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SHORT COMMUNICATION

Altered Rubisco activity and amounts of a daytime tight-binding inhibitor in transgenic tobacco expressing limiting amounts of phosphoribulokinase

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Abstract

Transgenic tobacco with RuBP-limited photosynthetic assimilation due to a 95% reduction in phosphoribulokinase activity, had higher specific activities of Rubisco in fresh extracts and after full activation, than in the wild type. Differences in the amounts of a daytime tight-binding inhibitor were sufficient to contribute significantly to these activity differences.

Key words: *Nicotiana tabacum*, transgenic plant, phosphoribulokinase, Rubisco, tight-binding inhibitor.

Introduction

Rubisco is the key enzyme of photosynthesis. Its activity is regulated by carbamylation (Lorimer and Miziorko, 1980), stromal metabolites (Foyer *et al.*, 1987) and in many species by the tight-binding inhibitor, 2-carboxyarabinitol-1-phosphate (CA1P) (Gutteridge *et al.*, 1986) produced in low light or darkness (Andralojc *et al.*, 1994). Recent work suggests a further level of control in the form of a tight-binding inhibitor distinct from CA1P and present during the daytime (Keys *et al.*, 1995).

In this study, differences in the amounts of this inhibitor between the wild type and transgenic tobacco with phosphoribulokinase (PRK) activity reduced to 5% of wild type, are sufficient to contribute significantly to the Rubisco activity differences also observed in these plants.

Materials and methods

Plant material

Wild type and transgenic tobacco plants with PRK activity reduced by an inverted PRK cDNA (Paul *et al.*, 1995) were grown in loam-based compost with fertilizer at 25 °C, 330 μmol photons m^{-2} s^{-1} over a 14 h photoperiod and with 70% relative humidity.

Rubisco activity and Rubisco protein

Samples from most recently fully expanded leaves of 5-week-old plants were freeze-clamped in liquid N_2 under the growing conditions during the middle of a photoperiod. Leaf tissue was extracted and initial and total activities measured as in Parry *et al.* (1993). Amounts of Rubisco were measured by ELISA, using monoclonal rabbit antisera raised against wheat Rubisco and developed with a goat anti-rabbit IgG peroxidase conjugate. The method was calibrated with purified tobacco Rubisco.

Extraction and HPLC- fractionation of Rubisco inhibitors

Leaf samples (0.5 g) were freeze-clamped as above and at the end of the dark period. Samples were ground using a pestle and mortar whilst still frozen and extracted in 1.5 ml of 0.46 M trifluoroacetic acid containing 10 mM 8-hydroxyquinoline as in Keys *et al.* (1995). As Rubisco inhibitors can be formed from degradation of RuBP (Paech *et al.*, 1978) and the wild type plants contain more RuBP than the plants with very low PRK activity (Paul *et al.*, 1995) extraction was also performed in the presence of exogenous RuBP added to twice the endogenous leaf RuBP content. To check that inhibitor could also be recovered using a different extraction procedure, some samples were extracted in methanol/chloroform/water (Redgwell, 1980).

For HPLC-fractionation, neutralized leaf extract was diluted with an equal volume of deionized water and applied via a 1 ml injection loop to a CarboPac PA1 column (4 × 250 mm) attached to a Bio-LC chromatography system (Dionex, UK). The

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column was pre-equilibrated in 0.1 M sodium acetate (pH 7, acetic acid). The flow rate was 1 ml min^{-1} throughout. The gradient programmer produced a linear 0.1 to 1.0 M sodium acetate gradient from 5 to 32 min after initiating a run cycle. Fractions were collected at 0.5 min intervals and 50–150 μl aliquots were tested for the presence of Rubisco inhibitors (see below).

Assay of Rubisco inhibitors

Up to 150 μl of fractionated and unfractionated leaf extracts of light and dark samples was incubated for 5 min in 0.5 ml 100 mM Bicine or TRIS buffer pH 8.2, which contained 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 10 μg purified wheat Rubisco, previously activated for 40 min at 37 °C in 100 mM Bicine/HCl pH 8.2, 20 mM $MgCl_2$, 10 mM $NaHCO_3$. After 5 min, 0.5 ml 100 mM Bicine/HCl pH 8.2 containing 20 mM $MgCl_2$, 0.66 mM RuBP and 10 mM $NaH^{14}CO_3$ (18.5 kBq μmol^{-1}) was added. After 5 min, the assay was stopped with 100 μl 10 M formic acid. Samples were oven-dried and ^{14}C incorporation determined by scintillation counting. Inhibition produced by extracts was related to that produced with CA1P. Assays were performed in TRIS and Bicine buffers because TRIS abolishes the inhibitory activity of the daytime inhibitor, but not of CA1P, whereas Bicine has no such effect (Keys *et al.*, 1995). Comparison of the inhibition produced in TRIS and Bicine is, therefore, a good diagnostic tool for distinguishing between the inhibitors.

Results

Rubisco activities

Both initial and total activities of Rubisco expressed on a Rubisco protein basis were higher in plants with 5% of wild-type PRK activity than in the wild type (Fig. 1A). The activation state in plants with a range of PRK activities was inversely related to the PRK content and approached 100% in plants with the lowest activities of PRK (Fig. 1B). To determine the factor that was limiting Rubisco activity in the wild-type plants, the possible involvement of a tight-binding inhibitor was investigated.

Quantification and characterization of inhibitors

Rubisco-inhibitory activity in illuminated leaves was decreased by up to 80% when assays were performed in TRIS buffer compared to Bicine buffer (Fig. 2A). This is characteristic of the daytime inhibitor in wheat leaves and demonstrates that the inhibition is not due to CA1P, which retains inhibitory activity in TRIS (Keys *et al.*, 1995). In Bicine buffer, Rubisco-inhibitory activity was equivalent to 12 nmol CA1P equivalents g^{-1} FW in wild-type plants and 5.5 nmol g^{-1} FW in plants with 5% PRK (Fig. 2A). In HPLC-fractionated samples, inhibition was associated with a peak emerging after 24.3 min, distinct from CA1P, but consistent with the retention time of a bisphosphate and identical to the retention time of the wheat inhibitor (Keys *et al.*, 1995). Alkaline phosphatase (bovine intestinal mucosa) totally abolished activity of the tobacco inhibitor. Moreover, partial abolition of inhibitory activity with CA1P phosphatase indicates structural similarity to CA1P (data not presented). Recovery of inhibitory activity following fractionation was approximately 50% (Fig. 2A, B) and the efficacy of the fractionated inhibitor was also attentuated by TRIS. No daytime inhibitor was detected in dark samples. Only trace amounts of CA1P were present during the day, but about 20 nmol g^{-1} FW was present at night in the wild type and transformant (Fig. 2C).

In the presence of a fixed concentration of fractionated daytime inhibitor, increasing concentrations of Rubisco resulted in a biphasic rise in carboxylase activity: a shallow gradient while the inhibitor concentration exceeded that of Rubisco catalytic sites, but a steep gradient once the concentration of catalytic sites exceeded that of the inhibitor. This behaviour demonstrates tight-binding kinetics and resembles that of CA1P and the daytime inhibitor in wheat and French bean (see Keys

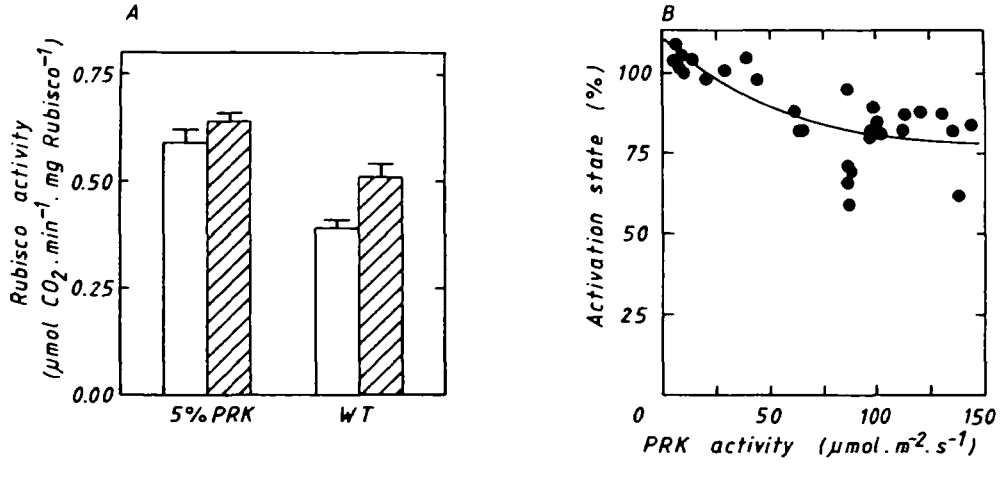

Fig. 1. Rubisco activities (μmol CO_2 min^{-1} mg^{-1} Rubisco) in transgenic tobacco with altered amounts of PRK activity. (A) Initial activities (open bars) and total activities (hatched bars) in plants with 5% of wild-type PRK activity and in wild type. (B) Rubisco activation state (initial activity expressed as a percentage of total activity) in plants with a range of PRK activities.

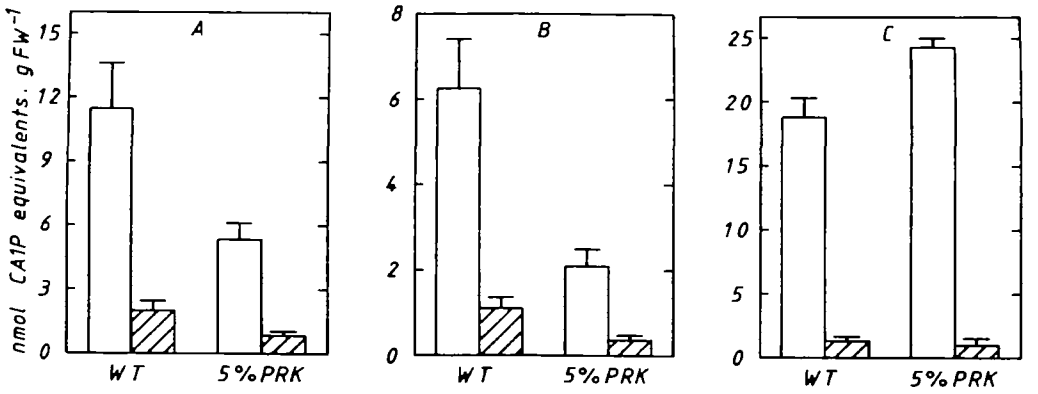

Fig. 2. Quantification of daytime inhibitor and CA1P in wild type and plants with 5% of wild-type PRK activity. (A) Amounts of daytime inhibitor in unfractionated daytime leaf extracts, assayed in Bicine (open bars) or TRIS (hatched bars); (B) amounts of daytime inhibitor in fractionated daytime extracts assayed in Bicine (open bars) or TRIS (hatched bars); (C) amounts of CA1P present at night (open bars) and during the day (hatched bars). Daytime inhibitor was not detectable at night.

et al., 1995). Addition of RuBP during extraction did not alter the amounts of daytime inhibitor and similar amounts of inhibitor compared to acid extracts were recovered following extraction in methanol/chloroform/water (data not presented).

Discussion

In RuBP-regeneration-limited transgenic tobacco, Rubisco activity is up-regulated. This challenges the assumption that Rubisco activity is always down-regulated when photosynthesis is limited by low RuBP (Sage, 1990; Sage *et al.*, 1990). Rubisco up-regulation was partly due to differences in carbamylation, where different concentrations of metabolites within the chloroplast stroma, particularly low 3-PGA (Paul *et al.*, 1995), may have facilitated carbamylation as a consequence of high pH (Mott *et al.*, 1984), and high ATP, which may have enhanced up-regulation by Rubisco activase (Portis, 1990). However, the investigations reveal a mechanism for regulating Rubisco activity in the light that does not depend directly on carbamylation. Evidence is provided that a tight-binding inhibitor of Rubisco in illuminated leaf extracts, distinct from CA1P and other compounds known to inhibit Rubisco, contributes to the regulation of Rubisco in the light. Assuming 3 g Rubisco m^{-2} and 14.2 nmol active sites mg^{-1} Rubisco, then there are 100 nmol active sites g^{-1} FW. Inhibitor was therefore present in quantities sufficient to occupy about 12% of the Rubisco active sites in the wild type and about 5% active sites in plants with 5% PRK. Work in this laboratory has demonstrated that the inhibitor is located in the chloroplast (H Lowe, A Keys, M Parry, unpublished observations). Small but consistent differences in amounts of inhibitor reported here are, therefore, of the order required to contribute to the small but consistent differences in total activities observed (Fig. 1).

By relating Rubisco activities to the amounts of inhibitor, the first evidence is provided that relates differences in the amounts of this inhibitor to differences in Rubisco activity *in vivo*. The inhibitor may provide further flexibility in the regulation of Rubisco, acting in concert with carbamylation to regulate Rubisco activity during the day.

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