

# Combined Effects of Light and Water Stress on Chloroplast Volume Regulation

Douglas C. McCain

Department of Chemistry and Biochemistry, University of Southern Mississippi, Hattiesburg, Mississippi 39406 USA

**ABSTRACT** A nuclear magnetic resonance technique was used to measure changes in the water content of *Acer platanoides* chloroplasts in leaf discs that had reached osmotic equilibrium with external solutions either in the dark or under exposure to light. Results showed that chloroplast volume regulation (CVR) maintained constant water content in the chloroplasts over a range of water potentials in the dark, but CVR failed when the water potential fell below a critical value. The critical potential was lower in the dark in sun leaves than in shade leaves. Upon exposure to intense light, CVR remained effective in sun leaves over the same range as in the dark, but it failed in shade leaves at all water potentials. Osmolytes are necessary for CVR, but KCl is relatively ineffective; increased concentrations of intracellular KCl did not fully support an increase in the range of CVR. The results indicate that leaves need reserve supplies of cytosolic osmolytes to maintain CVR at low water potentials, and a larger reserve supply is needed in leaves that are exposed to intense light.

## INTRODUCTION

Chloroplast volume regulation (CVR) is the process by which chloroplasts import or export osmolytes to maintain constant volume in a changing environment. Robinson (1985) was the first to demonstrate CVR. He showed that isolated chloroplasts retained a constant quantity of water over a range of water potentials, but they lost water when the water potential fell below a critical value. Photosynthetic oxygen production was sensitive to volume change; chloroplasts that were above or below an optimum volume evolved less O<sub>2</sub>. Sen Gupta and Berkowitz (1988) confirmed these observations, and Santakumari and Berkowitz (1991) found that CVR extended to lower water potentials in chloroplasts isolated from plants that had experienced water stress.

Chloroplasts shrink upon exposure to light. Ions are transported from the stroma to the cytosol, and water follows osmotically (Nobel, 1969; Defilippis and Pallaghy, 1973). Coulter counter and packed-volume measurements have revealed a light-induced decrease of 15–30% in the volume of pea chloroplasts (Nobel, 1968; Zurzycki and Metzner, 1977), and light microscopy has been used to measure a 25% decrease in thickness along their minor axis (Nobel et al., 1969; Miller and Nobel, 1972). Stereological analysis has showed that sunflower chloroplasts occupy 30% of the palisade cell volume at night, but they contract to 21% of cell volume in daylight (Fagerberg, 1983). Chloroplast shrinkage also has been detected by changes in light scattering (Heber et al., 1986). The light-induced shrinkage is reversible in the dark (Lawlor, 1987).

Both CVR and light-induced shrinkage involve osmolyte transport through the envelope; therefore, an interaction between the two processes is possible. While CVR and light-induced shrinkage have been studied separately, no single study has focused on both simultaneously. This paper investigates the combined effects of light, water stress, and leaf type on chloroplast water content.

Nuclear magnetic resonance (NMR) was used to measure changes in chloroplast water content. The major advantage of this method is that it can detect chloroplast volume change *in vivo*. This is important because the effective range of CVR is not a property of chloroplasts alone, but of the entire plant. CVR may be affected by osmolyte compartmentalization, membrane interactions, turgor pressure, and other factors that cannot be duplicated easily *in vitro*.

## MATERIALS AND METHODS

Leaves were harvested from a Norway maple (*Acer platanoides* L. cv "Emerald Queen") growing on the University of Wisconsin-Madison campus. Leaves from this cultivar provide reproducible NMR spectra with relatively good peak resolution, unlike those from most other species and some other cultivars (McCain et al., 1984). Shade leaves were from the interior of the crown where direct sunlight almost never reached. Sun leaves developed where there was daily exposure to full sunlight, even though the leaf may have been shaded for part of the day. Two well resolved peaks of nearly equal amplitude are characteristic features of shade leaf spectra; sun leaves yield two peaks of unequal height (Fig. 1). In each leaf type, the peak on the right has been assigned to chloroplast water, while the left-hand peak is the signal from nonchloroplast water (McCain and Markley, 1985).

Typically, one fresh leaf was harvested each day; it was kept in distilled water to ensure that it was fully hydrated, and then a large number of discs (one for each of the various osmotic solutions and light treatments to be studied on that day) were removed from it over a short period of time. The shade leaf data reported here are averages of measurements made on 25 different leaves, and sun leaf data are from 9 leaves. Shade leaves complete their development in early June, but sun leaves continue to develop through midsummer (McCain et al., 1988); therefore, shade leaves were studied from June through August, and sun leaves only during August. Consistent results were obtained from leaves collected during three consecutive summers.

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Address reprint requests to Dr. Douglas McCain, Department of Chemistry and Biochemistry, University of Mississippi, Box 5043, Hattiesburg, MS 39406. Tel.: 601-266-4376; Fax: 601-266-5829; E-mail: dmccain@whale.st.usm.edu.

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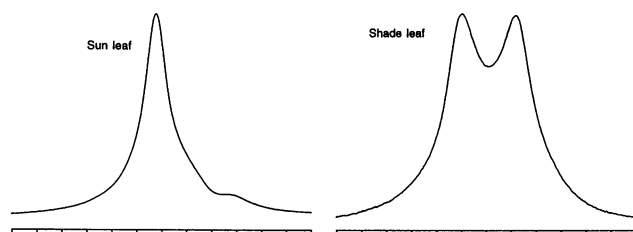


FIGURE 1 Typical  $^1\text{H}$  NMR spectra from sun and shade leaves of the *A. platanooides* cultivar "Emerald Queen". The peak on the right in each spectrum is the signal from water in the chloroplasts, while the left peak is from water in all the other leaf compartments. Tick marks on the horizontal scales register parts-per-million of magnetic field. Relative peak intensities show that chloroplasts contained a larger fraction of the total leaf water in shade leaves than in sun leaves.

Discs (4 mm diameter) were punched from near the centers of leaf blades, avoiding large veins. The discs were placed in a sample holder that was designed to ensure magnetic field homogeneity and to orient the leaf surface perpendicular to the applied magnetic field (McCain et al., 1984; McCain, 1986).  $^1\text{H}$  NMR spectra were obtained at 400 MHz on a Bruker AM-400 spectrometer (Billerica, MA). Samples were exposed to insignificant light intensities while in the bore of the magnet.

Initial spectra were obtained using fresh discs from fully hydrated leaves. Next, each disc was submerged briefly in an osmotic solution and allowed to float with its abaxial surface up so that stomata were exposed to air while the adaxial surface and the cut edge were in contact with solution. At measured time intervals, discs were lifted from the solutions, blotted dry with tissue paper, and reexamined under the same NMR acquisition parameters as before. Some samples were refloated and used again.

Aqueous solutions on which discs were floated included: KCl (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 M), polyethylene glycol, PEG-8000 (0.1, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, and 0.6 g PEG/g  $\text{H}_2\text{O}$ ), and mixtures containing both 0.1 M KCl and each of the first nine PEG concentrations listed above. A thermocouple psychrometer (Model SC10A, Decagon Devices, Inc., Pullman WA) was used to measure water potentials.

Some floating discs were kept in total darkness; others were placed over a white reflective surface and illuminated from above. Light from a slide projector was focused through a 500 ml round-bottomed flask filled with water that acted simultaneously as a lens and as a heat filter; a mirror reflected the light so that it fell vertically on the floating discs. A model LI-185B photodetector (Li-Cor, Inc., Lincoln, NE) was used to measure light intensity.

All data reported here were obtained at 295 K. Although solution temperatures increased by less than  $0.5^\circ$  upon exposure to light, the possibility that localized heating could have caused the "light effect" was investigated. No heat effect was found; discs that floated on osmotic solutions in darkness at 304 K showed the same behavior as those maintained in darkness at 295 K.

Light-induced chloroplast movements are well known in algae, but are seldom observed in higher plants where chloroplast positions are more dependent on leaf anatomy (Nagai, 1993). Stress-induced chloroplast movements in *A. platanooides* leaves have been detected by observing changes in NMR peak shapes (the observations will be reported elsewhere), but such movements occur only under extreme conditions and were not important in the measurements reported here. Light induces rapid conformational changes in thylakoid membranes; the changes can be detected by NMR (McCain et al., 1994), but not under the conditions of the present experiments.

Relative water content (RWC) can be computed as the total integral of the spectrum after treatment divided by the total integral from the fresh, fully hydrated sample. Defined in this way, RWC corresponds to the net fraction of the initial water content of the fresh leaf disc that remains in the sample after treatment. Integrals are proportional to water content because the water signal dominates the spectrum.

By analogy to RWC, relative chloroplast water content (RCWC) can be defined as the fraction of the initial water content of the chloroplasts that remains in the chloroplasts after treatment. The quantity of water in the chloroplasts is proportional to the integral of the chloroplast peak (if spins are allowed to recover thermal equilibrium between successive pulses), and, according to theory, it is also inversely proportional to peak separation. Integrals are reliable, but the theory that relates peak separations to water content must be applied with caution; to use peak separations, one must assume that the orientations of thylakoid membranes and the conformations of their molecular components remain unchanged by the treatment (McCain and Markley, 1985). The integral method was used for all data reported here, and the measurements were extrapolated to osmotic equilibrium.

Total integrals are easy to measure in an NMR spectrum when the spectral window is wide enough to reveal the true baseline. In shade leaves, the chloroplast peak integral was defined as the area to the right of the midpoint between the two peaks; although there is some overlap between peaks, the measurement provides a good approximation of the true chloroplast peak integral because the peaks have similar shapes and intensities. In sun leaves, however, the chloroplast peak integral is difficult to define because of significant overlap from the stronger nonchloroplast peak (Fig. 1). Sun leaf chloroplast integrals were measured by curve fitting, assuming two peaks of equal width but different amplitude; they may be less accurate than shade leaf chloroplast integrals.

The water content of a leaf disc slowly approaches an asymptotic value as the disc reaches osmotic equilibrium with the solution on which it floats. An earlier paper (McCain and Markley, 1992) demonstrated that experimental RCWC values approach equilibrium exponentially according to the function:

$$\text{RCWC} = F + (1 - F)\exp(-t/T)$$

where  $t$  is the total time that the discs have floated,  $T$  is a characteristic relaxation time, and  $F$  is the final equilibrium RCWC value. The same extrapolation function fits the new data reported here, but equilibrium is reached faster in the light than in the dark. The relaxation time was remeasured in the dark on PEG and on PEG + 0.1 M KCl and found to be 110 min, the same value as before (McCain and Markley, 1992), but in the light,  $T = 75$  min. RWC approaches equilibrium by the same function and with the same relaxation times as does RCWC.

Discs floating on concentrated KCl at first lost water by osmosis, but water later reentered as KCl diffused into the discs. This behavior resembles leaf-disc equilibration on glycerol solutions (McCain and Markley, 1992). As with glycerol, the time-dependent RWC and RCWC data could not be fitted to a suitable extrapolation function; instead, KCl measurements from  $t > 80$  min were averaged to estimate equilibrium values.

Discs that floated in the dark for a time on PEG and then were transferred to water recovered most of the water they had lost, but discs transferred from KCl to water recovered more than their initial water content, indicating that KCl had entered the cells. Discs that floated in the light on PEG and then were transferred to water never fully regained their initial water content, indicating they had lost some osmolytes. Discs that floated more than 2 hr at low water potential developed a thin ring of plasmolyzed cells at their periphery; the ring broadened with time and eventually (after  $\sim 1$  day) reached the center of the disc.

## RESULTS

Fig. 2 presents a small sample of the raw data to illustrate what was observed under varying conditions of light and water stress. For this experiment, eight discs were cut from the same leaf, and their initial spectra were recorded. The eight initial spectra were almost identical, and closely resembled the shade leaf spectrum in Fig. 1. Next, each disc was floated on one of four PEG solutions, either in the dark or in the light. NMR spectral changes began immediately

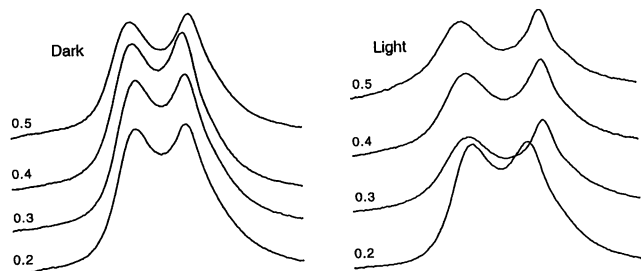


FIGURE 2 NMR spectra of eight different shade leaf discs after they had floated for 90 min on PEG solutions either in the dark or exposed to light at  $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Each spectrum is labeled with the concentration (in units of g PEG/g  $\text{H}_2\text{O}$ ) of the solution on which the disc floated. The discs had almost identical spectra before treatment, but different combinations of light and water stress altered the positions and intensities of the peaks.

and could be detected as soon as 5 min after flotation. The changes are mostly reversible in the early stages, but become irreversible after extended treatment with light or with high concentrations of PEG. After treatment for 90 min, a final spectrum was obtained from each sample. At 90 min, the spectra had nearly reached equilibrium.

As nonchloroplast compartments lose water, the peak on the left decreases in intensity but does not shift. When chloroplasts lose water, the peak on the right decreases in intensity and simultaneously shifts farther to the right. For example, one can see in Fig. 2 that chloroplasts retained water in the dark as the PEG concentration increased from 0.2 to 0.4 g/g (i.e., the peak on the right in the 0.4 g/g trace has the same height and occupies the same position as the corresponding peaks below it), but nonchloroplast compartments lost water under the same conditions (i.e., the peak on the left in the 0.4 g/g trace is slightly shorter than those below it). Both the chloroplast and the nonchloroplast compartments lost water in the dark on 0.5 g/g PEG (i.e., both peaks are shorter and the chloroplast peak has shifted to the right). Water loss was greater in leaf discs that were exposed to light.

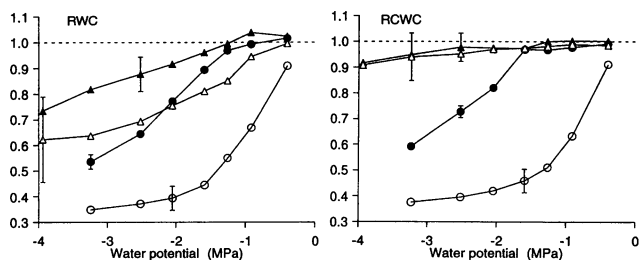


FIGURE 3 Comparing RWC (left) and RCWC (right) from sun leaves ( $\blacktriangle$ ,  $\triangle$ ) and shade leaves ( $\bullet$ ,  $\circ$ ) taken from the same tree and equilibrated to various water potentials on external PEG solutions. Filled symbols represent samples kept in darkness; open symbols were measured using samples that were exposed to light at  $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ . One error bar is reproduced for each data set to indicate the 95% confidence limit for all data points in the set. Dashed lines indicate where RWC or RCWC = 1.0, corresponding to no net gain or loss of water.

Fig. 3 compares RWC and RCWC data from sun leaf and shade leaf discs in equilibrium with PEG solutions at various water potentials. Error bars are longer in sun leaf than in shade leaf data because fewer sun leaf samples were used and because it was more difficult to measure chloroplast peak integrals from sun leaves. Error bars are longer for illuminated samples because individual leaves responded somewhat differently to light. In general, RWC and RCWC data follow the same trends, but there are significant differences. In shade leaves in the dark, for example, the leaf disc lost 11% of its initial water content after the water potential was reduced to  $-1.6 \text{ MPa}$  ( $\text{RWC} = 0.89 \pm 0.03$ ), but chloroplasts retained almost all their water ( $\text{RCWC} = 0.97 \pm 0.02$ ). One can identify the effective range of CVR as the range of water potentials over which RCWC remained near 1.0, i.e., the range of water potentials over which chloroplast water content was maintained near its initial value. CVR was effective in shade leaves between  $-1.6 \text{ MPa}$  and the experimental upper limit of  $-0.4 \text{ MPa}$ . CVR began to fail in shade leaves in the dark somewhere between the data points at  $-1.6$  and  $-2.1 \text{ MPa}$ , and it failed completely in the light where chloroplasts lost as large a fraction of their water as did the entire sample (i.e.,  $\text{RCWC} = \text{RWC}$ ). Light had little or no effect on RCWC in sun leaves where effective CVR extended to below  $-2.5 \text{ MPa}$ .

Plotting RCWC as a function of RWC (Fig. 4) allows the effective range of CVR to be identified as the range of RWC values over which RCWC remains near 1.0, i.e., where chloroplast water content was maintained regardless of leaf water content. Data points that lie near the horizontal dashed line (at  $\text{RCWC} = 1$ ) indicate where CVR was completely effective, and points near the diagonal dashed line ( $\text{RCWC} = \text{RWC}$ ) show where CVR failed completely; points between the two lines correspond to partially effective CVR.

Figs. 5 and 6 present RWC and RCWC data from shade leaves at equilibrium with KCl and with PEG + 0.1 M KCl solutions. On PEG + 0.1 M KCl, the dark behavior is similar to that on PEG alone (Figs. 3 and 4), and in the light, RWC is slightly larger on PEG + 0.1 M KCl than on PEG alone. The range of cellular osmoregulation (i.e., where  $\text{RWC} = 1.0$ ) extends to lower water potentials in leaf discs

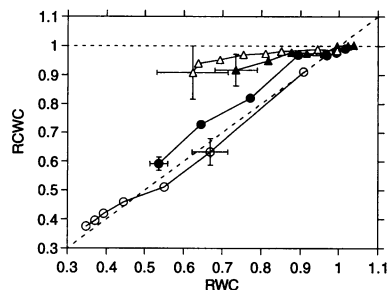


FIGURE 4 Data from Fig. 3 replotted to show RCWC as a function of RWC. Symbols have the same meanings as in Fig. 3. Data points would lie on the horizontal dashed line if  $\text{RCWC} = 1.0$ , and on the diagonal dashed line if  $\text{RCWC} = \text{RWC}$ . Error bars indicate the 95% confidence limits in both variables.

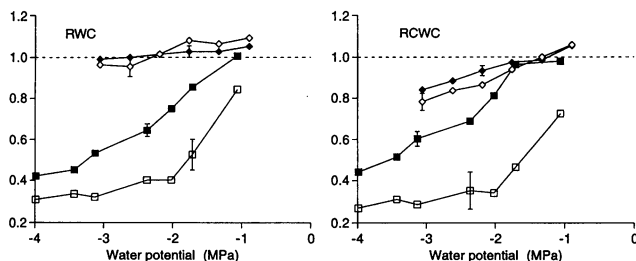


FIGURE 5 RWC (left) and RCWC (right) data from shade leaf samples that had equilibrated on external KCl ( $\blacklozenge, \blacklozenge$ ) and on PEG + 0.1 M KCl solutions ( $\blacksquare, \square$ ). Filled symbols are from samples kept in darkness; open symbols represent samples that were exposed to light at  $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Error bars show the 95% confidence limit.

on KCl. Chloroplasts maintain a larger volume at low water potentials on KCl than on PEG, but Fig. 6 shows that CVR is ineffective on KCl; indeed, the data points fall below the diagonal line, indicating that chloroplasts lose a larger fraction of their water than does the whole disc.

The response to light and to increasingly negative water potentials was nonlinear in shade leaf discs on PEG (Fig. 7). The light-induced reduction in both RWC and RCWC saturated at high light intensity. The main effect of low intensity light ( $12.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) on RCWC was to reduce the CVR range. Note that some of the data in Fig. 7 (the data points representing both full darkness and highest light intensity) are reproduced from Fig. 3 for direct comparison with intermediate intensity data.

## DISCUSSION

### RWC

According to equilibrium thermodynamics, water moves through a semipermeable membrane in the direction of a lower water potential. Therefore, in leaf discs at equilibrium with a constant external water potential, water follows the osmolytes; it escapes when osmolytes are lost and enters when osmolytes are taken up. This principle allows us to interpret several features of the RWC data. For example:

(1) Lower RWC values were measured in the light than in the dark on PEG (Fig. 3). But PEG cannot pass through the cell membrane. Therefore, physiological osmolytes must have been lost from illuminated discs; in the dark, osmolytes either were retained within the disc or they were lost more slowly.

(2) When the external osmolyte was KCl alone, RWC remained near 1.0 in either light or dark at water potentials where discs lost water on PEG (Figs. 3 and 5). Therefore, KCl must have entered the leaf discs.

(3) RWC was almost identical at the same water potentials in the dark on PEG and on the mixed osmolyte PEG + 0.1 M KCl (Figs. 3 and 5). The intracellular  $\text{K}^+$  concentration in *A. platanooides* shade leaves is about 0.2 M (McCain and Markley, 1989). Therefore, KCl did not enter a leaf disc against a concentration gradient.

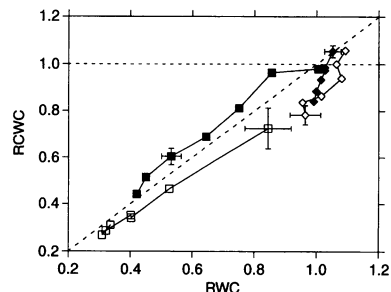


FIGURE 6 Data from Fig. 5 replotted to show RCWC versus RWC. Symbols are the same as in Fig. 5, and dashed lines the same as in Fig. 4.

(4) RWC was significantly higher in the light on PEG + 0.1 M KCl than on PEG alone (Figs. 3 and 5). Therefore, osmolyte loss was reduced in the light when the external medium contained KCl. Since external KCl inhibited osmolyte loss and since KCl cannot pass through the membrane against a concentration gradient, then either  $\text{K}^+$ ,  $\text{Cl}^-$ , or both ions together must be among the osmolytes that escaped from leaf discs in the light. A similar effect has been observed in *Arabidopsis* where light opens  $\text{K}^+$  channels to depolarize the plasma membrane (Spalding and Goldsmith, 1993).

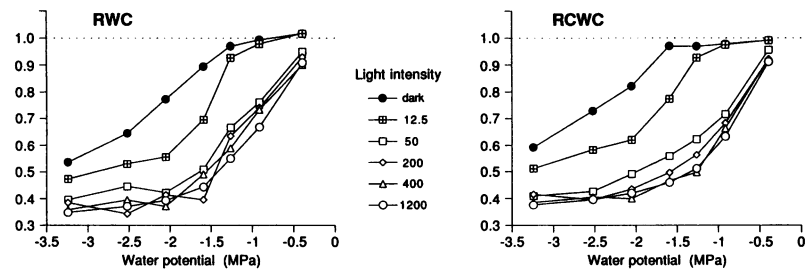
### CVR

New data in this paper confirm the previous demonstration of CVR by NMR techniques (McCain and Markley, 1992). But, in addition, by using integrals instead of peak shifts, RWC has been measured simultaneously with RCWC. The availability of RWC data raises the question: Which is the better operational definition of CVR? Is it maintenance of constant chloroplast water content in response to changing water potential or in response to changing cell water content? I suggest that cell water content may be the more fundamental variable, since plants may respond to decreasing water potential in other ways than loss of water; e.g., they may acquire or synthesize more osmolytes. Therefore, for this discussion, CVR will be defined as a process that maintains RCWC as close as possible to 1.0 regardless of RWC. By this definition, CVR is "effective" at data points that lie near the horizontal dashed lines in Figs. 4 and 6, and it "fails" at points near the diagonal lines. Points that lie between the two dashed lines indicate that chloroplasts retained a larger fraction of their osmolytes than did the nonchloroplast compartments and that CVR was partially effective.

The data support three novel conclusions:

(1) CVR failed in shade leaves in the dark at RWC values where it was effective in sun leaves. The difference between leaf types may be related to a difference in reserve supplies of cytosolic osmolytes, i.e., osmolytes that are available to be imported into chloroplasts for CVR. As the water potential decreases, CVR can be effective only as long as suitable osmolytes can be obtained from other compartments or

FIGURE 7 Comparison of equilibrium RWC (left) and equilibrium RCWC (right) from shade leaf discs that were exposed to different light intensities while floating on PEG solutions. Filled symbols represent samples kept in darkness; open symbols are data from samples that were illuminated at various light intensities. Symbols are listed in the center where light intensities are expressed in units of  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Dashed lines show where RWC or RCWC = 1.0.



synthesized within the chloroplasts; it must fail after the osmolyte reserve has been exhausted. RWC curves indicate that osmolarities initially were similar in sun and shade leaves; i.e., in both leaf types water loss became significant below about  $-1.3$  MPa (Fig. 3). Chloroplasts contain about half the total leaf water in *A. platanoides* shade leaves, but only about one-sixth of the total in sun leaves (McCain et al., 1988, 1993). Together, these two factors (similar initial osmolarities, different relative compartment volumes) insure that the reserve supply of osmolytes is larger in sun leaves than in shade leaves (comparing tissue samples containing equal volumes of chloroplasts). A quantitative analysis of this effect would require additional information about turgor pressures and the relative stiffness of cell walls in sun and shade leaves.

(2) CVR failed in shade leaves when they were exposed to light, but RCWC in sun leaves was almost unaffected by light intensity. The light effect in shade leaves was nonlinear and saturated at relatively low light intensity (Fig. 7). Similar behavior was reported by Miller and Nobel (1972), who found that shrinkage of pea chloroplasts was half-saturated at 50 lux.

The difference indicates that sun leaf chloroplasts either had access to a larger supply of whatever osmolytes did not escape from the disc in the light, or were able to retain more osmolytes in the light, or both. If it is a matter of access, then sun leaves need a larger reserve supply of cytosolic osmolytes to protect against loss of CVR in intense light at low water potentials; indeed, as noted above, sun leaves do have a larger reserve supply than shade leaves.

Chloroplasts in shade leaves normally contain a larger fraction of leaf water than those in sun leaves (McCain et al., 1988, 1993), but the shade leaves used in these experiments were extreme examples of their type and had unusually large chloroplast water fractions. The crown was so dense that they almost never were exposed to full sunlight, or even to sunflecks. Therefore, when CVR failed in strong light in shade leaves, it did so under conditions that were outside the range to which they were adapted.

(3) KCl was relatively ineffective as an osmolyte for CVR, especially in the light. The only time that RCWC was found to be significantly less than RWC was in samples where one may reasonably infer an elevated cytosolic KCl concentration (i.e., on KCl in dark or light where KCl must have entered the discs, and on PEG + 0.1 M KCl in the light where cytosolic KCl concentrations could not have fallen

below 0.1 M, in contrast to discs on PEG where more KCl may have escaped). Apparently, chloroplasts were unable to import sufficient KCl to maintain constant volumes at low water potentials. This observation is consistent with reports that light induces  $\text{K}^+$  transport out of chloroplasts (Nobel, 1969; Defilippis and Pallaghy, 1973).

Decreased ionic concentrations help to promote optimum photosynthetic rates. Stromal  $\text{K}^+$  concentrations need to be reduced at high light intensities to prevent damage from excessive  $\text{H}^+$  accumulation in the lumen (Dilley et al., 1987). Also, it has been shown that reducing the KCl concentration from 0.15 to 0.05 M in an artificial stroma medium increases  $\text{CO}_2$  fixation rates by more than 25% (Kaiser et al., 1986).

Perhaps photosynthetic efficiency may be enhanced by the interaction of CVR with light-induced ion export. If ions are removed selectively from chloroplasts upon exposure to light, then water must follow and the chloroplasts must shrink unless CVR can replenish the lost osmolytes. If CVR imports non-ionic osmolytes, the net result will be lower stromal concentrations of ions and higher stromal concentrations of non-ionic osmolytes in the light than in the dark. CVR could perform the same function even if it were only partially effective, although in that case chloroplasts would shrink in the light. In this way, CVR may help to regulate the composition of the stromal medium to promote optimum photosynthetic rates.

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