

# PEPC-mediated carbon fixation in transmitting tract cells reflects style–pollen tube interactions

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## Summary

**Styles nurture and guide pollen tubes to the ovules. The styles of *Nicotiana tabacum*, a C<sub>3</sub> plant, contain a concentric strand of transmitting tract cells replete with well-developed chloroplasts. It is shown that the chloroplasts have normal ultrastructure and electron transport ability. However, they were found to be devoid of Rubisco, the key enzyme responsible for carbon fixation in C<sub>3</sub> plants. Nevertheless, non-invasive fluorescence techniques showed a light-driven photosynthetic flux. Carbon fixation via phosphoenol pyruvate carboxylase (PEPC) into malate was demonstrated, the latter accumulating during stylar development. Characterization of stylar PEPC *in vitro* and *in vivo* revealed apparent  $K_m$  values consistent with bicarbonate as a rate limiting factor for photosynthetic flux. Presumably, in the closed confines of the intact style, respired CO<sub>2</sub> is the source of carbonate. Enhanced photosynthetic flux was detected following pollination, suggesting utilization of the additional respired bicarbonate and underlining metabolic interactions between the style and the elongating pollen tube.**

## Introduction

The style provides the conduit of sporophytic selection for the thousands of competing elongating pollen tubes. On their way towards the ovules, the pollen tubes traverse an extracellular matrix secreted by the elongated sausage-like cells of the transmitting tract. The matrix is a complex proteinaceous array of arabinogalactan proteoglycans (Fincher *et al.*, 1983; Sedgley *et al.*, 1985), (1-3)- $\beta$ -glucanases (Ori *et al.*, 1990), glyco- and lipoproteins (Heslop-Harrison, 1987). Low molecular weight compounds such as glucose and galactose are present in the matrix and are thought to supply energy for the growing tubes (Knox, 1984; Konar and Linskens, 1966; Kroh *et al.*, 1970). The transmitting tract is surrounded by a cortex of impermeable cells. The resulting structure could act as a pollen tube

guide. Recent findings indicate that the idea of a passive role for the style in pollen tube growth is simplistic, since inert latex particles move towards the ovules in the gynoecea of widely divergent species (Sanders and Lord, 1988). The translocation rate of the beads through the transmitting tract tissue is similar to that of growing pollen tubes. The mechanism responsible for bead movement in the style is not known but the phenomenon suggests an active participation of style components. However, to date, only elementary bioenergetic aspects of stylar metabolism have been documented.

The chlorophyllous nature of the transmitting tract cells in some species, and the presence of starch-containing plastids in many others, have been noted (Bell and Hicks, 1976; Jensen and Fisher, 1969). Photosynthetically active tissue could alleviate the necessity for nutrient import into the style (Halevy, 1987; Kinet *et al.*, 1985). However, the role of stylar photosynthesis appears optional as pollen tube growth and fertilization are not known to be light-dependent phenomena. We were intrigued by the presence of seemingly normal chloroplasts in styles of *Nicotiana tabacum*, a C<sub>3</sub> plant. Our investigations, reported here, revealed the absence of ribulose biphosphate carboxylase (Rubisco) activity in transmitting tract cells. We show that photosynthesis in these cells, in a fashion partly analogous to C<sub>4</sub> and CAM plants, is functionally coupled to PEPC-mediated 'dark reactions', which result in the formation of malate.

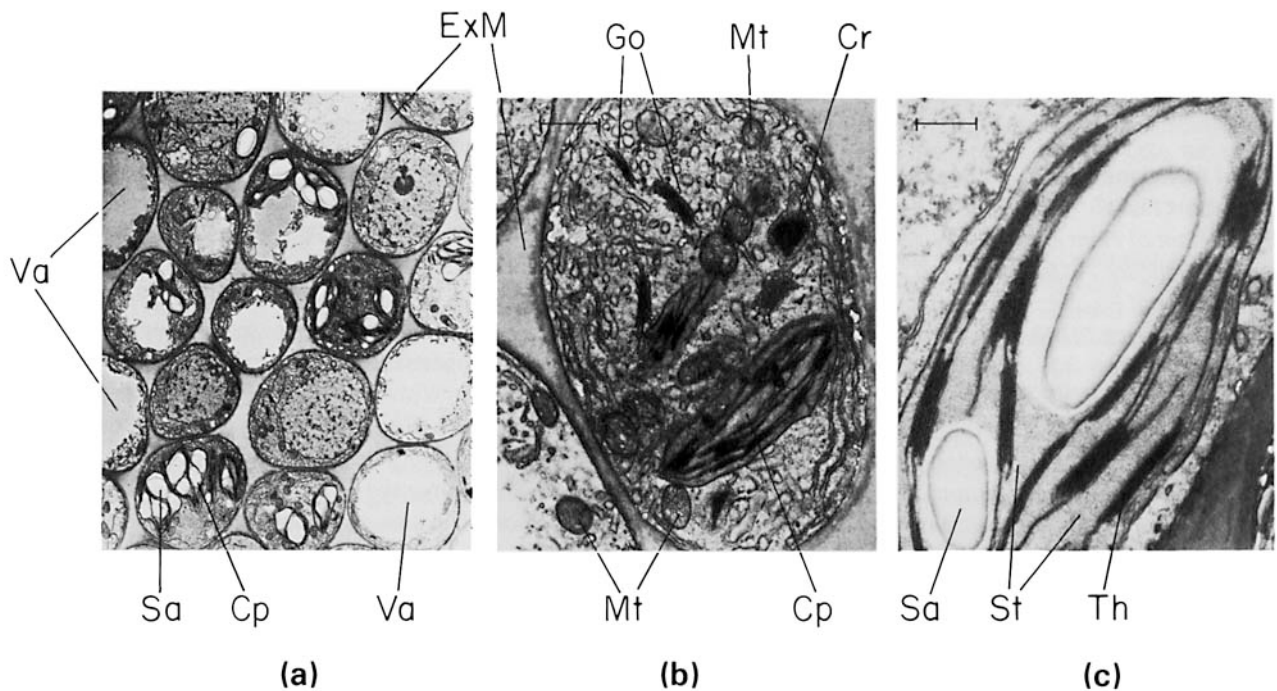
## Results

### *Ultrastructure analysis of style tissue*

The tissue of the transmitting tract of many of the *Solanaceae*, including tobacco, appears as a thin shaft of green chloroplast-containing cells extending from beneath the stigma surface to the ovules. The cells are surrounded by electron-dense proteinaceous material, through which the pollen tubes grow (Figure 1a). A thin primary cell wall, slightly more electron dense than the extracellular matrix, envelopes the cells (Figure 1b). The cytoplasm of the transmitting tract cells contains well-developed endoplasmic reticulum, Golgi bodies and vesicles. The presence of these organelles is in agreement with the major secretory function of these cells (Figure 1b). Almost all cells contain at least one large vacuole that occupies half or more of the cell (Figure 1a). Well-differentiated mitochondria are prominent and are presumably involved in the metabolic upkeep of the intense cellular activities (Figure 1b). The

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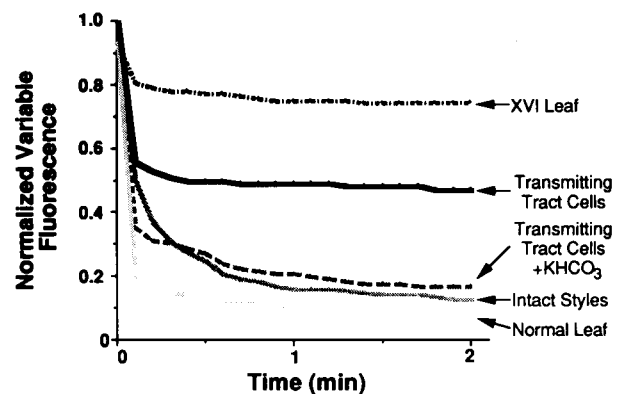


**Figure 1.** Transverse section of cells comprising the styler transmitting tract tissue viewed by electron microscopy. Abbreviations: Cp, chloroplast; Cr, crystal body; ExM, extracellular matrix; Go, golgi complex; Mt, mitochondria; Sa, starch granule; St, stromal space; Th, thylakoids; Va, vacuoles. Bars in plates a–c represent 3.6, 0.7 and 0.3  $\mu\text{m}$ , respectively.

function of the numerous starch-engorged chloroplasts (20–30 chloroplasts per cell) is less clear (Figure 1a). They appear to have normal stacked thylakoid and stroma lamellae (Figure 1b and c). However, their presence in cells situated 10 or more cell layers below the style surface is enigmatic. The absence of stomata and lack of contiguous air spaces, which are normally associated with photosynthetically competent cells, call into question the involvement of styler chloroplasts in orthodox photosynthesis.

*Isolated transmitting tract tissue displays unusual light-induced chlorophyll fluorescence but retains normal photosynthetic electron transport capacity*

To establish the photosynthetic potential of styler chloroplasts, we examined key parameters of light-dependent reactions and so-called ‘dark reactions’ of photosynthesis. A facile non-invasive method of investigating photosynthetic potential is the measurement of light-induced fluorescence transients (Krause and Weis, 1984). Chlorophyll a fluorescence in a leaf is influenced by events that are directly or indirectly related to photosynthetic light reactions. After the onset of illumination, dark-adapted normal leaves will in the course of a few minutes minimize variable fluorescence emission (Figure 2). Leaves in which photosynthetic flux is inhibited will show high variable fluorescence with no or little adjustment. Fluorescence was



**Figure 2.** Comparison of slow fluorescence kinetics in normal tobacco organs and mutant XVI tobacco leaves. Detached organs were subjected to 3 min of dark adaptation prior to measurement. Leaves were detached at the petiole and styles at their base. Sets of five styles were aligned side-by-side and examined together. Transmitting tract cells were isolated from approximately 20 styles and were maintained in 10 mM phosphate buffer (pH 7) in the dark, with or without 10 mM  $\text{KHCO}_3$ . Representative measurements of variable fluorescence using actinic light of 650 nm are presented. Data are normalized to maximal fluorescence such that  $F_m = 1$ , and  $F_0 = 0$ .

used as a probe to examine photosynthesis in isolated transmitting tract cells. The process of isolation does not visibly damage transmitting tract cells as judged by microscopic examination of their continuing active cyclosis. The variable fluorescence remained unusually high compared with normal leaves, indicating inefficient utilization of light (Figure 2). Similar data were obtained over a wide range

of actinic light intensities ( $2\text{--}60 \mu\text{E m}^{-2} \text{sec}^{-1}$ ; data not shown), suggesting that the difference is not merely a result of different saturation kinetics of photosynthesis. One could argue that the differences in variable fluorescence observed here (Figure 2), are due to an imbalance in the distribution of light between photosystems I and II. This possibility was ruled out by determining that the variable fluorescence obtained with actinic light of 436 nm (absorbed equally well by both photosystems) is identical to that obtained with actinic light of 650 nm (absorbed mainly by photosystem II; Canaani and Malkin, 1984).

Inefficient photosynthesis can originate from limited electron flow capacity. We therefore analysed electron transport capacity in thylakoids isolated from transmitting tract cells and from leaves (Table 1). Electron transport rates, through photosystem II or both photosystems I and II together, were measured by monitoring oxygen evolution with ferricyanide as an electron acceptor or by monitoring oxygen uptake with methyl viologen as an electron acceptor, respectively. In each case the electron transport rates, measured in photosynthetic membranes isolated from styles or leaves, were similar (Table 1). The activities were suppressed by the addition of diuron, a specific inhibitor of photosystem II (Table 1). Thus light reactions appear normal and other determinants of the elevated fluorescence observed in isolated transmitting tract cells were sought.

#### Transmitting tract cells lack Rubisco

The inefficient light utilization by isolated transmitting tract cells could originate from lack of dark reactions. Efficient dark reactions act as a sink for reduced carriers formed in the light reactions thereby avoiding loss of light energy as fluorescence (Dietz *et al.*, 1985). Therefore, we analysed styles and leaves for the presence of Rubisco, which is a key component of dark reactions. It is responsible for carbon fixation in all photosynthetic organisms and thus

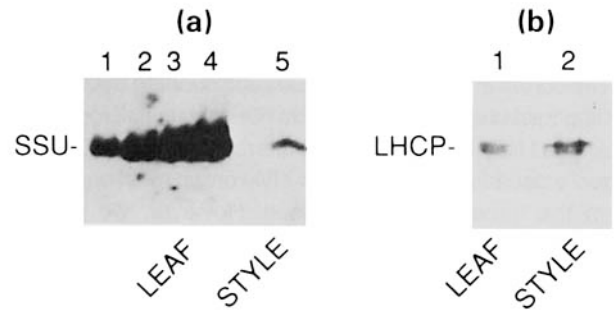
**Table 1.** Rates of photosynthetic electron transport through photosystem II (PS II) or photosystem I and photosystem II together (PS II + PS I) measured in isolated thylakoids

	PS II <sup>a</sup>		PS II + PS I <sup>b</sup>	
	H <sub>2</sub> O → Ferricyanide		H <sub>2</sub> O → Methyl viologen	
	-Diuron	+Diuron	-Diuron	+Diuron
Leaf	78	0	51	0
Style	72	0	60	0

Values measured are in  $\mu\text{mol O}_2 \text{mg}^{-1} \text{chlorophyll h}^{-1}$ .

<sup>a</sup>The reaction to ferricyanide was carried out in the presence of 1  $\mu\text{M}$  ferricyanide and 5 mM  $\text{NH}_4\text{Cl}$ . Diuron concentration was 30  $\mu\text{M}$ . The average of three experiments is presented.

<sup>b</sup>The reaction to methyl viologen was carried out in the presence of 50  $\mu\text{M}$  methyl viologen and 5 mM  $\text{NH}_4\text{Cl}$ . The average of four experiments is presented.



**Figure 3.** Quantitative analysis of the small subunit of Rubisco and the light harvesting complex protein by immunoblot analysis.

(a) Lanes 1–4 contain 10, 50, 250 and 2500 ng of soluble extract from tobacco leaves; lane 5 contains 10 000 ng of soluble extract from transmitting tract cells. Lanes 4 and 5 were derived from samples containing equal amounts of chlorophyll. The immunoblot was developed with antibody specific for the small subunit (SSU) of Rubisco.

(b) Lane 1 contains 2500 ng of total leaf proteins; lane 2 contains 10 000 ng of total extract from transmitting tract cells. Lanes 1 and 2 contain equal amounts of chlorophyll. The immunoblot was developed with antibody specific for the light harvesting complex protein (LHCP).

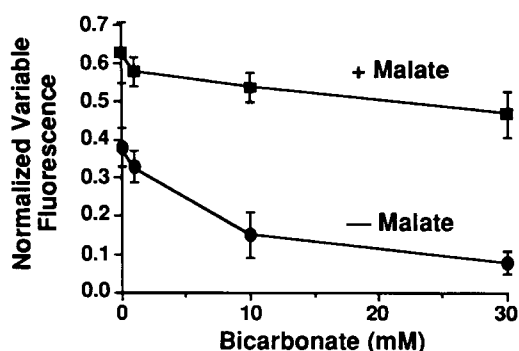
plays a role in draining reduced constituents produced during photosynthetic electron transport. As a comparison, the tissues were examined for the presence of light-harvesting complex (LHC II). Soluble and membrane fractions were prepared from transmitting tract cells and leaves and analysed by immunoblotting. When equal chlorophyll levels of membrane-bound protein were fractionated on denaturing PAGE, no difference was detected in the amount of LHC between styles and leaves (Figure 3b). This is consistent with the similarity of photosynthetic electron transport capabilities of both tissues (Table 1). However, less than 0.1% of the amount of Rubisco small subunit could be detected in the soluble protein extracts from transmitting tract cells as compared with leaves (Figure 3a). As expected from this result, we could not detect any Rubisco activity in stylar extracts measured by  $\text{H}^{14}\text{CO}_3^-$  incorporation into 3-phosphoglycerate (data not shown). The deficiency of Rubisco in transmitting tract cells would result in the accumulation of reduced constituents, e.g. NADPH. The concurrent depletion of  $\text{NADP}^+$ , the acceptor for electrons from light reactions would inhibit electron transport and cause high variable fluorescence. To confirm this supposition, we analysed the variable fluorescence kinetics of leaves from a heterotrophic tobacco mutant, XV1, which lacks Rubisco (Fluhr *et al.*, 1985). The maternally inherited chloroplast-based mutation has been traced to a single amino acid change in the large subunit of Rubisco, that prevents proper assembly. The green leaves of this mutant have normal electron transport capacity (Avni *et al.*, 1989). We observed that dark-adapted leaves of XV1 also had high unchanging variable fluorescence (Figure 2). Thus, the absence of efficient dark reactions could by analogy be

responsible for the enhanced fluorescence in isolated transmitting tract cells.

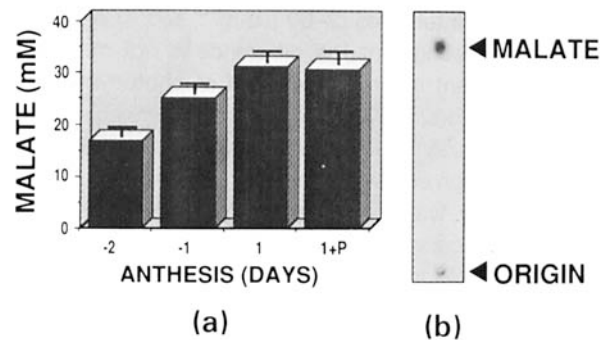
The cortex and epidermal tissues surrounding the transmitting tract are translucent and do not contain chloroplasts (Bell and Hicks, 1976). Thus, an alteration in fluorescence signal emanating from the whole style organ must originate from the transmitting tract tissue. However, we unexpectedly found that the fluorescence decay in intact styles was similar to that of normal leaves (Figure 2). We conclude that the mere detachment of transmitting tract tissue from the style, without noticeable damage, disrupted the ability of the cells to utilize photosynthetic light. This observation, seemingly in contradiction with our conclusion that it was the lack of Rubisco that attenuated the photosynthetic flux, was resolved in the following experiments.

*Stylar phosphoenol pyruvate carboxylase (PEPC) mediates the bicarbonate-dependent fluorescence depression*

We surmised that there is a difference between ambient levels of CO<sub>2</sub> in the air and internal levels within the intact style. The effect of different CO<sub>2</sub> concentrations was tested by supplying the isolated cells with additional bicarbonate. The application of 10 mM bicarbonate reduced the variable fluorescence in isolated cells to that observed in intact styles (Figure 2). When the steady state level of fluorescence (achieved after 2–3 min of light) was examined, a bicarbonate-dependent depression of variable fluorescence was observed (Figure 4). Hence, high levels of CO<sub>2</sub> are necessary for photosynthesis in the isolated Rubisco-deficient transmitting tract tissue. Using the depression in relative fluorescence shown in Figure 4 as a calibration curve we estimate the internal concentration of bicarbonate realized in the intact style (normalized



**Figure 4.** Effect of bicarbonate and malate on the steady state variable fluorescence of isolated transmitting tract cells. Experimental measurements were carried out as described in Figure 2 except actinic light was 436 nm. The levels of variable fluorescence after 3 min of actinic light are shown. Average and standard error of three independent experiments are shown. Isolated cells were pre-incubated with or without 30 mM malate and 0, 1, 10 and 30 mM of KHCO<sub>3</sub>.



**Figure 5.** Accumulation of malate in styles and isolated transmitting tract cells.

(a) Styles were harvested 2 days before (–2), 1 day before (–1) or on the day of anthesis. On the day of anthesis styles were isolated either without or 12 h after pollination (1 and 1+P, respectively). Malate concentration in the total style organ and standard error of five experimental measurements for each developmental stage are shown.

(b) Autoradiograph of TLC-fractionated alcohol soluble extracts of transmitting tract cells. Cells were incubated for 10 min in the light in the presence of 1 mM KH<sup>14</sup>CO<sub>3</sub> at a specific activity of 0.5 mCi mmol<sup>–1</sup>. Extracts were fractionated on a TLC plate as described in the Experimental procedures. The following organic compounds were tested and resolved: fumarate, pyruvate, oxalate, succinate, malate, citrate, aspartate, and phosphoglycerate.

variable fluorescence of 0.2, Figure 2) as grossly between 1 and 10 mM.

The carboxylation of phosphoenol pyruvate (PEP) catalysed by PEPC to obtain oxaloacetate (OAA) and the subsequent reduction of OAA by NADPH to yield malate offer a good alternative pathway for CO<sub>2</sub> fixation in styles. The recycling of NADP would allow photosynthetic flux and minimize fluorescence emission. We therefore assayed for malate using a quantitative spectrophotometric assay. Indeed, malate was found to be a major organic acid constituent accumulating during development, reaching levels of 30 mM in total intact styles on the day of anthesis (Figure 5a). The calculated levels of malate in transmitting tract cells were 75–120 mM. No change in malate levels was detected 12 h after pollination on the day of anthesis. Labelling of isolated transmitting tract cells with KH<sup>14</sup>CO<sub>3</sub> showed 60–70% of the acid non-volatile radioactivity incorporated into malate (Figure 5b). Additional malate-derived products were detected in extracts of transmitting tract tissue on the day of anthesis by HPLC. The analysis showed total soluble amino acids accumulating to levels of 8 mM. The major amino acids were asparagine, serine, glutamine and aspartate, constituting 35, 18, 11 and 9% of the total, respectively (data not shown).

Coupling between photosynthetic light reactions and PEPC-mediated reactions in isolated transmitting tract cells was confirmed by examining the effect of the addition of malate together with bicarbonate. PEPC is located in the cytoplasm and non-sequestered malate acts as a specific and potent feedback inhibitor of PEPC from many plant sources (O'Leary, 1982). Indeed, when malate was

**Table 2.** Characterization of apparent enzymatic parameters of PEPC estimated in desalted crude stylar extracts or in washed intact transmitting tract cells in the light and dark

Extract or tissue type	$K_m$ PEP (mM)	$K_m$ $\text{HCO}_3^{-1}$ (mM)	$V_{\max}$ ( $\mu\text{mol min}^{-1}$ )	$K_i$ malate (mM)
Enzyme extract	0.25	0.51	23 <sup>b</sup>	12
Intact cells <sup>a</sup>				
Light	—	2.7	20 <sup>c</sup>	—
Dark	—	3.7	15 <sup>c</sup>	—

<sup>a</sup>The reaction measuring carbon fixation into malate in transmitting tract cells did not necessitate the exogenous addition of PEP over the whole range of bicarbonate tested (0–50 mM).

<sup>b</sup> $V_{\max}$  is expressed per mg protein.

<sup>c</sup> $V_{\max}$  is expressed per mg chlorophyll.

added to isolated transmitting tract cells the bicarbonate-induced decrease in the variable fluorescence was nullified (Figure 4). Without added bicarbonate, the levels of variable fluorescence as a result of malate addition were higher. Presumably, malate inhibits the low rate of carboxylation afforded by the ambient amounts of  $\text{CO}_2$  present in the atmosphere or respired by the tissue. The addition of malate to thylakoids isolated from transmitting tract cells had no effect on fluorescence (data not shown). Thus the effect of malate in whole cells was not directly on photosynthetic electron transport and can be ascribed to the inhibition of dark reactions in transmitting tract cells. As we have shown that malate actually accumulates in styles (Figure 5a), it must be sequestered.

Crude soluble extracts of styles with low molecular weight metabolites removed, were assayed for PEPC to ascertain that PEPC activity was indeed present and to quantitate the outstanding kinetic properties of this enzyme. Analysis of style extracts provided apparent  $K_m$  values for PEP and bicarbonate and apparent  $K_i$  values for malate (Table 2). The apparent  $K_m$  values measured in style extracts are in the range found for other  $\text{C}_3$ -type plants, and significantly less than those obtained for  $\text{C}_4$  plants (Ashton *et al.*, 1990; Hatch, 1987). We note that both the apparent  $K_m$  value for bicarbonate and the apparent  $K_i$  value for malate are in the concentration range where we observed effects on the variable fluorescence. This supports our conclusion that stylar chloroplasts are functionally coupled to PEPC-mediated dark reactions.

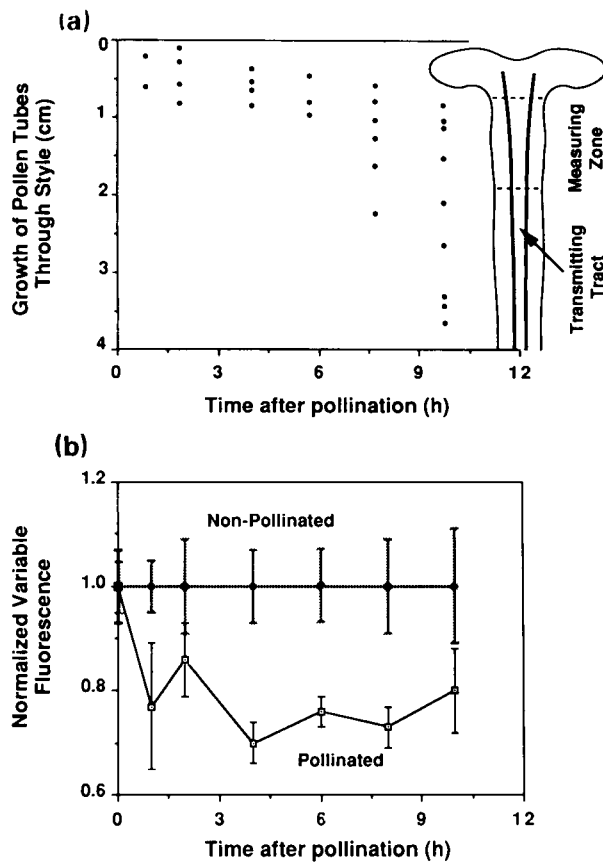
It is important to establish the relevance of the potential PEPC activity measured *in vitro* to the activity in the intact cell. This was ascertained by labelling isolated transmitting tract cells with [ $^{14}\text{C}$ ]bicarbonate and by quantifying [ $^{14}\text{C}$ ]malate formation. Whole cell measurements of the apparent  $K_m$  for exogenously added bicarbonate differ somewhat from the *in-vitro* measurements (Table 2) and indicate that additional factors control bicarbonate fixation. The relatively high *in-vitro* and 'whole cell' apparent  $K_m$

value for bicarbonate (0.5–3 mM) is consistent with the observation that cells exposed to atmospheric levels of  $\text{CO}_2$  show high unadjusted levels of variable fluorescence. Indeed, exogenous concentrations of 1 mM bicarbonate and more were necessary to stimulate photosynthetic flux (Figure 4).

The PEPC pathway, especially in CAM plants, operates in the dark. We therefore measured the kinetics of malate accumulation of isolated transmitting tract cells in light and dark. Malate synthesis was found to occur in the dark, albeit at a slightly reduced rate (Table 2). We conclude that not only the chloroplast but also other sources of reducing power, e.g. the mitochondria, can contribute to malate formation. Thus, although stylar chloroplasts require malate synthesis to release reducing potential, malate synthesis does not necessarily require chloroplast-based light reactions.

#### *Increased total stylar respiration as a result of pollination reduces variable fluorescence*

The high photosynthetic rate supported by transmitting tract tissue within intact non-pollinated styles indicates high cellular concentrations of bicarbonate. Presumably, the source of bicarbonate is the very active respiratory output of transmitting tract cells within the closed confines of the style. We would expect that in pollinated styles the additional respiration of hundreds of elongating pollen tubes, or pollination-induced enhancement of stylar respiration, directly influence the homeostatic metabolic status of the style. The increased rate of  $\text{CO}_2$  production associated with pollen tube growth should therefore be reflected in a depression of variable fluorescence (increased photosynthetic flux). To test this, styles were detached from flowers before dehiscence and hand pollinated. The variable fluorescence was recorded during the growth of tubes through the style (Figure 6a) and compared with the variable fluorescence of detached non-



**Figure 6.** Steady state variable fluorescence in pollinated and non-pollinated styles.

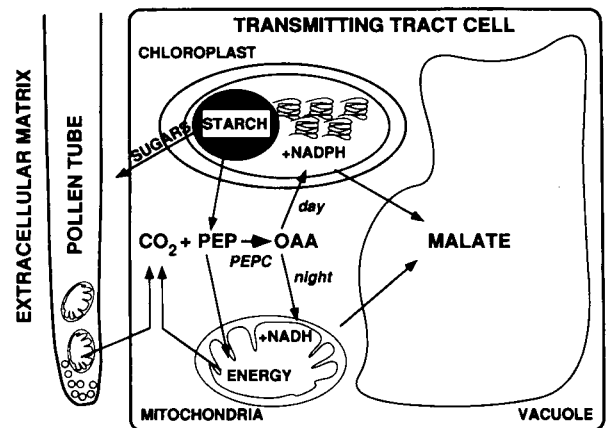
(a) Transverse sections of pollinated styles were stained with sirofluor and viewed microscopically. The extent, in cm, of pollen tube growth was measured. Dots represent individual styles and are the average of two independent measurements. The schematic drawing of the style illustrates the position of the fluorescence probe. Hand pollination was performed immediately after the first fluorescence measurement ( $t = 0$ ).

(b) Fluorescence measurements were carried out as described in Figures 2 and 4. The steady state levels of fluorescence after 3 min are recorded. All measurements were made in a zone that extended from 0.5 to 1.5 cm below the stigma surface. Sets of styles aligned side-by-side were measured. The average and standard error of six independent experiments are shown. Variable fluorescence at each time point was normalized to that of non-pollinated styles.

pollinated styles (Figure 6b). A measurable decrease (approximately 20%) in the relative variable fluorescence was apparent well before the bulk of pollen tips reached the point of measurement (Figure 6a). The relative variable fluorescence remained depressed after the bulk of pollen tubes had passed the measuring point and throughout the duration of the experiment. In analogy to the effect of bicarbonate shown previously (Figures 2 and 4), we propose that the increased photosynthetic flux is due to additional pollination-enhanced stelar metabolic activity.

## Discussion

This work shows that the chlorophyllous transmitting tract tissue from the  $C_3$  plant *N. tabacum* is devoid of Rubisco.



**Figure 7.** Proposed scheme of PEPC pathway in stelar tissue.

Starch is the source of PEP. PEP can be utilized for energy and fixation of respiratory  $CO_2$  into malate. PEPC regulates the conversion of PEP into OAA. The reduction of OAA into malate is dependent on mitochondrial functions in the dark and would be assisted by chloroplasts in the light. Accumulating malate is sequestered in the vacuole.

Nonetheless, styles are photosynthetically active. Using a non-invasive fluorescence technique to measure photosynthetic flux, we show that this flux depends on the availability of bicarbonate. Detection of incorporated radioactive tracers and quantitative malate measurements indicate PEPC-mediated carbon fixation in styles as depicted in Figure 7. Bicarbonate is fixed by PEPC to OAA which is subsequently reduced to malate. It is this reduction step which enables the chloroplasts to release accumulating reducing power and maintain low variable fluorescence. Without sufficient bicarbonate, malate production ceases, the photosynthetic apparatus quickly becomes reduced as NADPH accumulates and further photosynthetic flux is blocked. We conclude, from the stimulatory effects of bicarbonate and inhibitory effects of malate on photosynthesis, that stelar chloroplasts are functionally coupled to PEPC-mediated reactions which result in malate formation. PEPC is a cytoplasmic enzyme, hence the reduction of OAA, via the chloroplast NADP-malate dehydrogenase would call for transport of OAA to the chloroplast. A similar scenario has been suggested for  $C_3$  guard cell chloroplasts (Gotow *et al.*, 1985). We show that malate formation occurs both in the light and in the dark. While chloroplasts supply reducing energy in the light, in the dark other sources of reducing energy, e.g. mitochondria, would catalyse OAA reduction. Plant mitochondria have well-developed specific translocators for OAA which would facilitate its metabolism (Ebbighausen *et al.*, 1985).

Carbon fixation related to that found in styles occurs in stomatal guard cells of *Commelina communis*, *Tulipa gesneriana* and *Pisum sativum*. Their guard cells contain chloroplasts, but do not possess an efficient reductive pentose phosphate pathway (Raschke and Dittrich, 1977; Zeiger, 1983). While the similarities between guard cell

and transmitting tract cell metabolism appear striking, there are important differences. We have shown the direct dependence of stylar photosynthesis on the availability of bicarbonate. The control of malate synthesis in guard cells is more complex. In fact, in guard cells, malate accumulation and CO<sub>2</sub> concentration are related in a reciprocal manner (Mansfield, 1985).

The rather low affinity (high  $K_m$ ) of stylar PEPC for bicarbonate is in the normal range reported for C<sub>3</sub> plants (Ashton *et al.*, 1990). It explains why the fluorescence of isolated transmitting tract cells is high (low photosynthetic flux) when exposed to the atmosphere. The isolated cells respond to bicarbonate in the low millimolar range. It follows that in the intact style, where low fluorescence (high photosynthetic flux) is observed, a steady-state level of bicarbonate in the millimolar range is present. Thus, given the  $K_m$  of PEPC for bicarbonate (0.5–3 mM), the additional 20% elevation of photosynthetic flux observed in post-pollination styles is consistent with an increase in the bicarbonate concentration of less than 1 mM. The cuticular character of the style surface, the absence of stomata and lack of contiguous air spaces, would all contribute to the accumulation of respiratory CO<sub>2</sub>. The observed internal bicarbonate levels are only estimates as the minute dimensions of the tissue preclude facile direct measurement of bicarbonate levels in closed-type styles. However, they appear to be compatible with measurements obtained from other plants. Concentrations of over 2% CO<sub>2</sub> (approximately 3 mM bicarbonate based on pH 7) have been reported in the cavity of lily styles (Sfakiotakis *et al.*, 1972). In other respiring organs, such as fruits, concentrations of bicarbonate above 10 mM (>7%) have been recorded (Blanke and Lenz, 1989).

PEPC-mediated malate formation accounts for two remarkable structural features of the specialized transmitting tract cells; the prominent starch granules in the chloroplast and the large vacuole (Figure 1). The source of starch may be metabolic import. The breakdown of stored starch would have a dual function of supplying PEP as an energy substrate and as a substrate for PEPC (Schnabl, 1981). The resultant product of PEP carboxylation, malate, would be sequestered in the large vacuoles of the transmitting tract cells (Figure 7). We have shown that malate can inhibit transmitting tract PEPC activity, hence sequestration is a prerequisite to resolve potential feedback inhibition. Accumulation of malate in the vacuoles of guard cells (Robinson and Preiss, 1985), photosynthetic cells of CAM plants (Winter, 1985) and fruit tissue has been well documented (Blanke and Lenz, 1989). Clearly the capacity of transmitting tract vacuoles for malate accumulation is limited and its further metabolic utilization must be considered. Apparently, some malate is further transaminated into other useful intermediate compounds like aspartate and asparagine, which were detected in

elevated amounts. Thus, energy storage or supplying metabolic precursors may be a goal of PEPC-mediated malate formation. Alternatively, the presence of PEPC activity may enable these cells to maintain a homeostatic level of bicarbonate. High CO<sub>2</sub> concentrations can directly inhibit Krebs cycle enzymes (Ranson *et al.*, 1960; Wager, 1974) or indirectly influence cellular processes *via* effects on cytosolic pH (Latzko and Kelly, 1983). The proposed functions of PEPC in the styles are consistent with the long list of tentative capacities for the multifaceted PEPC.

## Experimental procedures

### Plant material

*N. tabacum* cv. 'Samsun' plants were grown in the greenhouse, in 18 h day, 26°C and 6 h night, 22°C diurnal cycles. Unpollinated styles were excised from flowers 1 day prior to anthesis. Pollen was taken from young flowers immediately following dehiscence. XV1 mutant was grown heterotrophically as described by Fluhr *et al.* (1985). Transmitting tract cells were isolated from freshly picked styles. Median longitudinal sections (halves) were prepared and the internal tissues gently excised with a scalpel. Cells were maintained on 10 mM phosphate buffer (pH 7) and used immediately.

### Microscopy

Visual inspection of pollen tube growth was based on sirofluor staining of callose (Stone *et al.*, 1984). Freehand transverse sections of pollinated styles were stained and viewed microscopically under 450–490 nm excitation, 510 nm dichromatic beam splitter and 520 nm barrier filter (Zeiss). Tissue sections for electron micrography were prepared as described in Ori *et al.* (1990). Electron micrographs were made with a Philips 410 microscope operated at 80 kV.

### Measurements of chlorophyll a fluorescence

Measurements were made using modulated light of low intensity (650 nm, 0.2  $\mu\text{E m}^{-2} \text{sec}^{-1}$ ) from a pulse chlorophyll a fluorometer (Walz, Effeltrich, Germany) operated at 100 kHz pulse frequency. Actinic light (650 nm or 436 nm, 40  $\mu\text{E m}^{-2} \text{sec}^{-1}$ ) and photosynthetically saturating light (white, 1000  $\mu\text{E m}^{-2} \text{sec}^{-1}$ ) were optionally added to the pulsed modulated light. Dark-adapted samples were first irradiated with the modulated light alone to determine the base fluorescence level ( $F_0$ ), this was followed by a 2 sec flash of saturating light to obtain the level of the maximal fluorescence ( $F_m$ ). Subsequently the samples were irradiated for 2 or 3 min in actinic light while the fluorescence was monitored continuously until a steady state fluorescence ( $F_t$ ) was achieved. The variable fluorescence ( $F_v$ ) which is independent of absorbance and light scattering was calculated at any time  $t$  according to Schreiber *et al.* (1986), as  $F_v = (F_t - F_0)/(F_m - F_0)$ .

### Protein gels and immunoblotting

Tissue samples were prepared, fractionated in denaturing gels and immunoblotted as described in Lotan and Fluhr (1990). Antibodies to LHCP proteins were described in Broido *et al.* (1991). Antibodies to the small subunit of Rubisco were described in Avni *et al.* (1989).

*Measurements of electron transport and enzyme activities*

Thylakoids were isolated and electron transport was measured as detailed in Avni *et al.* (1989). For measurements of enzymatic activities, tissues were ground frozen in 25 mM Tris-HCl, pH 8, 5 mM MgCl<sub>2</sub> and 1 mM DTT. The frozen slurry was thawed and low molecular weight contaminants were removed by passing the extracts through a BioRad P6 column (0.3 ml extract in 3 ml bed volume) equilibrated in grinding buffer. Rubisco and PEPC activity were measured by incorporation of [<sup>14</sup>C]bicarbonate (1.9 Bq mmol<sup>-1</sup>) using ribulose-1,5-bisphosphate and phosphoenolpyruvate as substrates, respectively. The assay for Rubisco was carried out as described by Keys and Parry (1990). The assay for PEPC activity was carried out according to Ashton *et al.* (1990). Thin layer chromatography of labelled compounds was carried out on cellulose TLC plates (Machery-Nagel DC-Fertigplatten Cel 300–25) and developed in diethyl ether:formic acid:water (7:2:1) as described by Myers and Huang (1968). Total malate concentrations were determined spectrophotometrically by the enzymatic reduction of NAD in the presence of malate dehydrogenase as described in Van Kirk and Raschke (1978).

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