## S27-010

# Can negligible levels of Rubisco activase be the reason for decreased Rubisco activity levels in guard cells?

S. Madhavan [1] , P. Echtenkamp[1], and K.W. Lee[2].

[1]*Department of Biochemistry, The Beadle Center,*

[2]*School of Biological Sciences, University of Nebraska, Lincoln, NE 68588*
*Fax: 402-472-7842; e-mail: msoundar@unlnotes.unl.edu*

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### Introduction

Although Rubisco is a normal and abundant enzyme in mesophyll chloroplasts, its presence and activity in guard cell chloroplasts has been a subject of controversy. Several studies using a variety of techniques have unequivocally confirmed not only the presence of Rubisco in guard cells (Vaughn and Vaughn, 1988), but also found it to be catalytically active (Reckmann *et al*, 1990). Based on a cell basis, the amount of Rubisco in guard cells is suggested to be in the range of 0.6 to 1.4 % whereas its activity is reported to be in the range of 0.35 to 0.5%, of a mesophyll cell. The absolute physiological significance of Rubisco, however, has still not been clearly understood in these cells.

It is now well known that Rubisco must be activated by $CO_2$ in order for it to catalyze the carboxylation of ribulose bisphosphate. Reckmann *et al* (1990) have reported that Rubisco in *P sativum* guard cells had a specific activity similar to that of mesophyll enzyme, but the absolute Rubisco activity in a guard cell was only 1:280 of that found in a mesophyll cell. This low activity may be due : a) to a low Rubisco content, and/or, b) to either the absence or an inactive functional activation mechanism in guard cells.

Rubisco activase is a nuclear encoded chloroplast protein that enables Rubisco to function under physiological conditions. This was first identified as a biochemical lesion in the *rca* mutant of *Arabidopsis* (Salvucci and Ogren, 1996) which lacked this enzyme. Rubisco appears to be largely inactive *in vivo* without an activase. Study of Rubisco in this mutant (Somerville *et al*, 1982) and transgenic tobacco plants with reduced rubisco activase levels (Mate *et al*, 1993), both showed that Rubisco could not achieve and maintain an adequate level of activity without an activase. Rubisco activase promotes 'activation' of Rubisco by overcoming the deleterious effects of tight binding sugar phosphates and low chloroplast $CO_2$ levels on catalysis and carbamylation (Salvucci and Ogren, 1996).

Rubisco activase has been detected immunologically in higher plant species (Salvucci et al, 1987), in two unicellular green algal species (Roesler and Ogren, 1990; McKay *et al*, 1991) and also in cyanobacteria (Friedberg *et al*, 1993). In many plant species Rubisco activase protein consists of two polypeptides, 41 and 45 kDa.

Mesophyll cells have Rubisco activase but no conclusive evidence for its presence in guard cells has yet been provided. Using both immunoblot and immunogold electron microscopy techniques in this study, we provide evidence for the presence or absence of

both Rubisco and Rubisco activase, in guard cells, mesophyll cells and bundle sheath cells of $C_3$ and $C_4$ plants.

## Materials and Methods

### *Plant Material*

Tree tobacco (*Nicotiana glauca* Graham) Broad bean (*Vicia faba* L.), spinach (*Spinacea oleracea*) and maize (*Zea mays* L.) plants were grown under natural light conditions at day/night temperatures of 22˚C/17˚C and at 60-70% RH in a greenhouse.

### *Protoplast Isolation*

Guard cell protoplasts (GCP) were isolated from *V. faba* leaf epidermal peels following the procedures of Shimazaki *et al* (1982) with slight modifications. Mesophyll cell protoplasts (MCP) were isolated following the procedures of Spalding *et al* (1992) from *V. faba* leaves free of abaxial epidermis.

### *Preparation of Extracts*

Crude leaf extracts were prepared by grinding them in 100 mM Tris-HCl buffer, pH 7.5 containing 10 mM $MgCl_2$, 14 mM 2-mercaptoethanol, 1 mM EDTA and 5% glycerol. The homogenate was filtered through four layers of cheesecloth and the filtrate was centrifuged at 27,000 g for 15 min. Protoplast extracts were also made following the above procedure except the homogenates were not filtered through layers of cheesecloth. Aliquots of the supernatants were used for SDS-PAGE.

### *SDS-PAGE and immunoblotting*

Protein samples prepared from various tissues were analyzed by SDS-PAGE (Laemmli, 1972) using Bio-Rad Ready Gels (10% Tris-Glycine Gel with 4% stacking gel). After electrophoresis, proteins on gels were electroblotted on to a nitrocellulose membrane for western blotting according to Towbin *et al* (1979). Polyclonal antibodies raised against Rubisco and Rubisco activase were used to probe the membrane.

### *Immunogold electron microscopy*

Leaf samples for electron microscopy were fixed ovrnight at 4°C 1 % gluteraldehyde and dehydrated in ethanol series before embedding in L.R. White resin. The sections on Formvar-coated nickel grids were incubated for 1 h in BSA (1%) to block non-specific protein binding sites on the sections. Sections were incubated for 1 h with either pre-immune serum or Rubisco/Rubisco activase antibodies (1:200). After extensive washing with PBS, the sections were incubated for 2 h in the respective secondary antibody conjugated with 20 nm gold. Sections were washed with PBS and distilled water prior to post staining with 2% aqueous uranyl acetate and lead citrate.

## Results and Discussion

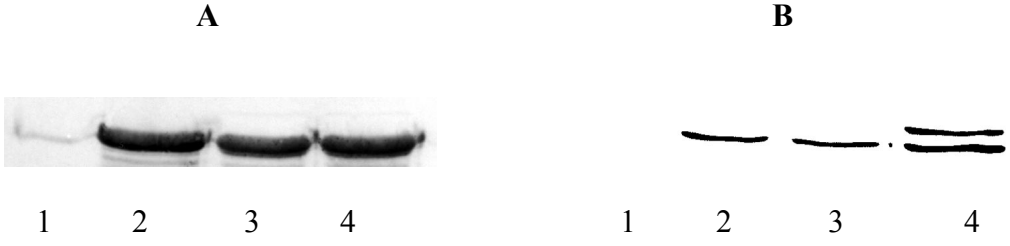


**Fig.1.** Immunoblots of tissue extracts from *Vicia faba* and spinach using anti-rubisco Ab (A) and anti-rubisco activase Ab (B). 1. *V. faba* GCP; 2. *V. faba* MCP; 3. *V. faba* Leaf ; and 4. Spinach leaf.

Immunoblots from SDS-PAGE of proteins in the crude extracts of spinach leaves and the GCP and MCP from *V. faba* distinctly showed a strong band (weak in GCP) around 55 kD corresponding to the large subunit of Rubisco (Fig 1A) when probed with anti-Rubisco polyclonal antibodies. This is consistent with earlier observations on the presence of Rubisco in guard cells. However, immunoblots of the same tissue extracts when probed with anti-Rubsico activase antibodies indicated its presence in all samples except in GCP (Fig 1B).

Figure 2 (below, after refs) shows the immunogold labeling of both Rubisco and Rubisco activase in mesophyll, bundle sheath and guard cells of *N. glauca* and *Z. mays*, respectively.

**2a&b** show the immunogold labelling of Rubisco and Rubisco activase, respectively, in a mesophyll cell chloroplast of the $C_3$ *N. glauca.* It is not surprising to see an abundance of Rubisco labelling when compared to Rubisco activase labelling, in these chloroplasts. Immunogold labelling of Rubisco in guard cells was distinct, though less abundant than in a mesophyll cell chloroplast (Fig **2c**). On the contrary, immunogold labelling was almost negligible for Rubisco activase, in guard cells (Fig **2d**). In the $C_4$ , *Zea mays*, both Rubisco and Rubisco activase are localized in the bundle sheath chloroplasts, as expected (Fig **2e** and **f**). Fig **2g** and **h** show the localization of Rubisco and Rubisco activase in guard cell chloroplasts of *Zea mays*. When compared to the *N. glauca* guard cells, *Zea mays* guard cells appear to have no Rubisco activase in their cells.

The abundance ratio of Rubisco to Rubisco activase, in general, in a mesophyll cell chloroplast of *N. glauca* is ~ 5:1, whereas the same ratio for guard cells appears to be ~10:1. Results from immunolabelling experiments clearly show a negligible amount of Rubisco activase in both, *N. glauca*, and *Zea mays* guard cells. This suggests the possibility that this may be a reason for a lower Rubisco activity in these cells. The reported inadequate levels of Rubisco activity in guard cells is consistent with the reported inadequate level of Rubisco activity in Arabidopsis mutants lacking Rubisco activase and transgenic tomato plants with reduced Rubisco activase levels.

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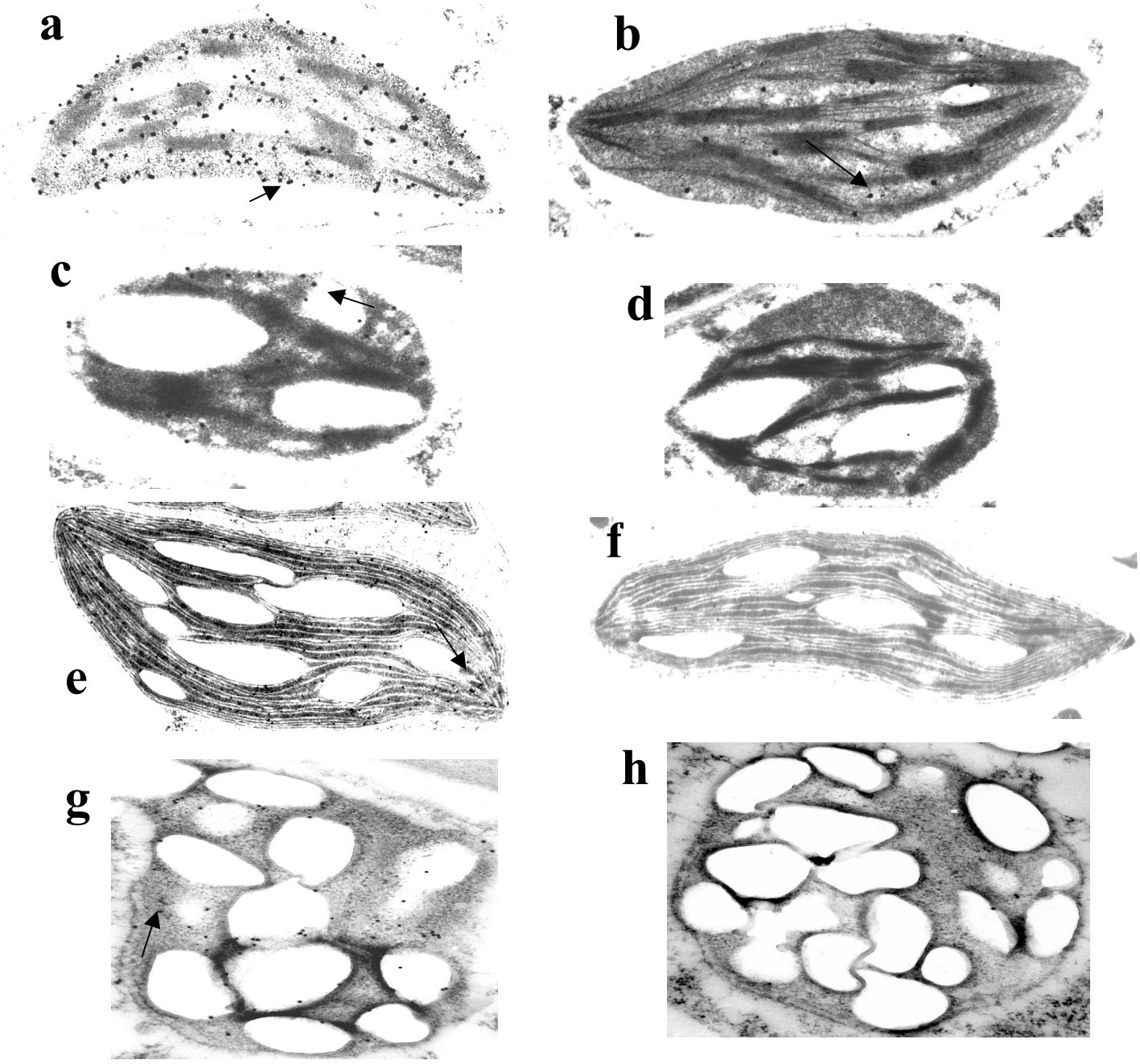


**Fig 2**. Immunogold labeling of Rubisco and Rubisco activase in chloroplasts of : 1. mesophyll cells (a & b) and guard cells of *N. glauca* (c & d); and 2. bundle sheath cells (e & f) and guard cells (g & h) of *Z. mays*. Gold labeling is indicated by an arrow in the respective panels.