The role of transient starch in acclimation to elevated atmospheric CO_2

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Received 23 April 1998

Abstract Although increased concentrations of CO_2 stimulate photosynthesis, this stimulation is often lost during prolonged exposure to elevated carbon dioxide, leading to an attenuation of the potential gain in yield. Under these conditions, a wide variety of species accumulates non-structural carbohydrates in leaves. It has been proposed that starch accumulation directly inhibits photosynthesis, that the rate of sucrose and starch synthesis limits photosynthesis, or that accumulation of sugars triggers changes in gene expression resulting in lower activities of Rubisco and inhibition of photosynthesis. To distinguish these explanations, transgenic plants unable to accumulate transient starch due to leaf mesophyll-specific antisense expression of *AGP B* were grown at ambient and elevated carbon dioxide. There was a positive correlation between the capacity for starch synthesis and the rate of photosynthesis at elevated CO_2 concentrations, showing that the capability to synthesize leaf starch is essential for photosynthesis in elevated carbon dioxide. The results show that in elevated carbon dioxide, photosynthesis is restricted by the rate of end product synthesis. Accumulation of starch is not responsible for inhibition of photosynthesis. Although transgenic plants contained increased levels of hexoses, transcripts of photosynthetic genes were not downregulated and Rubisco activity was not decreased arguing against a role of sugar sensing in acclimation to high CO_2.

Key words: Carbon dioxide; Photosynthesis; ADP-glucose pyrophosphorylase; Antisense; Sugar sensing; *Solanum tuberosum*

1. Introduction

The worldwide emission of gases derived from the burning of fossile fuels is seen as a major cause of global change mainly visible as an increase in tropospheric CO_2 levels [1,2]. Since CO_2 serves as the major source for organic carbon by assimilation through photosynthesis and since current CO_2 levels are limiting photosynthesis, one might expect elevated carbon dioxide to lead to a significant increase in plant growth and crop yield. However, this stimulation of photosynthesis is not always maintained during long term exposure to elevated carbon dioxide [3,4]. A central question is therefore how plants can and will adapt to increased atmospheric CO_2 levels. A multitude of investigations has been carried out in growth chambers, greenhouses and in field experiments (FACE) to study this question, including effects on growth and yield, and even ecosystems, with special emphasis on the interaction with environmental factors, such as light, temperature and nutrient supply (for reviews see [4,5]).

A common feature in many plants exposed to elevated CO_2 is an acclimation characterized by the accumulation of starch and soluble carbohydrates, and a reduction in photosynthesis. The inhibition of photosynthesis is often accompanied by a decrease of Rubisco activity [3,6] and of levels of the transcripts for *RBC S* and other genes involved in photosynthesis [7–11]. Since increased hexose levels are supposed to downregulate transcription of photosynthetic genes via a sugar sensing and signalling pathway [12–14], a direct link between the acclimation and sugar accumulation has been postulated [7,12,15]. An alternative explanation for the decreased expression of Rubisco and other proteins involved in photosynthesis, however, is that faster growth in elevated carbon dioxide accelerates leaf senescence due to ontogenetic drift [16] or because nutrients become limiting [17]. Two further explanations for the acclimation of photosynthesis relate more directly to the increase of sugars and starch in elevated carbon dioxide. One is that the accumulation of large starch grains in elevated carbon dioxide could physically disrupt the chloroplast (see [3] for references). Alternatively, the rate of photosynthesis in elevated carbon dioxide may become limited by the rate at which newly fixed carbon is converted to starch and sucrose, the main end products of photosynthesis [3,18]. One way to distinguish between these various explanations is to investigate the impact of a decreased rate of starch synthesis on photosynthetic acclimation to elevated carbon dioxide. Plants with a decreased capacity for starch synthesis will be less susceptible to inhibition due to physical effects of starch grains, and more susceptible to inhibition due to an inadequate capacity for the synthesis of carbohydrates. They might also be more susceptible to inhibition by sugar-mediated repression of gene expression if the lower rate of starch synthesis results in higher levels of sugars in the leaves, and in this case the inhibition of photosynthesis should be accompanied by a large decrease in the transcripts for and the activities of Rubisco and other enzymes involved in photosynthesis.

The key regulatory step in starch biosynthesis is catalyzed by ADP-glucose pyrophosphorylase (AGPase), a tetramer composed of B and S subunits. Respective genes have been cloned from potato [19,20]. The two gene classes differ in their expression patterns in different organs. One of the *AGP S* genes is strongly inducible by metabolizable carbohydrates (e.g. sucrose supplied to detached leaves). Leaf-specific antisense repression of the *AGP B* gene altered the timing of photosynthate export from leaves when the plants were grown in ambient CO_2. Photosynthesis was not significantly altered relative to wild-type plants, but as a result of the reduction of

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PII S0014-5793(98)00580-8

starch synthesis a higher portion of assimilated carbon was exported from the source leaves to the sink tissues during the light period. The altered leaf export characteristics did not change tuber yield [21,22].

To study the effects of elevated CO_2 on potato plants and to unravel the importance of transient starch, the overall rate of end product synthesis and sugar-mediated changes in gene expression for the acclimation of photosynthesis, wild-type and AGPase B antisense plants were exposed for 22 and 50 days to increased atmospheric concentrations (1000 ppm) of CO_2 and analyzed for growth and yield parameters, photosynthesis, carbohydrates and the expression levels of AGPase and genes involved in photosynthesis.

2. Materials and methods

2.1. Plant material, growth and transformation

Wild-type plants and transformants of *Solanum tuberosum* L. var. Désirée L700 *AGP B*, lines 9, 80 and 101 [27], were transferred from axenic cultures to soil. All physiological results (e.g. starch content) were consistent with the degree of inhibition of AGPase activity. Line 101 thus retained a higher rest activity as compared to the description in [27]. Growth chamber conditions were at 400 ppm (ambient) and 1000 ppm (elevated) CO_2, respectively, with a light intensity of 600 μE in a 12-h day/night cycle at 20°C with 60% rel. humidity in 2.5-l pots during the whole growing period.

2.2. RNA isolation and Northern blot analysis

Terminal leaflets of mature potato leaves were harvested 3 h after start of the light period from plants grown for 50 days in soil and RNA was isolated essentially as described in [28], followed by LiCl precipitation and 30 μg total RNA were separated on 1.5% formaldehyde agarose gels, blotted on GeneScreen (DuPont). Radioactive labelling of cDNA fragments was carried out using the High Prime-Kit (Boehringer, Mannheim) and [α-^{32}P]-dCTP. Hybridization was performed as described in [29], and detection was carried out on an imaging analyzer (Fuji BAS 2000, Fuji, Tokyo).

2.3. Photosynthetic measurements

Gas exchange was measured on terminal leaflets of mature leaves of 21- and 49-day old plants in the growth carbon dioxide concentration and ambient light, using the LI-6400 portable photosynthesis system (LI-COR, Inc.).

2.4. Physiological measurements

Samples for the determination of the leaf contents of soluble sugars, starch, nitrate, protein and enzymic activities were harvested 3 h after onset of light from 22- and/or 50-day old plants. Soluble sugars and starch were determined as in [30]; nitrate was determined from the same extracts using the nitrite/nitrate determination kit (Boehringer, Mannheim) according to the protocol supplied by the manufacturer. Protein content was analyzed according to [31], max. Rubisco activity was determined as in [32], AGPase activity measurements were performed as in [21].

3. Results

3.1. The effect of increased atmospheric CO_2 concentrations on plant growth and photosynthesis

These experiments used transformants with a leaf mesophyll-specific inhibition of AGPase due to antisense expression of *AGP B*. In three separate transformant lines, AGPase activity was decreased in lines 9, 80 and 101 to 12, 15 and 26%, respectively, of the activity in wild-type source leaves (WT; Fig. 1). Wild-type and transgenic plants were grown in ambient (400 ppm) or elevated (1000 ppm) CO_2 concentrations in a controlled high-light environment. Elevated carbon dioxide led to a 70% increase of shoot fresh weight and a 2-fold increase of tuber yield, compared to ambient conditions

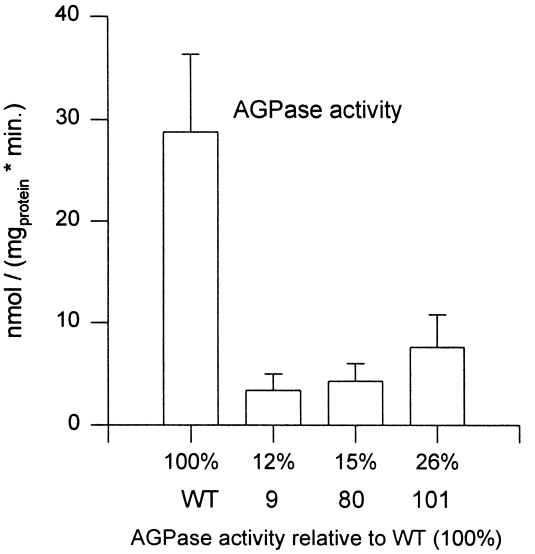

Fig. 1. AGPase activity in source leaves of control (WT, 100%) and *AGP B* antisense potato plants. Plants were propagated in tissue culture and transferred to growth chambers. AGPase activity was measured 50 days after transfer (DAT). Plants were grown under ambient (400 ppm) and elevated (1000 ppm) atmospheric [CO_2]. Results are given as mean ± S.E. ($n = 8$). Samples (four samples each growth condition) were taken three hours after onset of light. Plant lines (9, 80, 101) are ordered by their residual AGPase activity (12%, 15%, 26%, respectively) relative to control plants.

(Fig. 2). No significant and consistent differences with respect to growth were observed between wild-type and transgenic plants.

Net carbon dioxide uptake of potato WT and transgenic plants was monitored 21 and 49 days after transfer (DAT) of the plants from tissue culture into soil (Fig. 3A). When the plants were grown at ambient CO_2, no significant differences were detected between young and old plants, or between WT and transgenic plants. There was a slight trend to decreased rates of photosynthesis in ambient carbon dioxide in the transgenic plants and this correlated with the extent to which AGPase activity was reduced. In contrast, large differences in the rate of photosynthesis emerged when the plants were grown at elevated CO_2. In general, the net rates of CO_2 uptake were higher after 21 days than after 49 days in elevated CO_2. There were also marked differences between the WT and the antisense plants. After 21 days, all three antisense lines had significantly lower photosynthetic rates than WT plants. The extent of the inhibition correlated with the inhibition of AGPase activity. A similar trend was seen after 49 days although it was less marked because photosynthesis was also decreased in the WT plants. It is especially noteworthy that the young WT plants profited from enhanced carbon dioxide supply whereas older WT plants did not, and that transgenic plants with reduced AGPase activity did not profit from elevated carbon dioxide at either time point.

3.2. The effect of increased atmospheric CO_2 concentrations on Rubisco, protein and nitrate content of leaves

To analyze whether gross changes in the amount of Rubisco were responsible for the changes in photosynthesis, maximal Rubisco activity was determined after 50 days. No significant effect of elevated carbon dioxide on Rubisco activity could be detected. Rubisco activity was not reduced in the

transformants, even though they showed lower rates of photosynthesis in elevated carbon dioxide than WT plants (Fig. 4A).

The total protein content was significantly reduced in elevated carbon dioxide in WT and antisense plants (Fig. 4A). The transformants contained slightly more protein than the WT plants. Since potato plants assimilate nitrate mainly in their leaves, the increased photosynthetic activity at elevated CO_2 concentrations might affect the assimilation of inorganic nitrogen. Determination of the nitrate content in leaves showed that after 22 days wild-type plants contained reduced levels of nitrate in elevated carbon dioxide. The transgenic plants that had reduced photosynthetic activity at elevated CO_2 contained comparable nitrate levels in elevated and ambient conditions at 22 DAT (Fig. 4B). Clearly, neither WT nor antisense plants are nitrogen deficient at this stage. At 50 DAT the leaf content of free nitrate was below the detection level in all treatments, indicating that the plants have become nitrogen limited (data not shown).

3.3. *The effect of increased atmospheric CO_2 concentrations on carbohydrate content*

As expected, antisense repression of *AGP B* led to a reduced starch content in source leaves (Fig. 3B). The reduction of the starch content correlated with the inhibition of AGPase activity in the various transgenic lines. The decrease in starch content was positively correlated with a slight inhibition of net carbon dioxide uptake (Fig. 5). In elevated CO_2, the starch content increased about 10-fold in wild-type plants and to a smaller extent in antisense plants, and the decrease of starch was correlated with a quite large inhibition of net photosynthesis (Fig. 5). Sucrose levels were slightly increased in AGPase antisense plants compared to WT plants. Elevated CO_2 led to a 30% increase of sucrose in WT plants and the three

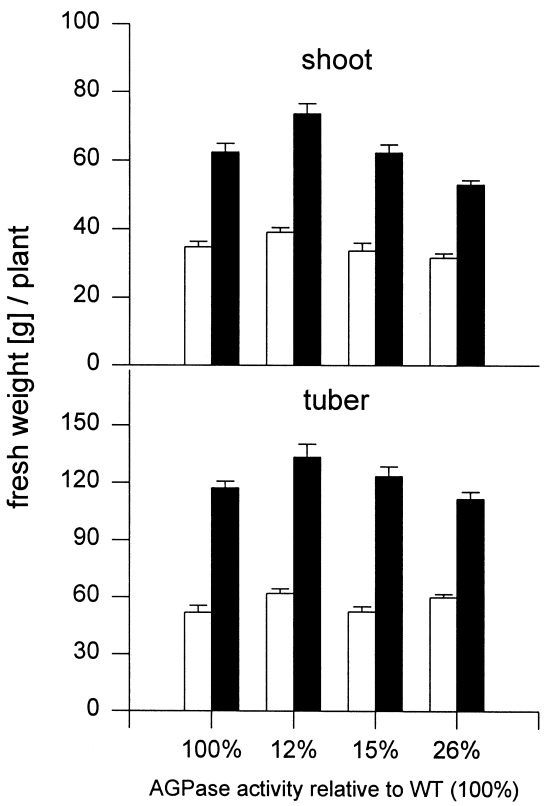

Fig. 2. Fresh weight of shoots and tubers of control (100%) and transgenic plants (see Fig. 1). Plants were grown under either ambient (open bars) or elevated (filled bars) atmospheric [CO_2] for 50 days. Results are given as mean ± S.E. (n = 13–16).

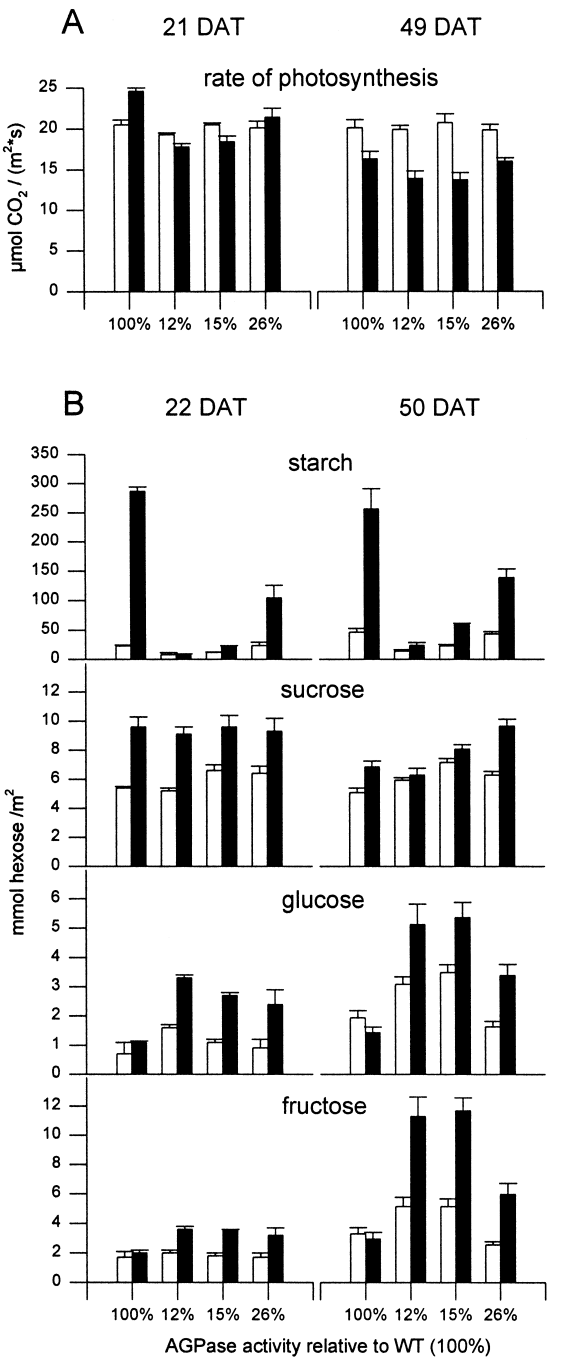

Fig. 3. A: Rate of photosynthesis of source leaves 21 and 49 days after transfer (DAT) from tissue culture. Results are given as mean ± S.E. (21 DAT: n = 3 for transgenic and n = 9 for control plants; 49 DAT: $n \geq 5$). B: Starch, sucrose, glucose and fructose contents of source leaves 22 and 50 days after transfer (DAT) from tissue culture. Samples were taken three hours after illumination. Results are given as mean ± S.E. (22 DAT: n = 3; 50 DAT: n = 10). Plants were grown under either ambient (open bars) or elevated (filled bars) atmospheric [CO_2]. Plant lines are ordered by their residual AGPase activity (see Fig. 1).

antisense lines (Fig. 3B). The effects were less pronounced at 50 DAT. Although hexose levels were slightly increased in the antisense plants at 22 DAT, and markedly at 50 DAT (Fig. 3B), the increase at older stages was not reproduced in other independent experiments (data not shown). Elevated CO_2 did not lead to an increase of hexoses in WT plants, but it did lead to an increase of hexoses in the antisense plants.

3.4. The effect of increased atmospheric CO_2 concentrations on gene expression

In WT plants the transcripts for *AGP B* and *AGP S* transcripts both increased in elevated CO_2 at 50 DAT (Fig. 6). Due to antisense repression, *AGP B* transcripts could not be detected in the antisense plants, and elevated CO_2 led to even higher levels of *AGP S*, although the magnitude of the rise did not correlate with the severity of inhibition of the *AGP B* expression.

In contrast to the expectations, *RBCS* and *CAB* transcripts did not display any significant reduction in source leaves when grown under elevated carbon dioxide, nor were they altered in the antisense plants even though photosynthesis was inhibited in them compared to wild-type plants. The absence of change in these transcripts is in striking contrast to the marked change in the transcripts for *AGP S* and *AGP B*.

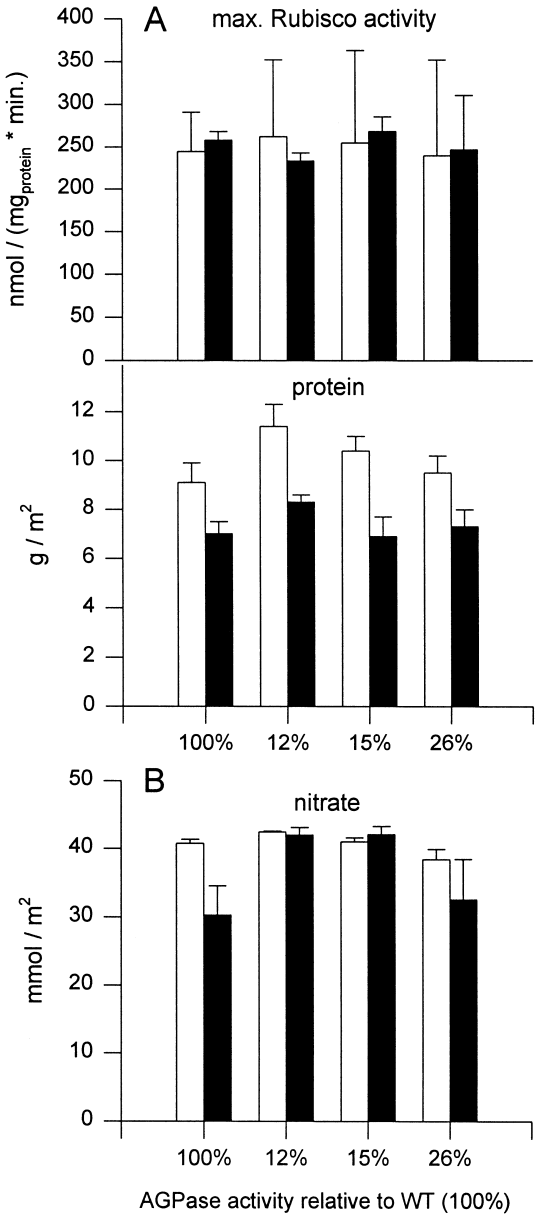

Fig. 4. A: Maximal Rubisco activity and protein content in source leaves of control and transgenic plants 50 DAT. Results are given as mean ± S.E. ($n = 4$ for max. Rubisco activity; $n = 8$ for protein content). B: Nitrate content in source leaves of control and transgenic plants 22 DAT. Results are given as mean ± S.E. ($n = 3$). Plants were grown under either ambient (open bars) or elevated (filled bars) atmospheric [CO_2]. Samples were taken three hours after illumination. Plant lines are ordered by their residual AGPase activity (see Fig. 1).

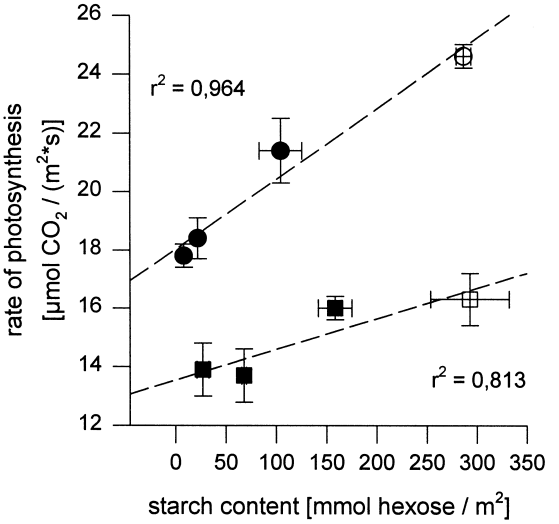

Fig. 5. Correlation between the rate of photosynthesis and starch content of source leaves. The data are taken from control (open symbols) and transgenic plants (filled symbols) grown under elevated atmospheric [CO_2] for either 22 (circles) or 50 days (squares, compare to Fig. 3). The dashed lines show the respective linear regressions.

3.5. Concluding remarks

Elevated carbon dioxide led to an increased rate of photosynthesis in young WT potatoes that resulted in higher levels of leaf carbohydrate, especially starch, and increased yield. In older WT plants, the rate of photosynthesis declined, as seen in other studies [10,16,23]. Antisense inhibition of *AGP B* expression modified the responses of leaf carbohydrates and photosynthesis. In ambient carbon dioxide, starch accumulation was markedly reduced, as expected from the 74–88% inhibition of AGPase activity, and there was a slight increase of hexose sugars but not of sucrose (see also [21,22]). In elevated carbon dioxide, starch accumulation was still strongly decreased compared to WT plants, there was a more marked increase of hexoses, and photosynthesis was inhibited compared to WT plants in elevated carbon dioxide and to antisense plants in ambient carbon dioxide. Crucially, this inhibition of photosynthesis was not accompanied by a decrease of Rubisco activity, or of the levels of the *RBC S* or *CAB* transcripts.

These results demonstrate that the acclimation of photosynthesis to elevated carbon dioxide is not caused by the accumulation of starch. They also show that acclimation is not due to a specific sugar-mediated decrease in the expression

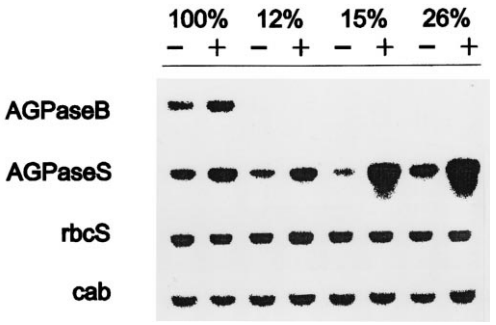

Fig. 6. Northern blot analysis of control and transgenic potato plants (see Fig. 1) grown under either ambient (−) or elevated (+) atmospheric [CO_2] for 50 days. Samples were taken from source leaves three hours after illumination. 30 μg of total RNA was loaded in each lane. Probes were cDNAs for *AGPase B*- and *S*-subunit of potato, the small subunit of Rubisco (*rbcS*) and the chlorophyll *a/b* binding protein (*cab*) of tobacco.

of *RBC S* and other genes involved in photosynthesis: the inhibition of photosynthesis in the antisense plants compared to WT plants at 21 DAT was not accompanied by a decrease of Rubisco activity, and the acclimation of photosynthesis in WT plants at 50 DAT was not accompanied by a decrease of the levels of the *RBC S* and *CAB* transcripts. Instead, the results show that the rate of photosynthesis in elevated carbon dioxide can become co-limited by rate of end product synthesis. Antisense lines with a strongly decreased rate of starch synthesis show a very slight inhibition of photosynthesis in ambient carbon dioxide, which became much more marked after growth in elevated carbon dioxide (Fig. 5). In this context, it is interesting that elevated carbon dioxide led to increased expression of *AGP S* and *AGP B*. Increased expression of AGPase will not only allow increased accumulation of carbohydrate, but may also allow higher rates of photosynthesis in elevated carbon dioxide.

Investigations of acclimation to elevated carbon dioxide have been dominated by investigations of changes in Rubisco, but in many cases the reported decline may be due to nutrient deficiency and accelerated senescence [10,16,17,23–25] rather than a specific adaptation of the photosynthetic apparatus. Although our results with potato cannot be automatically extrapolated to other species, they show that far more attention should be paid to the genes and proteins that are involved in end product synthesis. In this context, Socias et al. [26] provided indirect evidence that end product synthesis co-limits photosynthesis in bean plants growing in elevated carbon dioxide, and Micallef et al. [18] showed that tobacco transformants overexpressing maize sucrose phosphate synthase maintain higher rates of photosynthesis than WT plants in elevated carbon dioxide.

Acknowledgements: We would like to thank Birgit Brandl, Heike Deppner, Andrea Großpietsch and Monika Raabe for technical assistance in physiological analyses and Andrea Knospe for tissue culture work. This work was supported by the Schwerpunktprogramm of Deutsche Forschungsgemeinschaft 'Stoffwechsel und Wachstum der Pflanze unter erhöhter CO_2-Konzentration'.

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