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Induction of CAM in *Mesembryanthemum crystallinum* Abolishes the Stomatal Response to Blue Light and Light-Dependent Zeaxanthin Formation in Guard Cell Chloroplasts

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Facultative CAM plants such as *Mesembryanthemum crystallinum* (ice plant) possess C3 metabolism when unstressed but develop CAM under water or salt stress. When ice plants shift from C3 metabolism to CAM, their stomata remain closed during the day and open at night. Recent studies have shown that the stomatal response of ice plants in the C3 mode depends solely on the guard cell response to blue light. Recent evidence for a possible role of the xanthophyll, zeaxanthin in blue light photoreception of guard cells led to the question of whether changes in the regulation of the xanthophyll cycle in guard cells parallel the shift from diurnal to nocturnal stomatal opening associated with CAM induction. In the present study, light-dependent stomatal opening and the operation of the xanthophyll cycle were characterized in guard cells isolated from ice plants shifting from C3 metabolism to CAM. Stomata in epidermis detached from leaves with C3 metabolism opened in response to white light and blue light, but they did not open in response to red light. Guard cells from these leaves showed light-dependent conversion of violaxanthin to zeaxanthin. Induction of CAM by NaCl abolished both white light- and blue light-stimulated stomatal opening and light-dependent zeaxanthin formation. When guard cells isolated from leaves with CAM were treated with 100 mM ascorbate, pH 5.0 for 1 h in darkness, guard cell zeaxanthin content increased at rates equal to or higher than those stimulated by light in guard cells from leaves in the C3 mode. The ascorbate effect indicates that chloroplasts in guard cells from leaves with CAM retain their competence to operate the xanthophyll cycle, but that zeaxanthin formation does not take place in the light. The data suggest that inhibition of light-dependent zeaxanthin formation in guard cells might be one of the regulatory steps mediating the shift from diurnal to nocturnal stomatal opening typical of plants with CAM.

Key words: Blue light response — Guard cells — Ice plant — *Mesembryanthemum crystallinum* — Stomata — Zeaxanthin.

Abbreviations: A, antheraxanthin; DTT, dithiothreitol; PPFD, photosynthetic photon flux density; Rubisco, ribulose 1,5 bis-phosphate carboxylase/oxygenase; V, violaxanthin; Z, zeaxanthin.

Stomata of unstressed C3 and C4 plants open in the light and close in darkness, whereas stomata of facultative CAM plants shift from diurnal to nocturnal stomatal opening during stress-induced shifts from C3 metabolism to CAM (Osmond 1978, Ting 1987). Inhibition of the stomatal response to light in CAM plants is of interest for understanding this remarkable plant adaptation to stress and for understanding the mechanisms mediating photoreception and signal transduction in guard cells.

In attempts to explain the pattern of stomatal movements in CAM plants, a number of hypotheses have been advanced that invoke inhibition of light-dependent stomatal opening by external signals such as high intercellular leaf CO_2 concentrations (Cockburn et al. 1979) or temperatures (Kluge and Ting 1978). These hypotheses, however, can not account for the observation that when stomata isolated from leaves with CAM are kept under conditions that favor light-stimulated opening of stomata isolated from leaves with C3 or C4 metabolism, they fail to open in response to light (Jewer et al. 1981, Lee and Assmann 1992, Mawson and Zaugg 1994). These studies indicate that in addition to any prevailing effects of environmental conditions encountered by CAM leaves, the shift from diurnal to nocturnal stomatal opening in CAM plants could depend on regulatory mechanisms within the guard cells proper.

Facultative CAM plants provide a convenient experimental system with which to study how plant characteristics typical of C3 metabolism are altered upon CAM induction. A study of *Portulacaria afra* (Lee and Assmann 1992) has shown that stomata from plants in the C3 mode have the typical light responses associated with the two major photoreceptor systems in guard cells, photosynthesis and a specific blue light response (Zeiger and Field 1982, Tallman and Zeiger 1988, Poffenroth et al. 1992, Talbott and Zeiger 1993, Mawson 1993). Upon CAM induction, neither of these two light responses can be detected either in intact leaves or in isolated stomata (Lee and Assmann 1992). On the other hand, stomata isolated from *Mesembryan-*

themum crystallinum (ice plant) operating in the C3 mode respond to blue but not to red light (Mawson and Zaugg 1994). Upon induction of CAM, stomata from *M. crystallinum* no longer open in response to blue light (Mawson and Zaugg 1994).

Signal transduction of blue light in guard cells has been the subject of extensive investigation (Mawson 1993). Recent studies have shown that the xanthophyll, zeaxanthin, which functions in photoprotection in mesophyll chloroplasts (Adams III and Demmig-Adams 1992, Long et al. 1994), also plays a role in photosensory transduction in guard cells. Guard cell zeaxanthin content closely tracks incident radiation at the leaf surface (Srivastava and Zeiger 1995a) and is positively correlated with stomatal apertures (Srivastava and Zeiger 1995a). Furthermore, inhibition of zeaxanthin formation in guard cells in detached leaf epidermis (epidermal "peels") inhibits blue light-stimulated stomatal opening (Srivastava and Zeiger 1995b). Zeaxanthin has an absorption spectrum that matches the action spectrum of blue light-stimulated stomatal opening (Karlsson 1986, Quiñones et al. 1996) and the action spectra of intrinsic blue light responses of guard cell and coleoptile chloroplasts (Quiñones et al. 1996).

In the present study, we used enzymatically-cleaned epidermal tissue from ice plant leaves in the C3 mode to investigate whether guard cells from *M. crystallinum* operate the xanthophyll cycle. We then induced CAM and assessed whether changes in stomatal responses to light were paralleled by changes in xanthophyll cycle metabolism. Results show that guard cells from ice plant leaves with CAM are devoid of both their blue light response and their ability to de-epoxidize violaxanthin to form zeaxanthin upon illumination.

Materials and Methods

Plants—Plants were grown from seed in a greenhouse as described previously (Mawson and Zaugg 1994). After two months, ice plants in the C3 mode were moved from the greenhouse to a growth chamber. Plants were maintained on a 16 h light/8 h dark cycle at 75% R.H. at 25°C.

Treatments—C3 metabolism was maintained in half of the plants growing in the chamber by keeping them at a PPFD of 65 μmol m^{-2} s^{-1} of white light (white fluorescent + incandescent) and watering them with water. CAM was induced in the other half of the plants by watering them with increasing concentrations of NaCl (highest concentration = 400 mM) and illuminating them at a PPFD of 350 μmol m^{-2} s^{-1} (Winter and Von Willert 1972, Winter and Gademann 1991, Mawson and Zaugg 1994).

Measurement of nocturnal titratable acidity—Nocturnal titratable acidity was measured as described by Mawson and Zaugg (1994). Leaves were collected at 0830 PST, 30 min before lights came on in the growth chamber. A portion of a single leaf was weighed (0.15 to 0.65 g) and then blended in 50 ml of chilled deionized, distilled water for 2 min at top speed in a Waring blender. The slurry was filtered through a 40 μm × 40 μm mesh nylon net. The pH of 10 ml of the filtrate was recorded and the sample was then titrated to pH 7.0 with 10 mM NaOH. Nocturnal titratable acidity was calculated by multiplying by 50 the number of ml of 10 mM NaOH required to reach pH 7.0 and then dividing that number by the fresh weight of the tissue (g).

Light-induced stomatal opening in detached epidermis—Guard cell responses to white, red, or red and blue light were measured as changes in stomatal aperture under each light condition in epidermis obtained from leaves of plants with either C3 metabolism or CAM. For each experiment, a single leaf was collected at 0830 h PST. A small portion of the leaf was excised for measurement of nocturnal titratable acidity (see above). The remainder was deveined and the tissue between the veins was chopped to fine pieces with a razor blade. Depending on leaf age, leaf pieces were blended at top speed in a Waring blender for 10 to 25 s in 50 ml of chilled 10% Ficoll (w : v), 5 mM $CaCl_2$, 0.1% polyvinylpyrrolidone 40 (w : v; PVP 40), 10 mM ascorbic acid (Kruse et al. 1989). Epidermis detached by blending was collected on a 220 μm × 220 μm mesh nylon net and washed for 1 min under running deionized tap water. After the rinse, epidermal peels were transferred to 25 ml of a solution of 60 mM KCl, 0.01 mM $CaCl_2$ that was aerated continuously with compressed air. Peels were incubated for 4 h either in darkness or under 350 μmol m^{-2} s^{-1} of light from a projector bulb (Model DAH, General Electric, Cleveland, OH, U.S.A.). Temperature was maintained at 25°C by circulating chilled water and by aiming three fans at the light source. Red light (350 μmol m^{-2} s^{-1}) was supplied from the bottom of the dish. In dual beam experiments, 350 μmol m^{-2} s^{-1} of red light was applied from the bottom and 15 μmol m^{-2} s^{-1} of blue light was applied from the top of the dish. Heat from the red light source was removed through a water filter as described previously (Schwartz and Zeiger 1984). Red and blue light filters were of Plexiglas™ as described by Talbott and Zeiger (1993). At the end of the incubation period, apertures were measured microscopically at 400× using a microscope equipped with a digital video camera attached to a computer digitizing system (Talbott and Zeiger 1993). For each treatment, means were calculated from 30 measurements, 10 on each of three epidermal peels.

Isolation of guard cells—For measurement of the xanthophyll cycle in guard cells, epidermal and mesophyll cells were removed from detached epidermis enzymatically, so that only guard cells remained in the detached epidermis. Digestion of detached epidermis in a solution of cellulolytic enzymes in a hypotonic medium hydrolyzes cell walls of contaminating cell types faster than those of guard cells (Hudson et al. 1983). After 45 to 55 min of digestion, guard cells were the only viable cells still attached to the cuticle.

In order to obtain enough tissue for xanthophyll cycling experiments, four to eight leaves (4.4 to 6.6 g) were collected from leaves with either C3 metabolism or CAM and epidermis was detached by blending as described above. Prior to blending, a small portion of one leaf was used for measurement of nocturnal titratable acidity as described above. After blending, any remaining, unblended leaf fragments were removed from washed epidermis with forceps and the epidermal peels were transferred to 50 ml of a solution of 0.4 M D-mannitol, 1 mM $CaCl_2$, 1.6% Cellulase Onozuka RS (w : v), 0.012% Pectolyase Y-23 (w : v), 0.5% PVP 40 (w : v), and 0.3% bovine serum albumin (w : v). Peels were incubated for 45 to 55 min (depending on leaf age) at 30°C with orbital shaking at 75 excursions min^{-1}. At the end of the incubation period, peels were triturated 25 times with a 25 ml pipette modified to have a tip opening of 5 mm, collected on a 220 μm × 220 μm mesh nylon net, and washed with 1 liter of 0.4 M D-mannitol, 1 mM $CaCl_2$, pH 6.5. To remove adhering veins and vein

fragments from cleaned epidermis, peels were transferred with a spatula to a 50 ml conical plastic centrifuge tube containing 25 ml of 0.4 M D-mannitol, 1 mM $CaCl_2$, pH 6.5. Tubes were capped, shaken, and centrifuged at $70 \times g$ for 5 min. At the end of each centrifugation, peels floated on the surface of the solution, and vein fragments with adhering mesophyll cells were in the solution below. The solution was aspirated and the procedure was repeated five times. To ensure that peels were free of contaminants they were examined microscopically both with bright field microscopy and with epifluorescence microscopy. Chloroplasts of contaminating mesophyll cells were larger than those of guard cells and their red fluorescence was more intense than that of guard cell chloroplasts so that mesophyll chloroplasts could be readily detected. Only preparations free of visible contaminants were used for experiments. To assess the viability of guard cells, peels were examined microscopically for fragmentation of chloroplasts and for weakened chlorophyll fluorescence.

Measurement of light-induced zeaxanthin formation in isolated guard cells—Washed peels were transferred to 25 ml of 0.28 M D-mannitol, 60 mM KCl, 0.01 mM $CaCl_2$, 5 mM HEPES buffer, pH 6.5; the solution was aerated continuously with compressed air. After preincubation in darkness for 1 h, peels were either harvested or illuminated for an additional 4 h with 350 μmol m^{-2} s^{-1} of light from the projector bulb described above. Temperature was maintained at 25°C by circulating chilled water around incubation containers and by aiming fans at light sources. At the end of the incubation period, peels were harvested on nylon nets, transferred to 1.5 ml conical microcentrifuge tubes, and frozen at −80°C for 30 min. Pigments were then extracted on ice under dim light. Peels were homogenized with a pestle for 2 min in sand and dry ice in a mortar containing 0.5 ml of $NaHCO_3$-saturated acetone and 2 ml of hexane. Extracted pigments were transferred to a centrifuge tube on ice and the mortar and pestle were washed with 2.5 ml of ice cold 0.2 M KCl. The wash was transferred to the same tube and tubes were centrifuged at $5{,}000 \times g$ for 3 min. The entire green organic phase was collected and evaporated to dryness in a Speedvac™ (Model SC 100, Savant, Farmingdale, NY, U.S.A.). Dried samples were resuspended in acetone for HPLC analysis. Pigment extracts were chromatographed on a Beckman Model 334 Gradient Chromatograph (Beckman Instruments, Fullerton, CA, U.S.A.) equipped with an Alltech 4.6×250 mm Spherisorb ODS-1 5 μ column (Alltech, Deerfield, IL, U.S.A.; Gilmore and Yamamoto 1991). Samples were separated by elution with an aqueous mixture of acetonitrite : methanol : 0.1 M Tris-HCl buffer (pH 8.0, 72 : 8 : 3) for 4 min followed by a 2.5-min linear gradient of methanol : hexane (4 : 1) which was then held for an additional 8.5 min. Flow rate was 2 ml per min. Pigments were detected at 440 nm with a variable wavelength detector (Beckman Model 165) connected to an integrator (Spectra-Physics, San Jose, CA, U.S.A.). Standards were used to calibrate the instrument and to quantify pigments. To assess the viability of guard cells, peels were compared microscopically both at the beginning and at the end of the 4 h incubation period as described above.

Zeaxanthin formation in ascorbate-treated guard cells from plants with CAM—To determine whether chloroplasts of guard cells from plants with CAM had a functional xanthophyll cycle, isolated guard cells were treated with 100 mM ascorbate at pH 5.0 in darkness (Havaux and Gruszecki 1993). Guard cells were isolated as described above and incubated in darkness for 1 h in 25 ml of 0.4 M D-mannitol, 0.1 mM $CaCl_2$, 5 mM HEPES buffer, pH 5.0 or in 25 ml of 0.3 M D-mannitol, 100 mM ascorbate, 0.1 mM $CaCl_2$, 5 mM HEPES buffer, pH 5.0. Solutions were aerated continuously with compressed air. At the end of the incubation period, pigments were extracted and chromatographed as described. For comparison, guard cells isolated from leaves of plants possessing CAM metabolism were given light or dark treatments. After preincubation in darkness for 1 h in 25 ml of 0.4 M D-mannitol, 0.1 mM $CaCl_2$, 5 mM HEPES buffer, pH 6.5, peels were incubated for an additional 2 h in the same solution under 350 μmol m^{-2} s^{-1} of light from the projector bulb or for an additional 1 h in darkness. In all experiments, temperature was maintained at 25°C as described.

Statistical analysis—Changes or differences in stomatal apertures, in average percentages of individual xanthophylls (V, A, or Z) in the total xanthophyll pool (V+A+Z), and in total xanthophylls of guard cells from plants with C3 metabolism or CAM normalized to guard cell Chl content were compared by a two-tailed, unpaired t-test at the 0.05 level of significance. In ascorbate experiments, changes in Z were compared by one-way ANOVA followed by a Scheffe F-test at the 0.05 level of significance.

Results

Purity of isolated guard cells—One thousand microscopic fields from two separate preparations of isolated guard cells were examined with epifluorescence microscopy for contaminating epidermal and mesophyll cells. Because analysis of contaminants was performed at an early stage in the development of the method for isolating guard cells, preparations were washed only three times to remove veins instead of the six times used for all subsequent preparations (see Materials and Methods). No intact epidermal cells or mesophyll cells were seen (Table 1). Contamination with vein fragments showing very weak chlorophyll fluorescence was detected in 2.2% of fields examined (Table 1).

Light-stimulated stomatal opening in detached epidermis—As ice plants shifted from C3 metabolism to CAM, the nocturnal titratable acidity in leaf tissue increased significantly (Winter and Von Willert 1972, Mawson and Zaugg 1994) and the responses of stomata in epidermal peels to white light decreased with increasing nocturnal titratable acidity (Fig. 1). Stomata in epidermal peels from plants with C3 metabolism did not open in response to red light, but they did open in response to low fluences of blue light superimposed onto a red light background (Fig. 1). Stomata in epidermal peels from plants with CAM did not open in response to the same fluences of red light or to low fluences of blue light superimposed onto a red light background (Fig. 1). Bubbling the incubation solution with CO_2-free air had no significant effect on stomatal opening in epidermal peels illuminated with red light or with red and blue light (not shown).

Light-stimulated zeaxanthin formation in isolated guard cells—When guard cells from plants with C3 metabolism were illuminated for 4 h with white light, Z increased significantly from 6.2% to 15.2% of the total xanthophyll pool (V+A+Z; $t=-15.3$; $p=0.0006$; $n=3$; Fig. 2). The increase in Z was accompanied by significant 12% net decrease in V ($t=3.16$; $p=0.034$; $n=3$; Fig. 2) and a net in-

Table 1 Contaminants in preparations of isolated guard cells in enzymatically digested epidermis from leaves of *Mesembryanthemum crystallinum*

Experiment	Fields/peels examined	Mesophyll cells	Epidermal cells	Veins—no Chl fluorescence	Veins—some Chl fluorescence
1	500	0	0	13	3
2	500	0	0	10	19
Total	1,000	0	0	23	22

To remove veins, peels were washed by centrifugation three times. Two isolates prepared on separate days were examined with epifluorescence microscopy for chlorophyll fluorescence from mesophyll contaminants either attached to peels or associated with vein material in the preparations. On each day, 500 peels were examined at 200× and scored for contaminants.

crease of 3.0% in A, which was not statistically significant ($t=-1.095$; $p=0.335$; $n=3$; Fig. 2). When light-stimulated changes in xanthophylls from guard cells of C3 plants were compared on a Chl basis, in each of three separate experiments, V and A decreased, but Z increased. On average, V decreased from 333.0±5.0 to 152.5±3.2 ng (μg Chl)$^{-1}$ ($\Delta=-54.2\%$), A decreased from 39.0±2.4 to 28.8±2.9 ng (μg Chl)$^{-1}$ ($\Delta=-26.2\%$), and Z increased from 24.9±2.2 to 33.5±2.7 ng (μg Chl)$^{-1}$ ($\Delta=+34.5\%$).

When guard cells isolated from plants with CAM were illuminated for 4 h with white light, there was no significant change in the percentage of V, A, or Z in the xanthophyll pool. When light-stimulated changes in xan-

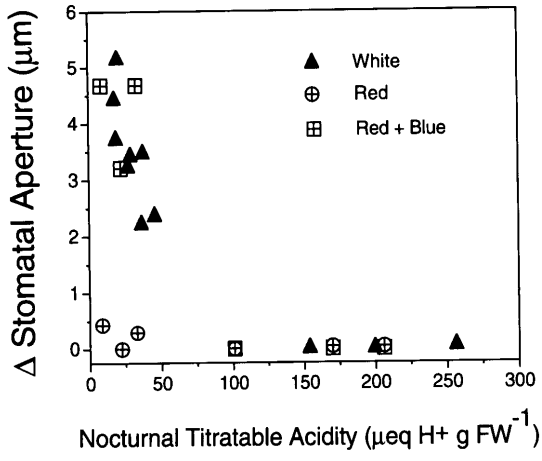

Fig. 1 Stomatal aperture in epidermis detached from leaves of *Mesembryanthemum crystallinum* as a function of leaf nocturnal titratable acidity. Epidermal peels were incubated in 25 ml of 60 mM KCl, 0.01 mM $CaCl_2$ for 4 h either in light or in darkness. Values are differences between means of light and dark treatments performed on the same day. For each treatment, means were calculated from 30 measurements, 10 on each of three epidermal peels. Light=350 μmol m^{-2} s^{-1} of white (triangles); 350 μmol m^{-2} s^{-1} of red (circles); or 350 μmol m^{-2} s^{-1} of red+15 μmol m^{-2} s^{-1} of blue (squares). Equation for a best fit line (not shown) through white light points (triangles) is $y=-4.107\log(x)+9.463$ with $r=0.956$.

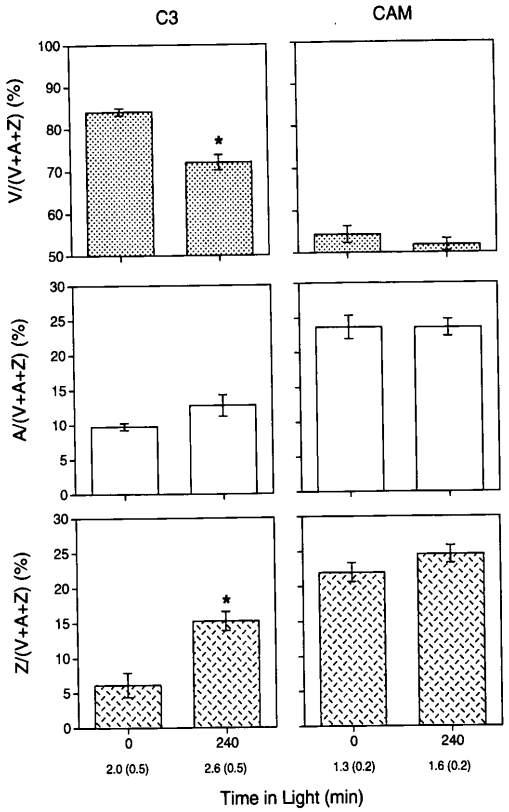

Fig. 2 Percentage of violaxanthin (V), antheraxanthin (A), or zeaxanthin (Z) in the xanthophyll pool (V+A+Z) in isolated guard cells from leaves of *Mesembryanthemum crystallinum* incubated for 1 h in darkness (0 time control) or for 1 h in darkness and then for 4 h under light (350 μmol m^{-2} s^{-1}). The average nocturnal titratable acidity of leaves of C3 plants was 28.2±2.2 μeq H^+ (g FW)$^{-1}$ (mean; SE of the sample mean; $n=3$); that of CAM plants was 203.2±5.1 μeq H^+ (g FW)$^{-1}$. *=significantly different from 0 time control (unpaired t-test; 0.05 level of significance). Numbers beneath time values are normalized chlorophyll (Chl) contents of isolated epidermis in ng Chl per ng lutein. Lutein was chosen as a standard because its content was nearly constant among treatments. Chl values are means and SE for 3 replicates of each treatment performed on separate days. There was no significant difference between means of C3 and CAM dark treatments or between means of C3 and CAM light treatments (unpaired t-test; 0.05 level of significance).

thophylls from guard cells of CAM plants were compared on a Chl basis, in each of three separate experiments, V, A, and Z decreased. On average, V decreased from 400.4±6.5 to 289.7±5.2 ng (μg Chl)$^{-1}$ (Δ=−27.6%), A decreased from 171.9±4.3 to 129.6±2.7 ng (μg Chl)$^{-1}$ (Δ=−24.6%), and Z decreased from 128.9±5.5 to 105.1±4.6 ng (μg Chl)$^{-1}$ (Δ=−18.5%).

On a Chl basis, total xanthophyll concentrations (V+A+Z) in guard cells from plants with CAM were≈1.8 times greater than those of guard cells from plants with C3 metabolism (t=−3.8; p=0.032; n=3). Initial concentrations of both Z and A were significantly greater in guard cells from CAM plants than in guard cells from plants with C3 metabolism (for A, t=−6.03; p=0.004; for Z, t=−1.07; p=0.04); differences in V were not statistically significant.

Stimulation of zeaxanthin formation by ascorbate in guard cells from CAM plants—When guard cells from plants with CAM were incubated for 1 h in darkness in 100 mM ascorbate, pH 5.0, Z increased significantly from 15.3% to 39.4% of the total xanthophyll pool (t=−6.15; p=0.0034; n=3; Fig. 3). The percentage of V in the xanthophyll pool decreased significantly from 66.5±0.6% to 43.9±3.1% (t=7.3; p=0.002; n=3). There were no significant changes in A. In seven separate experiments with light-treated guard cells from plants with CAM, the average percentage of Z was 18.0% (Fig. 3), a mean value not significantly different from that of untreated dark controls (n=3) of ascorbate experiments (t=1.22; p=0.26). On a Chl basis, Z increased significantly from 77.8±12.6 ng (μg Chl)$^{-1}$ to 161.1±14.8 ng (μg Chl)$^{-1}$ (p=0.008; n=3). V and A decreased significantly from 333.9±34.0 ng (μg Chl)$^{-1}$ to 180.8±20.0 ng (μg Chl)$^{-1}$ and from 90.1±4.6 ng (μg Chl)$^{-1}$ to 68.8±5.2 ng (μg Chl)$^{-1}$, respectively (p=0.018 for V and 0.038 for A; n=3). After exposure to light, the average Z concentration in guard cells from plants with CAM was 76.7±16.7 ng (μg Chl)$^{-1}$, a mean value not significantly different from that of guard cells from plants with CAM incubated for 2 h in darkness.

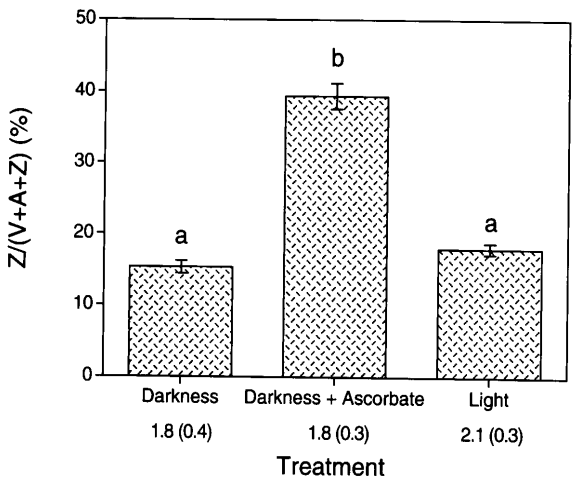

Fig. 3 Percentage of zeaxanthin (Z) in the xanthophyll pool (V+A+Z) in ascorbate-treated guard cells of enzymatically cleaned epidermis isolated from leaves of *Mesembryanthemum crystallinum* in the CAM mode. Peels were incubated in darkness for 1 h in a solution of 0.4 M D-mannitol, 0.1 mM $CaCl_2$, 5 mM HEPES buffer, pH 5.0 (darkness) or in a solution of 0.3 M D-mannitol, 100 mM ascorbate, 0.1 mM $CaCl_2$, 5 mM HEPES buffer, pH 5.0 (darkness+ascorbate). Values are means and SE from 3 experiments performed on separate days. For comparison, values are given for light-treated guard cells from leaves of plants possessing CAM metabolism (light; n=7). After preincubation in darkness for 1 h in a solution of 0.4 M D-mannitol, 0.1 mM $CaCl_2$, 5 mM HEPES buffer, pH 6.5, peels were incubated for an additional 2 h in the same solution under 350 μmol m^{-2} s^{-1} of light. The average nocturnal titratable acidity of leaves of CAM plants used for ascorbate experiments was 143.7±5.7 μeq H^+ (g FW)$^{-1}$. The average nocturnal titratable acidity of leaves of CAM plants used for light experiments was 215.0±3.8 μeq H^+ (g FW)$^{-1}$. Values beneath bar labels are mean normalized Chl contents of cleaned epidermis in ng Chl per ng lutein (see legend to Fig. 2). Same letter=means not significantly different, Scheffe F-test, 0.05 level; different letters=means significantly different, Scheffe F-test, 0.05 level.

Discussion

Ice plants watered with a NaCl solution under relatively high incident radiation (Winter and Gademann 1991, Mawson and Zaugg 1994) shift from a Rubisco-dependent C3 mode of carbon fixation to a CAM mode that depends on PEP carboxylase to catalyze primary carbon fixation (Thomas and Bohnert 1993). Nocturnal fixation of CO_2 into malate and a shift from diurnal to nocturnal stomatal opening are central features of the CAM adaptation. The onset of CAM, which can be monitored by an increase in leaf titratable acidity (Winter and Von Willert 1972, Mawson and Zaugg 1994), is paralleled by an inhibition of the stomatal response to light (Fig. 1; Osmond 1978, Ting 1987, Lee and Assmann 1992, Mawson and Zaugg 1994). The light insensitivity of stomata from epidermal peels indicates that the shift from diurnal to nocturnal opening in CAM plants depends, at least in part, on regulatory mechanisms within the guard cells proper (Fig. 1; Jewer et al. 1981, Lee and Assmann 1992, Mawson and Zaugg 1994).

Stomata from leaves in the C3 mode opened in response to white and blue light applied over background red light, but not in response to red light alone (Fig. 1). These data suggest that the stomatal response to white light is probably a response to the blue light portion of the white light spectrum. These results confirm those of a previous study (Mawson and Zaugg 1994) showing that stomata from *Mesembryanthemum* leaves in the C3 mode are devoid of red light responses. The red light response is diagnostic for photosynthetic activity in guard cells (Tallman and Zeiger 1988, Poffenroth et al. 1992, Talbott and Zeiger 1993). Lack of a red light response has been

reported for achlorophyllous guard cells of the genus *Paphiopedilum* (Zeiger et al. 1983) but is atypical of guard cells with seemingly normal chloroplasts, such as those of ice plant (Faraday et al. 1982). On the other hand, stomata from the facultative CAM species, *P. afra* showed a normal red light response (Lee and Assmann 1992) indicating that the lack of red light sensitivity found with *M. crystallinum* is not a general characteristic of CAM species. In contrast with the two documented instances of stomata responding to blue and not to red light, a converse instance of stomata responding to red but not to blue light has not been reported. Compared to blue and white light, stomatal responses to red light are consistently sub-optimal, suggesting that the blue light response has a key functional role in stomatal movements perhaps related to the quantitative sensing of incident solar radiation (Srivastava and Zeiger 1995a).

Guard cells from ice plant leaves in the C3 mode convert violaxanthin to zeaxanthin in the light. Recent studies have shown that in addition to the well documented role of the xanthophyll cycle in photoprotection (Long et al. 1994), zeaxanthin may function in blue light photoreception in *Vicia* guard cells and in corn coleoptiles (Quiñones and Zeiger 1994, Quiñones et al. 1996). The findings reported here show that guard cells from a facultative CAM species have a functional xanthophyll cycle, and that in the C3 mode, they convert violaxanthin into zeaxanthin in the light. Upon induction of CAM, the inhibition of light-dependent stomatal opening (Fig. 1) is accompanied by an inhibition of light-dependent zeaxanthin formation (Fig. 2). The observation that guard cells from leaves in the CAM mode can form zeaxanthin in the dark in the presence of ascorbate (Fig. 3) indicates that the lack of light sensitivity of the xanthophyll cycle is not a result of a loss of functional competence of the guard cell chloroplast. This is the first report of a specific change in a metabolic characteristic of a guard cell associated with CAM induction.

The absorption spectrum of zeaxanthin closely matches the action spectrum of blue light-stimulated stomatal opening (Quiñones et al. 1996), and inhibition of zeaxanthin formation by DTT specifically inhibits blue light-stimulated stomatal opening in *Vicia faba* (Srivastava and Zeiger 1995b). The observed inhibition of blue light-stimulated stomatal opening associated with the inhibition of zeaxanthin formation in *Vicia* guard cells (Srivastava and Zeiger 1995b) suggests that suppression of zeaxanthin formation associated with CAM induction in *Mesembryanthemum* guard cells could have a regulatory role in the shift from diurnal to nocturnal stomatal opening. Such a regulatory switch might operate in conjunction with other documented factors such as high CO_2 concentrations (Cockburn et al. 1979), and enhanced ABA (Ting 1981, Davies and Zhang 1989) and temperature sensitivities (Kluge and Ting 1978).

Despite lack of xanthophyll cycling, zeaxanthin concentrations in guard cells from ice plant leaves in the CAM mode were higher than those from leaves in the C3 mode, both in the light and in darkness (Fig. 2). Zeaxanthin in C3 mesophyll chloroplasts increases in the light (Demmig et al. 1987), and dark zeaxanthin levels have been reported to remain high in leaves exposed to low winter temperatures (Adams III and Demmig-Adams 1994, 1995, Adams III et al. 1995). This, however, was not the case in mesophyll cells of ice plant leaves in the CAM mode. In leaves with nocturnal titratable acidities = 300 to 330 μeq H^+ $(g\ FW)^{-1}$ taken from the chamber 30 min before the onset of illumination (0830 h), zeaxanthin was 3–5% of the total xanthophyll pool. Zeaxanthin remained low (< 10%) until early afternoon. At 1530 h, the time at which titratable acidity dropped to its minimum daily value (50 to 110 μeq H^+ $(g\ FW)^{-1}$), zeaxanthin increased to 30–40% of the total xanthophyll pool (Nouhi, Mawson and Zeiger unpublished). Similar observations of xanthophyll cycling in leaves of obligate CAM plants have been reported (Adams and Demmig-Adams 1996). These data indicate that the lack of xanthophyll cycling in guard cells from leaves in the CAM mode is not typical of their mesophyll chloroplasts. One possible explanation for the high concentrations of zeaxanthin and antheraxanthin found in these guard cells is that such an excess in de-epoxidized xanthophyll is required for photoprotection of guard cell chloroplasts under conditions in which stomata remain closed during the day. It has also been suggested that two zeaxanthin populations, one specialized for photoprotection and the other for sensory transduction, may exist in coleoptile chloroplasts (Zhu et al. 1995). A third intriguing possibility is that signal transduction of blue light by guard cell zeaxanthin requires xanthophyll cycling per se. Available data on the relationship between stomatal apertures and zeaxanthin does not allow us to distinguish between these alternative explanations, warranting its further investigation in both C3 and CAM species.

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