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The Mechanism of Calcium Action on Flower Induction in *Pharbitis nil*

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Summary

Calcium ions, calcium ionophores A23187 and ionomycin, as well as caffeine, stimulated the flowering of *Pharbitis nil* when applied just before and 2 h after starting a subinductive, 14-h long dark period. Effectiveness of all the mentioned compounds decreased over successive hours and when used from 6 h after the onset of the dark period they had no effect or inhibited flowering. Intracellular calcium modulators, ryanodine and thapsigargicin, which are active in animal cells had no effect on flowering. These results suggest that an increase in free Ca^{2+} before and during the first 2 h of the dark period increases the flowering responses, whereas after the 6th hour it decreases it. We postulate that the targets for calcium action are stomata, which are open before the dark period and remain closed during the first 4–5 h of the dark period. The significance of stomata in flower induction was confirmed in experiments with abscisic acid (ABA), a plant hormone which regulates stomatal movements. Treating the cotyledons of plants with ABA at the 8th and 10th hour of the dark period, resulted in plants with about 50 % less flower buds than the control, however, this phytohormone had no effect on flowering when used before the subinductive dark period.

Key words: Calcium, calcium ionophores, caffeine, flowering, Pharbitis nil, stomata.

Introduction

Friedman et al. (1989) have shown that calcium is involved in photoperiodic flower induction in *Pharbitis nil.* They found that application of the calcium ionophore A23187 reduced the length of the critical dark period. However, the calcium channel blocker, La^{3+}, as well as calmodulin inhibitors, chlorpromazine and W7, reduced the level of flowering (Friedman et al., 1989). Subsequently, Tretyn et al. (1990) published data showing that spraying of *P. nil* seedlings with La^{3+} and chlorpromazine only slightly inhibits this process, and is photoperiodically independent. Furthermore, both the calcium channel antagonist, nifedipine (Tretyn et al., 1990; Vince-Prue, 1994) and agonist, Bay K 8644 (Tretyn et al., 1990) had no significant effect on the flowering response in *P. nil.* However, Friedman et al. (1989), Tretyn et al. (1990) and Takeno (1993) have provided evidence that the calcium chelator, EGTA, applied before an inductive 16-h dark period, inhibits flowering.

Tretyn et al. (1990) pointed out that application of different compounds *via* spraying is not very effective. Furthermore plants with thicker cuticles respond to a lesser degree than seedlings with a thinner cuticle. Recently, Takeno (1993) showed that perfusion of *P. nil* seedlings with aqueous solutions of EGTA, La^{3+} and chlorpromazine inhibited flowering when applied before the inductive dark period.

However, they had no effect when used immediately after the 16-h dark period (Takeno, 1993). Although the perfusion method introduced by Takeno (1993) was very effective, control plants treated with a 16-h inductive dark period produced at least 50 % less flower buds per plant than those not perfused. Furthermore, removal of the root system led to an increase in stem length. In this paper we have examined the effect of intracellular calcium modulators on flowering in *P. nil* treated with a subinductive, 14-h, long dark period. We have developed a very effective technique of applying compounds on the adaxial surface of *P. nil* cotyledons using a soft paint brush.

Material and Methods

Plant material

Seeds of *P. nil* Choisy cv. «Violet» (Marutane Seed Co., Kyoto, Japan) were soaked in concentrated sulphuric acid for 40 min and washed overnight in running tap water. They were then sown in wet sterile vermiculite in plastic pots and grown for 5 days under continuous light (18.7 Wm^{-2}; cool-white fluorescent) at 25 °C.

Plant treatment

Aqueous solutions of all tested compounds were applied on the adaxial surface of the cotyledons using a soft paint-brush as indicated in each experiment. We have found that this method was more effective than spraying seedlings and does not decrease the number of flower buds produced per seedling (M. Cymerski, unpublished data). Distilled water was used for control plants.

Calcium chloride (Sigma, St. Louis, MO, USA), caffeine (Sigma) and ryanodine (Calbiochem, San Diego, Ca, USA) were prepared as aqueous solutions. Both calcium ionophores: A23187 and ionomycin, as well as thapsigargicin (all Calbiochem), were dissolved in dimethylsulfoxide (DMSO) as a stock solution and kept in darkness in a refrigerator. All solutions of used compounds were adjusted to pH 7.0 with KOH. A solution of abscisic acid (ABA, Sigma) was prepared in 10 mM 2-[N-morpholino]ethanesulfonic acid (Mes) buffer pH 6.15 containing 50 mM KCl (Trejo et al., 1993).

All treatments were given to 5-day-old seedlings at 24 °C. After the treatment, plants were returned to continuous light and grown at 25 °C. The number of flower buds per plant was counted 21 days after induction and the per cent of flowering plants (with 4 or more flower buds per plant), as well as per cent of plants with a terminal flower bud calculated. Ten plants were used for each treatment and each experiment was repeated at least three times. Data are presented as means ± standard error (SE).

Light microscopy and counting of stomata

Both an inverted (Olympus IMT-2) and a fluorescence light microscope (Olympus BH2) were used for observation of the cotyledon surface. When counting stomata either the abaxial or the adaxial surface of the cotyledon were covered with a thin layer of transparent nail-varnish. The varnish was immediately dried with a stream of cool air produced by a hair dryer. The dry varnish replica was removed from the surface, and afterwards observed and photographed using an Olympus IMT-2 light microscope fitted with an automatic exposure photomicrographic system (Olympus, model PM-10AK). Three randomly selected fields per replica were photographed. Each experiment was repeated three times. All stomata were counted after projection of negatives on a screen and the data presented as percentage of open stomata visible in one microscope field (magnification of the objective was 20×).

Secretion of an unknown substance on the adaxial surface of *Pharbitis* cotyledons was studied using an Olympus BH2 fluorescence microscope using both ultra-violet (wide band) and a violet (narrow band, 405 nm) excitation filters.

Scanning electron microscopy

For observation in the scanning electron microscope (SEM) the cotyledons were cut with a razor into 4 × 4 mm pieces. The samples were fixed for 4 h in 1 % (v/v) glutaraldehyde and 0.1 % (w/v) tannic acid in 0.1 M phosphate buffer pH 7.3 at 4 °C. The plant material was then treated for 1 h with 2 % (w/v) tannic acid in the buffer at 35 °C. Samples were collected and prefixed either under white light or dim green safelight depending on the experiment. After washing in phosphate buffer, tissues were post-fixed in 1 % (w/v) OsO_4 (12 h, 4 °C), dehydrated in ethanol and propanol and freeze dried in an Hitachi ES 2020 freeze dryer. Samples were subsequently coated with platinum using an Hitachi E 102 ion sputter and observed in an Hitachi S-2350 scanning electron microscope.

Results

After a 14-h suboptimal dark treatment ca. 70 % of *P. nil* plants flowered and ca. 50 % of them produced a terminal flower (data not shown). Control plants usually had four flower buds per plant.

Application of 1 mM $CaCl_2$ on the adaxial surface of the cotyledons just before the inductive dark period significantly increased both the percentage of flowering plants and the number of plants with a terminal flower (data not shown). Seedlings treated with Ca^{2+} before the dark period had about 50 % more flower buds than the controls (Fig. 1 A). Effectiveness of Ca^{2+} in stimulating flower induction decreased over successive hours of the dark period and at the 10th hour, plants treated with distilled water (control) and 1 mM $CaCl_2$ produced a similar number of flower buds.

The calcium ionophore A23187 (10 µM) stimulated the flowering response when applied before or during the first 6 h of a suboptimal dark period (Fig. 1 B). The ionophore A23187 was less effective when applied both at the 6th and 8th hour of the dark period. Seedlings treated with A23187 at the 10th hour had fewer flower buds (Fig. 1 B) including terminal ones and flowered to a lesser extent than the controls (data not shown).

Ionomycin (5 µM) was only active in the induction of the flowering response when used just before and during the first 4 h of the suboptimal dark period (Fig. 1 C); plants treated later had a similar or lower number of flower buds than the control (Fig. 1 C, and data not shown).

In the next experiments caffeine and ryanodine, compounds which in many types of animal cells release Ca^{2+} from internal stores were used. Caffeine (1 mM) was extremely active in flower induction (Fig. 1 D). Plants treated before the suboptimal dark period with 1 mM caffeine had almost 100 % flowering and most of them had terminal flowers (data not shown). These plants had the same number of flower buds as those after a 16-h-long optimal dark period (data not shown) and about 50 % more than plants kept in

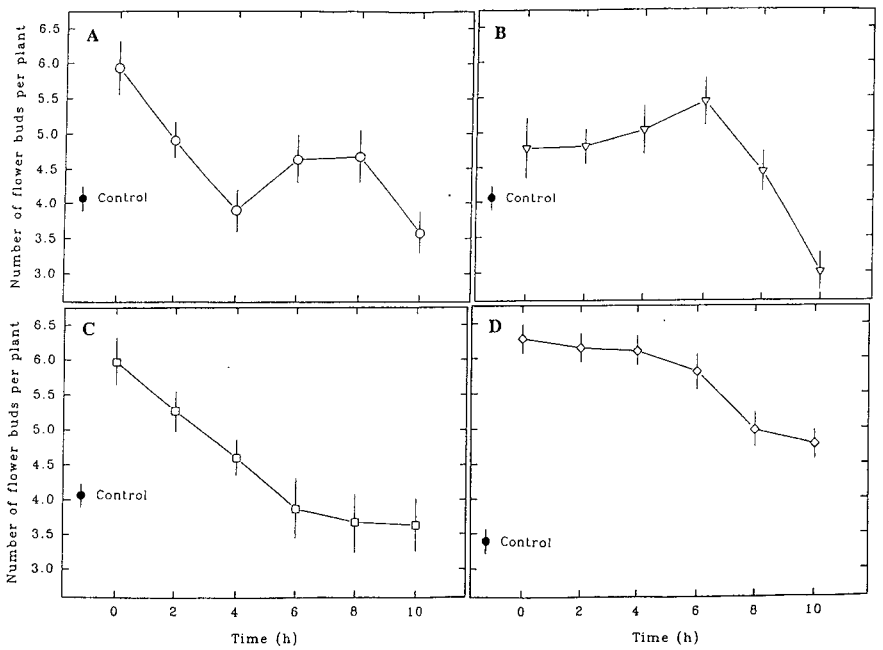

Fig. 1: The effect of calcium ions (1 mM) (A) and calcium ionophores A23187 (10 μM) (B) ionomycin (5 μM) (C) and caffeine (1 mM) (D) on flowering (number of flower buds ± SE) in *Pharbitis nil*. All substances were applied on the adaxial surface of cotyledons before or during the first 10 h of a subinductive, 14-h dark period.

darkness for 14 h (Fig. 1 D). Caffeine was active before and during the first 6 h of the subinductive dark period after which its activity decreased (Fig. 2). Plants treated with caffeine after a 14-h, long dark period had the same number of flower buds as the controls (data not shown).

Ryanodine (50 μM), which has the same proposed mechanism of action as caffeine had no effect on the flower response (data not shown). Moreover, thapsigargicin (1 and 10 μM), an inhibitor of animal calcium pumps, only slightly stimulated flowering when applied to the cotyledons at the 2nd hour of a suboptimal dark period (data not shown).

Because diffusion of exogenous Ca^{2+} and calcium ionophores into plant tissues is very slow and restricted to short distances we speculated that the epidermis is the target for their action. During light microscopic analysis we observed that stomata are abundant on both the abaxial and the adaxial sides of *P. nil* cotyledons (Fig. 2 A–B). They can occupy ca. 15–20 % of total cotyledon surface area. There are about 20 % less stomata on the abaxial than on the adaxial surface (compare Fig. 2 A with Fig. 2 B). The shape of epidermal cells of both sides of the cotyledons are different (compare Fig. 2 A with Fig. 2 B). However, stomata present on both sides of the cotyledon are very similar in size and shape (Fig. 2 C–D).

It is well known that exogenously applied calcium and calcium ionophores inhibit stomatal opening and promote stomatal closure (Kearns and Assmann, 1993). Therefore, we have tested the effect of Ca^{2+} and A23187 on this process in *P. nil*. Using a light microscope we have studied the number of open stomata on the adaxial cotyledon surface: just before and during an inductive, 16 h dark period, as well as 30 min after its completion (see Materials and Methods).

At the beginning of an inductive dark period about 60–70 stomata were counted in one microscopic field. However, at the end of this period there were only ca. 45–55 stomata present in the same area as a consequence of lateral expansion (all observations were at magnification 20×). Enlargement of epidermal cells parallels the increase of cotyledon area.

About 95 % of the observed stomata of light-grown seedlings were open (Fig. 3). However, 2 h after starting the dark period only 21 % of them remained open. At the 4th hour of darkness only a limited number of open stomata (ca. 7 %) were observed (Fig. 3). From the 6th, throughout 8th, until 10th hour of the studied period the number of open stomata increased from 25 to 51 % up to 60 % (Fig. 3), respectively. During the next 4 h a steady decrease of the percentage of open stomata was observed (Fig. 3). At the end of the inductive dark period 33 % of the stomata were open and this increased to 69 % after 30 min white light (Fig. 3).

Abscisic acid is a factor regulating stomatal movements (Kearns and Assmann, 1993). Therefore in our further experiments we have tested the effect of this hormone on flower induction. Plants treated with ABA (Fig. 4) just before and at the 2nd hour of the dark period had the same number of flower buds as the controls. Application of the hormone from the 4th hour onwards inhibited the flowering response. Plants treated with ABA at the 8th and 10th hour of the dark period had about 50 % less flower buds than the controls (Fig. 4).

Calcium chloride, A23187, ABA and red light (660 nm, 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) used at the 8th hour of the dark period stimulated stomatal closure. Stomata treated in this way stay closed until the end of a subinductive and an inductive dark period, the remaining 6 and 8 h, respectively. The red light effect on stomata was cancelled out by a 10 min far-red light pulse (764 nm, 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$). In this case the number of open stomata was the same or even higher then in controls (data not shown).

The significance of the stomata in the flowering response could be associated with the extrusion of an unknown material produced inside the cotyledons. Figures 5 A–B shows

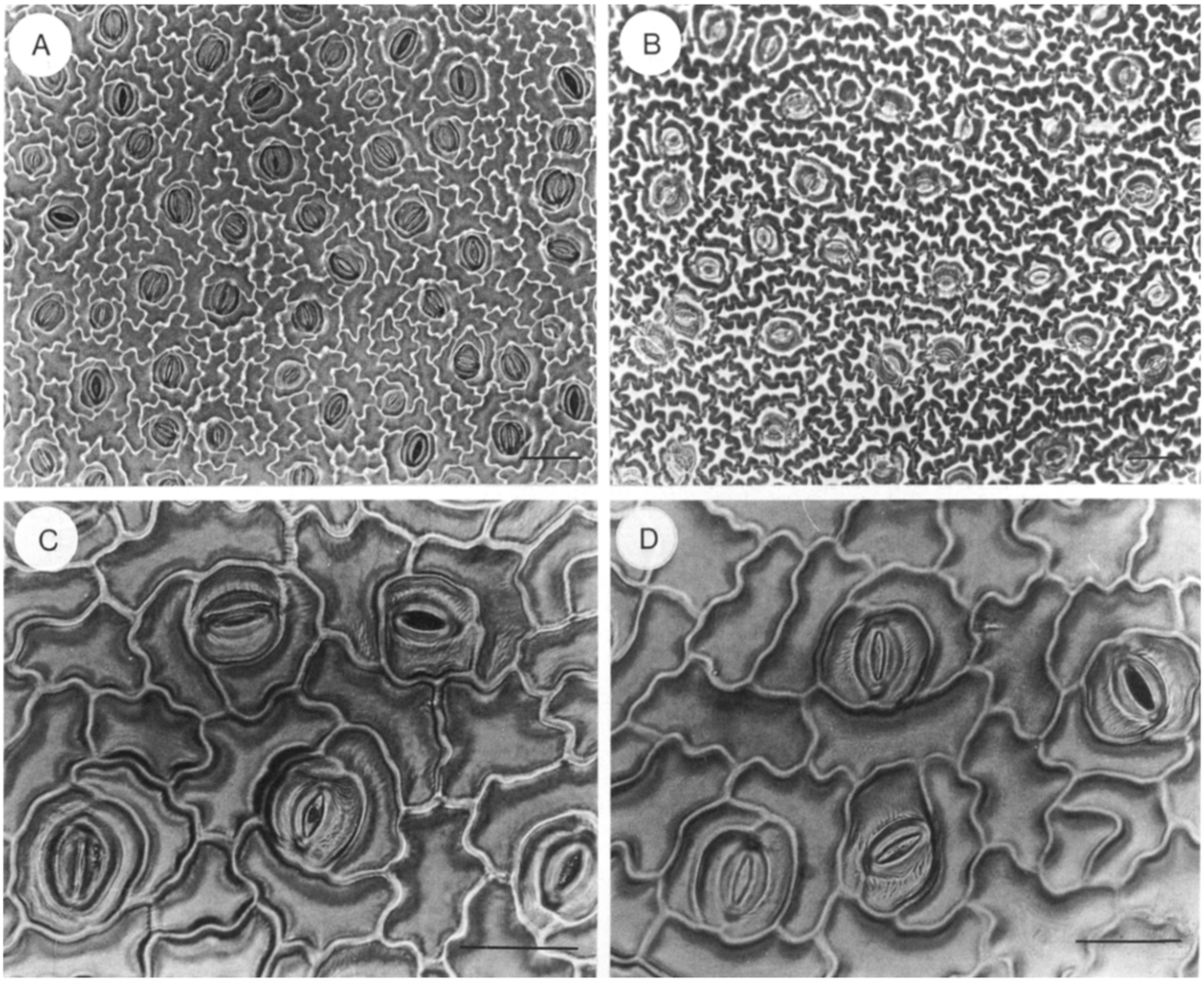

Fig. 2: Light microscope pictures showing the appearance of the surface (A–B) and stomata (C–D) on the abaxial (A, C) and adaxial (B, D) side of *Pharbitis nil* cotyledons just before a subinductive dark period. (Bar = 25 µM).

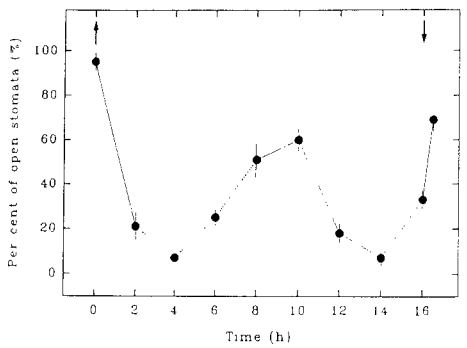

Fig. 3: Percentage of open stomata before, during and 30 min after an inductive dark period. The nail-varnish technique (see Materials and Methods) was used for visualizing the appearance of stomata. Arrows show when light was turned off (↑) and on (↓).

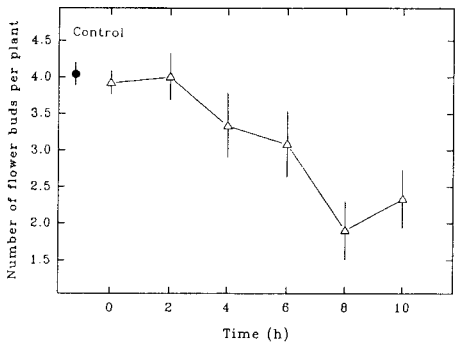

Fig. 4: The effect of abscisic acid (10 µM ABA + 50 mM KCl) on flowering (number of flower buds ± SE) in *Pharbitis nil.* The ABA was applied with a soft paint-brush to the adaxial surface of cotyledons before or during the first 10 h of a subinductive, 14-h dark period.

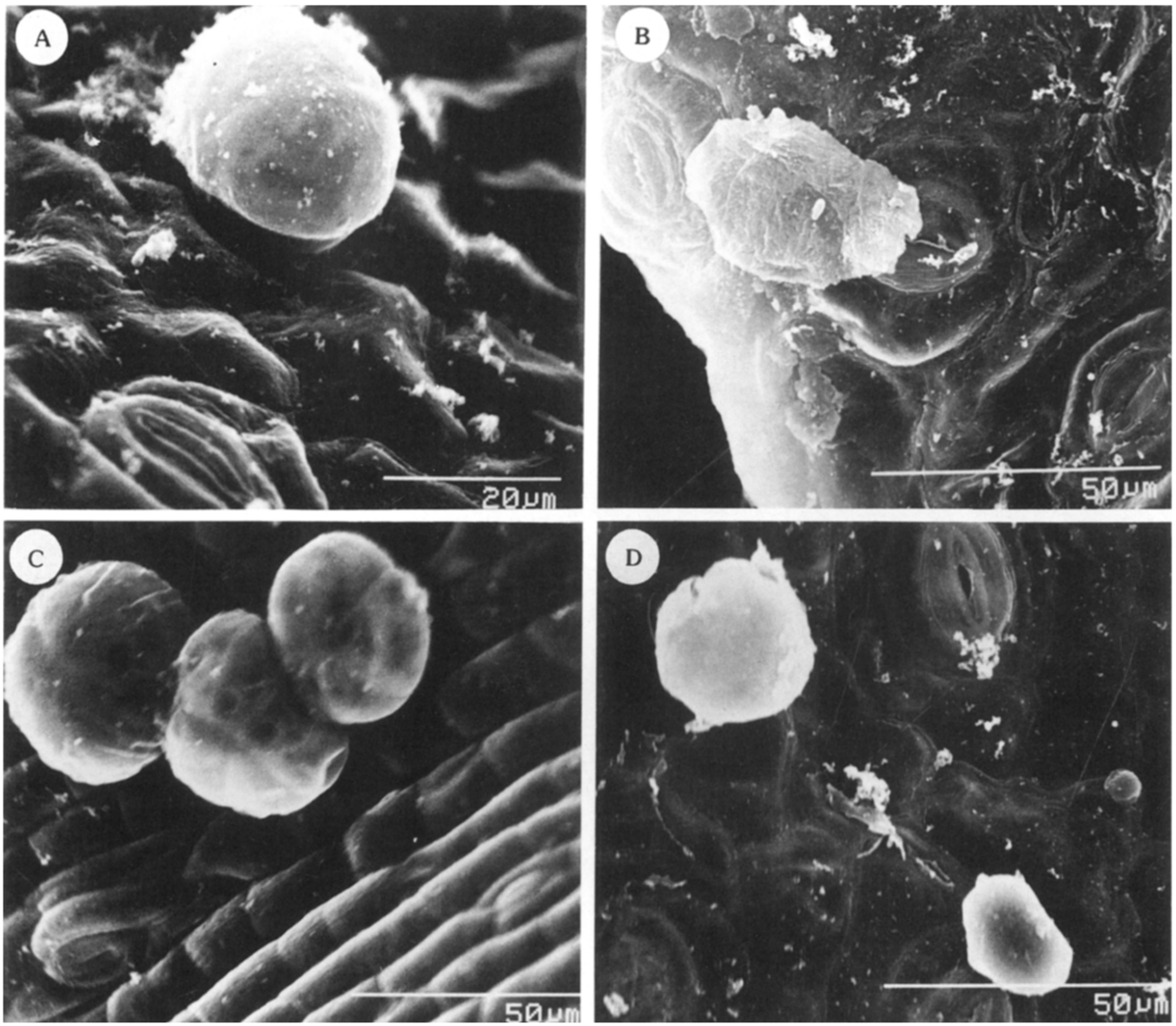

Fig. 5: Scanning electron microscope pictures showing apparent extrusion of unknown material from within the cotyledon through stomata to the adaxial surface at the 8th hour of the dark period (A–B). Similar material was observed on the upper side of the petiole (C). Droplets of different size observed in the proximity of open stomata (D).

stomata through which such material is apparently extruded to the surface. This material was rarely observed (using SEM) in light-grown, non-induced plants. However, during the subinductive dark period, especially the second part, the number of droplets observed on the surface of the adaxial side of cotyledons increased very significantly (data not shown). This substance was also found on the upper surface of the cotyledon petioles (Fig. 5 C). Sometimes, extrusion of the material to the surface of the cotyledon occurred through clefts which appeared between the epidermal cells (Fig. 5 D). In cotyledons observed under a fluorescence microscope (excitation at 405 nm) the above mentioned droplets (observed by SEM) appeared as a red-fluorescent material surrounding spherical structures present inside tissues of the organ (data not shown). This material was especially visible under a fluorescence microscope using a UV light for excitation (wide band, 270–330 nm filter).

Discussion

It was proposed that during the inductive dark period an increase of intracellular free calcium concentration ($[Ca^{2+}]_i$) occurs (Friedman et al., 1989). It is generally accepted that the extracellular free calcium level ($[Ca^{2+}]_o$) is about three orders of magnitude higher than $[Ca^{2+}]_i$ (Evans et al., 1991; Gilroy et al., 1991). Experimentally, it is possible to increase $[Ca^{2+}]_i$ by raising the $[Ca^{2+}]_o$. In such circumstances Ca^{2+} can cross the plasma membrane and enter the cytoplasm *via* diffusion or passage through specific calcium channels (Tsien

and Tsien, 1990; Evans et al., 1991; Gilroy et al., 1991). Calcium ionophores which increase membrane transport of the cation are frequently used for manipulation of $[Ca^{2+}]_i$ (Felle et al., 1992). Inhibition of the calcium pumps both at the plasma membrane and endomembranes, by specific inhibitors (e.g. thapsigargin; Thastrup et al., 1987) can also lead to an increase of $[Ca^{2+}]_i$. Such compounds as caffeine and ryanodine are well known agents which in animal cells release calcium from its intracellular stores, thus icreasing $[Ca^{2+}]_i$ (Tsien and Tsien, 1990).

We have shown previously that the calcium channel agonist, Bay K 8644 has no effect on flower induction in *P. nil* (Tretyn et al., 1990). Therefore, in the present work we have examined the effect of calcium, calcium ionophores: A23187 and ionomycin, thapsigargicin (an analog of thapsigargin), caffeine and ryanodine on the photoperiodic induction of *P. nil.*

Calcium ions and the calcium ionophore A23187, and to a lesser degree ionomycin, very significantly increase flowering when applied just before and during the first 2 h of a subinductive dark period. The lower effectiveness of ionomycin compared to A23187 might be because ionomycin was used at relatively lower concentrations than A23187. Furthermore, ionomycin-mediated calcium transport through membranes is strictly pH dependent. Because the pH in the cell wall is lower than 7.0 this ionophore might fail to form complexes with Ca^{2+} under such conditions.

Of the two compounds, caffeine and ryanodine, which can release calcium from its internal stores (Tsien and Tsien, 1990) and increase $[Ca^{2+}]_i$, only caffeine was active in flower induction. Beside treatment with either caffeine or ryanodine an increase in $[Ca^{2+}]_i$ can be also induced *via* inhibition of Ca^{2+}-ATPase (Felle et al., 1992). However, thapsigargicin, an inhibitor of animal Ca^{2+}-ATPase, (Thastrup et al., 1987) had no effect on flowering of *P. nil.*

Both scanning electron and light microscopic studies have shown that under natural conditions flowering in *P. nil* may be directly correlated with functioning of the stomata (Table 2). The convex shape, as well as the configuration of the epidermal cells may lead to accumulation (especially in the proximity of the stomata) of applied solutions on the adaxial side of the cotyledon (for details see Fig. 3 B and 3 D). We postulate that stomata can also be a target for calcium modulator action. It is in agreement with previously published data concerning the effect of the calcium chelator, EGTA on flowering in *P. nil.* Friedman et al. (1989), as well as Tretyn et al. (1990) have shown that spraying of the plant with EGTA before an inductive dark period inhibits flowering. Application of EGTA, Ca^{2+}, and calcium ionophores modulate stomatal opening and closure (Gilroy et al., 1991; Kearns and Assmann, 1993). Gilroy et al. (1991) have shown that an increase in exogenous free Ca^{2+} from 20 µM to 1 mM induces a steady rise in $[Ca^{2+}]_i$ which leads to stomatal closure within 30 min. A similar effect was observed when epidermal strips of *Commelina communis* were treated with 10 µM A23187 (Gilroy et al., 1991). Stomatal closure was also observed after releasing Ca^{2+} and inositol 1,4,5-trisphosphate (IP_3) from their caged forms (Gilroy et al., 1990).

Abscisic acid is a hormone, which can regulate stomatal closure (Kearns and Assmann, 1993). Irving et al. (1992) and McAinsh et al. (1992) provided evidence that ABA-induced an increase in $[Ca^{2+}]_i$ which preceded stomatal closure. Here we have shown that ABA decreased the flowering response in *P. nil* if applied when the stomata were open during the second part of the subinductive 14-h dark period (Fig. 4). We have shown that after a transition of *P. nil* seedlings from light to darkness, an increase in $[Ca^{2+}]_i$ and closure of the stomata occurs. More rapid closure of the stomata induced by treating them with Ca^{2+}, calcium ionophores and caffeine can lead to a decrease in the length of the critical dark period. Our hypothesis is in agreement with a conclusion made by Friedman et al. (1989) who also pointed out that Ca^{2+} play a significant role during the early stages of the inductive dark period.

Under natural conditions release of Ca^{2+} from internal stores (vacuole or endoplasmic reticulum) appears to precede stomatal closure (Gilroy et al., 1990, 1991; Kearns and Assmann, 1993). Photolysis of caged IP_3 and stomatal closure occurred even in guard cells pretreated for 15 min with 1 mM La^{3+} (Gilroy et al., 1990). Therefore, if stomata are important for flower induction, calcium channel blockers would not be expected to have a significant influence on flower induction. This is in agreement with results published previously in which it was shown that three calcium channel blockers; verapamil, nifedipine and La^{3+} had no specific effect on the flowering response in *Pharbitis* (Tretyn et al., 1990). The closure of stomata during the first 2 h of the inductive dark period described in the present paper is probably a result of changes in photosynthetic CO_2 metabolism of guard cells (Kearns and Assmann, 1993). It was possible to speed up this process by treating the adaxial epidermis with Ca^{2+}, calcium ionophores and caffeine and ABA.

It is well established that phytochrome controls flower induction in *P. nil* (for details see Vince-Prue, 1994). A 5 min red light pulse given at the 8–9th hour of an inductive dark period prevents flowering. However, when *P. nil* seedlings were irradiated with red light in the middle of an inductive dark period and simultaneously sprayed with a cytokinin no inhibitory effect of the active form (Pfr) of phytochrome on flowering was observed (Vince-Prue, 1994). In this paper we have shown that a red light pulse given at the 8th hour of the dark period stimulated stomatal closure. However, Irving et al. (1992) have provided evidence that cytokinin stimulates stomatal opening. Taking both these observations into account we speculate that stomata are the target for phytochrome action. Studies concerning this possibility are currently in progress.

We propose that during the second part of the subinductive dark period an unknown substance, which is synthesized inside the cotyledons is transported to the surface of the cotyledons through open stomata. Dark-induced closure of the stomata can synchronize synthesis of this substance. We now have evidence that synthesis of this substance takes place in special glandular structures. Their structure and chemical composition are currently under investigation.

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