

Sugar and Organic Acid Accumulation in Guard Cells of *Vicia faba* in Response to Red and Blue Light¹

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Changes in neutral sugar and organic acid content of guard cells were quantitated by high-performance liquid chromatography during stomatal opening in different light qualities. Sonicated *Vicia faba* epidermal peels were irradiated with $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ of blue light, a fluence rate insufficient for the activation of guard cell photosynthesis, or $125 \mu\text{mol m}^{-2} \text{s}^{-1}$ of red light, in the presence of 1 mM KCl, 0.1 mM CaCl₂. The low-fluence-rate blue light stimulated an average net stomatal opening of 4.7 μm in 2 h, whereas the saturating fluence rate of red light stimulated an average net opening of 3.8 μm in 2 h. Under blue light, the malate content of guard cells increased to 173% of the initial level during the first 30 min of opening and declined as opening continued. Sucrose levels continuously rose throughout the blue light-stimulated opening, reaching 215% of the initial level after 2 h. The starch hydrolysis products maltose and maltotriose remained elevated at all times. Under red light, guard cells showed very little increase in organic acid or maltose levels, whereas sucrose levels increased to 208% of the initial level after 2 h. Total measured organic metabolite concentrations were correlated with stomatal apertures in all cases except where substantial malate increases occurred. These results support the hypothesis that light quality modulates alternative mechanisms of osmotic accumulation in guard cells, including potassium uptake, photosynthetic sugar production, and starch breakdown.

From the earliest observations that starch in guard cell chloroplasts breaks down during the day and accumulates in the dark (Lloyd, 1908) until the late 1960s, carbohydrates were regarded as the primary osmotica modulating stomatal opening. The discovery of the important role of potassium uptake for stomatal opening (Imamura, 1943; Fujino, 1967; Fischer, 1968; Fischer and Hsiao, 1968), together with the identification of malate as the main counterion for potassium (Allaway, 1973; Shimada et al., 1979), led to the replacement of the starch-sugar hypothesis by the potassium-malate theory to explain stomatal opening. Subsequent reports of a lack of guard cell Rubisco activity (Outlaw et al., 1979) or a lack of functional significance for it (Reckman et al., 1990) have further weighed against the involvement of carbohydrates in guard cell osmoregulation.

A growing body of evidence that the PCR/P does operate in guard cells, and that alternative osmoregulatory pathways can be selectively stimulated by manipulating light quality, requires a reevaluation of the role of carbohydrates in sto-

matal opening. *Vicia* guard cells contain Rubisco and PCR/P enzyme activities (Zemel and Gepstein, 1985; Shimazaki et al., 1989). Guard cells of *Vicia* have been shown to have red light-dependent CO₂ uptake and O₂ evolution (Shimazaki and Zeiger, 1987), fluorescence transients characteristic of photosynthetic carbon fixation under green but not blue light (Mawson and Zeiger, 1991), and the ability to fix ¹⁴CO₂ into products of the PCR/P at significant rates under red light (Gotow et al., 1988). An action spectrum for malate accumulation in *Vicia* guard cells shows that malate synthesis is stimulated primarily by blue light (Ogawa et al., 1978). In isolated epidermal peels of *Vicia* and *Commelina*, red light-stimulated opening saturates at $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ and is DCMU sensitive and KCN insensitive, whereas blue light-stimulated opening saturates below $25 \mu\text{mol m}^{-2} \text{s}^{-1}$ and is KCN sensitive and DCMU insensitive (Schwartz and Zeiger, 1984).

There is strong indirect evidence that *Vicia* guard cells can utilize sugars as well as potassium-malate as osmotica, depending on light quality. Histochemical analysis showed that red light-stimulated opening in isolated epidermal peels of *Vicia* was not accompanied by either disappearance of starch or uptake of potassium (Tallman and Zeiger, 1988). Under blue light there was marked disappearance of starch. In the early phase of opening under blue light there was potassium uptake, but this potassium was no longer detected as opening proceeded. Based on these results and the DCMU sensitivity of the red light opening response, the authors proposed that red light-stimulated opening was modulated by sugars derived from photosynthesis. Blue light-stimulated opening was associated with potassium uptake, but the transitory nature of the response led to the proposal that sugars derived from starch breakdown can also make a significant contribution to opening at later stages of the blue light-stimulated opening. Recently, direct analysis of carbohydrates in *Vicia* guard cells by HPLC demonstrated the accumulation of sugars, principally Suc and Fru, during opening under both red and blue light, with only red light-stimulated sugar accumulation being inhibited by DCMU (Poffenroth et al., 1992).

Measurement of metabolites in guard cells, particularly sugars, has been hampered by the difficulty of obtaining sufficient quantities of guard cells free from mesophyll contamination. In this study, we used HPLC separation combined with high-sensitivity electrochemical and UV detection to monitor sugar and organic acid levels in *Vicia faba* guard cells from epidermal peels sonicated to remove contaminating mesophyll and epidermal cells (Ogawa et al., 1978). The

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Abbreviation: PCR/P, photosynthetic carbon reduction pathway.

present work confirms the involvement of carbohydrate in osmoregulation of guard cells in isolated epidermal peels and quantifies changes in specific sugars and organic acids during opening in red and blue light. In addition, we were able to determine levels of less abundant carbohydrates, particularly maltose and maltotriose, which are specific products of starch breakdown, and thus gain insight into the metabolic paths used for osmoregulation.

MATERIALS AND METHODS

Plant Material

Seeds of *Vicia faba* L. cv Long pod (W. Atlee Burpee & Co., Warminster, PA) were planted in pots of autoclaved soil (Soil Mix 16F, BD White, Culver City, CA) and grown in a greenhouse under natural light. The plants were watered every other day and fertilized once a week with commercial fertilizer (Spoonit, Morrison's Orchard Supply, Yuba City, CA). All experiments used recently matured leaves from the 3rd and 4th nodes of 5-week-old plants.

For epidermal peel preparation, three to four leaves per time point were harvested early in the morning and placed for 1.5 h in the dark, wrapped in wet paper towels. Epidermal peels from the abaxial side were carefully stripped by hand from interveinal regions into 0.1 mM CaCl_2 (peels from four leaves in 30 mL of solution) under dim light. Peels were sonicated on ice in small batches for 26 to 32 s at 50% power with a Cole-Parmer sonic homogenizer (series 4710, 300 W, 20 kHz). The exact sonication time was determined empirically according to guard cell viability and varied between sets of plants. After sonication, the peels were rinsed in distilled water and incubated for 1 h in the dark in a solution containing 1.0 mM KCl and 0.1 mM CaCl_2 . In this pretreatment and in subsequent light treatments, the incubation solutions were aerated with compressed air.

Light Treatments

Following preincubation, epidermal peels were divided into three portions for dark, red light, and blue light exposure. Peels were transferred to fresh 1 mM KCl, 0.1 mM CaCl_2 in 14-cm Petri dishes held in a clear plexiglass circulating water bath at 25°C. The peels could be kept in the dark using opaque dishes, illuminated with red light through a red filter mounted on the bottom of the dish (50% cutoff 595 nm, No. 2423 plexiglass, Rohm and Haas, Hayward, CA), illuminated with blue light through a blue filter mounted on the top of the dish (470-nm maximum, half-band width 100 nm, No. 2424 plexiglass, Rohm and Haas), or illuminated with red and blue light simultaneously from both sides. The light sources were Sylvania DAH 500-W incandescent projector bulbs for blue light and Sylvania 300-W 300PAR56/2MFL Cool Lux flood lamps for red light (GTE Products Corp., Winchester, KY). Peels received 125 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of red light and/or 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of blue light. Light fluence rates were measured with a Li-Cor quantum sensor (Li-Cor Inc., Lincoln, NE). All light treatments were run concurrently, and peels were sampled from the dishes at appropriate intervals.

Measurement of Stomatal Apertures

Aperture measurements were made with an Olympus BH-2 microscope connected to a Javelin JE2362A digital imaging camera. Image processing was handled with an IBM PC-based MV-1 image analysis board (Metrabyte Corp., Taunton, MA) and JAVA image analysis software (Jandel Scientific, Corte Madera, CA). The system allows rapid measurement of stomatal apertures and is accurate to within 0.4 μm .

HPLC Quantitation of Guard Cell Metabolites

After light exposure, the peels were rapidly frozen at -80°C to stop further reaction. Thereafter, peels were frozen and thawed twice to ensure that guard cells were ruptured, and cell sap was expressed from the peels at 5°C. The peels were washed in 100 μL of cold distilled water. The combined wash and cell sap was passed through a 0.45- μm Nylon filter, freeze dried, and analyzed by HPLC without further handling. This procedure allows both rapid preparation and a minimum of sample handling. The cell sap from 4 to 6 mg dry weight of peels (three to four leaves) provides enough sample for organic acid and carbohydrate determinations at one timepoint.

A Rainin 81-20 HPLC system (Rainin Instrument Co., Woburn, MA) equipped with a 2.6 \times 220 mm polypore H anion-exchange chromatography column (Alltech Associates, Deerfield, IL) was used for organic acid analysis. Samples were separated by isocratic elution with 0.018 M sulfuric acid at 85°C. A Dynamax UV-1 UV absorbance detector operating at 210 nm was used to quantitate organic acids according to calibration curves prepared with known standards. Sensitivity limits ranged from 0.5 to 1.0 nmol/ μL , depending on the specific acid.

For carbohydrate measurements, we used a Dionex 2010 HPLC system equipped with a SugarPak 6.5 \times 300 mm cation-exchange column (Waters Chromatography, Milford, MA), which provides a good separation of major monosaccharides and key di- and trisaccharides. Samples were eluted isocratically with 50 mM Ca-EDTA at 85°C. Carbohydrates were detected with a Dionex PADII electrochemical detector, which is both highly specific for carbohydrates and highly sensitive (0.2–0.4 nmol/ μL). Because maltose and Suc have similar elution times, maltose was determined after treating a portion of the extract with 10 units/ μL of invertase (Sigma Chemical Co).

Dry weights of peel samples were determined and used, along with empirical values for stomatal density and weight/unit surface area, to normalize results on a fmol/guard cell pair basis. Changes in metabolite levels, expressed as percentages of the initial content, were calculated for each experiment and then averaged over all experiments. Average initial metabolite content can be found in Table I.

RESULTS

Stomatal Opening in Red and Blue Light

As reported previously, the light-stimulated opening of stomata in isolated epidermal peels varied as a function of light quality. Exposure to low-fluence-rate blue light (10 μmol

Table 1. Initial values for aperture and metabolite content of guard cells

Measurements were taken from guard cells in sonicated epidermis after a 1-h dark pretreatment. Values are the average \pm SE for seven experiments.

| Aperture | Metabolites | Concentration |
|-----------------------------|----------------------|----------------------|
| $5.23 \pm 0.22 \mu\text{m}$ | | fmol/guard cell pair |
| | Maltotriose | 0.79 ± 0.17 |
| | Maltose | 11.1 ± 2.9 |
| | Suc | 78.0 ± 13.1 |
| | Glc | 30.1 ± 7.3 |
| | Fru | 47.6 ± 10.3 |
| | Ara-Gal ^a | 37.4 ± 2.4 |
| | Xyl | 14.6 ± 5.1 |
| Malate | 97.1 ± 14.3 | |
| Citrate | 16.5 ± 3.5 | |
| Pyruvate | 5.9 ± 1.6 | |

^a Not separated.

$\text{m}^{-2} \text{s}^{-1}$) resulted in larger aperture increases than exposure to saturating-fluence rates of red light ($125 \mu\text{mol m}^{-2} \text{s}^{-1}$). Figure 1 shows the results of three separate opening experiments. These data have a small SE for each time point and show a clear response pattern to the different light treatments, but there is a large variation in absolute aperture values. Such variation is commonly found in measurements of stomatal responses despite controlled experimental conditions (Outlaw, 1982), and we attribute it primarily to differences in the growth histories of the plants. Because of the large differences in absolute aperture values, the standard errors of the average of all experiments are substantially larger than the difference between treatments, thus obscuring the unambiguous response pattern to light quality seen in all experiments. On the other hand, the average values of all six experiments (Figs. 2 and 3) are fully consistent with the response pattern observed in each experiment (cf. Figs. 1 and 2). Additional statistical support for the interpretation of the data is provided by the analysis presented in Figure 4.

Two hours of exposure to low-fluence rates of blue light caused average net opening of $4.7 \mu\text{m}$ (190% of average initial aperture), with $3.3 \mu\text{m}$ of that opening occurring during the 1st h (Fig. 2). Saturating fluence rates of red light caused average net opening of $3.8 \mu\text{m}$ (172% of initial aperture) in 2 h, with $2.4 \mu\text{m}$ occurring during the 1st h. Control stomata kept in the dark under the same conditions showed constant apertures during the 1st h and slight opening at later times.

Metabolite Changes during Opening under Different Qualities of Light

At the end of the dark pretreatment, the predominant carbohydrate in the guard cells was Suc (Table I). Substantial quantities of Glc and Fru were present, with Fru always exceeding Glc content. The monosaccharides Ara and Gal, whose combined levels approximated that of Fru, were also detected but are not discussed further because their levels generally co-varied with Fru. The monosaccharide Xyl and the starch breakdown products maltose and maltotriose were

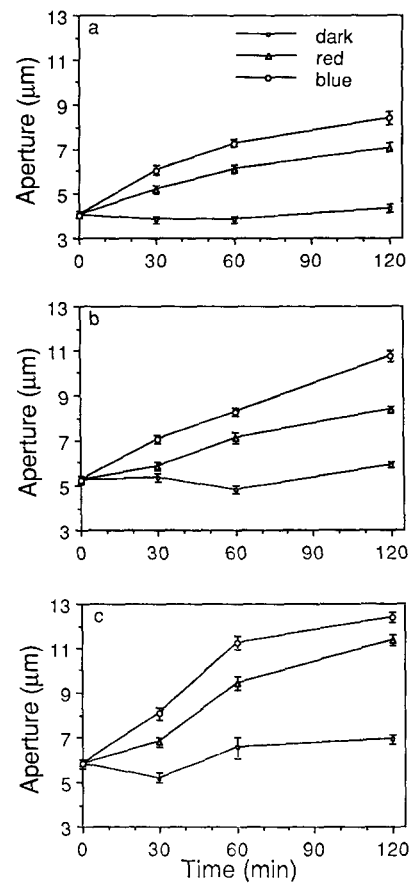


Figure 1. Stomatal opening in response to different light qualities. Sonicated epidermal peels were incubated 1 h in 1 mM KCl and 0.1 mM CaCl_2 in the dark and then exposed to light at time zero. Treatments were a dark control, $125 \mu\text{mol m}^{-2} \text{s}^{-1}$ of red light, and $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ of blue light. Panels a through c show results from three separate experiments. Each point represents 30 aperture measurements. Bars are the standard error of the average.

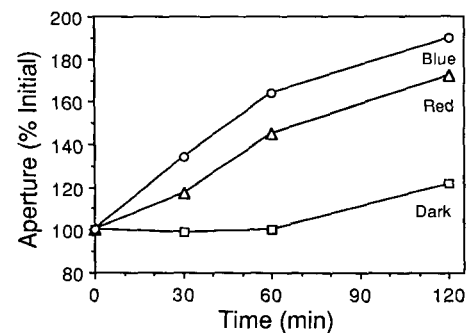
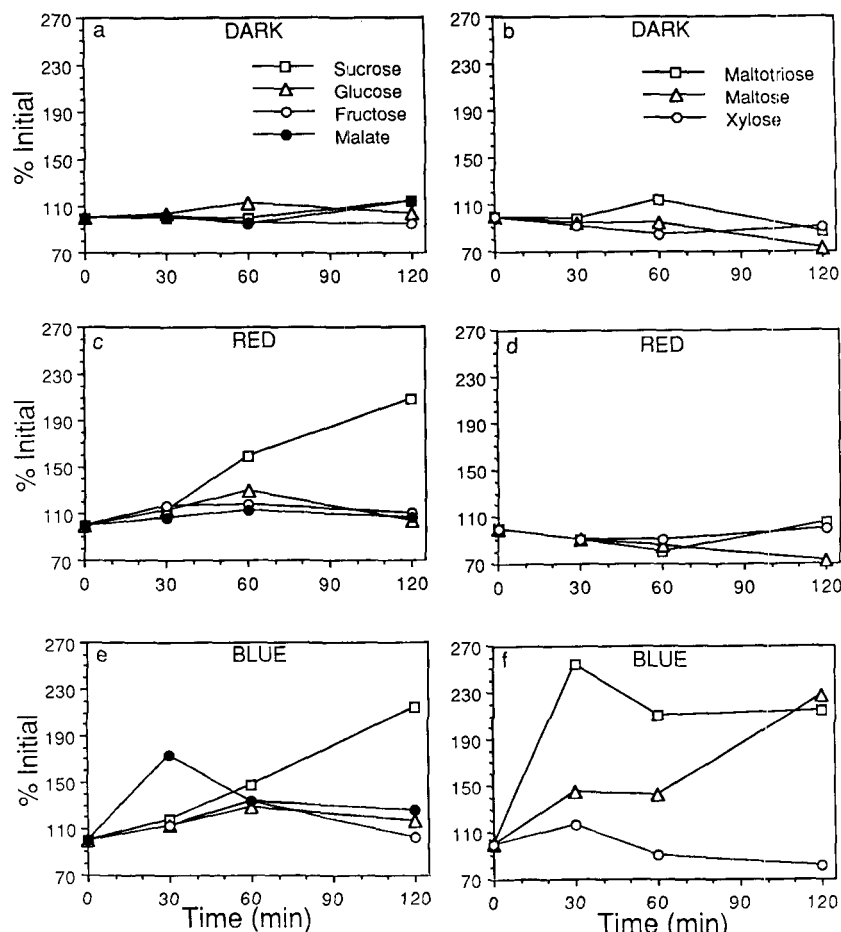


Figure 2. Stomatal opening in response to different light qualities. Aperture values were converted to percent of the initial value and averaged over six experiments. Average initial aperture value can be found in Table I.

Figure 3. Guard cell content of some major (left) and minor (right) carbohydrates and organic acids as a function of time in darkness (a and b), 125 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of red light (c and d), and 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of blue light (e and f). Contents are average values of six experiments calculated on a per guard cell pair basis and are shown as the percent of the initial content (see Table I). Only the major components make a significant contribution to the osmotic potential of the cells.



present at low levels. Malate was the predominant organic acid found in guard cells. Malate content exceeded that of Suc by 24% on average. Citrate was present at levels 16% those of malate. Minor amounts of pyruvate were also detected.

Peels incubated in the dark after the 1-h dark pretreatment did not show any consistent pattern of changes in metabolite pools (Fig. 3, a and b). After 1 h of treatment, there was a small increase in Glc, accompanied by a modest increase in maltotriose, pointing to a slight increase in the rate of starch breakdown. The lack of an increase in maltose levels perhaps indicates a slower rate of maltotriose consumption. At 2 h, both Suc and malate levels rose, perhaps accounting for the small degree of opening seen at this time in the dark-treated peels (Fig. 2).

Under red light, the Suc content of guard cells rose steadily after an apparent lag period seen at 30 min of treatment (Fig. 3c). After 120 min in red light, Suc content had increased to 208% of its initial value. The monosaccharides Glc and Fru also increased up to 60 min of treatment, followed by a minor decrease at later times. Judged by their pool size at 30 min, the initial rate of increase of Glc and Fru seems faster than that of Suc, suggesting a precursor-product relationship. In contrast to Suc, malate content rose only 13% by 60 min and declined thereafter. Of the minor metabolites (Fig. 3d), maltose and maltotriose pools declined, indicating the absence of

significant starch breakdown. The level of xylose, a minor monosaccharide used in cell wall synthesis and unlikely to be involved in the main biosynthetic paths of osmoticum production, is shown as a reference.

Under blue light, a different picture of osmotic accumulation emerged. In the early time periods, malate accumulated as the main organic component (Fig. 3e). By 30 min in blue light, malate increased to 173% of its initial level. Thereafter, malate levels declined, and by 120 min they were 125% of the initial value. It is interesting that levels of pyruvate, a possible product of malate decarboxylation, increased 1.5- to 2-fold when malate levels decreased (data not shown). Suc, Glc, and Fru levels also increased during opening under blue light. As in the red light treatment, the monosaccharides also reached a maximum at 60 min of blue light. There was a steady accumulation of Suc under blue light, and, at 120 min, Suc levels reached 215% of their initial value and constituted the major organic osmoticum present in the guard cell. In contrast with the red light treatment, the maltose and maltotriose content of guard cells in blue light increased rapidly within 30 min of exposure and remained elevated throughout the treatment (Fig. 3f), indicating an increased rate of starch breakdown. Maltotriose, in particular, remained at more than double its initial level.

The relative contribution of carbohydrates and malate to the organic osmoticum of the guard cells incubated under

blue or red light is presented in Figure 4. Values shown are the average percentage increase in carbohydrate or malate over initial levels, measured in six experiments. The relative contribution of carbohydrate and malate remained relatively constant under red light, with carbohydrates contributing over 80% of the organic osmotica throughout the opening time course. Under blue light, the $79 \pm 12\%$ contribution of malate seen at 30 min drops to $16 \pm 3.3\%$ by 120 min.

Addition of low-intensity blue light to a red light treatment switched guard cell osmoregulation from a red light pattern to a blue light one. Table II shows the results of an experiment in which peels were exposed to a saturating fluence rate of red light for 60 min. At the end of this period, Suc levels in guard cells increased, whereas malate, maltose, and maltotriose levels remained unchanged. Low-intensity blue light added to the red light irradiation caused a rapid increase in malate with a concomitant appearance of the starch breakdown products maltose and maltotriose. Suc, Glc, and Fru levels declined upon addition of blue light, possibly reflecting conversion of these carbohydrates to malate and/or their use as a respiratory substrate to provide energy for potassium uptake, which presumably accompanies malate biosynthesis.

Except for the early time points under blue light, cellular levels of organic osmotica correlate well with aperture in all treatments (Fig. 5a). Comparison of the sum of all significant carbohydrates and organic acids with aperture has an r^2 value of 0.90. The correlation does not hold at early points in blue

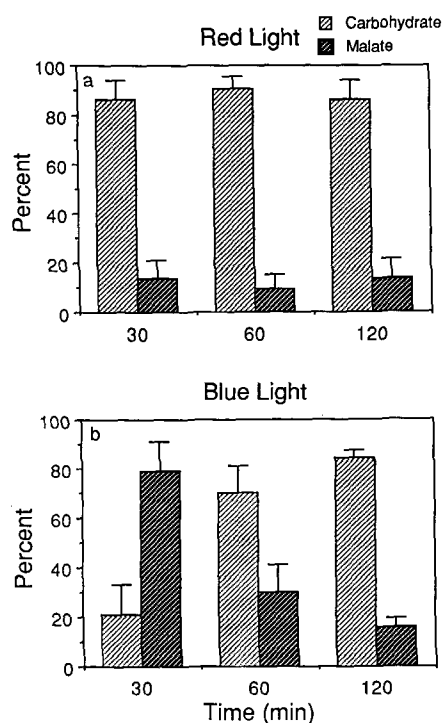


Figure 4. Effect of light quality on the relative contribution of malate and carbohydrate to guard cell osmoregulation. Values shown are the composition, in percent, of the organic osmotica accumulated in response to $125 \mu\text{mol m}^{-2} \text{s}^{-1}$ of red light (a), and $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ of blue light (b) at each time point and are the averages and se values of six experiments.

Table II. Changes in aperture and metabolite levels upon addition of blue to red light

Guard cells in sonicated epidermal peels were exposed to $125 \mu\text{mol m}^{-2} \text{s}^{-1}$ of red light. After 60 min, $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ of blue light was added to the red light. Values are percent of the initial aperture or metabolite content.

| | 60 min Red | +30 min Added Blue |
|-------------|------------|--------------------|
| Aperture | 124 | 157 |
| Metabolites | | |
| Maltotriose | 101 | 115 |
| Maltose | 103 | 116 |
| Suc | 121 | 110 |
| Glc | 107 | 84 |
| Fru | 108 | 90 |
| Malate | 100 | 144 |

light opening (Fig. 5b), probably reflecting the fact that the calculations do not include the osmotic contribution of potassium. Malate and potassium are likely to be major osmotica at the onset of the blue light treatment (Fig. 3e). Calculated osmotic totals are likely to be underestimates because of losses during extraction and the assumption of 100% survival used

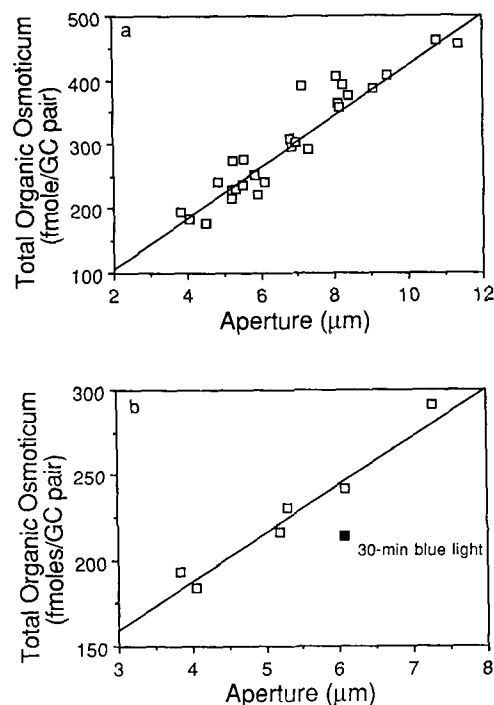


Figure 5. Correlation between total organic osmoticum in a guard cell pair and aperture values. Total organic osmoticum was calculated by adding the Suc, Glc, Fru, Ara, Gal, malate, and citrate content on a per guard cell basis. a, Correlation using points from several experiments and all light treatments ($r^2 = 0.90$). b, Correlation using points from a single experiment. An r^2 value of 0.95 is obtained using points from blue light treatments longer than 30 min, and all dark and red treatment time points (\square). The point from the 30-min blue-light treatment (\blacksquare) falls below the line.

in the calculation of number of stomata from stomatal density (measured survival after sonication was 90–95%).

DISCUSSION

HPLC analysis of metabolites clearly shows that isolated guard cells in epidermal peels can use both carbohydrates and organic acids as osmotica. These observations support previous results showing that potassium content in *Commelina communis* guard cells was insufficient to account for the observed opening and the proposal that sugars might constitute a second source of osmotica (MacRobbie and Lettau, 1980). Biochemical measurements with open and closed stomata in *Vicia* leaves found higher levels of Suc and hexose in open stomata (Outlaw and Manchester, 1979). Both malate and sugars were found to increase in isolated epidermal strips of *Commelina benghalensis* during fusicocin-stimulated stomatal opening, with sugars providing an estimated 60% of the total osmoticum (Reddy et al., 1983). A previous HPLC study found that stomata of isolated *Vicia* epidermal peels opened under both red and blue light showed increases in Suc and Fru (Poffenroth et al., 1992). The authors calculated that sugars accounted for 56 to 61% of the osmotica needed to sustain the increase in aperture under both red and blue light, although such calculations depend on estimations of guard cell volume and the required increase in osmotic potential per micrometer of opening.

The simultaneous measurements of sugars and organic acids reported in this work confirm that carbohydrates can be a significant source of osmotica. At the onset of the light treatments, Suc, Glc, and Fru accounted for 51% of the measured organic osmoticum, whereas malate and citrate accounted for 37%. After 2 h under either red or blue light, Suc, Glc, and Fru accounted for 58 to 60% of the total organic osmotica of the cell and 75 to 86% of the increase in organic osmotica. Malate accounted for only 25 to 28% of the total organic osmotica at this time.

The light quality regimens used in this study are known to activate alternative osmoregulatory pathways. Low-intensity blue light ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) has long been known to stimulate starch breakdown, to be more effective at stimulating opening than red light (Mouravieff, 1958), and to be below threshold for guard cell photosynthesis (Schwartz and Zeiger, 1984; Shimazaki and Zeiger, 1985). Stomatal opening in blue light was shown to be initially accompanied by rapid malate accumulation, most likely acting as a counterion for potassium (Shimada et al., 1979), the uptake of which is also stimulated by low-intensity blue light (Hsaio et al., 1973). However, the measurements of malate content presented here show that malate accumulation was transitory, with levels reaching maximum at 30 min and then declining until at least 2 h. This pattern of potassium-malate accumulation is supported by histochemical studies showing transient potassium accumulation in *Vicia* stomata opening under blue light (Tallman and Zeiger, 1988).

In contrast to malate, Suc increased steadily under blue light, and, at 2 h, it accounted for the majority of light-stimulated increase in organic osmotica within the guard cell. Monosaccharide levels also increased for at least 1 h, but do not constitute a major component of the net increase in

osmotic potential and more likely indicate additional carbohydrate supply for the Suc synthesis pathway. The large increase in the levels of maltose and maltotriose indicate that the higher carbohydrate levels seen under low-intensity blue light are derived from starch. Maltose and maltotriose are products of the hydrolytic pathway of starch degradation. Both the hydrolytic and phosphorylytic pathways of degradation operate in mesophyll chloroplasts (Peavey et al., 1977; Stitt and Heldt, 1981). Similar starch degradation pathways have been postulated for *Vicia* guard cell chloroplasts on the basis of enzyme localization studies (Robinson and Preiss, 1987). Biochemical and histochemical measurements indicate a decrease in starch content of guard cells opening in response to fusicocin and blue light (Reddy et al., 1983; Tallman and Zeiger, 1988). Since in sonicated peels there are no viable cells other than guard cells to act as outside sources of carbohydrate (Ogawa et al., 1978), and cell wall autolysis is not a significant source of sugars (Poffenroth et al., 1992), we conclude that both malate and Suc are formed from starch breakdown products in the blue light treatment. Use of monosaccharides as respiratory substrates is also likely to occur during blue light-stimulated opening, as indicated by the ability of KCN to inhibit this opening (Schwartz and Zeiger, 1984).

During opening in red light, guard cells accumulated primarily Suc. In contrast to blue light opening, only small increases in malate were observed, the maximum average malate level being 113% of the initial value. Glc and Fru levels increase as they do in the blue light treatment. Because maltose and maltotriose levels actually decline, there is no evidence for starch breakdown under red light. Given the lack of other sources of carbohydrates in isolated peels, we conclude that the opening observed in red light was supported mainly by an increase in Suc derived from photosynthetic carbon fixation in guard cells. This conclusion is supported by histochemical evidence showing no decrease in starch content and no accumulation of potassium in *Vicia* guard cells opening in red light (Tallman and Zeiger, 1988), by the fact that red light opening is inhibited by DCMU (Schwartz and Zeiger, 1984), and by the finding that DCMU prevents Suc and Fru accumulation in *Vicia* guard cells opening in red but not in low-level blue light (Poffenroth et al., 1992).

Results from the dual-beam experiments indicate that the activation of the photosynthetic carbon fixation pathway by red light can be overridden by addition of low-intensity blue light. Addition of blue light caused a change from Suc to malate accumulation, and this change was accompanied by increases in maltose and maltotriose levels. Thus, added blue light appears to shift the primary osmoregulatory mechanism of the guard cell away from the photosynthetic carbon metabolism pathway to the starch-malate (potassium) pathway. Histochemical evidence for this shift was obtained in experiments (Tallman and Zeiger, 1988) in which blue light added to red light caused additional stomatal opening, disappearance of starch, and the accumulation of potassium in *Vicia* guard cells. Similar metabolic shifts have been reported with red-light-irradiated *Chlorella* cells upon addition of low-intensity blue light (Miyachi et al., 1978).

Our findings support the concept that stomata possess at

least three pathways for osmoregulation: accumulation of Suc through photosynthetic carbon fixation; uptake of potassium (and chloride), balanced by malate derived from starch breakdown; and accumulation of Suc derived from starch breakdown. The first mechanism is stimulated in red light, whereas the latter two are stimulated by low-intensity blue light. The red light-stimulated photosynthetic pattern of osmotic accumulation can be shifted to a starch-malate (potassium) pattern by addition of blue light to background red illumination, perhaps partially explaining the failure of many researchers to see photosynthetic patterns in guard cells treated with white light.

Although light quality is a useful tool to separate these mechanisms, we do not expect it to be a key regulatory feature in natural conditions. Instead, sensory transduction of environmental signals should result in the regulation of key metabolic switches, which would modulate the expression of the different osmoregulatory pathways. Because we observed (potassium) malate accumulation only during the initial, rapid phase of blue light-stimulated opening, it is possible that the potassium malate pathway is used primarily in stomatal responses requiring rapid opening, whereas longer-term responses involve carbohydrate metabolism. Analysis of guard cell osmoregulation in response to different environmental signals should prove rewarding for a detailed characterization of the role of the three osmoregulatory pathways in stomatal movements.

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