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Sucrose: a solute that accumulates in the guard-cell apoplast and guard-cell symplast of open stomata

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Abstract Stomatal conductances of *Vicia faba* leaves were recorded over a day. Coordinately, (a) guard cells dissected from leaflets were assayed, providing total sucrose content, and (b) guard cells dissected from rinsed epidermes were also assayed, providing symplastic sucrose content. Compared with that of pre-dawn samples, apoplastic sucrose content increased 4.8–5.2 × (2 experiments), reaching 1,130–1,300 fmol·guard-cell-pair^{-1} at midday, when conductance was highest (ca. 0.13 mol·m^{-2}·s^{-1}); symplastic sucrose content increased 2.5–3.5 ×, reaching 350–390 fmol·guard-cell-pair^{-1}. Thus, there is a correlation between transpiration and guard-cell sucrose content, particularly that portion localized to the apoplast. Moreover, apoplastic sucrose is apparently a source of guard-cell nutrition and, possibly, osmoticum.

Key words: Gas exchange; Guard cell; Stoma; Sucrose; *Vicia faba*

1. Introduction

CO_2 uptake by plants occurs through stomata, which are embedded in the epidermis. The aperture size of these pores, which are non-selective with respect to gas exchange, is regulated to achieve a balance between the requirement for CO_2 and the requirement for water conservation. Each stomatal pore is defined by the space between a pair of parallel guard cells. The pore size increases when guard cells take up water and curl because of cell-wall constraints [1]. Water movement between the guard cell and the surroundings is driven by the net effect of differences in the solute concentration and the hydrostatic pressure in guard cells compared with the surroundings. Thus, guard-cell-solute-concentration fluctuations – in the range of several hundred millimolar – are the essence of stomatal physiology and consequentially control other basic processes such as energy exchange, nutrient ascent, and, as mentioned, photosynthesis and loss of water. This latter is especially important as water is usually the most limiting resource for a terrestrial plant. Identification of the guard-cell solutes that fluctuate in concentration and elucidation of the mechanisms and regulatory processes involved have been major foci in guard-cell biochemistry and biophysics (e.g. [2,3]). Although certain discrepancies disturbed them, earlier workers usually subscribed to the so-called Classical Theory, exemplified by [4]. According to this theory, the abundant and osmotically inactive starch in guard-cell chloroplasts, which are functionally reminiscent of amyloplasts [5], is degraded to sugars, which drives stomatal opening. These sugars would be the osmoticum that drives stomatal movements. The Classical Theory was discounted upon the discovery several decades ago that, under myriad conditions, potassium accumulation by guard cells was sufficient to cause stomatal opening, and stomatal closure was accompanied by the loss of potassium (history in [1,6,7]). Notwithstanding, occasional reports indicated that an explanation of stomatal movements based solely on fluctuations in potassium salts was inadequate. Thus, MacRobbie and Lettau [8] observed that the measured changes in potassium salts could not account for the osmotic changes measured, particularly at some aperture sizes. Subsequent investigations [9–11] also failed to show the obligatory relationship between aperture size and potassium concentration. Corroboratively, elevation of guard-cell sugars [12–14] and a unique pattern of sucrose-metabolizing enzymes in guard cells [15] have been the additional impetuses for our reinvestigation of the role of sucrose and potassium in stomatal movements. In the experiments reported here, both greenhouse- and growth-chamber-grown plants were investigated over a daily time course of normal stomatal movements in planta. We found that the guard-cell symplastic sucrose concentration increased when stomata open, and, contrary to expectations, we also found that the apoplastic solution that bathes the guard cell increased even more.

2. Experimental

2.1. Plant materials and sampling

Vicia faba L. cv 'Longpod' plants were grown in 1-l pots in Metro-Mix 220 soilless potting medium in an air-conditioned, shaded greenhouse or in a growth chamber. The third fully expanded bifoliate of three-week-old plants was used in all experiments.

Greenhouse-grown plants were cultured in the summer (~13 h light period) in Tallahassee, FL, USA (latitude, 30° 23′ N). On the day of sampling, the RH was 74% (range = 51–92%); the temperature was 25°C (range = 22–28°C), and the light intensity was 370 μmol·m^{-2}·s^{-1} at the mid-morning sample and 495 μmol·m^{-2}·s^{-1} at the mid-afternoon sample. (These values were approximately 60% less than the maximum value on a full-sun day.) At the time pre-dawn samples were taken, the light intensity at this urban greenhouse was ≈8 μmol·m^{-2}·s^{-1}. For each time point (Fig. 2), two or three greenhouse-grown plants were pre-selected for sampling several days before the experiment. Selected bifoliates were horizontal, unshaded, and not in contact with other plants. Two manipulations were made on each bifoliate, in order: a conductance measurement (see section 3.1) was made on one leaflet and a 0.5 × 0.5 cm disc free of major veins was punched from the other leaflet. This punch (and all other samples subsequently used for histochemical analysis) was immediately frozen by immersion in a liquid nitrogen slurry.

Growth-chamber-grown plants were cultured under defined conditions (60% RH; 16-h day; 600 μmol·m^{-2}·s^{-1} photosynthetic photon flux density, provided by a mixture of incandescent and fluorescent lamps; 25/20°C day/night temperature regime). For both experiments and each time point (Figs. 1 and 3), growth-chamber-grown plants were

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pre-selected as described above. Four manipulations were made on each bifoliate, in order: a conductance measurement was made on one side of one leaflet, and, immediately, a punch from the opposite side of this leaflet was collected for histochemical analysis. Abaxial epidermal peels were taken from both sides of the other sister leaflet. One peel was rinsed to remove apoplastic contents before it was processed for histochemical analysis. (The wash-out procedure was based on unpublished kinetic studies (Springer, S.A. and Outlaw Jr., W.H.) of ^{14}C-sucrose uptake and wash-out.) The other peel was stained with 0.03% Neutral red and was used for stomatal-aperture estimations.

Histochemical samples were stored at −80°C until they were freeze-dried. For sucrose analysis, individual guard-cell pairs were dissected out of the lower epidermis of whole-leaf samples or out of epidermal peels and were assayed individually. For potassium and chloride estimations, freeze-dried epidermal pieces were used directly (see section 2.3). General aspects of these histochemical procedures may be found in [16–18].

2.2. Sucrose analysis

Sucrose assays were conducted according to the procedure of Jones et al. [19] except the buffer BES was substituted for imidazole, and the initial extraction volume was 0.5 μl.

2.3. Potassium and chloride analyses

Elemental analyses were performed by use of a FISONS Delta energy dispersive X-ray spectrometer interfaced with a JEOL 840 scanning electron microscope operated at 10 kV.

2.4. Conductance measurements

Conductance measurements were made with the LI-1600 Steady State Porometer (LI-COR, Inc., Lincoln, NE). Preliminary experiments showed that up to four reproducible measurements could be made from a single leaf area.

3. Results

3.1. Diel cycle of stomatal conductance

Conductance was nil before the onset of the light period in growth-chamber-grown plants (Experiment I, Fig. 1). Over the next several hours, conductance increased to a maximum value at 11.00 h (0.12 $mol \cdot m^{-2} \cdot s^{-1}$), and thereafter declined to 0.03 $mol \cdot m^{-2} \cdot s^{-1}$ at 22.00 h and 0 $mol \cdot m^{-2} \cdot s^{-1}$ at 23.30 h. A virtually identical trend for conductance was obtained in a replicate experiment (not shown; 11.00 h: 0.14 $mol \cdot m^{-2} \cdot s^{-1}$; 22.00 h: 0 $mol \cdot m^{-2} \cdot s^{-1}$). Both complete experiments were in confirmation of several preliminary observations (not shown). As a less precise measurement, stomatal aperture sizes were quantified on epidermal strips (Fig. 1). In these experiments, the maximum aperture size, reached at 11.00 h, was 8.3 ± 0.5 (S.E.) μm. Thus, the trend in aperture-size changes mirrored that of conductance changes in both experiments. Importantly, aperture sizes measured on strips are larger than those measured in planta (see e.g. [14]) because peeling ruptures many epidermal cells and thus relieves the back-pressure they exert on guard cells. Also importantly, the rate and extent of stomatal opening were less in these experiments than in comparable ones [12] in which two stimuli (viz. low $[CO_2]$ + light) were applied simultaneously.

Only conductance measurements were made on greenhouse-grown plants (Fig. 2). Conductance was nil at the pre-dawn sampling time, reached a high value near midday, and declined by early afternoon.

3.2. Sucrose contents of guard cells dissected from whole leaf and from rinsed epidermal peels over a diel cycle

In growth-chamber-grown plants, the sucrose contents of guard cells dissected from whole leaflet were significantly higher than those of guard cells dissected from rinsed epidermal peels at all times (Fig. 1). Initially, the whole-leaf guard-cell sucrose content was 356 ± 27 (S.E.) $fmol \cdot guard\text{-}cell\text{-}pair^{-1}$, which is similar to the control ('closed stomata') values (77 $mmol \cdot kg^{-1}_{(dry)} \approx 460$ $fmol \cdot guard\text{-}cell\text{-}pair^{-1}$) of Outlaw and Manchester [12], who made their measurements on detached leaflets in midmorning. Initially, the rinsed-peel guard- cell sucrose content was 140 $fmol \cdot guard\text{-}cell\text{-}pair^{-1}$, indicating that 39% of the sucrose of guard cells in closed stomata was

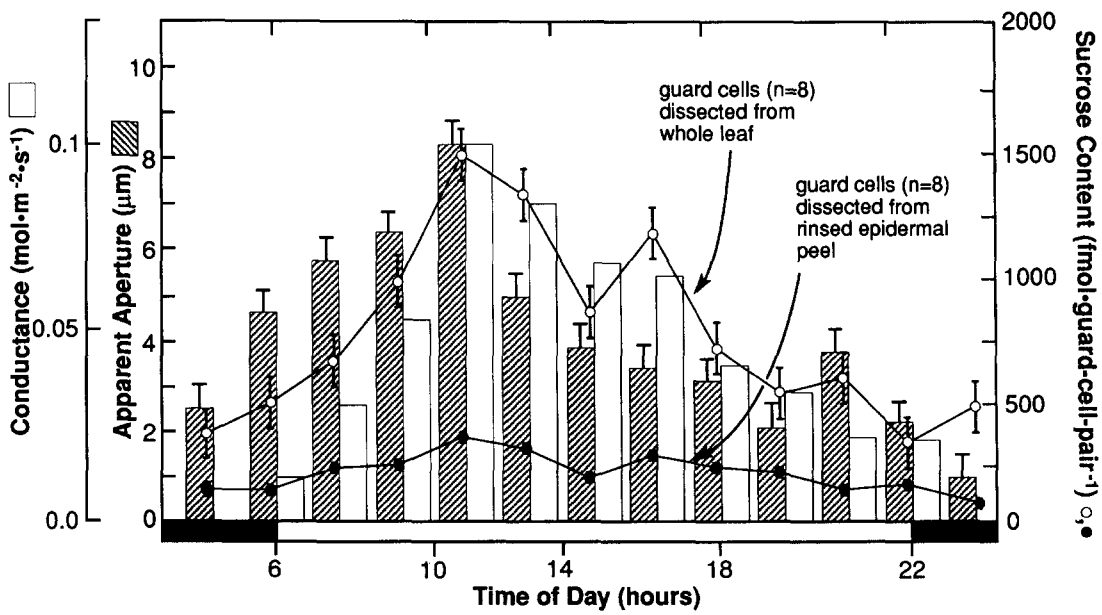

Fig. 1. The time course for stomatal conductance (open bars) and stomatal-aperture size (hatched bars) of growth-chamber-grown *Vicia faba* plants over a 24-h day. The sucrose contents of individually dissected guard cells from frozen-dried whole-leaf samples (apoplast + symplast, open circles) and the correlate values for those dissected from rinsed epidermal peels (symplast, closed circles) are superimposed on the histogram. Error bars are S.E.

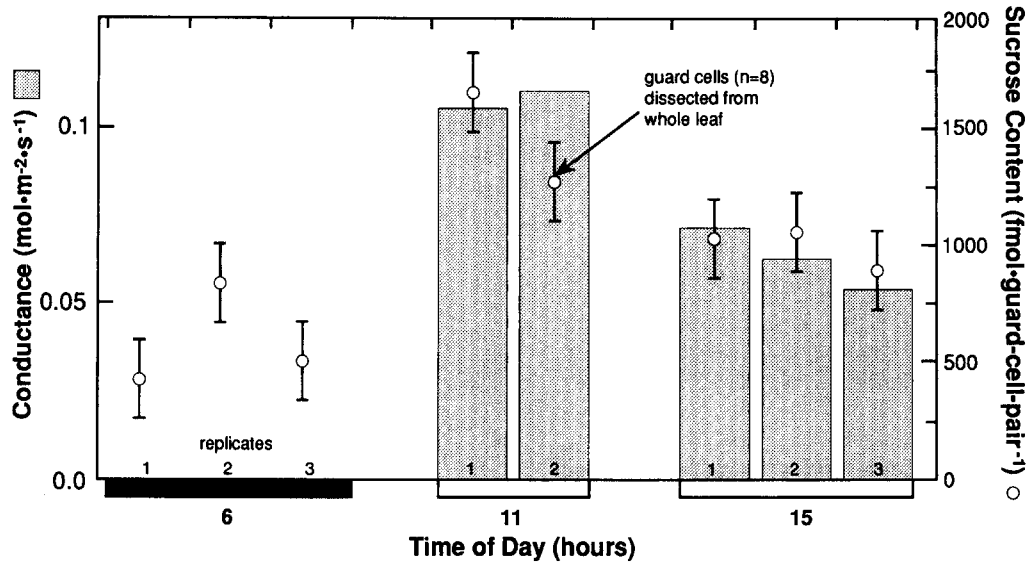

Fig. 2. Stomatal conductance (bars) of greenhouse-grown *Vicia faba* plants. Three samples from different plants were harvested at 06.00 h (essentially dark); two, at 11.00 h; and three, at 15.00 h. The sucrose contents of individually dissected guard cells from frozen-dried whole-leaf samples (apoplast + symplast, open circles) are superimposed on the histogram. Error bars are S.E.

symplastic (see section 3.3). In both types of guard-cell samples, the sucrose content increased dramatically as conductance increased (Fig. 1). The maximum value for guard cells dissected from whole leaflet, 1,475 fmol·guard-cell-pair^{-1}, coincided with the maximum value for conductance at 11.00 h; 24% of guard-cell sucrose was symplastic at this time. Following the midday maxima for sucrose content and conductance, the sucrose content of both types of guard-cell samples declined and reached approximately the pre-dawn levels by 22.00 to 24.00 h. The data for Experiment II (not shown) were virtually identical (at 05.30 h, the sucrose content was 383 ± 20 fmol·guard-cell-pair^{-1}, of which 29% was symplastic; at 11.00 h, the sucrose content was 1,690 ± 70 (S.E.) fmol·guard-cell-pair^{-1}, of which 23% was symplastic).

The sucrose contents of guard cells dissected from whole leaflet of greenhouse-grown plants were assayed at three times over the diel cycle (Fig. 2). Qualitatively, the changes of guard-cell sucrose content of these plants resembled those of growth-

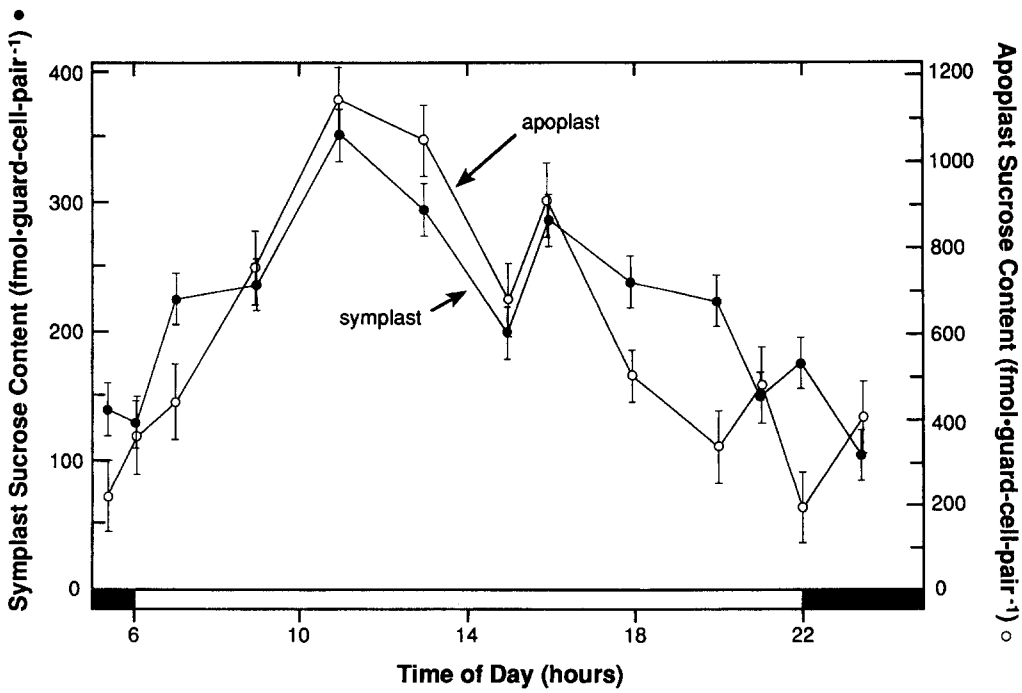

Fig. 3. The time course of changes in the guard-cell apoplast sucrose content (open circles) and in the guard-cell symplast sucrose content (closed circles). Error bars are S.E.

chamber-grown plants; i.e. the pre-dawn values were relatively low, the midday values were high, and a decline was evident by early afternoon.

3.3. Symplastic and apoplastic sucrose contents of guard cells over a diel cycle

Fig. 3 shows sucrose data transformed from Figure 1 (growth-chamber-grown plants). The sucrose of guard cells dissected from rinsed peels – because this sucrose is not readily diffusible – was assumed to be symplastic guard-cell sucrose, as implied above. The sucrose of guard cells dissected from whole leaflet includes that in both the guard-cell symplast and the guard-cell apoplast. Thus, values for the latter were obtained by subtraction (Fig. 3). (The directly obtained values – the symplastic ones – permitted the conventional method of obtaining statistical variations, whereas the apoplastic values were calculated by a routine that incorporated all the errors associated with the measurement of whole-leaf- and peel-derived guard-cell sucrose content.) Plotted on separate scales that permit easy comparisons, the changes in the symplastic and apoplastic sucrose pools were approximately parallel, with a somewhat greater increase in the apoplast.

3.4. Symplastic potassium and chloride contents of guard cells over a diel cycle

Intrigued by the reports that guard-cell potassium content declines over the diel cycle [20,21] and that changes in guard-cell potassium content are not coordinated always with changes in stomatal aperture [8–11], we assayed semiquantitatively for K and Cl in guard cells of growth-chamber-grown plants. Taken together, our semiquantitative elemental analyses revealed K and Cl fluctuations but the analyses did not show a reproducible correlation between the amounts of these elements and sucrose (or stomatal apertures).

4. Discussion

Ordinary epidermal peels are no longer used as a proxy for guard cells in the study of carbon metabolism because of confounding contamination from other cell types [22] and similar early studies have been discredited on this basis. Notwithstanding, the values obtained with peels over the course of a day should be relative and represent maximum limits when calculated to a guard-cell basis. Given these reservations, the most detailed published investigation to date of sucrose contents of 'guard cells' over a daily cycle [23] provides a reference for comparison. In brief, Pearson [23], who assayed for sucrose in unwashed epidermal peels of *Vicia faba* and *Commelina communis*, found a consistent increase, which peaked midafternoon (ca. 1,350 fmol·guard-cell-pair^{-1}, according to our conversion of his data to a basis comparable to ours). Although he found a strong relationship between time of day and 'guard cell' sucrose content, the relationship between aperture and sucrose content was weak (r^2 = 0.46 for *Vicia* and r^2 = 0.29 for *Commelina*). The experimental approach of Outlaw and Manchester [12] was different from Pearson's approach [23] and also provides results that complement those presented here. These workers [12] made a technically unambiguous measurement of guard-cell-sucrose content of stomata that were stimulated to open under non-photosynthetic conditions. The values thus obtained were, on average, only one-half of those reported here. Thus, the guard-cell-sucrose-concentration increase is time- and photosynthesis-dependent over a daily time course. We hypothesize that this sucrose increase results from evaporation of apoplastic sap in the guard-cell wall and is, therefore, a correlate of transpiration and photosynthesis in the mesophyll. In any case, an important present contribution is that the guard-cell apoplastic solution varies dramatically over the course of a day.

Two groups [13,14] have incubated detached sonicated peels under conditions that stimulate stomata to open. (Sonication of detached epidermal peels is a method to destroy epidermal cells but leave guard cells intact.) Their sucrose measurements over a 2- or 4-h time course also revealed that the guard-cell sucrose contents increase during stomatal opening, but the measured increases differed in detail from those reported here. They were symplastic measurements only, and the sucrose increase (which exceeds those we report by up to 3×) cannot be attributed to a mesophyll source. These differences may imply that considerable flexibility in the extent and the means by which guard cells metabolize carbohydrates awaits discovery. Particularly interesting is the possibility that guard cells have alternative osmoregulatory solutes, as outlined in the Introduction. Our results, however, indicated that the sucrose concentration *within* guard cells reached only approximately 40 mM, which is only a small fraction of the osmolality fluctuation that causes stomatal movements. We speculate that other experimental conditions, for example, higher photon flux or greater vapor pressure difference, would possibly amplify the increases we observed.

Our assays for guard-cell sucrose contents over a daily time course were highly reproducible, but comparable assays for potassium were not. Our results for Experiment I (a rapid increase in K^+, followed by a decline even as stomata remained open) are reminiscent of those reported by Talbott et al. [21], whereas those of Experiment II (a less rapid increase in K^+, which trailed the aperture-size increase) are reminiscent of those of Laffray et al. [10]. The important role of K^+ in stomatal movements [24] is unquestioned, but its role as the sole osmolyte that effects stomatal movements deserves reevaluation.

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