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Stimulation by Light of Rapid pH Regulation in the Chloroplast Stroma in Vivo as Indicated by CO_2 Solubilization in Leaves[1]

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Leaves of *Brassica oleracea, Helianthus annuus,* and *Nicotiana rustica* were exposed for 20 s to high concentrations of CO_2. CO_2 uptake by the leaf, which was very fast, was measured as a transient increase in the concentration of oxygen. Rapid solubilization of CO_2 in excess of that which is physically dissolved in aqueous phases is proposed to be caused by bicarbonate formation in the stroma of chloroplasts, which contain carbonic anhydrase. On this basis, pH values and bicarbonate accumulation in the chloroplast stroma were calculated. Buffer capacities were far higher than expected on the basis of known concentrations in the chloroplast stroma. Moreover, apparent buffer capacities increased with the time of exposure to high CO_2, and they were higher when the measurements were performed in the light than in the dark. During prolonged exposure of leaves to 16% CO_2, calculated bicarbonate concentrations in the chloroplast stroma exceeded 90 mM in the dark and 120 mM in the light. The observations are interpreted as indicating that under acid stress protons are rapidly exported from the chloroplasts in exchange for cations, which are imported. The data are discussed in terms of effective metabolic pH control by ion transport, first across the chloroplast envelope and, then, across the tonoplast of leaf mesophyll cells. The direct involvement of the vacuole in the regulation of the chloroplast pH in leaf cells is suggested.

In living cells, proton concentration is an important factor influencing enzyme activities and membrane energization. Internally generated acid is under metabolic control and is usually balanced by base, but acid may also be introduced from outside the cellular system. For example, acidic air pollutants such as SO_2 can act on cells by decreasing cellular pH (Laisk et al., 1988a, 1988b; Veljovic-Jovanovic et al., 1993; Heber et al., 1994). Very high concentrations of CO_2, which dissolves in water to form carbonic acid, inhibit photosynthesis of isolated chloroplasts even though CO_2 is a substrate for photosynthesis (Werdan et al., 1975; Wagner et al., 1990). However, in vivo, no inhibition of photosynthesis in leaves is observed at comparable concentrations of CO_2 (Heber et al., 1994).

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Apparently, pH homeostasis can be maintained in vivo but not in vitro. Stabilizing mechanisms control pH in different leaf cell compartments (Wagner et al., 1990; Gout et al., 1992; Heber et al., 1994). Damage or metabolic inhibition caused by proton accumulation results only when these mechanisms are overtaxed. We are interested in knowing how much acid load can be tolerated by plant leaves.

Differences of pH exist not only between the vacuole and cytoplasm of mesophyll cells but also, at least in the light, within the cytoplasm between cytosol and the chloroplast stroma (Yin et al., 1990), although the inner envelope of isolated chloroplasts has been shown to be somewhat permeable to protons (Werdan et al., 1975; Bligny et al., 1990). The stroma of illuminated chloroplasts may approach pH 8.0 (Heldt et al., 1973; Laisk and Oja, 1988; Wu and Berkowitz, 1992a), whereas cytoplasmic pH is maintained between 7.0 and 7.5 (Spanswick and Miller, 1977; Kurkdjian and Guern, 1989; Bligny et al., 1990). A light-driven transport of protons out of isolated chloroplasts into the suspending medium has been observed by several authors (Heber and Krause, 1971; Heldt et al., 1973; Enser and Heber, 1980; Demmig and Gimmler, 1983). Maintenance of high stromal K^+ and pH are coincident, and K^+/H^+ counterflux is known to occur across the chloroplast envelope (Demmig and Gimmler, 1979, 1983; Huber and Maury, 1980; Maury et al., 1981). However, the system of transport proteins in the chloroplast envelope that facilitates and regulates K^+ and H^+ fluxes is not well understood. The existence of separate uniport channels for H^+ and K^+ has been suggested (Wu and Berkowitz, 1992a, 1992b). Movement of H^+ through a proton channel was speculated to be regulated by an electroneutral K^+ countercurrent through the K^+ uniport pathway. An ATPase is known to be a major component of the total intrinsic protein of the chloroplast envelope (Heldt, 1969; Nguyen and Siegenthaler 1983; Coves et al., 1988; Douce and Joyard, 1990). The H^+-transporting role of this ATPase has been documented (Douce et al., 1973; Robinson, 1985), and it has been suggested that the energy-dependent K^+ flux across these membranes occurs as a consequence of primary H^+ pumping (Wu and Berkowitz, 1992a, 1992b; Berkowitz and Peters, 1993).

Most of the above-described work has been carried out with isolated chloroplasts and envelope vesicles. The re-

ported K^+ and H^+ transport rates have been slow. Little is known about the in vivo system, i.e. about ion fluxes across the chloroplast envelope in leaves and their role in pH control. Such control is of primary importance for photosynthesis because electron flow through PSII is regulated by the intrathylakoid pH (Siebke et al., 1990; Krause and Weis, 1991), which depends not only on light but also on the pH of the chloroplast stroma. Recent observations of a fast recovery of photosynthesis of leaves after an initial inhibition by very high CO_2 concentrations (20% or more in air) suggest fast pH regulation in the chloroplasts of leaves (Heber et al., 1994).

In vivo, the pH of the chloroplast stroma may be calculated from the Henderson-Hasselbalch equation on the basis of equilibrium CO_2 and bicarbonate concentrations (Segel, 1976), which can be measured with modern leaf gas-exchange methods. CO_2 has already been used as a pH probe in isolated chloroplasts (Werdan et al., 1975). With the development of rapid-response gas-exchange techniques (Oja et al., 1986), it became possible to measure the total amount of dissolved gaseous CO_2 and bicarbonate and its light-induced changes in the chloroplasts of leaves (Oja et al., 1986; Hauser et al., 1995; Oja and Laisk, 1995a). Results have been confirmed by other techniques with leaf protoplasts (Heineke and Heldt, 1988).

In this report we further develop the gas-exchange technique to measure CO_2 solubilization and desolubilization in leaves and apply it to the study of pH-stabilizing processes in the chloroplast stroma of intact leaves. We used CO_2 to generate an external acidic load on chloroplasts, measured the bicarbonate pools accumulated in the chloroplast stroma under different CO_2 concentrations, and calculated the corresponding pH shifts.

MATERIALS AND METHODS

The novel principle used in this work is based on the measurements of changes in O_2 concentration caused by uptake or evolution of CO_2 during solubilization or desolubilization of this gas in a leaf (Oja and Laisk, 1995b). When CO_2 is removed from a stream of gas containing CO_2, O_2, and N_2, the concentrations of O_2 and N_2 (amounts per unit volume) increase. They decrease when CO_2 is added back to the stream of gas by CO_2 evolution from the leaf. For O_2, such an increase or decrease in concentration was measured and related to the amounts of removed or added CO_2. Leaves used were from potted plants of *Nicotiana rustica* (tobacco), *Helianthus annuus* (sunflower), and *Brassica oleracea* (cabbage) that were grown in a greenhouse with a supplement of additional light (500 μmol m^{-2} s^{-1}) during daylight hours.

The system used for rapidly changing CO_2 concentration and measuring O_2 concentration in a gas stream contained two open (flow-through) gas lines, called channels A and B. A zirconium oxide O_2 analyzer (S-3A/I; Ametek, Pittsburgh, PA) was used as a detector for changes in CO_2 concentration. It was connected to the leaf chamber (4.2 × 4.2× 0.3 cm^3), which permits laminar gas flow. At first the leaf chamber was set into channel A with a stream of gas containing either no CO_2 (dark experiments) or 450 ppm CO_2 (experiments performed in the light). Rapid changes from channel A to channel B with a gas stream containing variable amounts of CO_2 were facilitated by a special four-way switch. CO_2 concentrations were varied in channel B from 1 to 16%. O_2 concentrations were set equal in both channels by adding a corresponding amount of N_2 into the flow of channel A while CO_2 was added in channel B. Gas flows and concentrations were adjusted by flow controllers (Tylan General, Eching, Germany) so that switching the empty chamber (without leaf) from channel A to B and back did not cause an appreciable signal on the O_2 analyzer. A small transient caused by replacement of N_2 with CO_2 in the O_2 analyzer was taken into account, and corresponding corrections were made. With a leaf in the chamber, CO_2 was taken up from the stream after switching from channel A to channel B. This caused a transient increase in the O_2 concentration, proportional to the rate of CO_2 uptake. Correspondingly, the O_2 concentration decreased proportionally to the rate of CO_2 evolution when the chamber was returned to channel A. The amount of dissolved CO_2 was found by integrating the transient uptake and evolution peaks. The recording was integrated during the first 6 s to determine only rapidly solubilized or desolubilized CO_2.

Measured CO_2 consisted of dissolved gaseous CO_2 plus the CO_2 derived from bicarbonate. The CO_2/bicarbonate conversion is considered to be fast in C_3 plants only in the chloroplast stroma, where carbonic anhydrase (EC 4.2.2.1) is present (Tsuzuki et al., 1985; Burnell et al., 1990). Calculations showed that solubilization of CO_2 as gas (or desolubilization, with catalyzed conversion to or from bicarbonate) was practically complete in whole cells, including the vacuoles, within 4 s (Oja and Laisk, 1995b). Uncatalyzed bicarbonate formation was slow. Thus, with carbonic anhydrase catalyzing CO_2/bicarbonate conversions, the integral represents the amount of gaseous CO_2 dissolved in whole cells plus the amount of bicarbonate in the chloroplast stroma.

At the beginning of experiments and during their course, transpiration was measured as dilution of O_2 in the gas stream B by the water vapor released from the leaf (in Fig. 1, see change E in O_2 concentration upon incorporation of the leaf into the cuvette). By this procedure the stomatal resistance could be calculated from the increase in O_2 concentration. During the experiment, transpiration was measured by replacing the leaf chamber by an equivalent tube without leaf and recording the change in O_2 concentration. In Figure 1, the stomatal resistance for CO_2 was 4.2 s cm^{-1}. It was calculated using the formula

$$r_s = \frac{1}{\left(F \times O_2 \times \left(\frac{1}{LC} - \frac{1}{EQ}\right) \times \frac{1}{A} \times \frac{1}{M}\right)} \times D,$$

where F means the flow rate in channel B in μmol O_2 s^{-1}. LC and EQ are oxygen concentrations in percent in air and in channel B with the leaf chamber (LC) or equivalent (EQ). A is the leaf area in cm^2, M the concentration difference of water vapor between leaf and chamber in μmol ml^{-1}, and D the ratio of diffusion coefficients for water and CO_2 in

air. Stomatal opening was checked routinely during long experiments and CO_2 solubility measurements were carried out only when r_s was below 10 s cm^{-1}.

Chl was determined according to the method of Arnon (1949). Water content of leaves was measured by drying. The CO_2 solubility in water is given by the Bunsen coefficient. The effect of ionic strength of the cell medium on solubility was taken into account (Segel, 1976), and the coefficient used was 0.8 mL CO_2 (mL of aqueous solution)$^{-1}$ at 22.7°C, the temperature at which the experiment with darkened leaves was carried out. In the light, leaf temperature increased by only 0.8°C, since the leaf was glued by starch paste to the thermostated glass window of the leaf chamber.

pH values and bicarbonate concentrations in the chloroplast stroma of the leaves could be calculated from CO_2 uptake transients such as shown in Figure 1 using the Henderson-Hasselbalch equation:

$$pH = pK + \log (HCO_3^-/CO_2).$$

The water and the Chl content per leaf area, the stroma volume of 40 μL mg^{-1} Chl (Oja et al., 1986; Winter et al., 1993), and the solubility of CO_2 in water served as the basis for the calculations. The pK of the reaction

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$$

was taken to be 6.1 at the ionic strength of the chloroplast stroma (Yokota and Kitaoka, 1985).

RESULTS

Figure 1 shows an example of changes of O_2 concentration produced by the solubilization of CO_2 when a cabbage leaf was suddenly transferred from a gas stream without CO_2 to another stream with 8% CO_2 (positive spikes) and back (negative spikes).

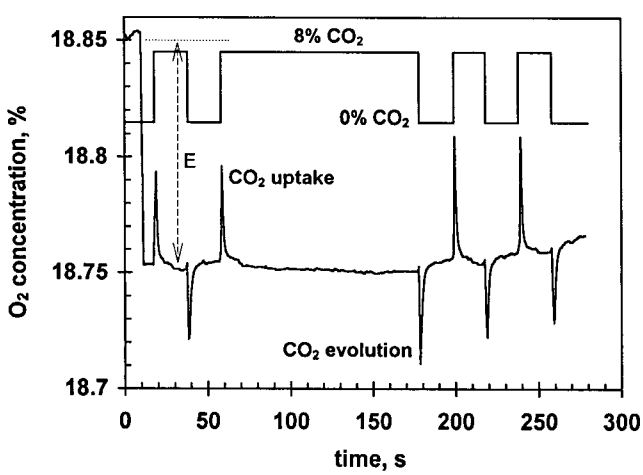

Figure 1. Transients of CO_2 uptake by a cabbage leaf (positive spikes) from a gas stream containing 8% CO_2, 18.75% O_2, 0.1% water vapor, and N_2 and release of CO_2 (negative spikes) after uptake into a gas stream without CO_2 but containing 18.75% O_2, 0.1% water vapor, and N_2 as revealed by changes in O_2 concentration. Difference E shows the amount of leaf transpiration. Switchings between the gas streams A (0% CO_2) and B (8% CO_2) are shown in the upper part of the figure. For further explanation, see text.

When the leaf chamber was switched from channel A (without CO_2) to channel B (with CO_2), CO_2 uptake caused a transient increase in O_2 concentration. A change from channel B to A resulted in the desolubilization of CO_2, which caused a transient decrease in the O_2 concentration of the gas stream. It should be noted that the transients were not completely symmetrical. The rapidly solubilized CO_2 slightly exceeded the rapidly desolubilized CO_2, especially when exposure to high CO_2 was prolonged. Apparently, after prolonged exposure to high CO_2, not all of the rapidly solubilized CO_2 was liberated as fast as it was taken up. This was also seen in other experiments (not shown). The reason for observed hysteresis is not yet clear. Hatch (1991) has reported inhibition of carbonic anhydrase by high concentrations of CO_2, but the kinetics of CO_2 release in Figure 1 is not consistent with such an explanation. To avoid the asymmetry of CO_2 uptake and release, and to characterize the status of the leaf prior to exposure to high CO_2, only uptake transients were used in the following experiments to calculate pH values and accumulation of bicarbonate in the chloroplast stroma.

Figure 2 demonstrates the influence of compartmental volumes (stroma as part of them) on calculated bicarbonate accumulation and pH in the compartment(s), where carbonic anhydrase is localized, when a cabbage leaf was exposed to CO_2 at concentrations increasing, from left to right, from 1% to finally 16% in the gas phase. Obviously, the higher the CO_2 concentration, the lower the pH and the higher the final bicarbonate concentration. However, there was also a considerable influence of compartmental volumes on calculated pH and bicarbonate concentration. Large compartmental volumes go with relatively low pH and low bicarbonate concentrations and vice versa. The calculations of the data shown in Figure 2 were based on compartment volumes of 30, 40, 60, and 80 μL mg^{-1} Chl. Because carbonic anhydrase has been reported to be localized only in the chloroplast stroma (Graham et al., 1984; Tsuzuki et al., 1985), we used a stroma volume of 40 μL mg^{-1} Chl in all further calculations (Oja et al., 1986; Winter et al., 1993).

Figure 3 shows results of such calculations for CO_2 uptake measurements. Leaves, still attached to the plant, were enclosed in the leaf chamber. For measurements in the dark, stomata were opened by exposing them for a prolonged period to CO_2-free air in the dark. For measurements in the light, stomata were opened under illumination (1700 μmol m^{-2} s^{-1}) in air with 450 ppm CO_2. After stomatal opening had stabilized, CO_2 measurements were started. During the experiments, CO_2 concentrations in channel B were changed, usually starting with 0.5% CO_2. Data presented in Figure 3 were calculated from the CO_2 uptake spike following the transition of the leaf to channel B. Time of exposure to CO_2 in channel B was 20 s. Between the exposures to high CO_2 the leaf was kept in CO_2-free air (dark) or 450 ppm CO_2 (light) for 5 min. This was sufficient to relax the effects caused by the previous exposure. The same procedure was subsequently performed with 1, 2, 4,

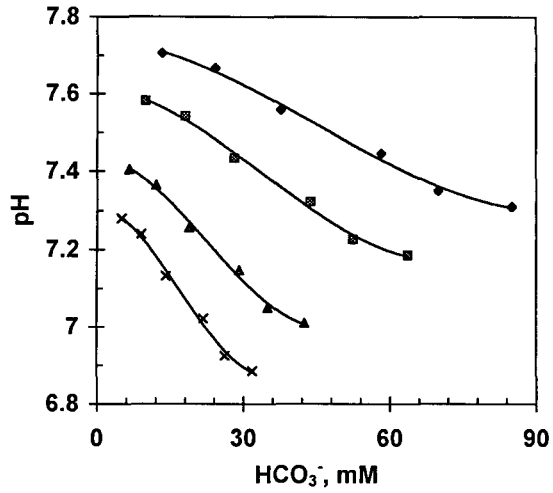

Figure 2. pH as a function of bicarbonate accumulation at different volumes of a compartment that contains active carbonic anhydrase. The curves are based on measurements from a cabbage leaf that was exposed to CO_2 in the light for 20 s. CO_2 concentrations were, from left to right, 1, 2, 4, 8, 12, and 16% in the gas phase. Compartmental volumes are 30 (diamonds), 40 (squares), 60 (triangles), and 80 μL mg^{-1} Chl (crosses). It is assumed that the stroma volume of chloroplasts, which contain carbonic anhydrase, is 40 μL mg^{-1} Chl.

8, 12, and 16% CO_2. Thus, data in Figure 3 represent CO_2 uptake curves of leaves in a state largely uninfluenced by the previous exposure in the dark and in the light.

Measurement in the dark (filled symbols in Fig. 3) revealed an initial pH of the chloroplast stroma from 7.6 to 7.75, depending on the source and the state of the particular leaf. The maximum CO_2 concentration of 16% caused the accumulation of 35 mM HCO_3^- in a senescing sunflower leaf, which caused the pH to decrease to 6.9 (filled triangles). In a cabbage leaf, 57 mM HCO_3^- was accumulated under 16% CO_2, and the pH decreased to only 7.15 (filled squares).

In the light (open symbols), the pH was usually less decreased by high CO_2 concentrations than in the dark and more bicarbonate accumulated in the chloroplast stroma. Similar results were obtained with a tobacco leaf (not shown). Under light conditions and in the presence of 16% CO_2, the cabbage leaf accumulated 71 mM bicarbonate in the stroma.

It was also observed that exposure to 0.5 and 1% CO_2 often decreased the pH more in light than in darkness. At first sight, this appears to disagree with the notion that pumping of protons from the chloroplast stroma into the intrathylakoid space causes alkalization of the chloroplast stroma in the light (Heldt et al., 1973; Oja et al., 1986). However, carboxylation of ribulose bisphosphate in the light by high CO_2 counteracts this effect, resulting in the formation of phosphoglyceric acid, which, in effect, can decrease the pH of the chloroplast stroma in the light compared to darkness.

In the experiment shown in Figure 4, which was carried out with a cabbage leaf, a short (20 s) loading was followed by a 120-s exposure to high CO_2. The routine of this experiment is shown as an example in Figure 1. The average of

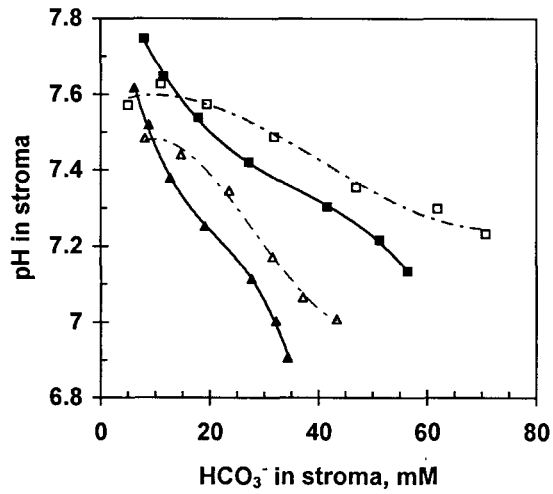

Figure 3. Changes of pH and accumulation of bicarbonate in the stroma of the chloroplasts of a cabbage leaf (squares) and a senescing sunflower leaf (triangles) after gassing the leaves with CO_2 at increasing concentrations (from left to right): 0.5, 1, 2, 4, 8, 12, and 16%. The leaves were darkened (filled symbols) or illuminated at a photon flux density of 1700 μmol m^{-2} s^{-1} (open symbols). Exposure times to high CO_2 were 20 s, and the leaf was exposed to CO_2-free air (dark) or air containing 450 ppm CO_2 (light) for 5 min before each exposure. It is assumed that the stroma volume of chloroplasts, which contain carbonic anhydrase, is 40 μL mg^{-1} Chl.

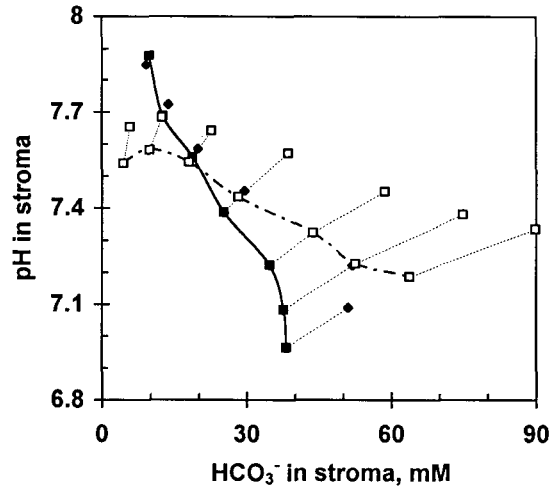

Figure 4. Effects of light and time of exposure to CO_2 on pH and accumulation of bicarbonate in the stroma of the chloroplasts of a cabbage leaf after gassing the leaf with the following CO_2 concentrations (from left to right): 0.5, 1, 2, 4, 8, 12, and 16%. An example of the routine of the experiment is shown in Figure 1. The average of the two first positive spikes, as in Figure 1 for 8% CO_2, and the average of the two last positive spikes (measured after the prolonged exposure of 120 s to high CO_2) are joined with a thin dashed line. Filled symbols, Experiment carried out in the dark; open symbols, experiment carried out under a photon flux density of 1700 μmol m^{-2} s^{-1}. Between the measurement cycles, the leaf was exposed to CO_2-free air (dark) or 450 ppm CO_2 (light) for 5 min. It is assumed that the stroma volume of chloroplasts, which contain carbonic anhydrase, is 40 μL mg^{-1} Chl.

the two first positive spikes in Figure 1 was used for the calculation of the unaffected CO_2 uptake curve (Fig. 4) and the average of the two last positive spikes was used to calculate the CO_2 uptakes obtained after the prolonged (2 min) exposure to high CO_2. A time interval of 5 min was used to relax the effects from previous exposures before the next CO_2 concentration was applied. Presentation of the data as titration curves (sequence of different CO_2 concentrations, Fig. 4) shows that after prolonged exposure to high CO_2 the pH of the stroma had become less sensitive to external CO_2. More bicarbonate accumulated and pH decreased to a lesser extent than before the prolonged exposure to high CO_2.

Figure 5 shows direct recordings of O_2 concentration changes in response to CO_2 uptake transients at exposures to 16% CO_2. The data are from the experiment shown in Figure 4. The curves demonstrate the sensitivity and noise level of the indirect method of measuring CO_2 solubilization by changes in O_2 concentration. It is also evident from the recordings that low solubilization of CO_2 is not caused by stomatal closure. If this were so, the curves with the smaller area should be flattened and shifted to the right compared to the curves with the greater area. In fact, stomata were only a little more closed in the dark experiment (filled squares) than in the light experiment (open symbols). This is apparent from the slight shift to the right in the position of the maximum of the curves. It is also important to note that the curves of Figure 5 reflect the kinetics of CO_2 uptake rather than the response time of the measuring system, which was faster than the relaxation of the observed transient.

The results of these experiments show that the ability of the chloroplast stroma to resist external acidification changed during the exposure to high CO_2. The response of the chloroplasts to high acidic load was further studied in an experiment in which a cabbage leaf was exposed to 16%

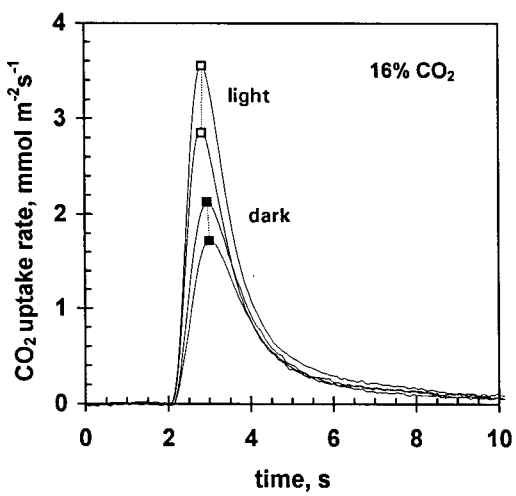

Figure 5. Recordings of rates of CO_2 uptake from a gas stream containing 16% CO_2 as shown by changes in O_2 concentration after subjecting a cabbage leaf to the CO_2 uptake cycles shown in Figure 4. Data points are denoted as in Figure 4. It is assumed that the stroma volume of chloroplasts, which contain carbonic anhydrase, is 40 μL mg^{-1} Chl.

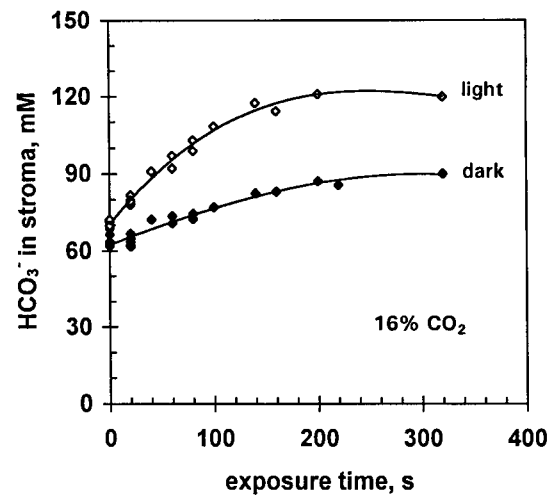

Figure 6. Bicarbonate accumulation in the chloroplast stroma after subjecting a cabbage leaf to a sequence of exposure times to 16% CO_2. Zero time shows bicarbonate accumulation when CO_2 is applied. The duration of the subsequent exposure was as shown on the abscissa and then CO_2 was removed. After 20 s, it was applied again, and the spike was measured. The leaf was exposed to 0 (dark) or 450 ppm CO_2 (light) for 5 to 10 min before the next cycle, with a longer exposure, was started. The degree of relaxation from the previous exposure is indicated by multiple data points at time 0. It is assumed that the stroma volume of chloroplasts, which contain carbonic anhydrase, is 40 μL mg^{-1} Chl.

CO_2. The first data point in Figure 6 at zero exposure time was obtained by switching the leaf to 16% CO_2. Other points were obtained by switching CO_2 off at the time indicated on the abscissa and on again for only 20 s. Uptake was measured during the 20-s exposure. After each such cycle, the leaf was exposed to 0 (dark) or 450 ppm CO_2 (light) for 5 to 10 min to relax the effect of the previous exposure to high CO_2. Completeness of the relaxation is indicated by the scattering of the multiple data points at zero exposure time.

The amounts of bicarbonate accumulating when CO_2 was switched on again after the prolonged exposure were calculated from the CO_2 uptake data and are shown on the ordinate of Figure 6. Bicarbonate levels increased as exposure times were increased until saturation was reached at about 120 mM bicarbonate in the light and about 90 mM in the dark after about 200 s of exposure to high CO_2. The rate of increase was faster in the light (0.43 mM s^{-1} or 39 μmol mg^{-1} Chl h^{-1}) than in the dark (0.17 mM s^{-1} or 15 μmol mg^{-1} Chl h^{-1}), as calculated from the initial slopes of the curves in Figure 6. Based on different and more qualitative evidence, the proton/cation exchange across the chloroplast envelope facilitating bicarbonate accumulation was estimated to have a capacity of about 40 μmol mg^{-1} Chl h^{-1} (Heber et al., 1994).

DISCUSSION

In earlier work on pH changes in the stroma of chloroplasts in vivo we measured CO_2 uptake by or evolution from leaves directly by a CO_2 gas-exchange measurement

(IRGA) method (Oja et al., 1986; Laisk et al., 1989; Wagner et al., 1990; Hauser et al., 1995). In this study, we instead used changes in O_2 concentration that result when CO_2 is either taken up from or is added to a gas mixture containing O_2. The new method using highly sensitive O_2 measurements (see original traces in Fig. 5) has the important advantage that it responds only to CO_2 taken up by or released from the leaf tissue (Oja and Laisk, 1995b). The IRGA method made it necessary to discriminate between dissolved CO_2 and gaseous CO_2 carried over from the leaf chamber and the intercellular air space outside the leaf tissue. Both are measured together, and discrimination must be done on a kinetics basis, which leaves an unavoidable margin of error. The sensitivity and time resolution of the O_2 measurements are high enough to distinguish between CO_2, which is rapidly dissolved in leaf tissue (and in the presence of carbonic anhydrase is rapidly converted to bicarbonate) and slowly absorbed CO_2 (Fig. 5). The latter probably reflects the CO_2/bicarbonate conversion in those leaf compartments that do not contain carbonic anhydrase but are sufficiently alkaline to accumulate bicarbonate. In the present work, attention was focused on rapidly reacting CO_2 and on CO_2 that dissolves in the tissue in the gaseous form.

The main observation shown and discussed in this communication is that chloroplasts, which in vitro are incapable of significant pH regulation (Wagner et al., 1990), already respond rapidly in vivo to acid loads in the dark, but they respond even faster in the light. The apparent buffer capacity of the chloroplast stroma as revealed by plots of pH versus acid consumption (which corresponds to calculated bicarbonate accumulation assuming that bicarbonate formed within 4 s after CO_2 becomes available is retained in the chloroplasts) is high and not constant but changes with the time of exposure of leaves to an acid load. Light also increases the apparent buffer capacity. The data of Figures 3 and 4 show that the response of the stromal pH to external acidification is not the same in the dark and in the light, and the response is influenced by a preceding exposure to external acid. This is unexpected if one considers a cell simply as a compartmented aqueous system containing solutes with different buffering properties (pK values). Rather, acidification in response to an acid load is smaller in the presence of light than in darkness. It is also smaller after extended exposure to high CO_2 than after brief exposure.

In mesophyll cells of leaves of C_3 plants (as used in the present work), the outer layer of cytoplasm occupies a volume that is much smaller than that of the large central vacuole. More than half of the cytoplasmic space is taken up by the chloroplasts, which are the dominant organelles in the mesophyll (Winter et al., 1993). When CO_2 was switched on for only short periods (20 s) after long intervals, and its concentration was gradually increased during measurements, the concentration of bicarbonate that accumulated during the first 4 s under high CO_2 increased also. The highest levels, with 16% CO_2, were about 71 mM in a cabbage leaf (Fig. 3). The apparent buffer capacities calculated from these measurements range from about 39 mM per pH unit in the dark for the sunflower leaf to 154 mM per pH unit in the light for the cabbage leaf. After an exposure to CO_2 for 2 min, bicarbonate accumulation increased both in the dark and in the light (but more in the light, Fig. 4), and the apparent buffer capacities were as large as about 59 and 238 mM per pH unit in the dark and in the light, respectively. On the other hand, titration with acid of isolated chloroplasts revealed buffer capacities in the range of only about 35 mM per pH unit (Pfanz and Heber, 1986). The main buffers in the chloroplast stroma are phosphate and phosphate esters, with pK values close to 7.1. Owing to the transport properties of the chloroplast envelope (Heber and Heldt, 1981), their concentration is kept constant in the chloroplast stroma (close to 20 or 25 mM; concentrations calculated from Gerhardt et al., 1987). Although the concentration of proteins is very high in the chloroplast stroma, their contribution to buffering cannot be very large because pK values of weakly acidic or basic groups of the proteins are outside the pH range of the stroma.

Increases in apparent buffer capacities of chloroplasts in vivo far above buffer capacities measured after isolation and effects of light and exposure to high CO_2 on buffering indicate the involvement of ion transport or metabolic factors. The nature of the involvement is not yet entirely clear. Accumulation of bicarbonate up to levels of 120 mM (Fig. 6) requires the presence of corresponding concentrations of counterions. Only part of them can be freed from buffers inside the chloroplasts by protonation reactions, as shown by the comparatively low buffer capacity of isolated chloroplasts. The remainder must come from outside the chloroplasts, i.e. from the cytosol. However, the volume of the cytosol is smaller than that of the chloroplast compartment (Winter et al., 1993). H^+/K^+ exchange is known to be possible across the chloroplast envelope (Douce et al., 1973; Wu and Berkowitz, 1992a), but it is impossible to pump all of the cytosolic K^+ (approximately 100 mM) into the chloroplasts that would be necessary to account for observed maximum bicarbonate accumulation in the chloroplasts if pH regulation were solely a cytoplasmic affair. Moreover, if the cytosol were the place where excess protons from the chloroplasts could be dumped, acidification problems would be compounded there. In fact, the cytosolic pH is also effectively regulated, as shown by the response of pyranine to acid loads after this fluorescent pH indicator has entered the cytosol of leaves (Heber et al., 1994). The only compartments in the mesophyll capable of absorbing excessive proton loads and capable of providing cations to the chloroplasts (and, by inference, to the other cytoplasmic compartments) are the apoplast and the large central vacuole of the mesophyll cells (Sze, 1984).

The apoplast contains about 5 to 12% of the total leaf water content (Winter et al., 1993). Cation concentrations are between 15 and 20 mM in the apoplast (Speer and Kaiser, 1991). Although ATP-dependent export of protons into the medium by suspension cultures of sycamore cells has been observed (Gout et al., 1992), energized proton/cation exchange across the plasmalemma can, in our short-time leaf experiments, account for only a small part of the

necessary proton export and cation import. It should perhaps be added here that on a much larger time scale than that used in our experiments such a mechanism might indeed be very important to relieve acid stress produced by air pollutants, such as SO_2, which can no longer be overcome by intracellular pH regulation.

The rapid pH regulation indicated by the experiments of Figures 3, 4, and 6 therefore appears possible only if the vacuole is the main compartment to receive protons from the chloroplasts (and other cytoplasmic compartments). Only the vacuole can supply cations in exchange for H^+ in the amounts necessary to account for observed bicarbonate accumulation. Involvement of the vacuole in cytoplasmic pH regulation has already been observed in suspension cultures of sycamore cells by Gout et al. (1992).

The main questions are how protons are transferred from the chloroplasts into the vacuole and how vacuolar cations are imported into the chloroplasts. If the pH of vacuoles is far below that of the cytosol, there is little doubt that proton export must be energized. Release of cations in an electroneutral fashion could be the consequence of pumping of protons into the vacuole (Sze, 1984). However, if pH differences between cytosol and vacuole are small, pumping of protons with the expense of metabolic energy may not be necessary. The production of protons during CO_2 solubilization in the chloroplasts could trigger fast proton/cation exchange processes across both the chloroplast envelope and the tonoplast.

The light dependence of bicarbonate accumulation in the chloroplast stroma would be consistent with an energy requirement of acid export from and cation import into the chloroplasts that involves the vacuole. However, measured rates of CO_2 solubilization are already disturbingly high in the dark. In the experiments of Figure 3, CO_2 equivalents of more than 70 mM bicarbonate reached the chloroplasts within the measured uptake time of 4 s after CO_2 was supplied at a concentration of 16% in the gas phase. In the long-exposure experiments of Figure 6, the accumulated bicarbonate reached a concentration of 120 mM in the light. Maximum uptake rates were reached less than 1 s after admission of CO_2 to the leaf (Fig. 5). When averaged over a measuring time of 4 s, maximum CO_2 uptake rates corresponding to bicarbonate accumulation were about 3600 μmol mg^{-1} Chl h^{-1} in the dark. They were even higher in the light. At the maximum flux, 1 s after CO_2 was made available, uptake rates were actually much higher than the average values for 4 s (see curves in Fig. 5). The question of how the very high rates of bicarbonate accumulation in leaves upon sudden exposure to high CO_2 concentrations must be seen in relation to transmembrane exchange between H^+ and cations will be considered in a subsequent contribution.

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