Glucose Localization in Maize Ovaries When Kernel Number Decreases at Low Water Potential and Sucrose is Fed to the Stems

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INTRODUCTION

Reproduction in angiosperms typically involves the conversion of a vegetative shoot apex to a floral meristem. Photoperiodic or other signals trigger the conversion, and the potential number of florets is laid down. Subsequent growth determines the fraction of the potential florets that develop into flowers, and eventually to fruits and seeds. Significant dry mass accumulates in fruits and seeds, and depends on a supply of photosynthetic products.

The full dry mass accumulation potential is often unrealized because environments are unfavourable, particularly for water supply (Boyer, 1982). Limited water causes water potentials ($\Psi_w$) to decrease, often inhibiting photosynthesis. If these conditions develop around the time of pollination, they cause particularly large losses in reproduction (Salter and Goode, 1967). In maize, several reproductive steps can be altered and ovary abortion can occur, decreasing kernel number (Westgate and Boyer, 1985, 1986b; Boyle et al., 1991; Zinselmeier et al., 1995a, b; Zinselmeier et al., 1999; Saini and Westgate, 2000; Setter et al., 2001). Feeding sucrose to the stems was found to partially prevent the abortion and restore kernel number (Zinselmeier et al., 1995a, 1999). The restoration illustrates the central role of photosynthesis in kernel development and further indicates that low $\Psi_w$ was not inherently lethal. Abortion had a biochemical cause.

Zinselmeier et al. (1999) reported a breakdown of ovary starch under these conditions and attributed the abortion to sugar starvation or possibly an abnormal distribution of sugars. Andersen et al. (2002) observed abortion in the presence of ovary starch, and Schussler and Westgate (1994) found abortion to be related more to the delivery of sugars than to the release of sugars from reserves in the parent plants. Acid invertase, the main enzyme to process sucrose, had less activity at low $\Psi_w$ than at high $\Psi_w$ (Zinselmeier et al., 1995b; Zinselmeier et al., 1999; Andersen et al., 2002). The activity was not fully restored by feeding sucrose to the stems (Zinselmeier et al., 1999). Intermediates for starch biosynthesis downstream of the invertase step were depleted at low $\Psi_w$ and not fully restored by the sucrose feeding, which implicated acid invertase as a limiting step in starch biosynthesis (Zinselmeier et al., 1999).

Because glucose is an immediate product of invertase activity and also of starch breakdown, it serves a central role in ovary metabolism that could be disrupted at low $\Psi_w$. Therefore, the following investigation was devoted to measuring glucose concentration and distribution in maize ovary.
ovaries undergoing abortion at low $\Psi_w$. Invertase activities also were localized in the same tissues.

**MATERIALS AND METHODS**

**Plant material and growth conditions**

Maize hybrids (Zea mays L. ‘DE2 X H99’) developed at the University of Delaware (Hawk and Weldekidan, 1999) were grown in 22-L pots containing 15-59 kg oven-dry soil mix, composed of Evesboro loamy sand, loamy substratum (coated, mesic, Typic Quartzipsamments) amended with peat and sand in volumes of 1:1:1. Dolomitic limestone (275 g per pot) was added to the mix to adjust the pH to 6.9. Two seeds were planted in each pot and the soil mix was saturated with nutrient solution (4 mM KNO$_3$, 6 mM Ca(NO$_3$)$_2$·4H$_2$O, 2 mM MgSO$_4$·7H$_2$O, 2 mM KH$_2$PO$_4$, 0.5 $\mu$M CuSO$_4$·5H$_2$O, 10 $\mu$M MnSO$_4$·H$_2$O, 2 $\mu$M ZnSO$_4$·7H$_2$O, 25 $\mu$M H$_2$BO$_3$, 0.5 $\mu$M H$_3$MoO$_4$ and 50 $\mu$M Fe-citrate according to Hoagland and Arnon (1950) and allowed to drain. The pots were placed in a controlled environment chamber (Environmental Growth Chambers, Chagrin Falls, Ohio, USA) with day/night temperatures and relative humidities of 30/20 °C ± 1 °C and 40/95 % ± 5 %, respectively. Cool-white fluorescent lamps provided a 14-h photoperiod with a photon flux of 850–1000 $\mu$mol m$^{-2}$ s$^{-1}$ at the top of the canopy. After 2-5 weeks, seedlings were thinned to one per pot and supplied with the same nutrient solution until drainage. Nutrients were supplied twice a week. Supplemental KNO$_3$ (12 mM) was supplied during the night, using the method of Zinselmeier et al. (1999) modified from Boyle et al. (1991). At the end of the dark period between each of the 5 days after water was withheld, the infusion was carried out at the upper part of one internode, starting with the ear internode and moving to one internode above and three below the ear internode on the successive days. The plants received between 35 and 45 mL sucrose solution (5.3–6.8 g of sucrose) each day, which was sufficient to provide the daily gain in dry mass normally produced by photosynthesis (Jurgens et al., 1978). For some infusions, especially at the lowest $\Psi_w$, two sites were used in the same internode to achieve an acceptable total uptake.

**Stem infusions**

For the plants at low $\Psi_w$ whose stems were fed with sucrose, a sucrose solution from sugarcane (0.438 M, $\Psi_S$ = −1.1 MPa) was infused into a well freshly made in an internode, using the method of Zinselmeier et al. (1999) modified from Boyle et al. (1991). At the end of the dark period between each of the 5 days after water was withheld, the infusion was carried out at the upper part of one internode, starting with the ear internode and moving to one internode above and three below the ear internode on the successive days. The plants received between 35 and 45 mL of sucrose solution (5.3–6.8 g of sucrose) each day, which was sufficient to provide the daily gain in dry mass normally produced by photosynthesis (Jurgens et al., 1978). For some infusions, especially at the lowest $\Psi_w$, two sites were used in the same internode to achieve an acceptable total uptake.

**Water potential, photosynthesis, water usage and green leaf measurements**

Leaf $\Psi_w$ and net photosynthesis were measured on the unshaded third leaf from the top of the plant. This leaf was fully green throughout all treatments. At 3–4 h into the photoperiod, net photosynthesis was measured using a LI-Cor LI-6400 photosynthesis meter (Li-Cor Inc., Lincoln, Nebraska, USA). The measured area was exposed to a red (670 nm) LED light source on the instrument at a PFD of 700 $\mu$mol m$^{-2}$ s$^{-1}$. The leaf then was sampled by removing a disc (4·2 cm$^2$) and determining $\Psi_w$ by isopiestic thermocouple psychrometry, according to the method of Boyer and Knippling (1965) and Boyer (1995). Water usage was monitored gravimetrically for the whole plant by weighing the pot–soil–plant system. The amount of healthy leaf area that was designated day ±5, the plants were hand-pollinated on day 0, and water was resupplied on day 1. All of the plants from which water was withheld gradually developed low $\Psi_w$, and sucrose was fed to the stems of half of them (one-third of the total plant population) each day, as in Zinselmeier et al. (1999). The remaining one-third of the plants were high $\Psi_w$ controls, watered with 750 mL in the morning and 750 mL in the afternoon (approximating the loss from transpiration).
was determined by measuring the area of each leaf and visually estimating the percentage of the area having a healthy green colour. The area of each leaf and area of each green portion were measured separately, and the total green area for the whole plant was expressed as a percentage of the total leaf area for the plant (a completely healthy plant had green area of 100%).

Invertase localization

During the 6-d treatment period, fresh ovaries along with subtending pedicels were removed from the ear and sections were hand-cut longitudinally (<0.1 mm thick) in phosphate buffer (0.38 M, pH 5.1) for assay of insoluble acid invertase in vivo. The slices were washed for 3 h with constant stirring using at least ten changes of water to remove endogenous sugars and other soluble molecules in the apoplast. Individual slices were exposed to 1 mL of reaction medium containing 0.24 mg mL⁻¹ nitroblue tetrazolium, 0.14 mg mL⁻¹ phenazine methosulfate, 25 units mL⁻¹ acid invertase, 5 mg mL⁻¹ glucose oxidase and 5 mg mL⁻¹ sucrose for 1 h at 25 °C in phosphate buffer, 0.38 M, pH 5.1 (Dahlqvist and Brun, 1962; Doehlert and Felker, 1987). In the reaction, β-D-glucose formed from sucrose hydrolysis was converted by glucose oxidase to gluconolactone and peroxide. The peroxide with phenazine methosulfate created a redox couple that reduced the nitroblue tetrazolium to an insoluble precipitate at the site of the reaction, thus localizing the activity (Dahlqvist and Brun, 1962; Doehlert and Felker, 1987). Sections were washed briefly with water, fixed in a 4% solution of formaldehyde, viewed under a dissecting microscope, and imaged with a digital camera (Nikon CoolPix 990). Control sections were exposed to the same medium without sucrose for the same periods of time. In maize ovaries, invertase is the only sucrose-cleaving enzyme that produces free glucose, and the low pH ensured that the activity was mostly from acid forms of the enzyme. Alkaline forms typically have pH optima between 7-0 and 7-8 (Sturm, 1999) and were not investigated. The thickness of the hand sections did not allow the activity to be localized inside individual cells but, because the sections were alive and thoroughly washed, all the glucose-producing activity was outside of the plasma membrane and wall-bound (insoluble acid invertase).

Soluble acid invertase activity was localized similarly, except the sections were placed in liquid nitrogen after being cut. The freezing disrupted cell membranes after which the sections were gently thawed at room temperature and washed as above. The sections were exposed to the reaction medium for 3 h instead of 1 h to allow time for sucrose to penetrate the cells and glucose to diffuse out. The sections were then fixed and imaged as above.

This soluble invertase assay depended on the concept that, after thawing, small solutes diffused rapidly in and out of the cell interior. This rapid diffusion is demonstrated in measurements of osmotic potential by vapour pressure methods in plant tissues (thermocouple psychrometry, Boyer, 1995). After freezing and thawing in liquid N₂, the vapour pressure developed from the apoplast solution indicates that solute in the apoplast arrives within minutes. Conversely, large solutes such as proteins and nucleic acids diffuse comparatively slowly out of the cell. Baron-Epel et al. (1988) showed in soybean that wall pores hindered the diffusion of proteins larger than about 67 kD, and did not permit proteins of 120 kD or larger to move through the cell walls. The soluble invertases of maize tend to be tetrameric and are reported to have molecular weights around 200 kD in their native form, but often aggregate further to over 7000 kD in the frozen state, embedded in OCT compound (Ted Pella, Redding, CA, USA) at −20 °C, sliced at −20 °C in a microcryotome to a thickness of 20 μm, transferred at −20 °C to a lyophilizer, lyophilized at −20 °C, and mounted on a slide with the reaction mix at −20 °C. The cryostat section and reaction mix were allowed to warm to initiate the reaction. Care had to be taken to prevent warm air from contacting the slide during all transfers because water condensed on the cold tissue and provided a path for glucose migration. Condensation was prevented by transporting the tissue in a covered Petri dish on a pre-cooled Al heat sink.
Endogenous glucose was localized in situ according to the method of Borisjuk et al. (1998) and Walenta et al. (2000) but using the glucose detection reagent 10-acetyl-3,7-dihydroxyphenoxazine (Amplex™ Red reagent, Molecular Probes, Eugene, OR). In the assay, endogenous β-D-glucose is converted by glucose oxidase to D-glucono-1,5-lactone and hydrogen peroxide at pH 7.4, which inhibits the activity of endogenous acid invertase to about 5% of its activity at pH 5 (Shannon and Dougherty, 1972). The pH optimum for both the soluble and insoluble acid invertases is approx. 5.0 (Shannon and Dougherty, 1972; Doehlert and Felker, 1987).

The released hydrogen peroxide was detected using the Amplex Red, which is normally non-fluorescent but is converted by the peroxide in a 1:1 stoichiometry to fluorescent resorufin (excitation at 563, emission at 587 nm) (Zhou et al., 1997). The spectra resulted in very little interference from tissue auto-fluorescence. To maintain the 1:1 stoichiometry, the initial concentration of Amplex Red was greater than the final concentration of hydrogen peroxide. If the final concentration of hydrogen peroxide became greater than for Amplex Red, resorufin became further oxidized to a non-fluorescent complex compound, terminating the 1:1 stoichiometry (Zhou et al., 1997).

A microscope slide containing a circular casting mould (Sigma Depth Module E, 1.3 mm depth) was prepared by filling the mould with a liquid gel mixture consisting of 10% Difco gelatin (BD Biosciences 211868, Franklin Lakes, NJ, USA), 5% polyethylene glycol (400 MW; Sigma P-3265, St Louis, MO, USA), glucose oxidase (Sigma G-9010) at a final concentration of 1.8 units mL⁻¹, horseradish peroxidase (Sigma P-8125) at a final concentration of 1.5 units mL⁻¹, and Amplex Red Reagent at a final concentration of 200 μM in 50 mM phosphate buffer at pH 7.4. After 30 min at room temperature in the dark, the resulting resorufin was quantified with a Shimadzu RF-1501 spectrofluorophotometer (Shimadzu, Kyoto, Japan) with excitation at 563 nm and emission at 587 nm. The absolute glucose content of individual cyrosections was determined from the resorufin produced from standard solutions of glucose (Sigma G-8270). Background fluorescence from a no-glucose control reaction was subtracted from each assay determination. The assay was linear from 5 μM to 45 μM glucose. Both the cyrosection and glucose standard assays were performed in triplicate.

**RESULTS**

**Physiological responses and ovary abortion**

For a few days before and after pollination, floret and embryo development are affected if $\Psi_w$ becomes low in maize (Westgate and Boyer, 1986b). Provided pollination occurs, the susceptibility resides in the female florets and generally involves irreversible cessation of ovary or embryo development (abortion) soon after fertilization takes place (Westgate and Boyer, 1986b). In the following experiments, $\Psi_w$ was typically −0.45 MPa in controls, but withholding water for 6 d after the silks first appeared (5 d before pollination) caused leaf $\Psi_w$ to decrease to −1.45 MPa by the day of pollination (Fig. 2A). Rewatering 1 d later caused $\Psi_w$ to recover fully by day 2. Westgate and Boyer (1986a) reported that silk $\Psi_w$ parallels leaf $\Psi_w$ under these conditions, but reaches slightly less extreme values.

The effects of low $\Psi_w$ were seen in plant water use, which decreased from about 1.4 L d⁻¹ in the controls to about 0.25 L d⁻¹ at low $\Psi_w$ on day 0. Water use recovered fully by day 4 (Fig. 2B). Net photosynthesis showed a similar response, diminishing to near zero on day 0 and recovering fully by day 4 (Fig. 2C). These recoveries in water use and net photosynthesis were slower than the recovery in $\Psi_w$, probably reflecting a delay in full stomatal opening as an after-effect of the low $\Psi_w$ (Beardsell and Cohen, 1975). There was little evidence of damage to the plant leaves. About 70% of the total leaf area remained a healthy green colour (Fig. 2D). The loss of green leaf area was mostly at the bottom of the plant and did not progress further after rewatering. Feeding sucrose to the stems did not change the response of leaf $\Psi_w$, plant water use, net photosynthesis, or percent green leaf during the water deficit (Fig. 2). These results indicated that the parent plant remained alive and able to resume physiological activity when $\Psi_w$ recovered.

However, many ovaries aborted at low $\Psi_w$, and at maturity kernel numbers were only about 15 ± 3 (s.e. for...
9–11 plants) per ear. Controls at high $Y_w$ contained nearly 500 fully developed kernels on each ear. Feeding sucrose to the stems prevented most of the abortion, and kernel numbers were 340 ± 15 per ear (68% of controls).

Ovary invertase activity

In maize ovaries, acid invertases have lower activities at low $Y_w$ than at high $Y_w$ (Zinselmeier et al., 1995b, 1999; Andersen et al., 2002). In order to localize the enzymes more fully in maize ovaries, the insoluble activity was measured in vivo in fresh sections and the soluble activity in situ in frozen/thawed sections. The insoluble invertases appear to be ionically bound to cell walls while the soluble forms appear to be in the cytoplasm (Doehlert and Felker, 1987; Sturm and Chrispeels, 1990). Activity for insoluble acid invertase was lacking in the nucellus (Fig. 3A) but was present in the upper pedicel tissue. By contrast, activity for soluble acid invertase was restricted to the nucellus (Fig. 3C), and no activity was detected in the pedicel tissue. If substrate sucrose was omitted from the assays, no activity was observed for either enzyme (Fig. 3B, D).

Figure 3 shows that the base of the spherical nucellus sits in a cup that includes some pedicel vascular tissue. The hand-cut sections used for Fig. 3 often included part of the side of the cup. In those sections, the nucellus and pedicel overlapped and the border between the soluble and insoluble invertases was blurred. Occasionally, hand sections passed through the centre of the nucellus without including any of the side tissue of the cup. In those sections, which were used in Fig. 3, there was no tissue overlap and the border between soluble and insoluble invertase was sharp.

If the assays for Fig. 3 were run for an additional 2 h or more, the localization of invertase activities become blurred as many sections showed precipitate in all the surrounding tissues. The precipitate began to be visible on the walls of the reaction vial. This non-specific staining apparently occurred because the first glucose produced by the enzymes consumed nearby assay constituents, which precipitated. This localized the enzyme activity, but glucose formed afterwards probably diffused farther into the medium (which was unstirred) before assay constituents were encountered and precipitation occurred. This later precipitate was deposited farther from the site of enzyme activity.
remained trapped in the cells while the insoluble enzyme in the present work. After this treatment, the soluble enzyme freezing and thawing allowed high activities to be expressed reported high invertase activities after tissue freezing, and 
et al. 2002). Zinselmeier (5 %) caused low invertase activities, also reported by 2 % formalin) or a brief exposure to dimethyl sulfoxide treatments to disrupt membranes using a ®xative (1 h in membranes by freezing and thawing the cells. Other ally to surrounding tissues.

In Fig. 3, assay times were adjusted to a minimum time for visible precipitate to form without spreading non-specifically to surrounding tissues. The assay for soluble invertase relied on the disruption of membranes by freezing and thawing the cells. Other treatments to disrupt membranes using a fixative (1 h in 2 % formalin) or a brief exposure to dimethyl sulfoxide (5 %) caused low invertase activities, also reported by Sergeeva and Vreugendhil (2002). Zinselmeier et al. (1999) reported high invertase activities after tissue freezing, and freezing and thawing allowed high activities to be expressed in the present work. After this treatment, the soluble enzyme remained trapped in the cells while the insoluble enzyme apparent before freezing (Fig. 3A) had essentially disappeared (Fig. 3C), presumably after being released by the cell salts and washed away in the rinse.

Glucose localization

There is considerable evidence that sucrose is the main translocated sugar in maize, and the sucrose is hydrolysed in the pedicel apoplasm soon after it is released from the phloem termini when kernels develop (Shannon, 1968, 1972; Shannon and Dougherty, 1972; Felker and Shannon, 1980; ap Rees, 1984; Doehlert and Felker, 1987; Griffith et al., 1987a; Porter et al., 1987; Miller and Choure, 1992; Thomas et al., 1993; Xu et al., 1995; Cheng et al., 1996). Glucose is one of the hydrolysis products, so its location not only identifies the sugar distribution but also indicates where invertase is hydrolysing the incoming sucrose.

In order to test where sucrose was hydrolysed within young ovaries before kernels were present and whether low \( \Psi_w \) altered the hydrolysis, the glucose location was determined in median sections of ovary tissue. The ovaries were rapidly frozen and kept frozen throughout preparation of the sections in order to maintain glucose \textit{in situ}. The sections were gradually warmed in the presence of the assay mix and the fluorescence resulting from glucose oxidation was monitored. The time for measuring the fluorescence was determined from the diffusion rate for glucose and assay components during the warming, tested with a nylon filter soaked in glucose solution, blotted onto Whatman No. 1 filter paper, and air-dried (Fig. 4A). The reaction with glucose was observed (Fig. 4B–D), and after 0.5 min diffusion was minimal from the edge of the filter (Fig. 4B). At 1.5 min, glucose had proceeded about 0.3 mm from the edge of the filter (4C). By 3 min, the glucose had moved 0.7 mm from the edge (Fig. 4D). As a control, there was no detectable fluorescence when glucose was omitted from the assay (Fig. 4E). As a further control, glucose oxidase was omitted from the assay in the tissue extract, and the resoru®n fluorescence was at the background level observed without glucose. In the tissue sections, omitting glucose oxidase also resulted in negligible fluorescence. These controls validated the assay conditions, and a 1.5 min assay time was selected for all subsequent assays in order to give adequate time for colour development but minimal diffusion.

Glucose concentrations were low and uniformly distributed throughout the young ovary and subtending tissues on day ~5 before treatments were imposed (Fig. 5A, B). As the ovaries developed in control plants having high \( \Psi_w \), glucose began to accumulate in the upper pedicel tissues (Fig. 5C, E, G). The vascular tissue visible in the autofluorescence images shown below each glucose image (Fig. 5D, F, H, respectively) was surrounded by tissues accumulating glucose. The glucose concentration decreased abruptly at the border of the nucellus and remained low throughout the nucellar tissue (Fig. 5C, E, G).

If sucrose was fed to the stems of plants at low \( \Psi_w \), glucose could be detected in the same tissues as the controls, but concentrations were not as high around the phloem termini nor was the pedicel/nucellus gradient as steep (Fig. 5J–O). At low \( \Psi_w \), glucose failed to accumulate
around the phloem termini (Fig. 5P±U). The glucose gradient was negligible, and concentrations were low throughout the pedicel and ovary (Fig. 5R, S). Recovery was slow after rewatering (Fig. 5T, U).

The glucose concentrations were quantified with spectrofluorophotometer measurements in extracts from similar cryosections, confirming the high concentration in the control ovaries, intermediate concentrations at low $\Psi_w$ with sucrose infusion, and low concentrations at low $\Psi_w$ (Fig. 5V). On the day of pollination, there were 7 $\mu$g of glucose per cryosection in the controls, 5.5 $\mu$g per cryosection at low $\Psi_w$ when sucrose was fed to the stems, and 2 $\mu$g per cryosection at low $\Psi_w$.

It is worth noting that the images represent substantial amounts of glucose. On the day of pollination, the band of glucose in the control image (Fig. 5E) was about 2 mm wide or about 100 times that of the 20 $\mu$m cryosections.

Assuming 7 $\mu$g of glucose per cryosection on that day (Fig. 5V), the total glucose in the ovary was about 0.7 mg, which represents about 1-4 mg of hydrolysed hexose (glucose plus fructose). On the same day, whole ovaries of plants grown similarly by Zinselmeier et al. (1999) contained 1-4 mg of hexoses measured chemically.

In addition, starch was abundant in the ovaries on the day of pollination. It was located mostly in the pedicel tissues surrounding the phloem termini in the controls (Fig. 6A). No starch was visible in the nucellus. At low $\Psi_w$ with sucrose infused into the stems, starch was located in the same tissues but was slightly less abundant (Fig. 6B). At low $\Psi_w$, most of the starch was absent (Fig. 6C). This absence was caused by starch breakdown shown earlier by Zinselmeier et al. (1999) in a detailed chemical and histochemical analysis of ovary starch under similar conditions. On the day of pollination, starch in the controls accounted for about 0.4 mg or 11% of the ovary dry mass of 3.5 mg, but at low $\Psi_w$ on the same day, starch had broken down to 0.06 mg or about 2.4% of the ovary dry mass of 2.5 mg (Zinselmeier et al., 1999).

**DISCUSSION**

The changes in $\Psi_w$, water use and photosynthesis, as well as extensive abortion of the ovaries, confirmed the behaviour reported by Boyle et al. (1991) and Zinselmeier et al. (1995a, 1999) under similar conditions. The parent plant suspended certain metabolic and physiological activities for a few days at low $\Psi_w$ and recovered when water was resupplied. By contrast, ovary development did not resume when water was resupplied. This irreversibility distinguished development of the ovaries from that of the parent.

The results indicate that ovary abortion was linked to the availability of sugars in the ovary. Glucose decreased at low $\Psi_w$ and recovered only slightly in the ovaries when water was resupplied. Abortion was extensive. The glucose was partially maintained when sucrose was fed to the stems under these conditions, and abortion was partially prevented. The linkage between glucose and abortion was thus quite strong.

The glucose distribution in the ovaries at low $\Psi_w$ was consistent with a lack of sucrose delivery. When sucrose was fed to replace the sucrose missing because of decreased photosynthesis, the resulting glucose was in the usual locations with no evidence for a block in transport, but glucose concentrations were somewhat lower than in the controls. Zinselmeier et al. (1999) reported that sucrose concentrations were completely maintained in similar conditions. The full maintenance of sucrose but partial maintenance of glucose implies that a limitation existed in sucrose conversion to glucose (and fructose) at low $\Psi_w$. Zinselmeier et al. (1999) and Andersen et al. (2002) found markedly lower activities of soluble and insoluble...
FIG. 5. In vivo location of glucose in maize kernels around the time of pollination. Epi-fluorescence micrographs of ovary glucose are paired with UV autofluorescence images underneath to show ovary anatomy. Control: (A) low glucose concentrations are present throughout control ovaries on day −5. (B) UV image of ovary in (A) shows vascular tissues as light gray, and the outline of the ovary can be seen. (C) Glucose (red) beginning to accumulate in upper pedicel on day −2 in control ovary. Accumulation is around vessels visible in (D). (E and F, G and H) The glucose pool becomes large in the control ovary on day 0 and day 2, but is restricted to upper pedicel. Concentrations in the nucellus are low, creating a steep gradient between the upper pedicel and nucellus. Low $\Psi_w$ + Sucrose infusion: (J, L, N) glucose is present in the upper pedicel but at lower concentrations in (L) and (N) than in the controls at (E) and (G). (K, M, O) Vascular anatomy of (J, L, N) indicates glucose is localized in the same tissues as in controls. Low $\Psi_w$: Glucose (R) fails to accumulate in the upper pedicel (S). Where present (P), glucose appears to surround vessels (Q). After rewatering, glucose is slow to return to the ovary (T, U). Scale bar = 1 mm. Images are taken after 1−5 min (see Fig. 4). (V) Spectrofluorophotometric quantification of glucose fluorescence in (A) to (U). Open circles, control at high $\Psi_w$; closed squares, low $\Psi_w$ plus sucrose infused into stems; closed circles, low $\Psi_w$. The black bar on the abscissa indicates when water was withheld from the soil. The white bar indicates when infusion was started on day −4 and continuing each day to include day 0. Data are means ± s.e. for three cryosections from three ovaries from three different plants.
invertases under these conditions. Zinselmeier et al. (1999) observed a depletion of hexoses and all of the other downstream metabolite pools for starch biosynthesis, with only a partial maintenance when sucrose was fed to the stems. The present work shows that these effects included glucose, which confirms that low invertase activity was a limiting step in carbon processing by the ovaries at low Ψw (Zinselmeier et al., 1995b, 1999; Andersen et al., 2002).

Andersen et al. (2002) found a correlation between invertase activity and transcript abundance of the corresponding invertase genes in the ovaries, suggesting that at least partial control of invertase activity may lie at the transcriptional level. Sucrose and/or glucose concentrations have been shown to alter the transcriptional activity for certain invertases (Koch, 1996). However, some of the regulation could be post-transcriptional. A peptide in maize can act as an invertase inhibitor (Jaynes and Nelson, 1971; Bate et al., 2004) by binding non-competitively to acid invertase at low sucrose concentrations (about 1–2 mM in tobacco, Weil et al., 1994). When invertase is extracted without sucrose, it becomes susceptible to the inhibitor in vitro (Pressey, 1967; Jaynes and Nelson, 1971), and low sugar concentrations in vivo might have the same effect. However, in the in vivo (fresh tissue) and in situ (frozen/thawed tissue) assays conducted here, endogenous sugars surrounded the invertases until the water rinsing began, probably protecting the enzyme from inhibitor binding. During rinsing, the small peptide (17.7 kD in maize, Bate et al., 2004) should have been washed away, removing any inhibitor from the assay.

The rinsing was essential to remove endogenous sugars so that sucrose could be supplied externally for the enzyme assays. Rinsing also could have removed soluble invertase from the apoplast, but probably not from the cell interior because the native enzyme had a molecular weight too large to move through the walls. Its presence in the nucellus (in situ assay) confirms that a large activity resided inside the nucellar cells. The apparent absence of soluble invertase activity in the pedicel (in situ assay) indicates that apoplast invertase was the dominant sucrose hydrolysis enzyme in that tissue, much of it bound to the cell walls (in vivo assay). The possibility that soluble invertase could have been present and rinsed away strengthens this conclusion (e.g. mRNA for a soluble invertase was detected by Andersen et al., 2002, in the pedicel tissues).

Sugar stream regulation

In maize, sucrose synthase (Wittich and Vreugdenhil, 1998) and acid invertase are found in the ovary and pedicel, but the invertase is the only sucrose-processing enzyme producing free glucose as one of the products. Because this glucose localizes the activity of the invertase, the glucose images indicate that glucose was produced mainly in the upper pedicel tissues. The dry mass of glucose quantified in the images showed about 2 μg of glucose were accumulating per day in each cryosection of the control ovaries on the day of pollination (slope of data in Fig. 5V). With 100 cryosections in each ovary, the rate of glucose accumulation approximated to 200 μg per day, which was equivalent to about 400 μg of glucose plus fructose. The ovaries hydrolyse approx. 1000 μg of sucrose on the day of pollination, calculated from the increase in ovary dry mass reported by Zinselmeier et al. (1999). Therefore, of the sucrose processed in the ovaries, about 40% was contributing to the accumulating glucose pool and 60% was being converted to ovary starch and developing ovary structures. While several enzymes might contribute to this sucrose processing, Zinselmeier et al. (1999) measured activity of insoluble acid invertase capable of producing 0.6 μg of glucose equivalents per min in each ovary. This is equivalent to 860 μg of sucrose processed per day and indicates that the activity of invertase in the pedicel apoplast was sufficient to support most of the dry mass accumulation by the ovaries on the day of pollination.

Although Andersen et al. (2002) suggest that soluble invertase in the nucellus could sustain sucrose gradients across plasmodesmata to enhance phloem unloading and post-phloem transport, the present results are consistent with a large amount of sucrose hydrolysed in the pedicel rather than the nucellus. The phloem terminates in the pedicel tissues, and the glucose images and apoplast
location of the enzyme suggest that considerable sucrose was unloaded from the phloem and entered the pedicel apoplast, immediately was hydrolysed, and the product glucose accumulated in large concentration in the same tissues. While some sucrose might escape hydrolysis in the pedicel apoplast and move into the nucellus, soluble invertase in the nucellar cells would hydrolyse it there. The two enzyme forms were situated to ensure that no sucrose would remain unhydrolysed after carbon entered the nucellar cells.

The accumulating glucose created a gradient extending from the normally high concentration in the upper pedicel to the low concentration in the nucellus. The glucose clearly entered the nucellus because a low concentration was found there, and Diboll and Larson (1966) observed a small amount of starch in the embryo sac embedded in the nucellus (Kiesselbach, 1949). Other investigators studied later stages of kernel development when an embryo and endosperm were present, and Griffith et al. (1987b) observed glucose, fructose and sucrose entering the embryo, with glucose and fructose entering four- to six-fold faster than sucrose. Felker and Shannon (1980) reported a gradient of carbon between the pedicel and young kernel using radiological measurements. They suggest the gradient might help drive sugars from the pedicel into the embryo and endosperm. The present results without embryo or endosperm resemble these results for later stages and further indicate that, at low $\Psi_w$, the disappearance of the steep glucose gradient would decrease the tendency for glucose to move toward the nucellus.

It should be noted that glucose is a highly mobile solute in cells, and its immobilization and rapid imaging were essential for accuracy. Keeping the tissues frozen until assay and using a highly sensitive fluorescent glucose detection system minimized the assay time and thus diffusion in a fashion similar to the method employed by Borisjuk et al. (1998). Other methods that did not immobilize glucose or use a fluorescent assay (e.g. Kim et al., 2000) did not localize glucose reliably in the present work.

### Fate of glucose

Glucose formed in the upper pedicel seemed to have two fates. One was directed toward the embryo sac and surrounding ovary structures. After hydrolysis in the pedicel apoplast, the glucose moved to these structures and contributed to their considerable increase in dry mass. Diboll and Larson (1966) report that there are no plasmodesmata connecting the nucellus to the embryo sac. Glucose moving from the nucellus into the embryo sac thus had to cross a second apoplast. Later, when an embryo and endosperm eventually form inside the embryo sac, this second apoplast becomes a dominant feature of the transport path during kernel development.

The second use of glucose was for starch synthesis around the pedicel vasculature. The cellular path of sugars from the phloem to this starch cannot be determined from the present study, but a substantial portion of ovary glucose was directed to the starch in the controls (about 11% of the ovary dry mass). The starch could have supplied glucose for ovary development in the absence of photosynthesis, such as at night. Because the ovaries normally gained about 1000 $\mu$g of dry mass in 24 h and contained about 400 $\mu$g of starch (see Zinselmeier et al., 1999), the complete breakdown of starch would supply glucose for nearly half that time (i.e. one night). A similar role could be played when photosynthesis was inhibited at low $\Psi_w$ during the day. Photosynthesis was inhibited for several days at low $\Psi_w$ in the present work, and starch was depleted when it otherwise would be abundant. Glucose became low throughout the pedicel/ovary structures. When sucrose was fed to the stem, the starch pool was partially maintained and glucose concentrations remained moderately high in the pedicel. Zinselmeier et al. (1999) reported similar changes in the starch pool. Therefore, the buffering action of starch appeared to be inadequate to protect against a prolonged absence of sucrose from photosynthesis, and sucrose had to enter from the feeding in order to maintain the starch and glucose supply.

The starch depletion at low $\Psi_w$ was seen in individual ovaries clearly destined to abort. Its maintenance with sucrose feeding was apparent, and abortion was partially prevented. However, Schussler and Westgate (1994, 1995) found that high carbohydrate reserves in the parent plant did not prevent abortion in similar experiments, and Andersen et al. (2002) reported ovary abortion without starch depletion in maize exposed to low $\Psi_w$. Because starch was not monitored in individual ovaries in the work of Schussler and Westgate (1994, 1995) or Andersen et al. (2002), it was possible that starch was depleted in those ovaries destined to abort but not in the surviving ovaries. Assaying a bulk of ovaries may have obscured this difference due to the effect of averaging. Together, the work of Andersen et al. (2002), Schussler and Westgate (1994, 1995) and Zinselmeier et al. (1995b, 1999) identify the primary importance of the immediate sugar stream for ovary development. But the present results also suggest that starch breakdown supplies glucose to that stream and delays, if only for a short time, the onset of abortion.

### CONCLUSIONS

The events described in this work occurred mostly before embryo and endosperm were present in young maize ovaries. Nevertheless, the mechanism of sucrose processing had features similar to those described by others for later stages when embryo and endosperm are present and obtain sugar from an apoplast separating them from the parental tissues. In both stages of development, the hydrolysis of sucrose to glucose and fructose occurred mostly in the pedicel, apparently in the apoplast mediated by apoplast acid invertase rather than soluble acid invertase inside the cells, which was undetectable in the pedicel. This implied that, before embryo and endosperm were present, sucrose was unloaded from the phloem and moved to the pedicel apoplast where it was hydrolysed, and glucose accumulated.
Soluble invertase was abundant inside the nucellus cells and may act as a scavenger of sucrose escaping hydrolysis in the pedicel apoplast of young ovaries. Because of the invertase activity in the pedicel apoplast, a large glucose gradient developed between the pedicel and the nucellus. The gradient favoured glucose movement toward the nucellus. The gradient disappeared at low $\psi_w$ but was partially maintained if sucrose was fed to the stems. In the young ovaries, some glucose was used for starch synthesis in the cells of the pedicel and some for ovary development.

When sucrose arrival was diminished at the ovaries during several days of low $\psi_w$, the pedicel starch was broken down and nearly disappeared. The disappearance of the glucose gradient at about the same time is consistent with the loss in starch. The lack of a glucose gradient probably decreased sugar transport to the ovaries and suggests a role for sugars in triggering the ovary abortion that followed.

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