In vivo study of chloroplast volume regulation

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ABSTRACT This paper describes a new technique that can be used to study chloroplast volume regulation in vivo. Nuclear magnetic resonance spectroscopy was used to measure relative amounts of chloroplast water in *Acer platanoides* leaves as they dried in air, and also in leaf disks exposed to aqueous polyethylene glycol, sucrose, or glycerol. The chloroplasts retained a constant quantity of water as leaf water potentials varied between −0.05 and −1.90 MPa, indicating that volume regulation was effective throughout this range. The chloroplasts lost water when the water potential fell below −1.90 MPa, except when leaf disks were exposed to glycerol, suggesting that the lower limit of effective volume regulation is determined by physiological levels of osmotic solutes and that glycerol can be used for chloroplast osmoregulation.

INTRODUCTION

Robinson (1985) was the first to demonstrate chloroplast volume regulation. He incubated chloroplasts with sorbitol in labeled water and measured stromal volumes by a centrifugation technique. Results showed that chloroplasts maintained constant volumes over a range of water potentials. Robinson also found that photosynthetic oxygen production was sensitive to volume changes; chloroplast volumes much above or below the optimum evolved very little O_2.

Sen Gupta and Berkowitz (1988) isolated chloroplasts in media that matched the water potentials of water-stressed plants. They showed that stromal volume was maintained initially as increasing water stress reduced the relative water content of a leaf. Eventually, however, a critical water potential was reached below which volume maintenance was lost. Photosynthetic rates dropped suddenly at the same water potential as did chloroplast volumes. Santakumari and Berkowitz (1991) extended this work to show that critical water potentials were lower in plants that had previously experienced water stress.

Evidence summarized above suggests that chloroplast volume regulation is necessary for efficient photosynthesis and that plants adjust the range of volume regulation in response to environmental stimuli. It is possible that changes in the range of volume regulation are an important component of "drought hardening."

Techniques used to demonstrate volume regulation in isolated chloroplasts are difficult to apply to environmental studies because osmotic solutes in nonchloroplast compartments may be lost or diluted during chloroplast isolation. An in vivo technique is needed.

This paper reports the first experimental study of chloroplast volume regulation in vivo. We used hydrogen-1 nuclear magnetic resonance (^{1}H NMR) spectroscopy to measure changes in chloroplast water content in response to changing water potentials. Our main objective here is to describe the new NMR technique and to present data that show its capabilities; future papers will deal with environmental factors.

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MATERIALS AND METHODS

Shade leaves were harvested during July and August from a Norway maple (*Acer platanoides* L. cv Emerald Queen) growing on the University of Wisconsin-Madison campus. Rainfall had been adequate, and the tree had not been stressed. NMR samples were prepared by excising 4-mm disks from near the centers of leaf blades as distant as possible from large veins. The disks were placed in a sample holder designed to insure magnetic field homogeneity and to orient the leaf surface perpendicular to the applied magnetic field (McCain et al., 1984; McCain, 1986). ^{1}H NMR spectra were obtained on Bruker AM-400, AM-500, and AM-600 spectrometers (Billerica, MA) (operating at 400, 500, and 600 MHz, respectively); typically, we used 16 pulses and a 2-s recycle time. Results from all three instruments were essentially identical.

For one set of experiments, leaves were allowed to dry in air. At intervals of 20 to 30 min, leaf water potentials were measured by using a pressure chamber, and then new disk samples were excised. The disks were placed in the NMR sample holder within 5 s after excision, and spectra were recorded. In repeated sampling from the same leaf, we took new disks from areas that were separated by major veins from the locations of earlier samples. Measurements were made at 50 different water potentials in the range from −0.05 to −2.21 MPa.

For another set of experiments, initial NMR spectra were obtained from disks excised from fresh leaves. Next, the disks were briefly

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submerged in a test solution and then were allowed to float with their abaxial surfaces up while they were exposed to ambient room light. The leaf surface sheds water, so abaxial stomata were exposed to air; meanwhile, the adaxial surface and the cut edges were in contact with solution. Disks were shielded from air currents, and solutions were stirred frequently. At measured time intervals, the disks were removed from solution, blotted dry with absorbent tissue paper, replaced in the sample holder, and then reexamined under the same NMR acquisition parameters as before. After new spectra had been recorded, samples typically were resubmerged, refloated, and used again. No more than 3 min elapsed between the times that leaf disks were removed and then returned to the solution, and samples were exposed to dry air no more than 5 s at each transfer. A total of 120 leaf disks were used, and 460 NMR spectra were recorded.

The aqueous solutions on which leaf disks were floated included: pure water, sucrose (at concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.85, 1.0, 1.15, 1.3, and 1.6 M), glycerol (at 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, and 2.25 M), and polyethylene glycol (PEG-6000) (at 0.1, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, and 0.5 g PEG per g H_2O). All solutions were at room temperature, 22°C. Water potentials, Ψ, were calculated from solution concentrations according to published data (Michel, 1972; Michel, 1983; Weast, 1988).

RESULTS AND DISCUSSION

The ^1H NMR spectrum of a leaf is essentially the spectrum of the water it contains. Signals from hydrogen nuclei in solids and membranes are too broad, and signals from solutes are too weak to be significant. However, a leaf spectrum is more complex than the single, narrow peak that is the normal spectrum of pure water. Internal structures in the leaf distort the applied magnetic field, so that water molecules experience different average field strengths in different parts of the leaf. Water molecules inside the chloroplasts are exposed to fields from manganese ions bound to the thylakoid membranes. When the membranes are ordered, these effects can cause the signal from water in the chloroplasts to be displaced from that of water in the other leaf compartments (McCain and Markley, 1986). For example, all the spectra in Fig. 1 display two well separated peaks of nearly equal amplitude; these are the distinctive features of shade leaves from the *Acer platanoides* cultivar 'Emerald Queen' (McCain et al., 1988). In these spectra, the upfield peak (the peak on the right) has been assigned to water in the chloroplasts, and the downfield peak (on the left) to nonchloroplast water (McCain and Markley, 1986).

Emerald Queen shade leaves were chosen for this study because they exhibit unusually large chloroplast peaks with relatively good peak resolution, and because the spectra are highly reproducible. Sun leaves from the same cultivar give smaller chloroplast peaks (McCain et al., 1988), and other *A. platanoides* cultivars provide spectra that are not as well resolved. Leaves from other species typically yield poorly resolved spectra (McCain et al., 1984).

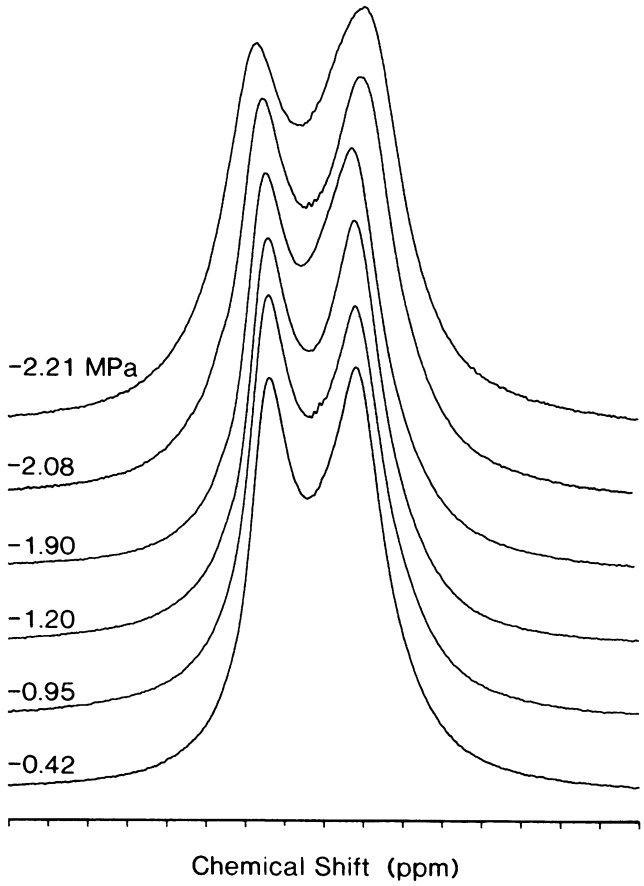

FIGURE 1 A series of NMR spectra obtained by repeated sampling from a single *Acer platanoides* leaf as it dried in air. Each spectrum is labeled with the leaf water potential as measured by a pressure bomb just before the sample was removed from the leaf. The lowest trace (−0.42 MPa) was recorded immediately after the leaf was harvested. Spectra displayed on successively higher traces were taken at ~30-min intervals. In each spectrum, the peak on the right has been assigned to water in the chloroplasts, and the peak on the left to nonchloroplast water. To simplify comparisons, all spectra were scaled to the same height; if absolute intensities had been used, one could have seen a gradual decrease in peak integrals as the leaf dried, but the decrease would not exactly parallel actual water loss in the leaf because different NMR samples had randomly different thicknesses. Tick marks on the horizontal scale are spaced apart 1.0 ppm in magnetic field; the chemical shift scale is not labeled because samples contained no reference signals that could be used to establish an origin. Note that the two peaks are almost equidistant in spectra from −0.42 to −1.90 MPa, but that the peaks diverge in traces recorded at lower water potentials.

Peak assignments rely on a theory that was published first in this journal (McCain and Markley, 1986). Since the original paper appeared, the theory has been confirmed by additional data. For example, NMR images

have demonstrated that the peak assigned to chloroplasts is derived from water located in the palisade and spongy mesophyll layers (but not in the epidermal layers), and that the distribution of this water over the cross section of the leaf is the same as the distribution of chloroplasts themselves, as observed by microscopy (McCain et al., 1991). Peak separations were found to be inversely proportional to chloroplast peak integrals, as predicted by the theory (McCain et al., 1989). Also, as predicted, peak separations increased as leaves accumulated manganese (McCain and Markley, 1989).

According to the theory (McCain and Markley, 1986), two different NMR techniques can be used to measure the relative water content of the chloroplasts. Peak separations, Δ (the distance along the horizontal axis between the tops of the two peaks), are inversely proportional to quantities of chloroplast water, and chloroplast-peak integrals are directly proportional to quantities of chloroplast water.

Fig. 1 shows a set of spectra taken from a single leaf as it dried in air. Peak separations, Δ, remained approximately constant as the water potential dropped from −0.42 to −1.90 MPa. The constant Δ indicates an unchanging ratio of chloroplast water to bound manganese, implying a constant quantity of chloroplast water (because no Mn is lost) and therefore constant chloroplast volume. Δ began to increase when the water potential fell below −1.90 MPa, showing that chloroplasts lost water on drying past this critical water potential.

Fig. 2 presents quantitative data from many experiments like those in Fig. 1. Over the range from −0.05 to −1.90 MPa, Δ remained constant, averaging 2.26 ppm with a standard deviation of 0.04 ppm. The average value of Δ has no particular significance because it is influenced by external factors such as: season, weather, manganese concentration in the soil, etc. (McCain and Markley, 1989). The important observation is that Δ remains constant over a well defined range in Ψ; this is strong evidence for chloroplast volume regulation.

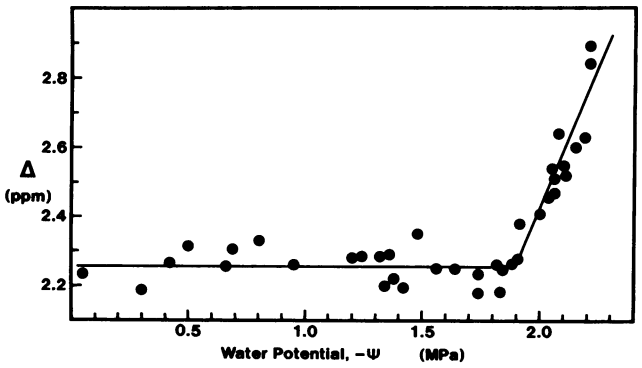

FIGURE 2 A plot of Δ (the separation between chloroplast and nonchloroplast NMR peaks) vs. Ψ (the leaf water potential) in air-dried leaves. The horizontal scale represents negative water potential; lowest values are to the right. Data were obtained from spectra similar to those shown Fig. 1; Δ is defined as the distance (in ppm) from one peak to the other measured at the highest points. Note that Δ remains constant (with some experimental scatter) down to −1.90 MPa, but it increases rapidly at lower water potentials.

Another way to vary water potentials is by floating leaf disks on osmotic solutions (Krizek, 1985). We began with disks from fresh leaves which had water potentials that were well within the range of chloroplast volume regulation. After initial NMR spectra had been obtained, the disks were floated on test solutions at known water potentials. Later, the disks were lifted from the solutions and used again to record NMR spectra. The process was repeated to give a series of spectra that showed how Δ and peak integrals changed with time. Our objective was to measure differences between the spectrum of each fresh disk and the spectrum of the same disk after it had reached equilibrium at a new water potential. Three solutes were chosen for their different membrane permeabilities. Glycerol can pass through both the cell membrane and the chloroplast envelope membrane; sucrose can penetrate the cell membrane but not the chloroplast envelope, and both membranes are impervious to PEG (Heber and Heldt, 1981).

Almost no changes were found in comparing spectra from fresh leaf disks with those from the same disks after exposure to moderate water potentials (between ~ −0.1 and −1.9 MPa) for periods up to 6 h, but large effects were observed in spectra from disks that had floated on pure water or on solutions with water potentials below −1.9 MPa. Fig. 3 shows progressive changes in spectra from disks that floated on concentrated aqueous PEG, sucrose, and glycerol solutions. On 0.5 g/g PEG (where Ψ = −3 MPa), integrals of both the chloroplast and the nonchloroplast peaks decreased with time while Δ increased; eventually, at very long exposure times, the PEG spectrum approached an equilibrium appearance similar to that of the 291-min trace. On 1.3 M sucrose (−5.1 MPa), the chloroplast peak integral decreased as Δ progressively increased toward equilibrium. However, in contrast to results from PEG, the integrated nonchloroplast-peak intensity did not change with time on sucrose; the height decreased, but the width increased so that the integral remained approximately constant. On 1.75 M glycerol (−5.1 MPa), the starting and final spectra were similar with regard to relative peak integrals and Δ, but intermediate spectra resembled those of sucrose with smaller chloroplast peaks and larger peak separations than at the start.

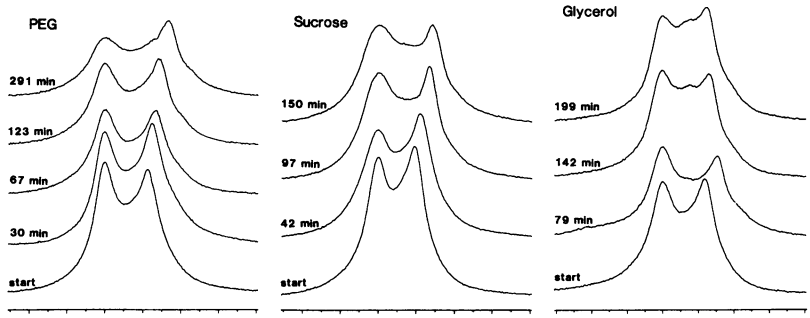

FIGURE 3 Three series of NMR spectra. Each series was obtained from a single *A. platanoides* leaf disk. Immediately after the starting spectra had been recorded, disks were floated on different solutions: one on 0.50 g PEG/g H_2O, another on 1.3 M sucrose, and the third on 1.75 M glycerol. The disks were removed at intervals to record additional spectra and then returned to the solutions. Labels show the total elapsed time each disk spent floating on its own particular solution. Note that peak separations increased with time on PEG and sucrose, while chloroplast peak amplitudes decreased. However, although peak separation increased and chloroplast peak intensity decreased during the first 79 min on glycerol, by 199 min the spectrum had returned approximately to its initial appearance.

We propose the following interpretation of the spectra shown in Fig. 3. Peak integrals indicate that nonchloroplast compartments lost water to concentrated PEG but not to sucrose or glycerol. Sucrose and glycerol can pass through cell walls and the cell membrane, but PEG cannot; therefore, osmoregulation is possible in the nonchloroplast compartments in the presence of sucrose and glycerol, but not with PEG. Sucrose and glycerol can be imported into the cell to balance the interior and exterior water potentials, but nonchloroplast water must be lost by osmosis to PEG solutions until equilibrium is established. Two different kinds of data (increasing Δ and decreasing chloroplast peak integrals) show that the chloroplasts lost water on PEG and sucrose. On glycerol, the chloroplasts lost water initially, but then they recovered it at equilibrium. Loss of chloroplast water indicates a loss of volume regulation which occurs when Ψ falls below -1.90 MPa. The glycerol effect (water loss from the chloroplasts, followed by recovery) may be explained as a consequence of unequal diffusion rates; at first, the chloroplasts lost water as water diffused out of the cells by osmosis and Ψ fell below -1.90 MPa; but then glycerol slowly diffused in, extending the range of chloroplast volume regulation beyond -1.90 MPa as it supplemented the physiological osmotic solutes. The glycerol effect was not seen on sucrose because, although sucrose diffuses more slowly than water, it cannot pass through the chloroplast envelope, so it cannot be used for chloroplast osmoregulation.

Quantitative analysis of data from floating leaf disks presents experimental problems. By measuring peak integrals, we should be able to determine the absolute water content of the chloroplasts. Unfortunately, this cannot be done accurately. To measure peak integrals, one must first locate a dividing point between the two peaks; however, in practice this requires a somewhat arbitrary choice because of peak overlap. Other sources of integral errors include long term variations in spectrometer sensitivity, variations in the position of the sample when it is replaced in the spectrometer, and the presence of broad background signals (from protons in solids and membranes) that elevate the baseline by an undetermined amount. An additional source of error for nonchloroplast water occurs when disks are blotted dry; some test solution always remains on the disk and contributes to the nonchloroplast peak. Attempts to fit experimental spectra by computer simulation do not improve matters because the lineshapes are somewhat irregular. For these reasons, we have chosen not to report quantitative integral measurements; only a qualitative analysis of integrals has been used. None of the problems with integrals seriously affects the accurate measurement of Δ.

We found some evidence of damage to the chloroplasts in stressed leaves. In the -2.21-MPa trace of Fig. 1, the chloroplast peak is broadened, and its shape has changed. The chloroplast peak also is distorted in the upper glycerol traces and in the 291-min PEG spectrum of Fig. 3. We have seen similar distortions in spectra from leaves that have been stressed by high or low temperatures or by high light intensities. Such effects probably indicate a loss of thylakoid order; according to our theory (McCain and Markley, 1986), the thylakoids must maintain a high degree of orientational order to insure a narrow, well resolved chloroplast peak. Our interpretation of the upper glycerol peak in Fig. 3, for example, is that a fraction of the chloroplasts has become disoriented in such a way as to produce a broad hump at the center of the spectrum, but that most of the chloroplasts remain about as they were. Effects such as

these are not reproducible. The spectrum is distorted differently in every sample; although, typically, exposure to mild stress causes only a small population of chloroplasts to become disoriented. In this study, distortions were seen only in leaves stressed well beyond the range of volume regulation, and they occurred only after peak shifts already had been detected. The first response of a leaf to decreasing water potential is reproducible; peaks begin to shift before the spectrum becomes distorted. Since peak shifts are the first observable effect of water stress, and since we use peak shifts to define the limits of volume regulation, we believe that our results are not adversely affected by secondary damage to the chloroplasts. Water stress has been reported to damage chloroplasts in other species (Giles et al., 1976).

The approach to osmotic equilibrium is slow, and extrapolation is required to find limiting values. For the following analysis, we define relative chloroplast water content (*RCW*) at time *t* as equal to the Δ from the initial spectrum divided by the Δ measured at *t*, where *t* is the total elapsed time that the disk had floated on solution (not including time that it was in the NMR spectrometer for measurement). This definition assumes that chloroplast water content is inversely proportional to peak separation as predicted by theory (McCain and Markley, 1986). Fig. 4 shows some *RCW* data fitted to the function:

$$RCW = F + (1 - F)\exp(-t/110).$$

The equation represents an exponential decay from an initial *RCW* value of 1.0 at $t = 0$ to a final equilibrium value equal to *F*. The function was chosen because it gave a good fit to experimental data (including much more data not shown in Fig. 4) and because water exchange typically follows an exponential decay law (Boyer, 1985; McCain and Markley, 1985). The 110-min exponential relaxation time (corresponding to a half-life of 76 min) seems to be valid for all our PEG and sucrose data; perhaps it represents the time required for water molecules to diffuse out of the disk (Boyer, 1985). Use of a fitting function provides several advantages. Experiments can be completed within a reasonable period of time because one need not wait for final equilibrium to determine *F*; measurements made after approximately one half-life are nearly as good. Also, shorter observation periods minimize potential problems that might result from long term changes in the sample. We were unable to find a suitable fitting function for glycerol data; instead, *RCW* data from $t > 180$ min were averaged to estimate equilibrium values.

Fig. 5 presents data showing relative equilibrium chloroplast water content. Each point is the average of several *F* values derived from least-squares fits as described above. The data show that equilibrium *RCW* increased substantially when leaf disks were exposed to pure water. The *RCW* remained almost unchanged near 1.0 at water potentials between ~ −0.1 and −1.9 MPa. Equilibrium *RCW* decreased sharply at water potentials below −1.9 MPa in PEG and sucrose, but not in glycerol. At moderate concentrations, *RCW* appears to be slightly larger in glycerol than in sucrose or PEG, but perhaps the difference is too small to be significant.

Our results allow us to confirm and extend the

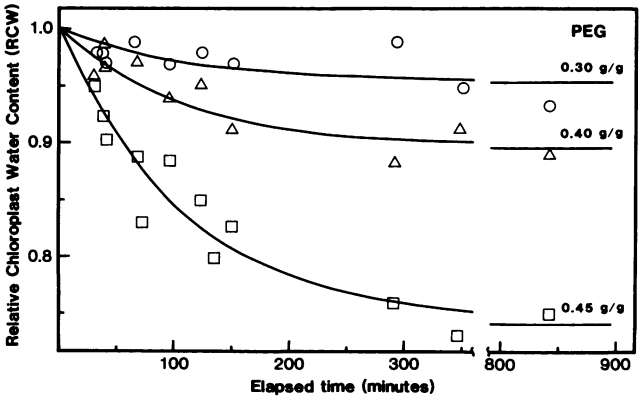

FIGURE 4 Relative chloroplast water content (*RCW*) vs. the total elapsed time that leaf disks spent floating on three different PEG solutions. Circles, triangles, and squares represent experimental data from solutions containing 0.30, 0.40, and 0.45 g PEG per g H_2O, respectively. There is a break in the time scale between 300 and 800 min. The curves are exponential decay functions derived from least-squares fits; they show that *RCW* approached equilibrium at long elapsed times. These data also show that much more chloroplast water was lost from leaf disks floating on 0.45 g/g PEG than on either of the two lower concentrations.

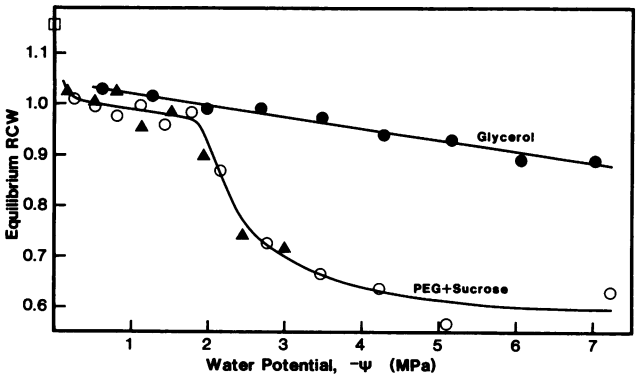

FIGURE 5 Relative chloroplast water content (*RCW*) of leaf disks in equilibrium with solutions of varying water potential, Ψ. The square on the left border at $\Psi = 0$ represents pure water. Open circles, closed circles, and triangles are data points for sucrose, glycerol, and PEG solutions, respectively. These data show that equilibrium *RCW* dropped sharply at water potentials below ~ −1.9 MPa in PEG and sucrose, but not in glycerol solutions.

conclusions of previous studies (Robinson, 1985; Gupta and Berkowitz, 1988). Data shown in Figs. 2 and 5 demonstrate that chloroplast volume regulation was effective in vivo in *Acer platanoides* shade leaves between limits of ~ -0.05 and -1.90 MPa. We also find that glycerol extends the range of volume regulation; this result suggests that the lower limit of effective regulation is determined primarily by the quantity of osmoticum available in the leaf and that glycerol can supplement the physiological supply of osmotic solutes.

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