The Plant Cell, Vol. 6, 1319–1328, September 1994

Two Transduction Pathways Mediate Rapid Effects of Abscisic Acid in *Commelina* Guard Cells

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Commelina **guard cells can be rapidly closed by abscisic acid (ABA), and it is thought that this signal is always transduced through increases in cytosolic calcium. However, when *Commelina* plants were grown at 10 to 17°C, most guard cells failed to exhibit any ABA-induced increase in cytosolic calcium even though all of these cells closed. At growth temperatures of 25°C or above, ABA-induced closure was always associated with an increase in cytosolic calcium. This suggests that there may be two transduction routes for ABA in guard cells; only one involves increases in cytosolic calcium. Activation of either pathway on its own appears to be sufficient to cause closure. Because the rates of ABA accumulation and transport in plants grown at different temperatures are likely to be different, we synthesized and microinjected caged ABA directly into guard cells. ABA was released internally by UV photolysis and subsequently caused stomatal closure. This result suggests a possible intracellular locale for the hypothesized ABA receptor.**

INTRODUCTION

Abscisic acid (ABA) was first isolated as a factor related to the control of abscission but is now indissolubly linked to plant water stress response and the development of desiccation tolerance (Trewavas and Jones, 1991). Studies on ABA biosynthetic and sensitivity mutants and the observed effects of exogenously added ABA have suggested that many morphological and physiological adaptations to water stress are at least in part under the control of ABA. These long-term adaptations are believed to result from changes in gene expression, and ABA-regulated genes have been cloned from a variety of plants. Many appear to be members of a coordinately regulated gene superfamily (Skriver and Mundy, 1990; Bray, 1991). The regulation by ABA of stomatal aperture is believed to be concerned with much shorter term variations in leaf water supply (Davies et al., 1994). Rapid variations in ABA concentration and factors that determine sensitivity to ABA help optimize the size of the aperture to ensure efficient use of water while allowing continued CO_2 transit and fixation. The exact relationship between the long-term and short-term effects of ABA are not understood, but it is known that guard cells are highly selective for the (+)-ABA enantiomer, whereas changes in growth, gene expression, and inhibition of amylase secretion are not stereoselective (Cummins and Sondheimer, 1973; Walton, 1983; Walker-Simmons et al., 1992).

The guard cell is a useful model system for studying the action of ABA because a physiological response of closure can be detected within 10 min of treatment. ABA-induced closure results from a loss of K^+ and other anions reducing turgor (see reviews by MacRobbie, 1992; Schroeder, 1992). The mechanism of action of ABA is of compelling interest and not yet fully understood; however, in a simplifying proposal, McAinsh et al. (1990) suggested that ABA induction of guard cell closure is transduced through increases in intracellular cytosolic calcium ($[Ca^{2+}]_i$). The evidence for this proposal comes from several sources. Release of Ca^{2+} inside guard cells by UV photolysis of loaded caged Ca^{2+} or caged inositol trisphosphate (caged IP_3) initiates closure (Gilroy et al., 1990). ABA and Ca^{2+} appear to act synergistically to induce stomatal closure by inhibition of inward-rectifying K^+ channels, activation of outward-rectifying K^+ channels, and activation of voltage-dependent anion channels (Keller et al., 1989; Schroeder and Hagiwara, 1989; Hedrich et al., 1990; Blatt 1992). Finally, increases in guard cell $[Ca^{2+}]_i$ can be detected after ABA treatment; these increases are assumed to result from increased opening of calcium channels (McAinsh et al., 1990, 1992; Schroeder and Hagiwara, 1990; Gilroy et al., 1991; Irving et al., 1992).

The published variations in the size and kinetics of the ABA-induced increases in $[Ca^{2+}]_i$ between individual guard cells are enormous; some guard cells fail to exhibit any detectable increase in $[Ca^{2+}]_i$ at all, even though closure was always observed (Gilroy et al., 1991; Irving et al., 1992; McAinsh et al., 1992). These observations can lead to legitimate questioning of the ABA/$[Ca^{2+}]_i$ hypothesis because the absence of an increase in $[Ca^{2+}]_i$ would fail to set in train the processes described above, which are directly dependent upon increases

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in $[Ca^{2+}]_i$. A relationship between $[Ca^{2+}]_i$ and ABA can still be maintained by proposing that in these cases, ABA changes the sensitivity of guard cells to $[Ca^{2+}]_i$. But sensitivity changes would clearly occur by a different molecular route than the opening of calcium channels. Both MacRobbie (1992) and McAinsh et al. (1992) have contended, however, that these represent simple technical problems in detection of the ABA-induced $[Ca^{2+}]_i$ change; in particular, the use of calcium-sensitive fluorescent dyes, which are only moderately fluorescent, might be responsible. Both authors concluded that there is a single transduction pathway for ABA; this pathway always operates through increases in $[Ca^{2+}]_i$.

We started this research in an attempt to better understand or even identify the sources of variation in the $[Ca^{2+}]_i$ response to ABA. There seemed to us two possible sources of variation that might be responsible. First, variations in water supply to plants or the temperature of growth are known to cause changes in accumulation of ABA and to alter the sensitivity of guard cells to ABA (Trewavas and Jones, 1991). The growth conditions of *Commelina* plants used for guard cell studies are often not reported, and greenhouse-grown plants, which are frequently used, can experience considerable temperature variations. In turn, this can cause uncontrolled water stress. Thus, we decided to investigate the effects of growth temperature on ABA-induced increases in guard cell $[Ca^{2+}]_i$. A second potential source of variation lies in the interaction between the guard cell and surrounding cells. The uptake and accumulation of ABA into cells is not a well-understood phenomenon but is believed to involve both differences in pH between different cell compartments and specific uptake carriers (Astle and Rubery, 1983, 1987; Slovik and Hartung, 1992a, 1992b; Slovik et al., 1992). The site of perception of ABA is not known, and both plasma membrane and intracellular locales have been suggested at different times (Kondo et al., 1980; Hartung, 1983; Hornberg and Weiler, 1984). Because ABA uptake, compartmentation, and indeed perception sites might be temperature dependent, we decided that an investigation involving different growth temperatures required a method of increasing the concentration of cytosolic ABA to a reproducible value. Therefore, we synthesized a new molecule for ABA studies; this molecule is a photoactivatable caged ABA, which was microinjected into guard cells. ABA was then released by UV photolysis. This enabled us to avoid any potential interaction between the guard cell and surrounding cells. Such fine control of the introduction of ABA to the cell as well as control of the growth conditions of the plant led us to conclude that temperature and ABA interact at the level of the $[Ca^{2+}]_i$ response.

RESULTS

Photoactivation of Caged ABA in Guard Cells Initiates Stomatal Closure

Caged ABA was synthesized and introduced into specific guard cells by microinjection. Two derivatives of ABA were synthesized: the 1-(2-nitro)phenylethyl ester (2-NPE-ABA) and the 4-nitrobenzyl ester (4-NBE-ABA). The chemical structures of these two derivatives are shown in Figures 1A and 1B. Only the 2-NPE-ABA contains a nitro group in the ortho position, which is necessary for UV photolability and is thus a genuine caged ABA. 4-NBE-ABA is not photolabile and was used as a control for any nonspecific UV activation of the ABA moiety.

To ensure that the ABA derivatives properly loaded into the cytoplasm, they were coinjected with Calcium Green-1, and the appearance of fluorescence was monitored in the cytoplasm of the guard cell. After an additional 30-min incubation period in which the loaded guard cells continued to open, a 30-sec UV photolysis of 2-NPE-ABA caused a loss of turgor that was measured as a decrease in half aperture that did not affect the uninjected companion guard cell (Figures 1C to 1E). Detectable closure commenced within 2 min of release of ABA inside the cell. We have photolysed 2-NPE-ABA in a total of more than 50 cells and observed closure in every case. The

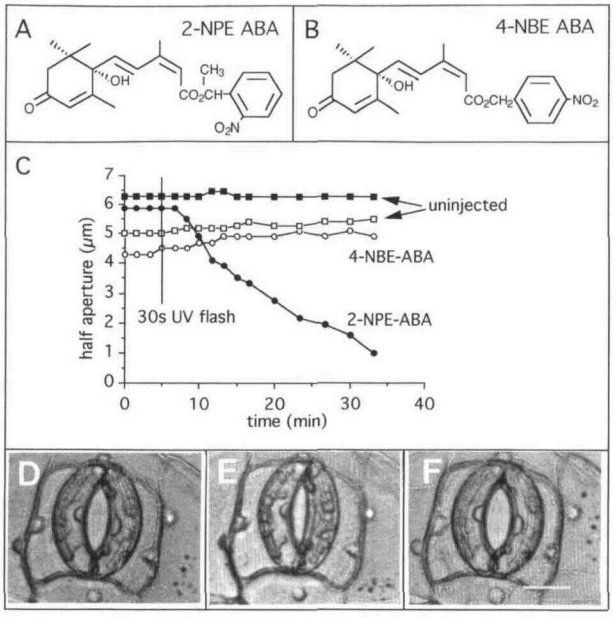

Figure 1. Stomatal Closure Induced by UV Photolysis of Caged ABA in the Guard Cell Cytoplasm.

Single guard cells in stomatal complexes were microinjected with Calcium Green-1 and 2-NPE-ABA or 4-NBE-ABA while being perfused in CO_2-free medium (50 mM KCl, 10 mM 2-[*N*-morpholino]ethanesulfonic acid, pH 6.15) at 25°C.

(A) The structure of 2-NPE-ABA, caged ABA.

(B) The structure of 4-NBE-ABA, a nonphotolyzable analog.

(C) The half-apertures recorded before and after a 30-sec UV exposure of the injected cells, which are represented by open and filled circles. Data are representative of at least four experiments.

(D) to **(F)** Bright-field images of the same stomatal complex in which the right-hand cell was loaded with 2-NPE-ABA 10 min before UV photolysis in **(D)**, 30 min after UV photolysis in **(E)**, and 1 hr after subsequent perfusion with 10 μM fusicoccin in **(F)**.

Bar in **(F)** = 10 μm.

detailed kinetics of closure (measured in four separate experiments) are similar to that shown in Figure 1C; a mean is not presented because of the differing apertures at the beginning of each experiment. Closure is unlikely to be the result of damage due to microinjection; the loss of turgor caused by UV photolysis of 2-NPE-ABA could be recovered by treatment with fusicoccin (10 μM; Figure 1F). Furthermore, temporal separation of loading and photorelease of ABA suggests closure could not be attributed to the microinjection procedure. The guard cell could be incubated after injection of 2-NPE-ABA for up to 1 hr with no deterioration in subsequent closing activity after photolysis. Closure is unlikely to be a result of the toxicity of the caging moiety because this is identical to that used for caged ATP, which has no detectable effect on stomatal closure in *Commelina* (Gilroy et al., 1990). We also injected subsidiary cells with 2-NPE-ABA and observed no effects on the aperture of the adjacent guard cells upon UV photolysis (data not shown). UV exposure of cells loaded with the nonhydrolyzable 4-NBE-ABA failed to induce any closure, and the cells continued to open (Figure 1C).

An external ABA concentration of 1 μM is sufficient to initiate closure in all *Commelina* guard cells (Weyers et al., 1987). We estimated the UV photolysis period necessary to ensure that the guard cell cytoplasm reached a concentration of ~1 μM ABA by two independent means. First, caged fluorescein, which is nonfluorescent until exposed to UV photolysis, was dissolved at 1 μM and sprayed as droplets into oil (as given in Gilroy et al., 1991). Drops of similar size to guard cells were selected and irradiated with increasing periods of UV light, and the released fluorescein was estimated photometrically. It was found that under the optical conditions used in this study, 10% of the caged fluorescein was released by photolysis for every 10 sec of UV light exposure. We then microinjected Calcium Green-1 into guard cells at the same concentration as caged ABA and estimated the total fluorescence after incubation with the calcium ionophore BrA23187 and 1 mM $CaCl_2$. The reproducibility of the microinjection was estimated from the Calcium Green-1 fluorescence of 50 injected guard cells and was found to be 25,500 ± 2,200 counts sec^{-1}. Calcium Green-1 solutions of different concentration were made up in 1 mM $CaCl_2$ and sprayed onto oil. Fluorescence of numerous drops of similar size to guard cells was measured, and a calibration curve of fluorescence versus dye concentration was constructed. A correction was incorporated into this calibration to account for the observation that the cytoplasm in *Commelina* is only 21% of the protoplast (Weyers and Meidner, 1990). From this calibration we were able to estimate the average Calcium Green-1 concentration to be just over 3 μM. A UV photolysis period of 30 sec of loaded, caged ABA at 3 μM would thus release ABA to a final cell concentration of ~1 μM, with the variance noted for the microinjection procedure.

In a second procedure, we microinjected Nitr-5 (caged Ca^{2+}) at the same concentration as caged ABA into guard cells together with Calcium Green-1. After 10-sec UV photolysis, immediate estimates were made of $[Ca^{2+}]_i$, which rose to just over 0.3 μM. Again, a 30-sec UV photolysis of incorporated caged ABA would release ABA to an approximate cell concentration of 1 μM. It should be appreciated that the behavior of these two caged probes may be different and that these concentrations will be approximations only. We have therefore used a 30-sec UV photolysis period for loaded, caged ABA throughout this study.

Photoactivation of Caged ABA inside Guard Cells Can Increase $[Ca^{2+}]_i$

Cytosolic free calcium concentrations, $[Ca^{2+}]_i$, were measured using single-wavelength photometry of Calcium Green-1 loaded by microinjection into individual guard cells simultaneously with the caged probes. Fluorescence signals were averaged over the whole of the loaded guard cell. Dual-wavelength dyes, such as Indo 1 or Fura 2, are easier to use for calcium measurements because the ratio procedure gives a direct readout of $[Ca^{2+}]_i$. However, these dyes require excitation in the UV range that would cause continued release of the caged compounds during the observation period. Also, Calcium Green-1 is the brightest of the calcium-sensitive fluorescent dyes and bleaches more slowly. It is therefore less likely to suffer difficulties with the detection of small increases in $[Ca^{2+}]_i$.

Figure 2 shows representative traces of the effects on $[Ca^{2+}]_i$ after photolysis of 2-NPE-ABA and 4-NBE-ABA in plants grown at 25°C. Controls showing the effect of no treatment or 30-sec UV exposure on Calcium Green-1 alone are also shown (Figures 2A and 2B). Photolysis of 2-NPE-ABA (Figure 2D) but not 4-NBE-ABA (Figure 2C) increased $[Ca^{2+}]_i$ within 30 sec in every guard cell examined (n = 8) and was followed by a decay to resting levels or slightly higher within 5 min. The immediate increase in $[Ca^{2+}]_i$ ranged from 200 to 1000 nM, with most values clustered around a 400 to 500 nM increase over resting levels. Figure 2E shows the effect of photolysis of Nitr-5 (caged Ca^{2+}) in the guard cell in place of 2-NPE-ABA. In all cases (n = 7), $[Ca^{2+}]_i$ eventually increased to over 1000 nM and stomatal closure occurred. The kinetics were different to the 2-NPE-ABA responses, with the increase in $[Ca^{2+}]_i$ continuing for up to 300 sec after the end of the UV pulse. This may be indicative of calcium-induced calcium release. Injection of caged ABA and Calcium Green-1 into subsidiary cells and subsequent UV photolysis produced no detectable increase in $[Ca^{2+}]_i$ in the subsidiary cell (data not shown).

The Magnitude of ABA-Induced $[Ca^{2+}]_i$ Increases Is Dependent on the Previous Growth Temperature of the Plants

To test the effects of different growth temperatures on subsequent ABA-induced increases in $[Ca^{2+}]_i$, *Commelina* plants were grown at day temperatures ranging from 10 to 40°C. Epidermal peels were removed at 25°C, and subsequent perfusion and experimentation continued at this temperature. After loading with Calcium Green-1 and 2-NPE-ABA, the kinetics of $[Ca^{2+}]_i$ changes were recorded using photon-counting photometry. Figure 3 illustrates traces that best represent the

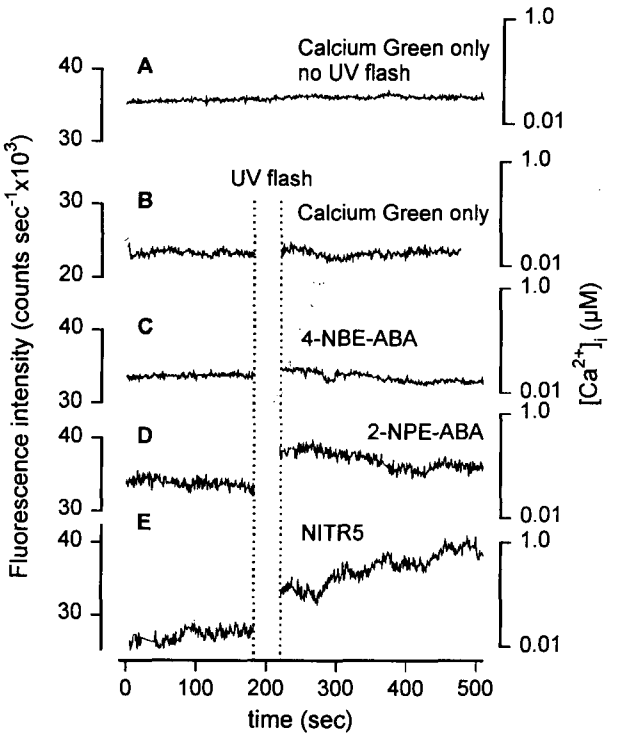

Figure 2. Photometric Measurements of Calcium Green-1 Fluorescence during Flash Photolysis of Caged Probes.

(A) A guard cell injected with Calcium Green-1 alone.
(B) A guard cell injected with Calcium Green-1 alone and exposed to a 30-sec UV pulse.
(C) Coinjection of Calcium Green-1 with 4-NBE-ABA.
(D) Coinjection of Calcium Green-1 with 2-NPE-ABA.
(E) Coinjection of Calcium Green-1 with Nitr-5.
Guard cells in stomatal complexes from plants exposed to 25°C for 48 hr were loaded by iontophoretic microinjection, and Calcium Green-1 fluorescence was monitored before and after a 30-sec UV pulse (360 ± 10 nm) while the cells were perfused at 25°C. The differences in initial fluorescence values are a result of the varying amount of Calcium Green-1 loaded.

average response recorded. We should emphasize that the degree of variation between cells in both the size of the $[Ca^{2+}]_i$ increase and the kinetics was small. The magnitude of the initial $[Ca^{2+}]_i$ transient, induced by photoactivation of 2-NPE-ABA, increased with the growth temperature, reaching more than 900 nM in guard cells grown at a 40°C day. In addition, Table 1 lists half-aperture values and the mean of the initial increases in $[Ca^{2+}]_i$ following the UV photolysis of 2-NPE-ABA for all the cells examined. Half-aperture measurements were taken typically 3 min before and at 5-min intervals after the UV photolysis.

No statistically significant changes in $[Ca^{2+}]_i$ were recorded in cells from plants previously grown at 10°C day/5°C night following UV photolysis of 2-NPE-ABA (Figure 3A), although a decline in half-aperture was still observed (Table 1). In plants grown at 17°C, four of 10 cells showed no detectable change in $[Ca^{2+}]_i$, and the remaining six showed slight increases lasting for ~2 min (Figure 3B). Most significantly, plants grown at 17°C and not watered for 3 days before the experiment showed no significant increases in $[Ca^{2+}]_i$, even though a decrease in half-aperture was still detected (Figure 3C and Table 1).

In contrast, $[Ca^{2+}]_i$ increased transiently in all cells from plants that had been preconditioned at temperatures of 25°C or higher. The kinetics of ABA-dependent $[Ca^{2+}]_i$ increases

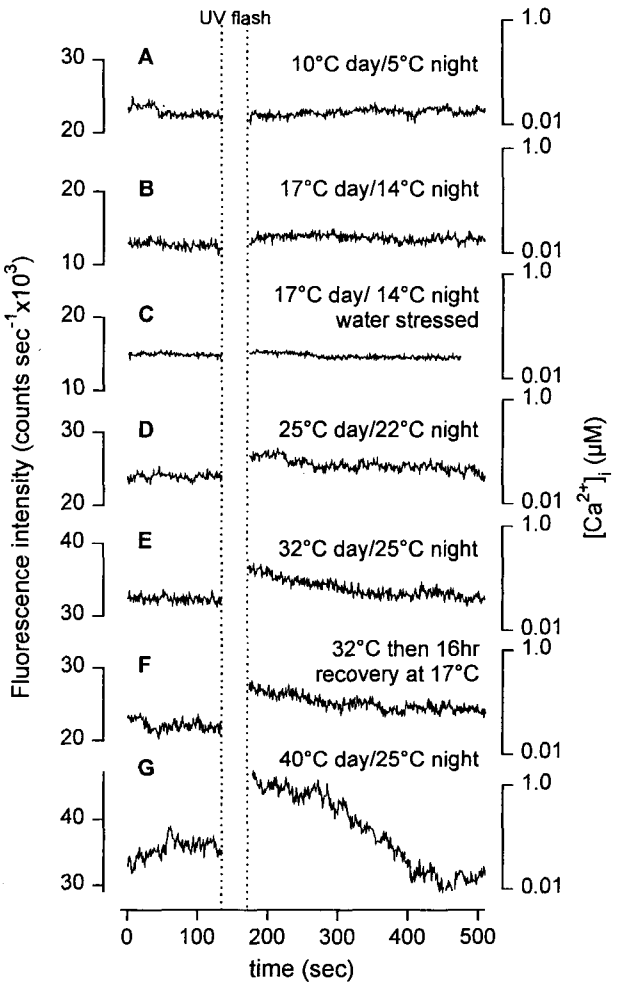

Figure 3. Calcium Transients Induced by Photolysis of Caged ABA in Guard Cells of *Commelina* Plants Grown at 10 to 40°C.

(A) to **(G)** Fluorescence of Calcium Green-1 in guard cells from plants pretreated at the labeled temperatures for 48 hr before the experiment. Individual guard cells in stomatal complexes from plants grown at varying temperatures or under water stress were microinjected with Calcium Green-1 and 2-NPE-ABA, and fluorescence was monitored before and after a 30-sec UV flash (360 ± 10 nm) while the cells were perfused at 25°C. The differences in the initial fluorescence values are a result of the varying amount of Calcium Green-1 loaded. Traces best represent the average for each day/night temperature combination.

Table 1. Temperature Dependence of Calcium Transients Induced by Photolysis of Caged ABA in Guard Cells

Temperature (Day/Night)	Initial Increase in $[Ca^{2+}]_i$ (nM; above Resting Levels)	Closure after 5 Min (μm)	Number of Replicates
10/5°C	15 ± 10	0.4 ± 0.2	5
17/14°C	120 ± 40	0.6 ± 0.2	10
17/14°C (Nonwatered, 3 Days)	10 ± 10	0.3 ± 0.2	6
25/22°C	400 ± 70	0.7 ± 0.2	8
32/25°C (with 16-hr Recovery at 17°C)	525 ± 70	0.6 ± 0.2	4
32/25°C	520 ± 80	0.9 ± 0.3	7
40/25°C	980 ± 90	1.9 ± 0.3	7

Individual guard cells in stomatal complexes from plants grown at varying day/night temperatures or under water stress were microinjected with Calcium Green-1 and 2-NPE-ABA. Fluorescence was monitored using photometry while cells were perfused at 25°C. ABA was released in the cytoplasm by a 30-sec UV pulse, and an immediate $[Ca^{2+}]_i$ increase was measured. The aperture 3 min before and 5 min after the UV pulse was used to determine the degree of closure. Each value represents the mean ± SE for the number of replicates shown.

were very uniform. After UV release of ABA, an immediate increase in $[Ca^{2+}]_i$ was followed by a decay back to resting levels within 2 to 5 min (e.g., Figures 3D to 3G). This pattern of response to release of caged ABA was maintained in plants grown at 32°C even after a 16-hr recovery period at 17°C (Figure 3F and Table 1).

Figure 4 shows that repeated photolysis of 2-NPE-ABA in the guard cell induced reproducible changes in $[Ca^{2+}]_i$ according to the previous growth temperature regime. We have selected two traces (of 47) that exemplify the two classes of guard cell response indicated in Figure 3 and Table 1. In guard cells from plants grown at temperatures of 25°C or above, repeated photolysis induced equivalent repetitive $[Ca^{2+}]_i$ increases that decayed to resting levels within 3 to 5 min (Figure 4B). In these cells, repetitive transients of $[Ca^{2+}]_i$ can be induced for up to three successive ABA releases but decline thereafter. In guard cells from plants grown at 10 or 17°C, repeated photolysis of caged ABA (in some experiments up to five times) failed to increase $[Ca^{2+}]_i$ above the values observed with a single photolysis (e.g., Figure 4A).

Figure 5A shows the effect of photolysis of caged ATP on $[Ca^{2+}]_i$ in a cell from a plant preincubated at a 40°C day/25°C night. This suggests that neither the caging moiety, released by the UV photolysis, nor the UV exposure itself is responsible for the large calcium increases seen when ABA was released in cells from plants exposed to higher temperatures. When cells from plants preincubated at a 40°C day/25°C night were treated with exogenous ABA (1 μM) in the perfusion buffer, $[Ca^{2+}]_i$ increased to over 600 nM (Figure 5B). This is a little lower than that resulting from internal release of ABA from caged ABA.

Rate of Guard Cell Responses to Exogenous ABA in Response to Previous Growth Temperature

The rate of stomatal opening under CO_2-free perfusion at 25°C in 50 mM KCl is similar irrespective of previous growth temperature of the plant (Figures 6A and 6B). Application of external ABA (1 μM) caused stomatal closure. The maximum rate of closure (from a regression fitted to the first 45 min after the addition of ABA) was slightly lower for plants treated at 10°C day/5°C night, but similar for all other temperature pretreatments (Figure 6C). The time taken for 50% closure appeared to decrease with increasing temperature of pretreatment (Figure 6D), although the effect was small. However, stomata from plants exposed to lower temperatures did not fully close during the period of the experiment (Figure 6A). Rates of closure agree well with those seen after release of ABA inside the guard cell (Table 1).

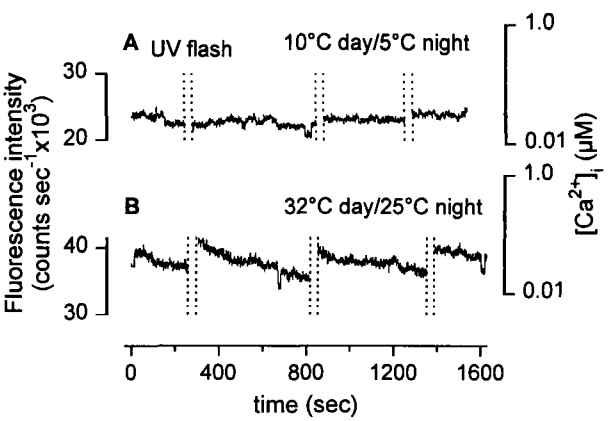

Figure 4. Calcium Transients in Guard Cells Induced by Repeated UV Photolysis of Loaded Caged ABA.

(A) Calcium Green-1 fluorescence of an injected guard cell of a plant preconditioned at 10°C day/5°C night.

(B) Calcium Green-1 fluorescence of an injected guard cell of a plant preconditioned at 32°C day/25°C night.

Individual guard cells in stomatal complexes from plants grown at the indicated temperatures were microinjected with Calcium Green-1 and 2-NPE-ABA, and fluorescence was monitored while cells were perfused at 25°C. ABA was released in the cytoplasm by repeated 30-sec UV (360 ± 10 nm) pulses (dotted lines).

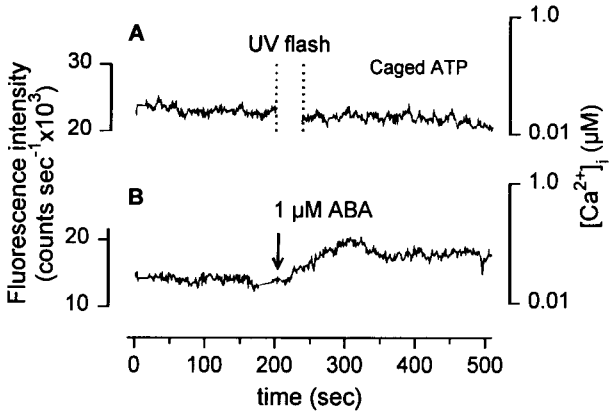

Figure 5. Effect of Caged ATP Photolysis and External ABA on Calcium in Guard Cells of *Commelina* Plants Grown at 40°C Day/25°C Night.

(A) UV photolysis of caged ATP in a guard cell of a plant preconditioned at 40°C day/25°C night.

(B) Calcium Green-1 fluorescence of an injected guard cell of a plant preconditioned at 40°C day/25°C night and exposed to exogenous ABA (1 μM).

Individual guard cells in stomatal complexes from plants grown at a 40°C day/25°C night were microinjected with Calcium Green-1 (and coinjected with caged ATP in **[A]**), and fluorescence was monitored while cells were perfused at 25°C. Caged ATP was released in the cytoplasm by a 30-sec UV (360 ± 10 nm) pulse, or exogenous ABA was added to the perfusion buffer.

DISCUSSION

Is ABA Perceived Internally in Guard Cells?

The release of ABA inside guard cells initiated closure (Figure 1). Because it is difficult to obtain a sufficient number of cells that can survive microinjection, we have not attempted a dose–response relationship that might clarify a similarity to externally added ABA. A notable feature of our results was that repeatedly subjecting caged ABA in guard cells of plants grown at 25°C to photolysis induced repeated Ca^{2+} transients (Figure 4). The implication is that cytosolic ABA either is rapidly conjugated or is removed to intracellular or extracellular compartments. Because the ABA-induced $[Ca^{2+}]_i$ transients only last 2 to 5 min, this provides a possible upper limit to a transit time of free ABA in the cytosol. Evidence for a vacuolar transport system for protonated ABA exists in *Commelina*, and guard cells can accumulate ABA up to a concentration of 6 mM (Baier et al., 1990; Brinckmann et al., 1990). Therefore, release of ABA inside the cell may lead to its rapid removal to the vacuole and thus effective inactivation. However, based on the complexity of signal transduction phenomena in guard cells, other types of multiple processes may be involved such as desensitization to ABA and regulation of various calcium transporters in the cell.

Our data do suggest that there is intracellular perception of ABA without prejudice to other possibilities. We cannot exclude the possibility that leakage of small amounts of ABA from the cell occurs, which could then occupy an outer facing plasma membrane receptor. However, our epidermal strips were continuously perfused, and the release of ABA inside one guard cell or in an adjacent subsidiary cell did not affect the turgor of the neighboring guard cell partner. Hornberg and Weiler (1984) used affinity labeling to demonstrate the presence of a putative plasma membrane receptor/binding protein, but this research still lacks confirmation. Hartung (1983) showed ABA initiation of guard cell closure in *Commelina* at pH 8 where penetration rates of guard cells by ABA are extremely low. However, Kondo et al. (1980) reported that ABA exerted little influence on the aperture of stomates in leaves from other species at pH 7 or above. Only at lower pH values where ABA more readily penetrates the guard cell membrane was closure observed.

The numbers of ABA receptors and their cellular locale are at present an open question. But if there are receptors both inside the cell and on the plasma membrane, they may regulate different cellular processes. This is clearly an intriguing

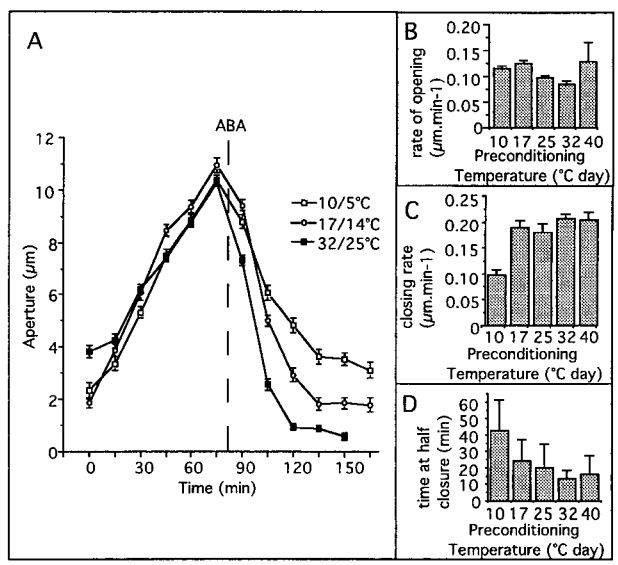

Figure 6. Effect of Different Growth Temperature on ABA-Induced Closure of Stomatal Guard Cells.

(A) Stomatal apertures on epidermal peels from plants of *Commelina* exposed to temperatures varying from (day/night) 10°C/5°C (□), 17°C/14°C (○), and 32°C/25°C (■) were opened in 50 mM KCl, high-light intensity (200 μE m^{-2} sec^{-1}), and low CO_2, and then ABA (1 μM) was added at the point indicated. The mean ± SE of three replicate experiments with 30 individual cells was measured in each experiment. The aperture values for stomata from 25°C/22°C and 40°C/25°C plants fell between the lines shown and have been omitted for clarity.

(B) Maximum rate of opening (mean ± SE).

(C) Maximum rate of ABA-induced closure (mean ± SE).

(D) Half-time for 50% closure (P value at 95% confidence).

area of study; the presence of multiple receptors might parallel observations on auxin where the auxin binding protein can be found both inside cells and positioned on the extracellular face of the plasma membrane (Barbier-Brygoo et al., 1989; Jones and Herman, 1993).

Two Transduction Routes for ABA in *Commelina* Guard Cells

The data published in this study support the hypothesis that ABA can be transduced in guard cells through increases in $[Ca^{2+}]_i$. However, changes in $[Ca^{2+}]_i$ are clearly not the only route whereby ABA induces closure. Because most guard cells in plants grown at day temperatures of 17°C or lower showed no detectable increase in $[Ca^{2+}]_i$ when challenged with ABA (Table 1) and whereas stomatal closure occurs rapidly with only slight alteration in rate (Figure 6), we feel there must be an independent route to closure that does not require increases in $[Ca^{2+}]_i$. There is the possibility that small undetected local changes in $[Ca^{2+}]_i$ are responsible for closure, yet artificial elevation of $[Ca^{2+}]_i$ to values less than 500 nM does not induce closure (Gilroy et al., 1990). With increasing growth temperatures, our data suggest that the guard cells respond to ABA with progressively larger increases in $[Ca^{2+}]_i$ (Figure 3). The ABA-induced $[Ca^{2+}]_i$ transients described in this study show remarkably little variation between different cells both in size and kinetics. Much of the variation observed in the past may have resulted from the use of plants in which growth conditions were not closely controlled.

We have previously released Ca^{2+} from caged Ca^{2+} or induced internal Ca^{2+} mobilization by releasing IP_3 from caged IP_3 in numerous *Commelina* guard cells (Gilroy et al., 1990). The experimental plants used at that time were grown in a greenhouse, which was usually maintained at temperatures between 15 to 20°C. These experiments involved observations of more than 50 cells. We did not observe variation in closure induced by artificial elevation of $[Ca^{2+}]_i$, providing the $[Ca^{2+}]_i$ exceeded a concentration of 500 to 600 nM. Below that $[Ca^{2+}]_i$ concentration, guard cells showed no aperture change (in figure 1 of Gilroy et al., 1990, an example was included in which elevation to 300 nM had no effect on aperture). Our observations suggest that a $[Ca^{2+}]_i$ response system is functional in guard cells of plants grown at relatively low temperatures but also challenges the significance of the small changes in $[Ca^{2+}]_i$ in some of the guard cells described here and by others (McAinsh et al., 1992). Only when plants were grown at temperatures of 32°C or above were routine increases in guard cell $[Ca^{2+}]_i$ of above 500 nM observed (Figure 3).

We can only speculate at the identity of a calcium-independent signal transduction route leading to guard cell closure. Blatt and Armstrong (1993) have indicated that changes in cytoplasmic pH might affect closure, but crucial experiments using UV photolysis of loaded caged protons have not yet been conducted. However, the outward-rectifying K^+ channel and the plasma membrane H^+-ATPase are both involved in closure and are affected by $[H^+]_i$ (Blatt, 1990, 1992; Blatt et al., 1990). It is known that an extracellular pH of 5.5 (compared to pH 6.8) can make *Commelina* guard cells much more sensitive to low concentrations of ABA (Paterson et al., 1988). Kondo et al. (1980) only observed effects of ABA at a pH of 7 or below, but in species other than *Commelina.* However, the relationship between extracellular pH and intracellular pH has not been clarified. Lowering the extracellular pH will increase the partitioning of ABA into the guard cell. On the other hand, acidification or alkalinization of external pH can be expected to alter the charge distribution on the extracellular face of every plasma membrane protein with unpredictable consequences. Hard conclusions are therefore difficult to reach without further measurements on the effects of ABA on intracellular pH measurements. These are currently underway in our laboratory.

Evidence for several transduction routes in plants in response to signals is beginning to emerge in the scientific literature. Raz and Fluhr (1992) showed that signal-regulated pathogenesis resistance could be induced either by an ethylene/calcium–dependent route or by an ethylene/calcium–independent route. Neuhaus et al. (1993) showed that phytochrome activated two independent pathways. One involved $[Ca^{2+}]_i$/calmodulin and led, for example, to the induction of chlorophyll binding protein gene expression. The other route, which caused the induction of anthocyanin biosynthesis, was independent of $[Ca^{2+}]_i$. Similar structures of control are appearing with respect to ABA regulation itself. Use of ABA-deficient or ABA-insensitive mutants has demonstrated that the expression of certain specific genes and proline accumulation in either cold acclimation or water stress can be induced both by ABA-dependent and ABA-independent routes (Stewart and Voetberg, 1987; Heino et al., 1990; Nordin et al., 1991; Butler and Cuming, 1993). We believe these control structures should provide flexibility in signal transduction mechanisms and patterns of response in plants that live in a variable environment (Trewavas and Jones, 1991).

We suggest that the effective flux through two ABA-induced transduction routes depends on the previous growth temperature. If this is the case, the physiological event of guard cell closure should depend on several, possibly overlapping, molecular processes that can be independently activated. $[Ca^{2+}]_i$ may not be the trigger for stomatal closure but rather an intermediate of one of two possible transduction routes. In using these two routes, the plant has found a mechanism for integrating information about both water status and temperature. If growth temperatures are high and water initially abundant, the plant will poise its guard cells to close more quickly when water becomes depleted. Greater release or slower uptake of $[Ca^{2+}]_i$ in response to ABA may be the mechanism by which closure rate is increased. This is a major advantage to survival in warmer areas. Such a refined interaction between just two factors—temperature and water status—is surely indicative of the complexity involved in signaling within the guard cell that interprets many environmental factors and acts upon them.

METHODS

Fluorescent Dyes, Caged Probes, and Chemicals

Calcium Green-1 was obtained from Molecular Probes (Eugene, OR) and made up to a 1-mM stock in HPLC grade water (BDH, Poole, Dorset, UK) at pH 7.0 with KOH. Racemic, caged ABA (2-NPE-ABA; 1-[2-nitro]phenylethyl ester of abscisic acid) and the nonhydrolyzable analog 4-NBE-ABA (4-nitrobenzyl ester of ABA) were synthesized by first generating a mixed anhydride from a reaction between commercial ABA (Lancaster Synthesis, Morecambe, Lancashire, UK) and isobutyl chloroformate and triethylamine (J.L. Ward and M.H. Beale, unpublished data). Reaction of this mixed anhydride with the caging groups 1-(2-nitro)phenylethanol or 4-nitrobenzyl alcohol was rapid in the presence of 4-dimethylaminopyridine. The caging group was designed around the photocleavable 1-(2-nitro)phenylethyl group, commonly used to cage phosphates (Walker, 1991), because these esters can be readily hydrolyzed as a result of the participation of the ortho nitro group in the ester bond cleavage. The components were purified using flash chromatography, and synthesis was characterized by ^{1}H-nuclear magnetic resonance. Caged ABA was made up as a 10 mM stock in methanol, vacuum dried, and frozen until use. Nitr-5 was obtained from CalBiochem (Nottingham, UK) and caged ATP and caged fluorescein from Molecular Probes. All care was taken to avoid exposing cage solutions to the light. Unless otherwise stated, all other chemicals were analytical grade and supplied by Sigma.

Plant Material

Plants of *Commelina communis* were grown from seed in Levingtons' M3 potting compost (Fisons, Ipswich, Suffolk, UK) at 17°C (day) and 14°C (night) with a 16-hr daylength (6 AM to 10 PM) at lighting levels of between 90 to 150 $\mu E\ m^{-2}\ sec^{-1}$ photosynthetically active radiation with mercury halide and tungsten filament lamps. All plants were regularly watered and maintained in trays with 20 mm of water to ensure that the compost remained damp. Plants to be water stressed were transferred to trays without water for a period of 3 days. To investigate effects on temperature preconditioning, plants were exposed to varying temperature regimes for 48 hr before use. Experiments were performed between 10 AM and 4 PM to minimize the effects of diurnal rhythms. The first fully expanded leaf on the main axis of 4- to 5-week-old plants was excised, peeled, and then floated on perfusion buffer (50 mM KCl, 10 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.15, with KOH), which encourages stomatal opening.

Microscope Sample Chamber and Perfusion System for Epidermal Peels

Epidermal strips were mounted, cuticle downwards, on a No. 1.5 coverslip (22 × 64 mm; Chance-Propper Ltd., Warley, UK) surrounded by a thin smear of paraffin grease ("soft white"; FSA Laboratory Supplies, Loughborough, UK) and secured with four coverslips around the edge, thus creating a small (5 × 10 mm) open chamber over the center of the peel (according to McAinsh et al., 1992). Perfusion buffer was passed continuously over this chamber (volume less than 100 μM) at a rate of 5 mL min^{-1} by gravity feed. The medium was supplied from a reservoir where it was aerated with CO_2-free air and maintained at 25°C in a temperature-controlled water bath. The medium was removed via an outlet tube attached to a vacuum pump.

Microinjection of Stomatal Guard Cells

Microelectrodes with tip diameters of ~0.3 μm were pulled from GC120 electrode glass (Clark Electromedical Instruments, Reading, UK) and back-filled with a small volume of 100 μM Calcium Green-1 with or without 200 μM caged ABA. The remainder of the electrode barrel was filled with 3 M KCl, and guard cells of stomata with apertures between 8 and 12 μm were iontophoretically microinjected until fluorescence within the cytoplasm could be seen (~10 sec). The electrode was removed after microinjection. The injected cells were allowed to recover for at least 30 min in the perfusion system. Criteria for the rejection of damaged cells (see Gilroy et al., 1991) and for the cytoplasmic location of the fluorescent dye were rigorously followed.

Measurement of Cytosolic Calcium Using Photometry of Calcium Green-1

The photometric system was based on a Nikon-Diaphot inverted microscope (Nikon UK Ltd., Shropshire, UK). The excitation wavelength was determined by interference filters (490 ± 10 nm or 360 ± 10 nm; Ealing Electro-Optics, Watford, UK) mounted in a motor-driven excitation filter wheel (Newcastle Photonic Systems, Newcastle-upon-Tyne, UK) in front of the Nikon 75-W Xenon epifluorescence lamp. A Nikon CF Fluor DL ×40, 160-mm, 0.85 numerical aperture, 0.37-mm working distance dry objective was used. Fluorescence from the dye was monitored during injection with a 510-nm dichroic mirror and 520-nm long-pass filter. A 400-nm dichroic mirror was used during experiments to maximize the amount of 360-nm UV light reaching the cell for photorelease of the cage and to reduce the level of photobleaching during excitation at 490 nm. Fluorescent light from the specimen passed to the microscope side port via a 525 ± 45-nm interference filter (Ealing Electro-Optics). Signals from the injected guard cell were selected using a Nikon variable rectangular aperture and passed to a photomultiplier (9924a; Thorn-EMI, Middlesex, UK) via a relay lens and Nikon PFX shutter system. The photomultiplier signal was passed to a photon counting box (Newcastle Photonic Systems) and analyzed on line with an Elonex 386 IBM-compatible computer (Elonex plc., Apsley Way, London, UK) using software supplied from Newcastle Photonic Systems. The autofluoresence signal from a noninjected stoma was recorded at the start of each experiment and subtracted from each time point. Calibration using the calcium ionophore BrA23187 (10 μM; CalBiochem, Nottingham, UK) and 2 mM Mn^{2+} was performed at the end of the experiment as described previously (Gilroy et al., 1990).

Measurement of Stomatal Apertures

Stomatal apertures were measured from epidermal peels grown under varying temperatures in the perfusion system at a constant 25°C at 15-min intervals during opening to ~12 μm and after application of 1 μM ABA using an adjustable Nikon Filar micrometer ×10 eyepiece with an accuracy of ±0.2 μm. In experiments using caged probes, half-apertures were measured normal to a line drawn between the junction of the injected and noninjected guard cells to the pore lip. The side port was closed during these measurements to prevent damage to the photomultiplier.

Photolysis System

Caged probes were photoactivated by exposure to 360-nm UV light from the 75-W epifluorescence illuminator via the 360 ± 10 nm interference filter for periods of 30 sec under computer control.

ACKNOWLEDGMENTS

We would like to thank Martin McAinsh and Tony Collins for their helpful discussions. This work was supported financially by grants from the Science and Engineering Research Council, the Agriculture and Forestry Research Council, and the Royal Society.

Received April 1, 1994; accepted July 1, 1994.

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