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Guard Cell Starch Concentration Quantitatively Related to Stomatal Aperture[1]

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ABSTRACT

Using quantitative histochemical techniques, the carbohydrate levels of guard cells from open and closed stomatal apparatus of *Vicia faba* L. were compared. To minimize experimental error, all comparisons were between leaflets of the same pair. Stomata on one leaflet were caused to open by light and reduced CO_2. The other leaflet, which was in darkness, had closed stomata. In one experiment, data were also collected on palisade parenchyma, spongy parenchyma, and epidermal cells.

Guard cell starch concentration was higher in the leaflets with closed stomata than in open stomata by 72 ± 16 millimoles per kilogram dry weight (anhydroglucosyl equivalents) ($N = 117$, $P < 0.02$). Variation in guard cell starch concentration from one part of a leaflet to another was small. The data are consistent with the hypothesis that starch degradation provides the carbon skeletons for anion synthesis in guard cells during stomatal opening.

Sucrose concentration was higher in guard cells when stomata were open than when they were closed in all three experiments (average difference = 45 ± 7 millimoles per kilogram dry weight [$N = 59$, $P < 0.01$]). The variability of sucrose concentration within test leaflets prevented an unequivocal interpretation of these results. When all data are considered, it appears that soluble sugars increase in guard cells when stomata of *Vicia faba* open.

Gas exchange between the environment and leaf interior is regulated by stomatal aperture. Stomatal opening results from guard cell swelling which is caused by increased osmotica in the cell. Most of the change in osmotic potential can be accounted for by K^+ influx (2, 6). Potassium uptake is balanced by Cl^- uptake (15, 18, 19) and organic anion synthesis (1, 14). Starch[2] decreases in guard cells as stomata open (for early references, see ref. 5). A current view is that starch is degraded to form precursors for anion synthesis (6, 13). Experiments were performed to test this hypothesis quantitatively. This paper reports the results of these experiments.

MATERIALS AND METHODS

PLANT MATERIAL

Vicia faba L. was used in all experiments. Tissue samples for experiments 1 and 2 were from the same leaflets used earlier for K^+ and organic anion analysis (14). Plants for all other experiments were grown in pots in a greenhouse where natural light was supplemented by General Electric cool-white fluorescent tubes to give a daylength of 14 h. Temperature was 22 to 27 C during growth. Conditions used for induction of stomatal opening and closing in the intact leaflets and sampling techniques have been described previously (14).

REAGENTS

Enzymes (except *Rhizopus* amyloglucosidase) which were used in the analytical procedures were supplied as $(NH_4)_2SO_4$ suspensions by Boehringer. SO_4^{2-} concentration was reduced as before (13). *Rhizopus* amyloglucosidase (grade II) and other biochemicals were from Sigma.

STARCH DETERMINATION

The principle used for starch determination has been described by Lust *et al.* (9). Details of the initial portions of the assay are given below.

Step 1. Guard cell contents were extracted in 20 nl of 0.2 N KOH containing 100 mM ethanol using the "oil well technique" (8). Incubation was for 20 min at 80 C. Starch standards were carried through all steps of the assay.

Step 2. One hundred nl of specific step reagent was added to the oil well droplet. The reagent was 100 mM sodium acetate (70 mM acid, 30 mM base) and 200 μg/ml amyloglucosidase (from *Rhizopus*, EC 3.2.1.3). Incubation was for 60 min at 30 C. This step was terminated by heating to 80 C for 20 min. Glucose standards were incorporated into some aliquots of this step and were carried through the remaining steps of the assay.

Step 3. Fifty nl of glucose reagent was added to the reaction droplet. The reagent was 360 mM Tris-HCl (50 mM acid, 310 mM base), 3.6 mM $MgCl_2$, 1 mM ATP, 0.1 mM $NADP^+$, 3.6 mM DTT, 0.2 μg/ml glucose-6-P dehydrogenase (from yeast, EC 1.1.1.49) and 3.6 μg/ml hexokinase (from yeast, EC 2.7.1.1). Incubation was for 30 min at 24 C.

Step 4. One μl of 0.1 N NaOH was added. The droplets were heated to 80 C for 20 min.

Step 5. The NADP in a 0.5-μl aliquot was amplified by an enzymic cycling technique (8).

Heating in 0.2 N ethanolic KOH was necessary to make amylaceous polymers susceptible to enzymic degradation. When wheat starch, corn starch, "soluble" potato starch, shellfish glycogen, and rabbit liver glycogen were heated in only 0.02 N NaOH, the end point of glucose release by *Aspergillus* amyloglucosidase was 34, 53, 55, 88, and 97%, respectively, of that obtained by acid hydrolysis (2 N HCl, 2 h). The present method yielded 100% of acid hydrolysis with these polymers. The *Rhizopus* amyloglucosidase preparation was apparently sufficiently contaminated with α-amylase to make addition of this enzyme unnecessary (10).

The enzyme concentration time factor was necessarily some-

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[2] Starch concentration is in anhydroglucosyl equivalents.

what arbitrarily chosen for guard cell analysis. However, with only 20 μg/ml of enzyme, the $t_{1/2}$ of the reaction with soluble potato starch was less than 5 min at 30 C. Thus, a large excess of enzyme was used to ensure hydrolysis of even the most enzymically resistant α,1-4, 1-6-glucan. No harmful effects of this large excess of enzyme were observed.

An incision through the wall of the dissected cells had to be made to allow diffusion of the enzyme or starch. (Most pores in higher plant cell walls do not exceed 40 Å [D. P. Delmer, personal communication]). When no conscientious effort was made to remove one end of the cell, one-third of the assays showed zero starch content (N = 33). Presumably, the other two-thirds of the cells were inadvertently nicked during dissection or fractured during freezing. The assay response in the experiments was an all or none phenomenon with no very low values.

SOLUBLE CARBOHYDRATE ANALYSIS

Microanalysis for sucrose was according to the method of Jones *et al.* (7). Microanalysis for total soluble hexosyl equivalents (defined here as glucose + fructose + 2X sucrose) was also according to Jones *et al.* except: (*a*) the extraction was in 0.02 N HCl at 80 C for 20 min (this procedure results in sucrose hydrolysis but not cellulose hydrolysis [as judged from tests with filter paper]); (*b*) invertase was omitted; and (*c*) glucose-6-P dehydrogenase was reduced to 0.05 μg/ml virtually to eliminate contaminating P-glucose isomerase activity. Typically, one aliquot of the extract was assayed for glucose (P-glucose isomerase omitted) and another aliquot was analyzed for glucose and fructose.

Sucrose analysis in $HClO_4$ extracts of whole leaflet was described earlier (7). Macroanalysis for total soluble hexosyl equivalents was also according to Jones *et al.* except P-glucose isomerase and invertase were omitted from the reagent and glucose-6-P dehydrogenase concentration was reduced. After the reactions with glucose were over, a fluorescence reading was made and P-glucose isomerase was added. A second reading was made after the reactions with fructose were over. Invertase was then added and a final reading was made after the reactions with sucrose were over.

It would have been more satisfactory to have been able to make sucrose, glucose, and fructose analysis on the same cell extract. A protocol for these determinations worked well on standard solutions (sucrose is not hydrolyzed by cold weak acid and neither glucose nor fructose is destroyed by cold alkali). However, extraction of guard cells was not complete unless the extracts were heated. Individual determinations on different cells were made for sucrose. Hexoses (endogenous and resulting from sucrose hydrolysis) were measured in another cell. Unfortunately, variability of the sugar concentration within a leaflet prevented simply subtracting out the components.

RESULTS

Guard cell starch concentration was lower in open stomata than in closed (Fig. 1). In experiment 3, guard cell starch concentration was assayed from several positions within each leaflet of the pair. The concentration of starch in guard cells varied only slightly within each leaflet (standard error of the means = 5 and 10 mmol/kg dry weight). The difference between the leaflets was large (54 ± 5 mmol/kg dry weight). In experiments 1 and 2, the decrease in guard cell starch when stomata opened was 63 ± 24 and 98 ± 20 mmol/kg dry weight. In the three experiments, the average decrease in guard cell starch concentration was 72 ± 16 mmol/kg dry weight. Epidermal starch concentration from both treatments was about 10 mmol/kg dry weight which was lower than any of the other tissues assayed. Starch concentration in the parenchyma was about 160 mmol/kg dry weight which is 2.5% of dry weight. In the palisade parenchyma, the starch content was the same in both treatments. However, in the spongy parenchyma, the leaflet in darkness had less starch.

The total soluble hexosyl equivalents concentration was much higher in guard cells of open stomata in experiments 1 [Δ = 245 ± 39 mmol/kg dry weight, $P < 0.01$) and 3 (Δ = 140 ± 40 mmol/kg dry weight, $P < 0.01$) (Fig. 2). In experiment 2, the concentra-

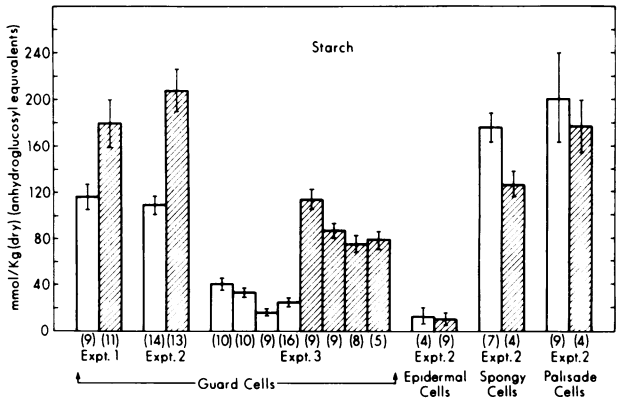

FIG. 1. Starch concentration in various tissues dissected from frozen-dried *Vicia* leaflet. Hatched bars are data from a leaflet with closed stomata. Open bars are data from a leaflet with open stomata. SE are indicated by vertical lines for the number of samples given in parentheses below the bars. Guard cells were assayed from various areas of the leaflets in experiment 3. $P < 0.02$, 0.01, and 0.01, respectively for experiments 1, 2, and 3 for the guard cell data.

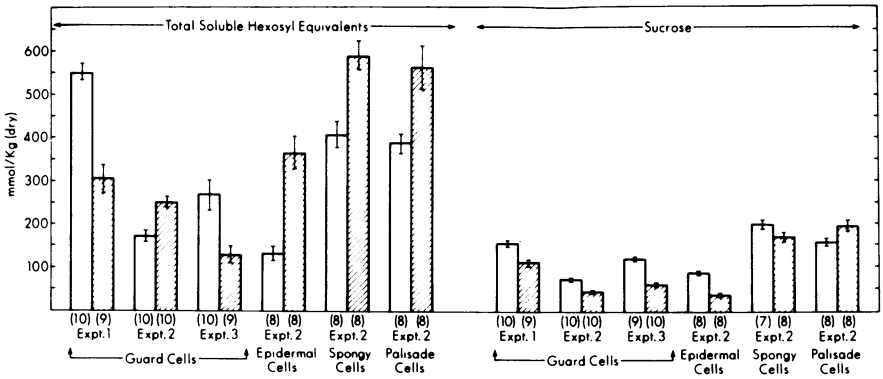

FIG. 2. Total soluble hexosyl equivalents (*i.e.* glucose + fructose + 2X sucrose) concentration and sucrose concentration in various tissues dissected from frozen-dried *Vicia* leaflet. Hatched bars are data for a leaflet with closed stomata. Open bars are data for a leaflet with open stomata. SE are indicated by vertical lines for the number of samples given in parentheses below the bars. $P < 0.01$ for all guard cell comparisons. Other experiments (see text) suggest that aberrant result for experiment 2 total soluble hexosyl equivalents concentration resulted from initial differences between leaflets of that pair.

tion was 30% lower in guard cells from the leaflet with open stomatal apparatus. To determine if the lower sugar concentration was a phenomenon restricted to guard cells in that leaflet, other tissues were also analyzed. The results show that the total soluble hexosyl equivalents concentration was also lower by an average of 42% in the other tissues. In three other experiments (data not shown), one leaflet of each pair was placed in darkness and the other leaflet was placed in light and reduced CO_2 (same protocol as used for the reported histochemistry experiments). Whole leaflet sucrose, glucose, and fructose concentrations were identical in the paired leaflets. Therefore, the difference between the two leaflets in experiment 2 was not owing to the pretreatment for stomatal opening and closing. If the guard cell data are normalized to account for the initial differences in the leaflets, a modest increase in the guard cell total soluble hexosyl equivalents concentration is observed in experiment 2 also.

Guard cell sucrose concentration was one-third higher ($\Delta = 45$ mmol/kg dry weight, $P < 0.01$) in leaflets with open stomata (Fig. 2). Sucrose concentration was also higher in the epidermis of the leaflet with open stomata. Sucrose concentration was about 180 mmol/kg dry weight in both parenchyma cell types with no correlation to stomatal aperture.

In six experiments (data not shown), leaflets were subsampled from four different areas and extracts were assayed for glucose, fructose, and sucrose concentration. Sometimes the sucrose concentration varied more than 100% within a leaflet. By contrast, neither glucose nor fructose ever varied by more than 60%; typically, the variation was no more than 10%.

DISCUSSION

Organic anion accumulation in guard cells during stomatal opening (14) may be accomplished by synthesis from endogenous reserves. On the other hand, anions may be transported from other parts of the leaf into guard cells. In several reports (*e.g.* 3, 20), no difference in malate concentration between epidermal peels with open or closed stomata was observed. Bowling (3) interpreted his results by proposing a malate switch hypothesis (3) to explain malate shuttle into guard cells. However, there are few direct data to support such transport. van Kirk and Raschke (22) have shown that the reduction in malate content of epidermal strips when stomata are induced to close by floating the strips on an ABA solution is due to the release of malate from guard cells. They concluded that malate release from guard cells during stomatal closure was a significant process.

There are two lines of support for the idea that malate is synthesized within guard cells: (*a*) starch grains are commonly seen to be reduced in guard cells during stomatal opening; and (*b*) experiments with epidermal peels have shown that stomata will open even when all epidermal cells *per se* have been destroyed (4, 21). In one of these reports (21), an increase in epidermal malate was seen. These observations suggest that the carbon skeleton for anion synthesis can be from an endogenous source. The purpose of the present investigation was to determine to what extent the degradation of starch could account for the carbon skeleton for anion synthesis. During stomatal opening, the concentration of guard cell malate and citrate increased by 72 mmol/kg dry weight and 47 mmol/kg dry weight, respectively, in the same leaflets used here for experiments 1 and 2 (14). One mol of starch (anhydroglucosyl equivalent) can be used to synthesize either 2 mol of malate or 1 mol of citrate. Degradation of starch in these experiments would have required 83 mmol/kg dry weight. The observed decrease in starch in experiments 1 and 2 was 81 mmol/kg dry weight. Thus, this report shows, for the first time, the quantitative relationship between guard cell starch concentration and stomatal aperture, and shows that the reduction in starch could have accounted for all organic anion synthesis, making the requirement for transport (3) unnecessary. Nevertheless, neither the experiments reported in this paper nor in the literature prove that, *in situ*, the precursors for anion synthesis come from starch. This remains an important question. The present paper does show that this pathway is quantitatively feasible.

The metabolite concentrations reported in this paper have been expressed as millimoles per kilogram dry weight. This convention has been adopted, in part, because the experimental protocol requires freezing and drying (to stabilize tissue contents). However, the results can be converted to a guard cell basis or to osmolarity because average guard cell dry mass (~ 6 ng, ref. 14) and guard cell volume (6, 13) are known. It can be calculated that the starch degradation shown in Figure 1 was sufficient to increase guard cell organic acid salts by 0.3 osmolar. Thus, starch reserves in *V. faba* guard cell chloroplasts may be very important in turgor generation for stomatal opening.

The soluble carbohydrate data can not be interpreted without some reservation owing to variation of sucrose within leaflets. However, it is extremely unlikely that all three experiments would show the same trend for both sucrose and total soluble hexosyl equivalents concentrations (see under "Results" for comments on experiment 2) if it were due solely to random variation. Additionally, on several hundreds of guard cell pairs dissected from paired *Vicia* leaflets (14), guard cells from open stomata weighed about 0.7 ng more than guard cells from closed stomata. Potassium and chloride uptake could account for about 0.2 ng of the observed increase. Sugars could account for some of the remainder of the increased weight. In other experiments, substantial exchange of sucrose between epidermis and the photosynthetic parenchyma was observed (11, 12). It is possible that guard cells *in situ* also exchange substantial amounts of sucrose with the surroundings. The few data in the literature on this point are mixed. Pearson (17) saw an increase of about 100% in sugar concentration in epidermal peels of *Vicia* and *Commelina*. The increase corresponded to increasing stomatal aperture during the day. Rutter *et al.* (20) found no differences in sugar content of epidermal peels of *Vicia*, *Tulipa*, or *Commelina* when stomatal aperture was changed. Nor did Pallas and Wright (16) find changes in glucose or sucrose in epidermal peels of *Vicia* which could be correlated to stomatal aperture. The data reported in this paper indicate that sugars increase in guard cells during stomatal opening. The role of these sugars needs to be investigated. There may simply be a mechanism to maintain sugar concentration in plant cells. If this is true, during stomatal opening, guard cells would either import or synthesize sugars. (However, this would change total leaf sugar pool less than 1%.)

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