Plant Biotechnology Journal (2003) **1**, pp. 43–50

Enhanced photosynthesis and growth of transgenic plants that express *ictB*, a gene involved in HCO_3^- accumulation in cyanobacteria

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Received 29 May 2002;
revised 5 September 2002;
accepted 12 September 2002.
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Keywords: growth, *ictB*, inorganic carbon, photosynthesis, RubisCO, transgenic plants.

Summary

Transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* plants that express *ictB*, a gene involved in HCO_3^- accumulation within the cyanobacterium *Synechococcus* sp. PCC 7942, exhibited significantly faster photosynthetic rates than the wild-types under limiting but not under saturating CO_2 concentrations. Under conditions of low relative humidity, growth of the transgenic *A. thaliana* plants was considerably faster than the wild-type. This enhancement of growth was not observed under humid conditions. There was no difference in the amount of ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) detected in the wild-types and their respective transgenic plants. Following activation *in vitro*, the activities of RubisCO from either low- or high-humidity-grown transgenic plants were similar to those observed in the wild-types. In contrast, the *in vivo* RubisCO activity, i.e. without prior activation, in plants grown under low humidity was considerably higher in *ictB*-expressing plants than in their wild-types. The CO_2 compensation point in the transgenic plants that express *ictB* was lower than in the wild-types, suggesting that the concentration of CO_2 in close proximity to RubisCO was higher. This may explain the higher activation level of RubisCO and enhanced photosynthetic activities and growth in the transgenic plants. These data indicated a potential use of *ictB* for the stimulation of crop yield.

Introduction

Plants that belong to the physiological C4 type or the Crassulacean acid metabolism groups, as well as many photosynthetic micro-organisms, possess various types of CO_2 concentrating mechanisms (Cushman and Bohnert, 2000; Hatch, 1992; Kaplan and Reinhold, 1999). These mechanisms enable them to raise the concentration of CO_2 in close proximity to ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) and hence overcome, at least partly, the low affinity of the enzyme for CO_2. In contrast, the majority of higher plants that belong to the C_3 group, including most crop plants, do not possess this ability. Therefore, under many environmental conditions plant photosynthesis is rate-limited by the concentration of CO_2 at the carboxylation site and/or by the activity of RubisCO. Attempts are being made to raise the apparent photosynthetic affinity of C_3 plants for CO_2 by various biotechnological approaches. These include a search for a RubisCO that exhibits an elevated specificity for CO_2 among natural photosynthetic populations (Uemura *et al*., 1997; Horken and Tabita, 1999; Tabita, 1999); site directed modifications of the enzyme (Cleland *et al*., 1998; Kostov *et al*., 1997; Ramage *et al*., 1998; Spreitzer and Salvucci, 2002) and expression of genes involved in C_4 metabolism within C_3 plants (Ku *et al*., 1999; Matsuoka *et al*., 2001; Nomura *et al*., 2000; Surridge, 2002). Characterization of a high-CO_2-requiring mutant of the cyanobacterium *Synechococcus* sp. strain PCC 7942 (hereafter *Synechococcus* PCC 7942) implicated *ictB* as a gene involved in inorganic carbon accumulation in this organism (Bonfil *et al*., 1998). IctB is highly conserved among cyanobacteria but its exact role is not known, since it was not possible to directly inactivate

it or its homologue *slr1515* in *Synechocystis* sp. strain PCC 6803.

Ability to stimulate photosynthesis and growth of tobacco by the expression of a single cyanobacterial gene encoding fructose-1,6/sedoheptulose-1,7-bisphospate phosphatase was recently demonstrated (Miyagawa *et al*., 2001). Apparently, the level of intermediates of the Calvin cycle was raised in the transgenic tobacco plants suggesting that, under the conditions of their experiments, photosynthesis was rate-limited by the level of ribulose 1,5-bisphosphate. In this study we show that expression of *ictB* from *Synechococcus* PCC 7942 enhanced photosynthesis and growth in C_3 plants due to a higher internal CO_2 concentration at the site of RubisCO and consequently higher enzyme activity in the transgenic plants.

Results

Expression of *ictB*

Transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* (tobacco) plants bearing *ictB* were raised after transformation with *Agrobacterium* (see Experimental procedures). Northern analyses performed on total RNA isolated from the wild-type and kanamycin-resistant transformed lines demonstrated that three transgenic *Arabidopsis* (A, B and C) and five (1, 3, 4, 8 and 11) tobacco plants expressed *ictB* to different extents (Figure 1). We did not detect a transcript of *ictB* in the wild-type plants. Southern analyses of DNA from the transgenic *Arabidopsis* and tobacco plants indicated that the construct bearing *ictB* was inserted in different sites within their genomes (not shown).

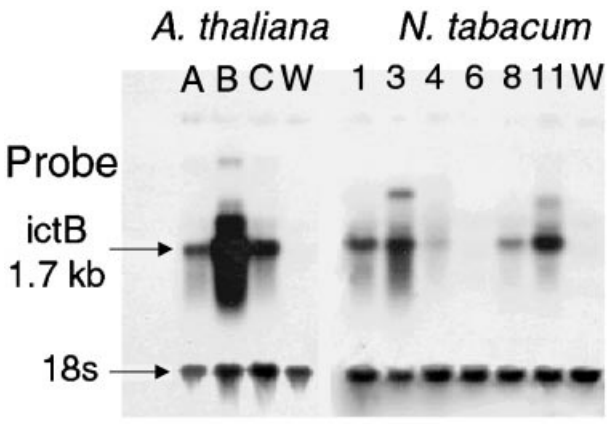

Figure 1 Northern blots of RNA isolated from transgenic and wild-type (W) *Arabidopsis* and tobacco plants hybridized to both *ictB* and 18S rDNA probes. 30 μg RNA was loaded in each lane. RNA was isolated as described in the Experimental procedures. Northern blot analyses on 1% agarose gels, was done as described by Mittler and Zilinskas (1992).

Photosynthetic performance

In *Synechococcus* PCC 7942, *ictB* is involved in the accumulation of inorganic carbon, and a mutant impaired in this gene demanded a high CO_2 concentration for photosynthesis. Therefore, we examined the rate of photosynthesis in the wild-types and the transgenic plants as it was affected by intercellular CO_2 concentration. Generally, despite the fact that the expression of *ictB* varied markedly between transgenic plants, in both *Arabidopsis* and tobacco (Figure 1), there was hardly any difference between their photosynthetic performances. Plants that expressed *ictB* showed similar photosynthetic characteristics, which differed markedly from those that did not express this gene. At saturating CO_2 levels, the photosynthetic rates of transgenic tobacco (Figure 2A) and *Arabidopsis* plants (Figure 2B) were similar to those found in their wild-types. This suggested that the ability to perform maximal photosynthesis was not affected by the expression of *ictB*. In contrast, under limiting intercellular CO_2 concentrations, the transgenic tobacco lines 1, 3 and 11 (Figure 2A) and *Arabidopsis* plants A, B (Figure 2B) and C (not shown) showed significantly higher photosynthetic rates than the wild-types. Notably, some of the transgenic, kanamycin-resistant plants which did not express *ictB* (cf. tobacco plant number 6, Figure 2A), exhibited either similar or sometimes even slightly lower photosynthetic rates than the respective wild-type. Stomatal conductances, measured by the Li-Cor 6400 or the Delta-T porometer (model MK3, UK), were lower in plants grown under the dry conditions but did not differ significantly between the wild-types and the transgenic plants (Table 1). These data confirmed that the higher photosynthetic rate at limiting intercellular CO_2

Table 1 Stomatal conductance in wild-type (WT) and transgenic *Arabidopsis* and tobacco plants. Plants grown under humid (70–75% relative humidity) or dry (25–30% humidity) conditions were used in these experiments

Plant	High humidity	Low humidity
Tobacco WT	686.8 ± 3.6	196.0 ± 1.2
Tobacco Plant 3	682.6 ± 4.5	196.7 ± 1.6
Tobacco Plant 11	684.3 ± 3.1	196.2 ± 1.2
Arabidopsis WT	597.9 ± 3.5	209.1 ± 1.3
Arabidopsis Plant A	598.4 ± 3.1	209.7 ± 1.7
Arabidopsis Plant B	599.5 ± 3.2	208.9 ± 1.3

The data are presented in mmole/m^2/s, as the average ± SE, n = 18.

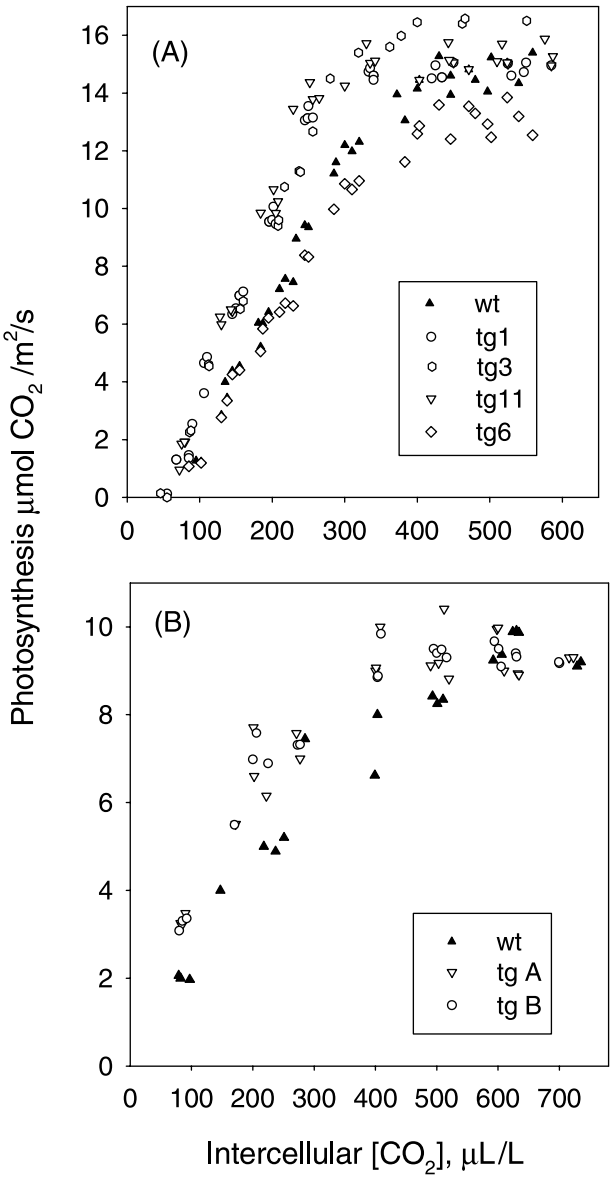

Figure 2 The rate of photosynthesis as affected by the intercellular concentration of CO_2 in wild-type (wt) and transgenic (tg) tobacco (A) and *Arabidopsis* (B) plants. The plants were grown under dry conditions. $n = 8$.

concentrations did not result from a higher conductance of CO_2 in the transgenic plants.

CO_2 compensation point

Involvement of IctB in the ability of *Synechococcus* PCC 7942 to accumulate HCO_3^- internally (Bonfil *et al*., 1998) raised the possibility that the higher photosynthetic and RubisCO activities (see below) at limiting CO_2 concentrations in the transgenic plants were due to an elevated CO_2 concentration in close proximity to RubisCO. Should this be the case, it would

Table 2 The CO_2 compensation points in wild-type (WT) and transgenic *Arabidopsis* and tobacco plants. The compensation points were deduced from measurements of the rate of CO_2 exchange over a range of CO_2 concentrations of 0–150 µL/L as described in Experimental procedures

Plant	CO_2 compensation (µL/L)
Arabidopsis Plant A	39.2 ± 1.0
Arabidopsis Plant B	41.0 ± 1.1
Arabidopsis WT	46.1 ± 1.1
Tobacco Plant 3	47.1 ± 1.4
Tobacco Plant 11	48.0 ± 1.6
Tobacco Wild-type	56.9 ± 1.6

The data are presented as the average ± SE, $n = 18$.

be expected to lower the CO_2 compensation point (i.e. the ambient CO_2 concentration, where the net CO_2 exchange is zero since CO_2 uptake in photosynthesis is equal to the sum of respiratory and photorespiratory CO_2 efflux). We examined the CO_2 compensation point in wild-type and transgenic *Arabidopsis* and tobacco plants by measuring CO_2 exchange in plants exposed to a range of CO_2 concentrations between 0 and 150 µL/L CO_2. In Table 2 we show that the average CO_2 compensation point was significantly ($P < 0.01$) lower in transgenic *Arabidopsis* and tobacco plants than in the respective wild-types. These data suggested that in both species the CO_2 concentration in close proximity to RubisCO was higher in the transgenic than in the wild-type plants. This is in agreement with the steeper initial slope of the curves relating CO_2 fixation to its concentration in the transgenic plants which express *ictB* than in the respective wild-types (Figure 2).

Activation state of RubisCO

The slope of the curves relating photosynthetic rate to intercellular CO_2 concentration (Figure 2) was steeper in the *ictB*-expressing plants than in their corresponding wild-types, suggesting an apparent higher affinity to CO_2 in the transgenic plants. This could be due to a higher level of RubisCO activity (Bainbridge *et al*., 2000; Mott and Woodrow, 2000; Poolman *et al*., 2000). We did not detect significant differences in the abundance of active sites of RubisCO per leaf surface area or per soluble proteins between the wild-types (tobacco and *Arabidopsis*) and their respective *ictB*-expressing plants. To examine the possibility that RubisCO activity (per active site) was higher in the transgenic plants, we exposed the neighbouring leaves of wild-types and of transgenic plants, of similar age, to identical ambient conditions of light intensity

Table 3 Rubisco activity in wild-type (WT) and transgenic tobacco plant number 3 grown in the high (70–75%) humidity chamber. RubisCO activity was determined with (*in vitro*) or without (*in vivo*) prior activation. The reaction was terminated after 1 min. Other conditions as described in Experimental procedures

Plant	RubisCO activity (nmol C fixed/nmol catalytic site/min)
WT, *in vitro*	105 ± 7
Transgenic, *in vitro*	103 ± 8
WT, *in vivo*	84 ± 7
Transgenic, *in vivo*	86 ± 6

n = 6, ± SD.

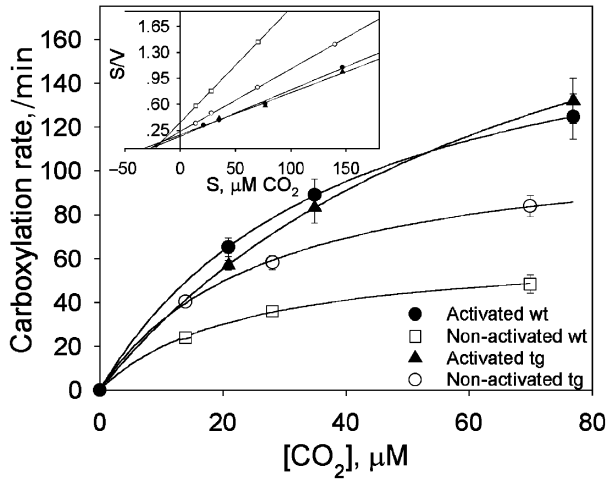

Figure 3 RubisCO activity *in vivo* (non-activated) and following *in vitro* activation in the wild-type (wt) and transgenic (tg) tobacco plant 3 grown under low humidity as affected by the CO_2 concentration during the assay. Rate of carboxylation is in nmol CO_2 fixed/nmol active sites/min. The inset provides the S/V vs. S plots of data from similar experiments. n = 6.

and orientation, temperature and relative humidity (either high or low) for several days. The leaves were excised 3 h after onset of illumination in the growth chamber and placed in liquid nitrogen (see Experimental procedures). These experiments were performed on wild-types and transgenic *Arabidopsis* and tobacco plants. As an example, we provide detailed results obtained in the experiments with the wild-type and transgenic line 3 of tobacco (Table 3, Figure 3).

Following *in vitro* activation by the addition of CO_2 and $MgCl_2$, where RubisCO activity was close to its maximum (Badger and Lorimer, 1976; Crafts-Brandner and Salvucci, 2000; Marcus and Gurevitz, 2000), there was no significant difference between the activities observed in the wild-type and transgenic plants maintained in either the humid (Table 3) or the dry conditions (Figure 3). These data confirmed that the insertion of *ictB* did not alter the intrinsic properties of RubisCO. Under the humid conditions, the RubisCO activity observed without *in vitro* activation (most likely closely resembling those *in vivo* just before the leaves were immersed in liquid nitrogen; Bainbridge *et al*., 2000; Crafts-Brandner and Salvucci, 2000), was about 85% of that of the *in vitro* activated enzyme in both the wild-type and transgenic plants (Table 3). In contrast, under the low humidity conditions, the *in vivo* activity of RubisCO was about 40% higher in the transgenic than in the wild-type plants over the entire range of CO_2 concentrations examined in the activity assays (Figure 3). In Figure 3 we show the activities of RubisCO exposed to different CO_2 concentrations in order to emphasize the consistency of the data, even at various CO_2 levels, rather than to provide a complete account of the kinetic parameters of activated and non-activated RubisCO from tobacco. Nevertheless, an analysis of the kinetic parameters from experiments similar to that depicted in Figure 3, performed with the wild-type and transgenic line 3, indicated that while the $K_m(CO_2)$ was scarcely affected by the expression of *ictB*, the V_{max} of carboxylation, *in vivo*, was significantly higher in the *ictB*-expressing plants. The higher *in vivo* RubisCO activity in the transgenic plants as compared with the wild-type (Figure 3), under the dry conditions where stomata conductance may limit CO_2 supply, is consistent with the steeper slope of the curve relating photosynthetic rate to intercellular CO_2 concentration (Figure 2). Naturally, the *in vivo* RubisCO activities were lower than those depicted by the *in vitro* activated enzyme (Figure 3, Table 3). The reduced *in vivo* RubisCO activity in the dry vs. the high humidity grown wild-type plants is possibly due to the lower internal CO_2 concentration imposed by the decreased stomatal conductance. These are also the conditions where the transgenic plants exhibited faster photosynthesis (Figure 2) and growth (see below).

Growth experiments

In view of the enhanced photosynthesis in the transgenic plants under CO_2 limiting conditions (Figure 2), we examined how their growth was affected by the relative humidity. There was no significant difference between the growth of wild-type or transgenic *Arabidopsis* plants maintained under high (70–75%) humidity (Figure 4). Under low humidity (25–30%), both wild-type and transgenic plants grew slower than in humid conditions, but the transgenic plants grew significantly faster ($P < 0.03$) than the wild-type. In Figure 4 we provide the relative growth rates (RGR) and the dry

weight accumulated over 18 days of growth. Enhancement of growth of the transgenic plants was observed throughout the growth period and not at a particular phase. Transgenic tobacco plants that expressed *ictB* also appeared to grow faster than the wild-type under low humidity. However, due to technical limitations (the size of the plants and the need to maintain them under identical conditions within the growth chamber), detailed growth experiments with enough plants to enable statistical analyses, were only performed on *Arabidopsis*.

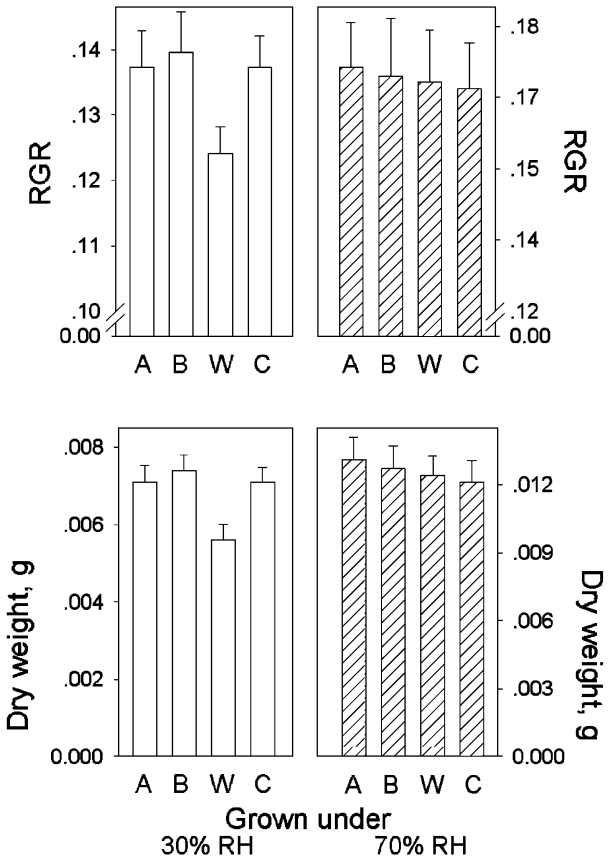

Figure 4 Growth of transgenic (A, B and C) and WT (W) *Arabidopsis* plants. Data are provided as the relative growth rate (RGR) and the average dry weight ± SD. The growth experiments were performed six times for 18 days each, n = 18. RH, relative humidity.

Discussion

Expression of a single gene from the cyanobacterium *Synechococcus* PCC 7942, *ictB*, enhanced photosynthesis and growth in C_3 plants. The increased photosynthetic rate at the limiting CO_2 level was most probably due to a higher RubisCO activity in the transgenic plants as opposed to another report where a higher level of fructose-1,6/sedoheptulose-1,7-bisphospate phosphatase raised the level of ribulose 1,5-bisphosphate and thereby stimulated photosynthesis (Miyagawa *et al*., 2001). In the absence of an independent method for directly determining CO_2 concentration in close proximity to RubisCO, we had to rely on measurements of the CO_2 compensation point (Table 2). The lower compensation point in transgenic plants expressing *ictB* suggested that the CO_2 concentration at RubisCO sites was higher than in the wild-types, but the mechanism involved is not yet known. As indicated (Introduction), while clearly involved in Ci accumulation in *Synechococcus* PCC 7942, the role of *ictB* in Ci uptake in cyanobacteria is not yet understood. Complete inhibition of Ci transport in a *Synechocystis* sp. strain PCC 6803 mutant which possesses a normal *slr1515* (a homologue of *ictB*), suggested that *slr1515* may not be essential for Ci transport in this organism (Shibata *et al*., 2002) which accumulates far less Ci internally than does *Synechococcus* PCC 7942. Nevertheless, it is most likely that the elevated activity of RubisCO in the transgenic plants was due to a higher CO_2 concentration that could enhance the enzyme activity, both as an activator and as a substrate. Apart from the concentration of CO_2, *in vivo* RubisCO activity is affected by several effectors and parameters including light intensity, pH, the levels of specific metabolites, magnesium concentrations and the activity of RubisCO activase (Badger and Lorimer, 1976; Bainbridge *et al*., 2000; Cleland *et al*., 1998; Crafts-Brandner and Salvucci, 2000; Uemura *et al*., 1997; Harrison *et al*., 2001; Kallis *et al*., 2000; Spreitzer, 1999). At this time it is not known whether any of these was affected by the expression of *ictB* in the transgenic plants and thus the reason(s) for the elevated activity of their RubisCO under low humidity and limiting CO_2 is not fully understood.

The level of *ictB* expression varied markedly between transgenic plants (Figure 1). Nevertheless, we did not detect statistically significant differences between *ictB*-expressing lines, within a given species, with respect to the CO_2 compensation point, RubisCO activity, photosynthetic performance or growth. It is possible that despite the different levels of transcription, the abundance of IctB was similar in the transgenic plants, or that a small level of IctB suffices. Our attempts to produce reliable antibodies to IctB to examine these possibilities were not successful. Those produced, directed to the hydrophilic region within this very hydrophobic protein, were not specific enough. Furthermore, in the absence of a reliable antibody, we could not examine the location of this protein in the transgenic plants and confirm that, as expected, the fusion to the transit peptide of the small subunit of RubisCO directed the protein to the chloroplast. Nevertheless, our data clearly suggested a potential use of *ictB* in raising the yield of C_3 plants, particularly under dry conditions where stomatal

closure may impose a CO_2 limitation and thus photosynthetic retardation.

Experimental procedures

Growth conditions and construction of transgenic plants

The plants used here were grown in controlled growth chambers (Binder, Germany). Tobacco (*Nicotiata tabacum*) plants were grown at 24 °C, light intensity was 350 μmol.photons/m^2/s, 12 h:12 h light : dark; *Arabidopsis thaliana* plants were grown at 21 °C, 200 μmol.photons/m^2/s, 8 h:16 h light : dark. The plants were grown in two growth chambers, the relative humidity was maintained at 25–30% in one chamber and 70–75% in the other. Transgenic tobacco and *Arabidopsis* expressing *ictB* were raised using a construct consisting of the 35S promoter fused to a DNA fragment encoding the transit peptide of the small subunit of RubisCO from pea, connected in-frame to *ictB*. This construct was inserted in *Agrobacterium* strains GV 3101 or LBA 4404 for *Arabidopsis* or tobacco transformations, respectively (Clough and Bent, 1998; Fraley *et al*., 1985). The *Agrobacterium* vectors contained a NOS terminator and a kanamycin-resistance encoding cassette.

Measurements of photosynthetic rate and CO_2 compensation point

CO_2 and water vapour exchange were determined with the aid of a Li-Cor 6400, operated according to the manufacturer's instructions (Li-Cor, Lincoln, NE). Saturating light intensities of 750 and 500 μmol.photons/m^2/s were used during the measurements with tobacco and *Arabidopsis*, respectively. The CO_2 compensation point was deduced from measurements of the rate of CO_2 exchange as affected by a range (0–150 μmole CO_2/L) of CO_2 concentrations. The CO_2 concentration where the curve relating net CO_2 exchange to concentration crossed zero CO_2 was taken as the compensation point.

Measurements of RubisCO activity

The plants were grown for 18 days under low or high relative humidity with temperature and light conditions as above. They were placed at a similar distance and orientation from the light sources to minimize possible differences between them due to unequal local conditions. The leaves were excised 3 h after the onset of illumination and immersed immediately in liquid nitrogen. Fifteen cm^2 of frozen leaves were ground in a buffer containing 1.5% PVP, 0.1% BSA, 1 mM DTT, protease inhibitors (Sigma) and 50 mM Hepes-NaOH pH 8.0. For *in vitro* activation, the extracts were centrifuged and aliquots of the supernatants were supplemented with 10 mM $NaHCO_3$ and 5 mM $MgCl_2$ (Badger and Lorimer, 1976) and maintained for at least 20 min at 25 °C. RubisCO activity was determined, either immediately or after the activation (Marcus and Gurevitz, 2000) in the presence of 20–150 μM $^{14}CO_2$ (6.2–9.3 Bq/nmole). The reaction was terminated after 1 min by 6 N acetic acid and the acid-stable products were counted in a scintillation counter (Marcus and Gurevitz, 2000). Time course analyses indicated that the RubisCO activities were constant for 1 min and declined thereafter, probably due to the accumulation of inhibitory intermediate metabolites (Cleland *et al*., 1998; Edmondson *et al*., 1990; Kane *et al*., 1998). Quantification of the amount of RubisCO active sites was performed as in Marcus and Gurevitz (2000).

Growth experiments

Wild-type and transgenic *Arabidopsis* plants were germinated and maintained for 10 days under humid conditions. To minimize possible variations in water supply between transgenic and wild-type plants, the seedlings were transferred to pots, each containing a wild-type and three different transgenic plants. Twelve pots were placed in each growth chamber (Binder, Germany) under equal light intensity and temperature. The relative humidity was maintained at 25–30% in one chamber and 70–75% in the other. Other growth conditions were as above. The plants were harvested after 18 days of growth, quickly weighed (fresh weight) and dried in the oven (dry weight). The growth experiments were repeated six times.

RNA isolation

For each sample, 1–2 g of plant material were ground in liquid nitrogen using a mortar and pestle and then transferred to 5 mL of Tris-HCl buffer (50 mM Tris-HCl, pH 8, 300 mM NaCl, 5 mM EDTA, 2% SDS, 2 mM Aurin tricarboxylic acid (ATA) and 14 mM β-mercaptoethanol) at room temperature, vortexed, and incubated on ice for 10 min. After adding 0.7 mL of ice-cold 3 M KCl the homogenates were vortexed and incubated on ice for 15 min. They were then centrifuged in a Sorvall SS-34 rotor at 10 000 r.p.m. for 10 min at 4 °C. The supernatants were transferred to new tubes containing 2 mL of 8 M LiCl and well mixed. The samples were incubated overnight at 4 °C and then centrifuged as described above.

The supernatants were discarded and the tubes were left upside down to drain for two min. The pellets were dissolved by vortexing in 0.4 mL of DEPC-treated water containing 0.5 mM ATA.

The RNA from each sample was extracted with 0.6 neutralized phenol in microfuge tubes and the upper aqueous phase was transferred to a new sterile tube containing 0.1 volume of 3 M Na-acetate and vortexed. Two volumes of cold ethanol were added, vortexed and incubated at –20 °C for 2 h. The samples were centrifuged for 30 min at 16 000 ***g*** in a microfuge. The pellets were rinsed with 1 mL cold 80% ethanol, centrifuged 10 min at 14000 r.p.m. and then the supernatants were discarded. The pellets were air-dried for 5 min and then resuspended in 25 μL sterile water containing 0.5 mM ATA.

Acknowledgements

We thank Dr Leonora Reinhold for helpful discussions. The research was partly supported by a grant from the Deutsch Foundation for Applied Sciences, the Hebrew University of Jerusalem.

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