Nakagawa et al., 2007) and pDESTOE (Booker et al., 2004). Transgenes were under the control of the CaMV 35S and FMV (Richins et al., 1987) constitutive promoters. Construct maps are shown in Supplementary Fig. S1 at JXB online. Full details of B2-TB, B2-FB, and FB-TB construct assembly can be seen in the Supplementary Materials and Methods at JXB online.

**Generation of transgenic plants**

The recombinant plasmids B2-TB, B2-FB, and FB-TB were introduced into wild-type tobacco (Nicotiana tabacum) L. cv Samsun or SBPase over-expressing tobacco cv. Samsun (Lefebvre et al., 2005) using Agrobacterium tumefaciens Ag1 via leaf-disc transformation (Horsch et al., 1985). Shoots were regenerated on MS medium containing kanamycin (100 mg l⁻¹), hygromycin (300 mg l⁻¹), and augmentin (500 g ml⁻¹). Kanamycin/hygromycin resistant primary transformants (T0 generation) with established root systems were transferred to soil and allowed to self fertilize.

**Plant growth conditions**

Wild-type tobacco plants and T1 progeny resulting from the self-fertilization of transgenic plants were germinated in sterile agar medium containing Murashige and Skoog salts supplemented with 1% (w/v) Suc (plus kanamycin 100 mg for the transformants) and grown to seed in soil (Levington F2, Fisons, Ipswich, UK) and lines of interest were identified by Western blot and qPCR. Wild-type plants used in this study were a combined group of WT and null segregants from the ictB over-expressing lines verified by PCR. Comparative
analysis of these groups can be seen in Supplementary Fig. S2 at JXB online. For experimental study, T2 progeny seeds were germinated on soil in controlled environment chambers at an irradiance of 130 μmol photons m$^{-2}$ s$^{-1}$, 22 °C, a relative humidity of 60%, in a 12h photoperiod. Plants were transferred to individual 8 cm pots and grown for 2 weeks at 130 μmol photons m$^{-2}$ s$^{-1}$, 22 °C, a relative humidity of 60%, in a 12h photoperiod. Plants were transferred to larger pots (17 cm across and 23 cm deep) and cultivated in a controlled environment greenhouse (16 h photoperiod, 25–30/20 °C day/night, and natural light supplemented with high-pressure sodium light bulbs, giving between 200–350 μmol m$^{-2}$ s$^{-1}$ (low light), 600–1 400 μmol m$^{-2}$ s$^{-1}$ (high light)) from the pot level to the top of the plant, respectively. Positions of the plants were changed daily and watered with a nutrient medium (Hoagland and Arnon, 1950). Four leaf discs (0.8 cm diameter), for the analysis of SBPase and FBPA activities were taken from the same areas of the leaf used for photosynthetic measurements, immediately plunged into liquid N2 and stored at –80 °C. Leaf areas were calculated using standard photography and ImageJ software (imagej.nih.gov/ij).

Protein extraction and Western blotting

Leaf discs sampled as described above were ground in liquid nitrogen and protein quantification determined (Harrison et al., 1998). Samples were loaded on an equal protein basis, separated using 12% (w/v) SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed using antibodies against SBPase and FBPA. Proteins were detected using horseradish peroxidase conjugated to the secondary antibody and the ECL chemiluminescence detection reagent (Amersham, Buckinghamshire, UK). SBPase antibodies are previously characterized in Lefebvre et al. (2005) and FBPA antibodies were raised against a peptide from a conserved region of the protein [C]-ASIGLENTEANRQA YR-amide, Cambridge Research Biochemicals, Cleveland, UK.

Determination of SBPase activity by phosphate release

SBPase activity was determined by phosphate release as described previously (Lefebvre et al., 2005). Immediately after photosynthesis measurement, leaf discs were isolated from the same leaves and frozen in liquid nitrogen. For analysis, leaf discs were ground to a fine powder in liquid nitrogen in extraction buffer (50 mM HEPES, pH 8.2; 5 mM MgCl$_2$; 1 mM EDTA; 1 mM EGTA; 10% glycerol; 0.1% Triton X-100; 2 mM benzamidine; 2 mM aminocaproic acid; 0.5 mM phenylmethylsulphonylfluoride; 10 mM dithiothreitol) and the resulting solution centrifuged for 1 min at 14 000×g at 4 °C. The resulting supernatant (1 ml) was desalted through an NAP-10 column (Amersham) and the eluate aliquoted and stored in liquid nitrogen. For the assay, the reaction was started by adding 20 μl of extract to 80 μl of assay buffer (50 mM TRIS, pH 8.2; 15 mM MgCl$_2$; 1.5 mM EDTA; 10 mM dithiothreitol; 2 mM SBP) and incubated at 25 °C for 30 min. The reaction was stopped by the addition of 50 μl of 1 M perchloric acid and centrifuged for 10 min at 14 000×g at 4 °C. Samples (30 μl) and standards (30 μl, PO$_4^{3-}$ 0.125–4 nmol) in triplicate were incubated for 30 min at room temperature following the addition of 300 μl of Biomol Green (Affiniti Research Products, Exeter, UK) and the $A_{420}$ was measured using a microplate reader (VERSAmax, Molecular Devices, Sunnyvale, CA).

Determination of FBPA activity

Desalted protein extracts were evaluated for FBPA activity as described previously by Haake et al. (1998).

cDNA generation and quantitative RT-PCR

Total RNA was extracted from tobacco leaf samples using the NucleoSpin® RNA Plant Kit (Macherey-Nagel, Fisher Scientific, UK). cDNA was synthesized using 1 μg total RNA in 20 μl using the oligo-dT primer according to the protocol in the RevertAid Reverse Transcriptase kit (Fermentas, Life Sciences, UK).

The PCR reaction contained 10 mM of each primer, 1.3× Taq polymerase buffer, 0.30 mM dNTPs. 1.5 units of Taq polymerase (BRL), and 2 μl of RT reaction mixture (100 ng of RNA) in a total volume of 25 μl. The final concentration was 4 ng μl$^{-1}$ of reaction mixture. The amplification reactions included 26 cycles of 30 s at 94 °C, 15 s at 60 °C, and 15 s at 72 °C. PCR products were fractionated on 1.5% agarose gel. Primers ietBf: AAGACAGCAGCAACAACTTC; NOSr: TGCCAATGTTTGAACGATCG were used to amplify the transgene.

Chlorophyll fluorescence imaging

Chlorophyll fluorescence measurements were performed on 3-week-old tobacco seedlings that had been grown in a controlled environment chamber at 130 μmol m$^{-2}$ s$^{-1}$ and ambient (400 μmol m$^{-2}$ s$^{-1}$) CO$_2$. Three days prior to chlorophyll fluorescence imaging, plants were transferred to the greenhouse and grown in natural irradiance with supplementary light to maintain the levels between 400–600 μmol m$^{-2}$ s$^{-1}$ PPFD at bench level. Chlorophyll fluorescence parameters were obtained using a chlorophyll fluorescence (CF) imaging system (Technologica, Colchester, UK; Barbagallo et al., 2003; Baker and Rosenqvist, 2004). The operating efficiency of photosystem II (PSII) photochemistry, $F_{q}/F_{m}'$, was calculated from measurements of steady-state fluorescence in the light ($F$) and maximum fluorescence in the light ($F_{m}'$) was obtained after a saturating 800 ms pulse of 5 500 μmol m$^{-2}$ s$^{-1}$ PPFD using the following equation $F_{q}/F_{m}' = (F_{m}' - F)/F_{m}'$. Images of $F_{q}/F_{m}'$ were taken under stable PPFD of 400 and 800 μmol m$^{-2}$ s$^{-1}$ PPFD (Oxborough and Baker, 1997; Baker et al., 2001). Measurements on tobacco seedlings were performed on 3-week-old plants grown in a controlled environment chamber with 130 μmol m$^{-2}$ s$^{-1}$ PPFD and ambient 400 μmol mol$^{-1}$ CO$_2$.

Gas exchange measurements

The response of net photosynthesis ($A$) to intracellular CO$_2$ concentration ($C_i$) was measured using a portable gas
exchange system (LI-COR 6400; LI-COR, Lincoln, NE). Illumination at 2000 μmol m\(^{-2}\) s\(^{-1}\) was provided by a red–blue light source attached to the leaf curve. Measurements of \(A\) were made at ambient CO\(_2\) concentration (\(C_a\)) of 400 μmol mol\(^{-1}\), before \(C_a\) was decreased step-wise to the lowest concentration of 50 μmol mol\(^{-1}\) and then increased step-wise to an upper concentration of 2000 μmol mol\(^{-1}\). Leaf temperature and vapour pressure deficit (VPD) were maintained at 25 °C and 1 ± 0.2 kPa, respectively. To calculate the maximum saturated CO\(_2\) assimilation rate (\(A_{\text{max}}\)), maximum carboxylation rate (\(V_c\)) and maximum electron transport flow (\(J_{\text{max}}\)), the C3 photosynthesis model (Farquhar et al. 1980), was fitted to the \(A/C_i\) data using a spreadsheet provided by Sharkey et al. (2007).

**Diurnal photosynthesis**

The diurnal response of leaf photosynthesis (\(A\)) and stomatal conductance (\(g_s\)) of a young expanding leaf (15–16 cm in length) were measured every 2 h between 06.30 h and 20.00 h. Measurements were made using a portable gas exchange system (LI-COR 6400). Light levels at each time point were set at the ambient light over the day: light levels ranged from 0 μmol m\(^{-2}\) s\(^{-1}\) to 350 μmol m\(^{-2}\) s\(^{-1}\), at mid-plant levels on the day of analysis. Measurements of \(A\) and \(g_s\) were recorded at steady-state (c. 2 min) and used to calculate intrinsic water-use efficiency (\(A/g_s=WUE\)). To estimate the integrated carbon gain (\(A\)') and water loss (\(g_s\)') over the measurement period, the area under the diurnal curve was calculated for each transgenic line.

**Statistical analysis**

All statistical analyses were done by comparing ANOVA, using Sys-stat, University of Essex, UK. The differences between means were tested using the Post hoc Tukey test (SPSS, Chicago).

**Results**

**Production and selection of tobacco transformants**

The full-length *Arabidopsis thaliana* (*Arabidopsis*) FBPA cDNA (At4g38970) and the putative-inorganic carbon transporter B (*ictB*) coding sequences (YP399376) (linked to the *Brachypodium distachyon* SBPase transit peptide (XP_003564625) were used to generate three cover-expression constructs driven by the CaMV 35S or FMV promoter; B2-FBPA, B2-ictB, and FB-TB in the vector pGWB2 (see Supplementary Fig. S1 at *JXB* online). Following leaf disc transformation of previously generated SBPase (At3g55800) over-expressing (lines 30 and 60) (Lefebvre et al., 2005) or wild-type (WT) tobacco plants. Primary transformants (57) (T0 generation) were rooted on hygromycin+kanamycin-containing medium, subsequently transferred to soil, and grown until maturity. Plants expressing the integrated transgenes were screened using RT-PCR (data not shown).

Western-blot analysis of T1 progeny from six selected lines (based on RT-PCR data) for transgenic tobacco expressing *ictB* (T), SBPase+FBPA (SF), SBPase+*ictB* (ST), and SBPase+FBPA+*ictB* (SFT) were carried out using WT and SBPase over-expressing lines (S) as a control. Western-blot analysis revealed a number of plants over-expressing SBPase for lines S, ST, and SFT and plants over-expressing FBPA in lines SF and SFT (Fig. 1a). No significant difference in Rubisco protein levels was observed between WT and the S and transgenic groups (see Supplementary Fig. S3a at *JXB* online).

Due to the difficulty in generating an antibody for *ictB*, semi-quantitative RT-PCR was used to detect the presence of the transcript in the *ictB*-expressing plant lines, T, ST, and SFT (Fig. 1b) and, while no transcript was detected in non transformed controls, transcript accumulation was easily observed in all transgenic lines selected for study.

In addition to Western-blot analysis carried out on T1 plants, total extractable SBPase and FBPA activity were analysed in newly fully expanded leaves of the T2 progeny used for the experimental determination of chlorophyll fluorescence and photosynthetic parameters. This analysis showed that these plants had increased levels of both SBPase and FBPA activity (Fig. 1c); SBPase activities ranged from 130–280% and FBPA activities in lines co-expressing FBPA, in addition to SBPase, displayed an increase of between 140% and 250%. The full set of assays showing the variation between plants for both SBPase and FBPA activities can be seen in Supplementary Fig. S3b, c at *JXB* online.

**Chlorophyll fluorescence imaging reveals increased photosynthetic efficiency in young transgenic seedlings**

In order to screen for potential changes in photosynthesis in 14-d-old seedlings (T2 progeny), chlorophyll \(a\) fluorescence imaging was used to examine the quantum efficiency of PSII photochemistry (\(F_m'/F_m\)) (Baker, 2008; Murchie and Lawson, 2013). Analysis of plants over-expressing SBPase alone did not show an increase in \(F_m'/F_m\). However, plants over-expressing FBPA and/or *ictB* in conjunction with SBPase (ST and SFT) had a significantly higher \(F_m'/F_m\) at an irradiance of 400 μmol m\(^{-2}\) s\(^{-1}\) when compared with either WT or SBPase over-expressing plants (Fig. 2a, b). At a higher light level (800 μmol m\(^{-2}\) s\(^{-1}\)) both the ST and SFT plants had a significantly higher \(F_m'/F_m\) compared with WT, S, and SF lines (Fig. 2c). From images taken during the fluorescence analysis of the seedlings, it was shown that the leaf area for SF, T, ST, and SFT plants was significantly larger than both WT and S (Fig. 2d). Differences in leaf area were also apparent in 8-d-old seedlings but, at this stage, no significant difference was observed between WT and S plants (see Supplementary Fig. S4 at *JXB* online).

**Photosynthetic CO\(_2\) assimilation rates are increased in mature plants grown in high light in the greenhouse**

Following chlorophyll fluorescence analysis, plants were moved into large pots and grown for a further 4 weeks in the
greenhouse, in natural light with supplementation providing light levels fluctuating between 600–1400 \(\mu\text{mol m}^{-2}\text{s}^{-1}\). The rate of CO\(_2\) assimilation \((A)\) was determined as a function of internal CO\(_2\) concentration \((C_i)\) in both the newest fully expanded leaf and in young expanding leaves (Fig. 3). In all the transgenic plants analysed in this study, the rate of \(A\) in developing leaves was significantly greater than that in WT plants at \(C_i\) concentrations above c. 300 \(\mu\text{mol mol}^{-1}\) (Fig. 3). This was accompanied by a significantly greater light-saturated rate of photosynthesis \((A_{\text{sat}})\) in all transgenic plants compared with the WT control (Fig. 4a). Further analysis of the \(A/C_i\) curves illustrated that the light- and CO\(_2\)-saturated rate of photosynthesis \((A_{\text{max}})\) was also significantly greater in all transgenic plants, with the exception of the plants over expressing of SBPase alone (Fig 4a, b). However, no significant enhancements of \(A_{\text{sat}}\) or \(A_{\text{max}}\) were observed between the different transgenic plants. Similarly the maximum rate of Rubisco carboxylation \((V_{\text{cmax}})\) and electron transport \((J_{\text{max}})\), calculated from the \(A/C_i\) curves, were statistically greater in all transgenic plants compared with the wild type in developing leaves, but again no significant differences between the transgenic lines was observed (Fig 4b, c).

In the fully expanded leaves of the same plants, the differences observed were more complex. No significant differences in \(A_{\text{sat}}\) or \(A_{\text{max}}\) were observed in plants over expressing SBPase alone compared with the WT (Figs 3, 4d). By contrast, plants over-expressing both SBPase and FBPA or SBPase and ictB or SBPase, FBPA, and ictB were all shown to have higher \(A_{\text{max}}\) than the WT (Fig. 4b). On the other hand, at ambient CO\(_2\) in saturating light \((A_{\text{sat}})\), plants over-expressing SBPase, FBPA, and ictB showed no significant difference in \(A_{\text{sat}}\) compared with the WT, however, the double expressing SF and ST had a significantly higher \(A_{\text{sat}}\) than the WT. Transgenic tobacco over-expressing T alone showed significantly higher \(A_{\text{max}}\) values compared with the triple over-expressing (SFT) plants, and SBPase alone, whilst the two double over-expressing lines were both significantly greater than plants over-expressing SBPase alone (Fig. 4d). \(V_{\text{cmax}}\) and \(J_{\text{max}}\) were statistically greater in all transgenic plants with the exception of those over-expressing SBPase alone, compared with the wild type and the single over-expressing SBPase plants (Fig. 4e, f). Furthermore, plants over-expressing ictB alone had a significantly high \(J_{\text{max}}\) when compared with the triple over-expressing (SFT) plants (Fig. 4f).

To investigate further the \textit{in situ} response of photosynthesis to the over-expression of ictB combined with increased enzyme activities (SBPase/FBPA), instantaneous measurements of \(A\), \(g_s\), and intrinsic water use efficiency (WUE\(_i\)) were measured in young expanding leaves of the wild type and transgenic lines. Lines over-expressing ictB (T), SBPase+ictB (ST) or SBPase+FBPA+ictB displayed an increase in photosynthetic rate of 19%, 16%, and 12%, respectively (based on measurements obtained between 09.00 h and 19.00 h) (Fig. 5a, b). These increases in \(A\) were accompanied by increased \(g_s\) (Fig. 5c, d) which resulted in lines over-expressing ictB in conjunction with either SBPase (ST) or SBPase+FBPA+ictB having a lower water use efficiency, possibly due to a greater stomatal conductivity (Fig. 5e, f). Interestingly no significant decrease in WUE was apparent in plants over-expressing ictB alone compared with the WT.
Increased SBPase and FBPA activity and expression of ictB stimulates growth in high light

The group of plants used for photosynthetic analysis described above were destructively harvested after 4 weeks further growth and the height, leaf number, and leaf area determined (Fig. 6). An increase in total biomass was also observed in all transgenic lines and, in the ST and SFT lines, a doubling of the dry weight was observed (Fig. 6g). These increases in total biomass were due to an increase in both leaf and stem dry weights (Fig. 6e, f). For example, plants over-expressing SFT showed an 88% and 124% increase in biomass for leaf and stem material, respectively.

The increases in total biomass for the SFT lines (103%) were significantly higher than S (+34%), T (+71%), and SF (+62%) indicating a positive effect of the additional transgenes (Fig. 6). The full set of data for these plants can be found in Supplementary Fig. S5 and Supplementary Table S1 at JXB online. Similar increases in height and leaf number were also evident in a non-destructive assessment of growth at an earlier stage in development (28 d after planting; see Supplementary Figs S6 and S7 at JXB online). The increase in above-ground biomass in these transgenic plants was not at the expense of reduced root growth (see Supplementary Fig. S8 at JXB online).
The impact of manipulation of the C3 cycle on photosynthetic CO2 assimilation and biomass when growth was in simulated shade

$A/C_i$ response curves were determined for plants over-expressing SBPase, SBPase+FBPA, SBPase+ictB, and SBPase+FBPA+ictB grown under simulated shade conditions (natural light, under shading at 200–350 μmol m$^{-2}$ s$^{-1}$ light intensity) (Fig. 7). These analyses revealed that $A_{sat}$ and $A_{max}$ were increased in developing leaves of transgenic plants compared with the WT plants and $A_{max}$ in the SFT plants was significantly greater than for SBPase alone (Fig. 8a). However, with the exception of the SFT plants, little or no difference in either $A_{sat}$ or $A_{max}$ was observed in fully expanded leaves.
Similarly, $V_c^{\text{max}}$ and $J^{\text{max}}$ were both enhanced in developing leaves of the majority of the transgenic plants compared with the WT (Fig. 8c, e). The SFT plants showed a further significant enhancement of $J^{\text{max}}$ over SB alone (Fig. 8a, e). By contrast, analysis of $V_c^{\text{max}}$ and $J^{\text{max}}$ in fully expanded leaves revealed little significant difference between transgenic and WT plants (Fig. 8d, f).

Significant differences in growth and biomass were also clearly evident in the transgenic plants grown in these simulated shade conditions (Fig. 9). This analysis revealed that height, leaf number, and leaf area were increased consistently in transgenic plants when compared with WT plants (Fig. 9). Total dry weight was increased by between 52% and 79% for lines S, SF, ST, and SF. The full set of data for low-light-grown plants can be seen in Supplementary Fig. S9 and Supplementary Table S1 at JXB online.

**Discussion**

The products of photosynthesis are the primary determinant of plant productivity and improving photosynthetic efficiency has been widely proposed as a key target for improving crop
Improving photosynthesis and yield

It has been shown previously that increasing the activity of two enzymes of the C3 cycle, SBPase (Lefebvre et al., 2005) and FBPA (Uematsu et al., 2012) in transgenic tobacco resulted in an increase in photosynthetic carbon assimilation and growth. Furthermore, the expression of the inorganic CO2 transporter B (ictB) in transgenic tobacco (Lieman-Hurwitz et al., 2003) also resulted in an increase in photosynthesis and biomass. These studies showed that the stimulation of photosynthesis was not seen under all experimental conditions, highlighting the need to consider different strategies to ensure success of this approach in a range of environmental conditions. These results, together with the analysis of the C3 cycle using a modelling approach (Poolman et al., 2000; Zhu et al., 2007), suggested that a re-engineering of multiple enzymes of the C3 cycle had the potential to achieve the gains in photosynthesis and growth required to provide sufficient food in the 21st century. In this study, it has been shown that increasing the levels of SBPase, FBPA, and ictB simultaneously in the same plant, compared with SBPase, FBPA or ictB alone or in pairs, resulted in a substantial and significant impact on photosynthesis and the biomass yield of tobacco grown in greenhouse conditions.

The plants in this study were grown in conditions as close to natural as possible; the pots containing the plants were grown in the greenhouse with natural lighting, plants were positioned such that, at maturity, a near-to-closed canopy was achieved and the temperature range was similar to the ambient external environment. Previously, it was shown that over-expression of FBPA in tobacco resulted in a stimulation of photosynthesis and growth when plants were grown under a square wave lighting environment and elevated CO2.

Fig. 5. The diurnal progression of CO2 assimilation, stomatal conductance, and water use efficiency in the WT and transgenic plants. Plants were grown in greenhouse conditions as in Fig. 3. (a) Leaf photosynthesis (\(A\)), (c) stomatal conductance, and (e) intrinsic water use efficiency (\(A/g_s=WUE_i\)) were monitored over a 14 h period. Each symbol represents the mean ±SE of five plants. For each transgenic group, two plants from each of four independent transgenic lines were evaluated and grouped together (eight plants per group). The integrated (b) carbon gain (\(\Delta\)), (d) water loss (\(g_s\)), and (f) total water use efficiency were calculated. An asterisk denotes a significant (<0.05) difference compared with WT plants. Lines over-expressing ictB (T), SBPase and ictB (ST), and SBPase, FBPA, and ictB (SFT) are represented.
conditions. However, the effect of FBPA over-expression was much less in plants grown in ambient CO₂ conditions (Uematsu et al., 2012). Interestingly, in this current study, it is shown that, even in ambient CO₂, over-expression of FBPA together with SBPase has a positive effect on growth in high light when compared with either WT plants or plants over-expressing SBPase alone. However, the cumulative effect of SBPase plus FBPA co-expression was not observed when plants were grown in low light under simulated shading. The over-expression of FBPA and SBPase would be expected to

Fig. 6. Growth analysis of WT and transgenic plants grown in greenhouse conditions. Plants were grown for 2 weeks at 130 μmol m⁻² s⁻¹ light intensity in long days (12/12 h days) before being transferred to the greenhouse and supplemented light (16/8 h days), at maximum 600–1400 μmol m⁻² s⁻¹ light intensity for an additional 4 weeks (6 weeks in total). Plants were harvested when the first sign of flower development became apparent. (a) Appearance of plants 6 weeks after planting. Lines over-expressing SBPase (S), SBPase and FBPA (SF), ictB (T), SBPase and ictB (ST), and SBPase, FBPA, and ictB (SFT) are represented. (b) Plant height, (c) leaf number, (d) leaf area, and (e–g) biomass. Results are representative of 4–5 plants from 3–4 individual lines (12–16 plants in total). Significant differences (<0.05) are represented by capital letters indicating if each specific line is significantly different from another. Lower case italic lettering indicates lines that are just below significance (>0.05 and <0.1). The percentage increases over the wild type are indicated.
increase the capacity for regeneration of the CO₂ acceptor molecule RuBP and this was evident from analysis of the A/Cᵢ response curves from the transgenic lines over-expressing these enzymes. Interestingly, in all of the lines in which Jmax was increased, a stimulation in Vcₘₐₓ was also observed, indicating an increase in the carboxylation rate of Rubisco. This may not be surprising as a stimulation in RuBP regeneration will increase the concentration of RuBP, thereby increasing the rate of carboxylation (Wullschleger, 1993; Price et al., 1998; Harrison et al., 2001). Rubisco activation state and RuBP levels were also shown to be higher in transgenic plants where photosynthetic capacity has been increased by the over-expression of SBPase (Lefebvre et al., 2005) or a bifunctional cyanobacterial FBPase/SBPase (Miyagawa et al., 2001).

It has been shown previously that the introduction of ictB into tobacco or Arabidopsis results in increased rates of Rubisco-limited photosynthesis together with increased growth [evident under low relative humidity (30%)]—this growth enhancement was, however, not observed under high relative humidity (70%). It is also shown here that, in high light, ictB (under 60% relative humidity) has the ability to increase photosynthesis and growth either when expressed on its own or together with SBPase or SBPase with FBPA. Although the additive effect of ictB with either SBPase or SBPase plus FBPA, was evident in the biomass gain, no enhancement of photosynthesis was detected in the mature leaves of these plants. The function of the ictB gene product has not been elucidated, but it has been proposed to be

Fig. 7. Photosynthetic responses of WT and transgenic plants grown in low light. Photosynthetic capacity of developing leaves (11–13 cm) and fully expanded leaves (leaf 7) from low-light, greenhouse-grown wild-type and transgenic plants. Plants were grown in supplemented, fluctuating light at a maximum of 200–350 μmol m⁻² s⁻¹ light intensity. Photosynthetic carbon fixation rates were determined as a function of increasing CO₂ concentrations at saturating-light levels. White circles represent the results from six wild-type plants, and black circles represent the results from eight SBPase over-expressing lines (Lefebvre et al., 2005). Grey circles represent 10–17 individual plants from 3–4 independent transgenic lines over-expressing SBPase+FBPA, SBPase+ictB, and SBPase, FBPA,+ictB.

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involved in accumulation of inorganic carbon in the cyanobacterium *Synechococcus* (PCC 7942), based on the isolation of a high CO$_2$-requiring mutant strain (Bonfil *et al.*, 1998; Kaplan and Reinfold, 1999). However, there was no evidence, from analysis of the A/C$_i$ curve, to support the suggestion that increased photosynthetic rates in the ictB over-expressing plants was only due to a stimulation of the carboxylation reaction of Rubisco. The A/C$_i$ curves were similar to those observed in transgenic plants with altered FBPA and SBPase levels that showed increased values of A$_{sat}$ and J$_{max}$. Measurements of photosynthesis revealed that the ictB plants had similar rates of photosynthesis over the diurnal period as did SBPase+ictB or ictB together with both FBPA and SBPase. Interestingly, in all the transgenic plants, the greatest increases in photosynthesis were observed after midday, when the stomatal conductance of WT plants was reduced. It is not clear if these differences were due to a direct effect on stomatal aperture brought about by changes in guard cell C3 enzymes (Lawson *et al.*, 2008, 2014; Lawson, 2009). However, the reduced stomatal conductance in WT plants did not appear to restrict photosynthesis as C$_i$ values were always greater than c. 270 μmol mol$^{-1}$. In the ictB plants, gains in photosynthetic carbon assimilation did not correspond to a significant decrease in WUEi. Previous studies on antisense tobacco plants with targeted reduction in photosynthetic enzymes have reported no differences in g$_s$ between WT and transgenic plants despite large decreases in assimilation rate (Quick *et al.*, 1991a, b; Hudson *et al.*, 1992; Price *et al.*, 1995) leading to significant reductions in WUE. Although the above studies were not specifically designed...
Fig. 9. Growth analysis of WT and transgenic plants grown in low light in the greenhouse. Plants were grown for 2 weeks at 130 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) light intensity in long days (12/12 h days) before being transferred to the greenhouse and supplemented light (16/8 h days), at a maximum 200–350 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) light intensity for an additional 5 weeks (7 weeks in total). Plants were harvested when the first sign of flower development became apparent. (a) Appearance of plants after 7 weeks. Lines over-expressing SBPase (S), SBPase and FBPA (SF), ictB (T), SBPase and ictB (ST), and SBPase, FBPA, and ictB (SFT) are represented. (b) Plant height, (c) leaf number, (d) leaf area, and (e–g) biomass. Results are representative of 4–5 plants from 3–4 individual lines (12–16 plants in total). Significant differences (<0.05) are represented by capital letters indicating if each specific line is significantly different from another. Lower case italic lettering indicates lines that are just below significance (>0.05 and <0.1). The percentage increases over the wild type are indicated.
to explore stomatal behaviour in these transgenic plants, subsequent research has confirmed that stomata in transgenic plants with impaired carboxylation (von Caemmerer et al., 2004), electron transport (Baroli et al., 2008) or Rubisco regeneration (Lawson et al., 2008) can achieve equivalent or even greater gs than WT plants and, therefore, have lower WUEs than the WT. The increase in gs in these plants was not due to a lowering of Ci, which remained >300 μmol mol−1 throughout the measurement period. Therefore, further work is needed to explore the mechanism(s) that link stomatal behaviour with mesophyll demands for carbon assimilation (Lawson, 2009; Lawson et al., 2014; Lawson and Blatt, 2014) particularly in those plants with alteration to A.

Simulated shading was also used to explore further the potential of our strategy to increase photosynthesis and yield in plants grown in suboptimal light conditions. Theoretical considerations would predict that, under low CO2 and high light, Rubisco activity would limit CO2 fixation in C3 plants. In low-light conditions, all of the transgenic lines showed a significant increase in photosynthetic CO2 assimilation when compared with the wild type. With only one exception (SFT Amax, Jmax), no significant difference was observed between transgenic lines expressing additional transcripts when compared with SBPase over-expressing lines. These data indicate that, in low-light conditions, light limitation impacts negatively on the benefits seen in photosynthesis observed in the plants expressing multiple transgenes grown in high light. Furthermore, these data imply that the photosynthetic efficiency of young expanding leaves may have a greater impact on plant development than the fully expanded leaves when plants are grown in low-light conditions. It is interesting that greater rates of photosynthesis in the transgenic plants were generally observed in fully expanded leaves in high-light-grown plants whilst, in plants grown under low-light conditions, the fully expanded leaves show limited increases in photosynthesis. These finding are consistent with earlier studies that showed that the main stimulatory effects of increased levels of SBPase occurred earlier in development (Lefebvre et al., 2005) and may also demonstrate the different limitations imposed on photosynthesis between developing and fully expanded leaves (Ölçer et al., 2001).

Chlorophyll fluorescence imaging, used to analyse plants at the seedling stage of development, demonstrated that the positive effect of multigene manipulation on the C3 cycle is evident early in development. The expression of SBPase, FBPA, and ictB in the same plant led to a further increase (11%) in Fv'/Fm′ compared with the increase over WT observed in plants expressing SBPase and FBPA together (2.8%) or plants expressing ictB alone (5.3%). These increases in photosynthetic rates translated into an increase in biomass in all the transgenic lines and, similar to previous studies, SBPase over-expressing lines showed an increase of ~25% in both leaf and stem biomass when grown in high light (Lefebvre et al., 2005). Furthermore, increases in biomass in high-light conditions were shown to be cumulative over the growing period, dependent on the number of transgenes expressed. For example, the increase in overall biomass for lines over-expressing SBPase+ictB was higher than for either SBPase or ictB individually. The same was true for plants over-expressing all three transgenes, with biomass increase being higher in these plants than that observed for either the S, T single- or the SF-double transgene plants. Interestingly, the total dry weight of the transgenic plants correlated well with the relative increases seen in photosynthesis in the young seedlings. These data provide evidence that chlorophyll fluorescence imaging can be used as a robust tool to allow the screening and identification of young seedlings with improved photosynthesis which results in improved yield at maturity.

Conclusion

In this study, it has been demonstrated that the over-expression of two C3 cycle enzymes leads to an increase in photosynthesis and a cumulative increase in overall biomass yield. It is also shown that over-expression of ictB, a protein proposed to be involved in inorganic carbon transport, in combination with SBPase (ictB and SBPase) or SBPase and FBPA (SBPase, FBPA, and ictB) brought about a further significant improvement in both of these parameters. Although there have been a number of publications with single gene manipulations very little data are available in relation to multiple target manipulation. Importantly, the work here also allowed a direct comparative analysis between the different manipulations, as all of the transgenic and wild-type plants were grown and assessed in parallel, identifying the best manipulations for introduction to crop plants. Although it is still necessary to address the issue of the importance of these manipulations in the field, the approach taken in this study provides strong evidence that multigene manipulation of photosynthesis can form an important part of the strategies to increase crop yield.

Supplementary data

Supplementary data can be found at JXB online.

Supplementary Materials and Methods. A detailed description of construct generation and primers.

Supplementary Fig. S1. Schematic representation of (A) the A. thaliana FBPaldolase (B2-FBPaldA) and (B) the Synechocystis PCC 6803 inorganic carbon transporter (B2-ictB) expression vectors; (C) shows the structure of a dual construct for the expression of both FBPaldolase and ictB.

Supplementary Fig. S2. Comparative analysis of wild type and null segregants used in this study.

Supplementary Fig. S3. Complete data set for enzyme assays in plants analysed and Rubisco protein levels in selected lines.

Supplementary Fig. S4. Leaf area at 8 d of development.

Supplementary Fig. S5. Complete data set for all transgenic lines grown in high light conditions evaluated at harvest.
Supplementary Fig. S6. Growth analysis of greenhouse-grown wild type (WT) and transgenic lines at 28 d after planting.

Supplementary Fig. S7. Complete data set for all transgenic lines evaluated at 28 d.

Supplementary Fig. S8. Root development of greenhouse-grown wild type (WT) and transgenic lines.

Supplementary Fig. S9. Complete data set for all transgenic lines grown in low light conditions evaluated at harvest.

Supplementary Table S1. The percentage increase over wild type for each parameter measured in low light- (200–350 μmol m⁻² s⁻¹) and high light- (600–1400 μmol m⁻² s⁻¹) grown plants.

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