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Short Communication

Organic Acid Changes in the Epidermis of *Vicia faba* and Their Implication in Stomatal Movement[1]

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ABSTRACT

Considerable evidence indicates that the increase in guard cell turgor resulting in stomatal opening is brought about by active K^+ uptake into guard cells. Only a small increase in inorganic anions appears to accompany the increase in K^+. A plausible explanation is that organic acids are produced within guard cells and act as counterions, whereas the H^+ produced are exchanged for K^+.

This hypothesis was tested by using different levels of ambient CO_2 in light to control stomatal aperture and at the same time measure changes in organic acid production in the epidermis of *Vicia faba*. Epidermal strips were used, quickly frozen in liquid nitrogen, and later extracted.

A positive correlation was found between stomatal resistance (r_s, indirect measure of stomatal aperture) and CO_2 level. With decreases in r_s, total titratable acidity increased. The organic acids, glyceric, malic, and citric, in the epidermis, as measured by gas chromatography of trimethylsilyl derivatives, increased. Changes in glucose or sucrose were not found. These analyses provided evidence that organic acid production in the epidermis is associated with stomatal opening.

The supporting role of starch in providing osmoticum for increasing guard cell turgor has been the subject of debate (12), although more recent evidence indicates that starch degrades with stomatal opening (10). In the classical scheme, sugars and their phosphorylated derivatives have been postulated as the active molecules bringing about turgor changes, with more recent emphasis on organic acid production (7, 9, 13). Overwhelming evidence is now available for an active (so-called pumping) K^+ (3, 6, 20) and possibly Na^+ uptake (11, 24) by the guard cell, increasing its turgor. However, there has been a general failure (2, 7, 11) to find equivalent amounts of inorganic anions (*i.e.*, Cl^-, SO_4^{2-}, and $H_2PO_4^-$) to offset the cation uptake into the guard cell. Since electroneutrality is no doubt maintained within the guard cell, one must surmise that anions of organic acids serve as counterions.

In these studies, we have attempted to determine whether there are changes in the sugar and organic acid contents of the epidermis and whether they relate to changes in stomatal aperture. Differences in stomatal condition were brought about by controlling ambient CO_2 level. Good correlation exists among ambient CO_2 concentration, stomatal aperture, and transpiration. Increasing the carbon dioxide content of air above normal (approximately 310 μl/l) reduces transpiration by causing stomata to close, whereas lowering the carbon dioxide content of air below normal causes stomata to open.

MATERIALS AND METHODS

Vicia faba "minor" was grown under controlled conditions in large flats containing fertile greenhouse soil (1:1 peat and soil) in chambers, type I (moderate infrared), as described in an earlier publication (16). The chambers were programmed for 25 C, 60% relative humidity for 14-hr photoperiods, and 20 C, 90% relative humidity for 10-hr nyctoperiods.

The carbon dioxide concentration in the chambers was maintained at 310 ± 15 μl/l and continuously monitored by Model 15A Liston-Becker infrared CO_2 analyzers. A control meter relay, in series with each recorder, controlled the injection of CO_2 into a chamber. Where CO_2 concentrations below ambient were used, a CO_2 scrubber population of *Zea mays* was grown in a chamber nearby, and the air was circulated between chambers using stovepipes and a blower while the injection system maintained the desired level of CO_2. A paired experimental method was used, whereby extracts from the epidermis of leaves from plants at 310 μl/l CO_2 were compared with extracts from the epidermis of leaves from similar plants at the test CO_2 level. In all instances, the imposition of the test CO_2 concentration started by 6 AM, the beginning of the light period, at least 3 hr before sampling. Indirect estimates of stomatal opening were made by continually recording plant transpiration from test flats with a recording balance (4). Direct microscopic observations of stomatal openings were made *in situ*.

Beginning at 9 AM, fully expanded leaves were picked in groups of 10 g from the 6- to 7-week-old plants, placed in a glass beaker, covered with wet paper towels, and then stripped of their lower epidermis under a bank of fluorescent lights. A 15- to 20-min period elapsed between picking the leaves and completing the stripping. Each strip was frozen immediately in liquid nitrogen. Epidermal tissue with any adhering cells (*e.g.*, bundle parenchyma) from the whole leaf was discarded. After stripping was complete (usually from 30 g of leaf), the tissue was refluxed in 95% boiling ethanol, decanted and washed with 95% ethanol, ground, and centrifuged. The extracts and washes were eluted through a Dowex 50-8X (H+)

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50- to 100-mesh column to remove basic components. After addition of the internal standards, diglycolic and *cis*-aconitic acids and inositol, the eluent was titrated to pH 8.5 with 0.01 N NaOH to determine total acidity and evaporated to dryness *in vacuo* at 40 C.

Trimethylsilyl derivatives were made by adding 1 ml of anhydrous pyridine to a sample, shaking 30 min, adding 0.6 ml of hexamethyldisilazane and 0.3 ml of trimethylcholorosilane, and shaking for an additional hour. After standing overnight, the sample was reshaken for an hour and the precipitate was allowed to settle. Aliquots of the solution were injected into a Perkin Elmer Model 881 gas chromatograph with a 3% SE-52 on AW-DMCS Chromosorb G 80–100 mesh (3 mm × 183 cm) column and a dual flame ionization detector. Sucrose was identified on 2% QF-1 on Anakrom SD 110–120-mesh copper columns. Other columns used in these studies for identification purposes, but not found as suitable, were: 3.8% SE-30 on Chromosorb W 80 to 100 mesh, 3% OV-17 on Chromosorb W 80 to 100 mesh, 3% OV-1 on Anakrom SD 100 to 110 mesh, 6% OV-17 on Gas Chrom P 100 to 140 mesh, 2.5% SE-52 on Chromosorb G 80 to 100 mesh, and 3% SE-52 on Anakrom SD 80 to 100 mesh. Careful analytical technique was required to reproduce results with the following parameters closely adhered to for the SE-52 column: Injection port temperature, 310 C; program temperatures, 70 to 210 C; program rate, 8 C/min; He flow rate, 60 ml/min; sample size, 1 to 2 μl; detector temperature, 225 to 250 C. Three determinations were made of each extract. Identification was made of α- and β-glucose, sucrose, glyceric, citric, and malic acids by comparison with trimethylsilyl derivatives of known compounds. Chromatographs were quantified by digital integration.

RESULTS AND DISCUSSION

In accord with earlier findings, the low CO_2 concentration of 160 μl/l (Table I) tended to increase transpiration of *Vicia faba,* in this instance 36%, by opening stomata, whereas concentrations of 500 and 1,000 μl/l decreased transpiration 9 and 18%, respectively, by stomatal closure. In general, CO_2 concentrations above normal decreased the total titratable acidity of the epidermis and the quantities of glyceric, citric, and malic acids therein (Table I). In two separate experiments comparing acidity changes and organic acid concentrations at 310 and 500 μl/l CO_2 (Table II), the concentrations of glyceric, citric, and malic acids decreased significantly. Tripling the ambient CO_2 concentration to 1,000 μl/l resulted in an approximate halving of both total acidity and glyceric, malic, and citric acids (Table I). The concentration of malic acid was severalfold lower than glyceric or citric. No statistically significant changes in sucrose or glucose were observed (Table I) that could be correlated with increases in CO_2 concentration.

At the low partial pressure of CO_2 (160 μl/l, Table I), the decrease in stomatal resistance, as indicated by the increase in transpiration, eventually caused leaf water content to decrease until leaves visibly wilted. The transpiration rate was measurably decreasing at the time of sampling. Microscopic observations near the end of picking showed that fewer than 50% of the stomata were open and had apertures less than 1 μm. Rather than terminate the experiment, chemical analyses were made, but as can be seen in Table I, there was lack of any significant trend. Malic, citric, and glyceric acid concentrations were slightly higher in the low CO_2 treated tissue (Table I, column 4).

The data offer evidence of organic acid changes in the epidermis associated with stomatal condition. Organic acid concentrations decreased in the epidermis with decreased transpiration or increased r_s since $T = 1/r_s + r_a$ where r_s is stomatal resistance and r_a boundary layer resistance. The latter was essentially constant in these studies. Since the leaves were held in constant conditions of temperature, irradiance, air flow, and VPD, the changes in transpiration rate are attributed to changes in stomatal aperture. One can surmise that the differences in organic acid concentration, reported in Table II, between ambient and above ambient CO_2 (column 7) are primarily indicative of changes within guard cells. First, because when water stress is negligible, the osmotic pressure of epidermal cells remains constant, whereas the guard cell osmotic pressure is directly correlated with aperture (19). Second, the lack of a stoichiometric relationship mentioned earlier between K^+ and inorganic anion uptake into the guard cell indicates organic acid anions within the guard cell are responsible for maintaining electroneutrality. It is well documented that the total milliequivalents of organic acid in plants are equal to the total milliequivalents of cations ($K^+ + Na^+ + Ca^{2+} + Mg^{2+}$) minus the total milliequivalents of inorganic anions ($NO_3^- + H_2PO_4^- + Cl^- + SO_4^{2-}$) (1, 22, 23). For example, Hiatt (5) has shown that the discrepancy between cation and anion uptake by low salt barley roots is equal to the increase in organic acid level in the sap. Third, the numerous mitochondria in the guard cell per unit volume (17, 21) would seemingly provide extraordinary ability for synthesis of Krebs cycle acids. Fourth, changes in neutral red accumulation suggest H^+ production within the guard cell during stomatal opening (14).

The changes in organic acid concentration found in these studies were ample to offset K^+ uptake into guard cells. Using stomatal density measurements of 6200/cm² (2) and an average value of 4.8×10^{-12} g eq of K^+ transported into a pair of guard

Table I. *Transpiration, Organic Acid, and Sugar Concentration in the Epidermis of Vicia faba as Related to Ambient CO_2 Concentration*

Experiment No.[1]	CO_2	Transpiration[2]	Glyceric, Citric Malic Acid	Total Acidity	Glucose	Sucrose
	μl/l	%	meq/0.1 g dry wt		mg/0.1 g dry wt	
II-71	160	136	0.13	0.27	1.0	0.7
II-72	310		0.10	0.32	0.8	0.8
II-59	500	91	0.06	0.24	0.5	0.6
II-58	310		0.09	0.33	0.6	0.8
II-63	1000	82	0.04	0.16	0.7	0.8
II-62	310		0.10	0.30	0.9	0.8

[1] II-71 and II-72, II-59 and II-58, and II-63 and II-62 were paired experiments.

[2] Daily transpiration of the population as a percentage of the day preceding at 310 μl/l Co_2.

Table II. *Organic Acid Content of Vicia faba Epidermis as Related to Ambient CO_2 Concentration*

Experiment No.[1]	CO_2	Glyceric	Citric	Malic	Total Acidity	Difference
	μl/l	μeq/0.1 g dry wt				
II-54	310	39	45	9	93	39
II-55	500	24**	24**	6*	54	
II-58	310	38	38	14	90	34
II-59	500	24*	25**	7*	56	

[1] II-54 and II-55 were paired experiments as were II-58 and II-59.

* Significantly different from control at 5%.

** Significantly different from control at 1%.

cells (7), gives 29.8 $\times$ 10^{-3} μg eq K^+/cm^2. The total change in glyceric, citric, and malic acids between 310 and 500 μl/l CO_2 (Table II, No. II-54 *versus* II-55) was 37 μ eq/0.1 g epidermal tissue. Based on dry weight estimates of 0.2 mg/cm^2 for our epidermal tissue, a difference of 78 $\times$ 10^{-3} μ eq/cm^2 of acid was found between strips containing open and only partially opened stomata. The organic acid content thus was severalfold in excess of the stoichiometric requirement.

The question arises as to what happens to the H^+ generated in organic acid synthesis? Its probable fate, as well as the generation of H^+ by the reduction of $NADP^+$ or cyclic photophosphorylation, has been discussed (14). Recapitulating, it appears that H^+ (in the guard cell) is exchanged for K^+ (in epidermal cells). Cation exchange for H^+ occurs in roots (8). Proton exchange has also recently been suggested by Mansfield and Jones (10) to be operative in stomatal movements. The work of Pekarek (18) tends to support H^+ exchange between guard and other epidermal cells. He detected that under normal conditions in the dark, guard cells of closed stomata were considerably more acidic than epidermal cells, whereas in light epidermal cells were slightly more acidic than guard cells.

In these studies the inability to find statistically significant changes in sugar concentration within the epidermis that were associated with stomatal changes questions the validity of the classical scheme involving sugars. However, modification of the scheme to include organic acid changes seems tenable. Possibly the sugars are primarily serving as intermediates in the generation of osmoticum, with starch as the reservoir. This is in accord with the inability to redemonstrate histochemically sugar changes in epidermal and guard cells associated with stomatal opening (15).

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