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Cytochemical and histochemical characterization of cotyledonary bodies from *Pharbitis nil* seedlings

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Summary. Cytological and histochemical characterization of the structures from which an obscure substance is secreted via open stomata to the abaxial surface of Japanese morning glory (*Pharbitis nil* Choisy cv. Violet) cotyledons has been carried out. Observation of intact cotyledons using the light microscope revealed randomly distributed semi-transparent structures. These structures, which were shown to be the same as those previously described as giant oil cells are referred to here as cotyledonary bodies. These bodies can be easily isolated and purified after enzymatic digestion of the cotyledons. Using different staining procedures we have confirmed that each cotyledonary body originates from an individual mesophyll cell during embryo development. Purified bodies consist of (*i*) a thick shell-like envelope; (*ii*) a transparent, hydrophilic zone; (*iii*) a hydrophobic core. Hydrophobic contents of the bodies were readily extracted with methanol and shown to contain fatty acids and phenolic compounds using the gas chromatography/mass spectrometry (GC/MS) technique. Methanolic extracts of cotyledonary bodies showed high fluorescence with two excitation and emission maxima. Using a fluorescence microscope we have shown that the bodies isolated from seedlings grown in continuous light, conditions non-inductive for flowering, and those grown under conditions inductive for flowering (a single 16 h, long dark period) have different fluorescence emission spectra. Different levels of free Ca^{2+} inside cotyledonary bodies isolated from light-grown and single dark-period treated *P. nil* seedlings were found using the fluorescent calcium indicator dye Fluo-3 under a confocal scanning laser microscope. On the basis of these observations we speculate that cotyledonary bodies could be involved in floral induction.

Keywords: Cytochemistry, Confocal scanning laser microscope; Flowering; Calcium indicator; Gas chromatography/mass spectrometry; Photomorphogenesis.

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Introduction

Light plays an important role in the regulation of plant growth and development. Operating via different photomorphogenic receptors it controls such processes as seed germination, de-etiolation and flowering (Kendrick and Kronenberg 1994). The mechanism of light-controlled transition from the vegetative to the generative stage in plants remains unresolved. On the basis of their light-dependent flowering response many plant species can be classified into two groups: short- and long-day plants. Flowering of short-day plants is stimulated by a short photoperiod (or long dark period), whereas that of long-day plants is accelerated by their exposure to long days. *Pharbitis nil* and *Sinapis alba* are two very convenient model species for the study of floral induction. *Pharbitis nil* is a short-day plant, which can be induced to flower by a single, long dark period (for review, see Vince-Prue 1994). However, flowering of *S. alba* is induced by a single, long-day treatment (for review, see Bernier et al. 1993). In both cases photoperiodic induction is initiated in the cotyledons or mature leaves. The floral stimulus which is produced or released in these organs is then transported to the apical meristem. The chemical nature of the floral stimulus remains unknown and there are probably alternative pathways involved (Bernier 1988, Bernier et al. 1993). It has been proposed that calcium is involved in the induction of flowering in *P. nil*. Friedman et al. (1989) and Tretyn et al. (1994) have shown that appli-

cation of the calcium ionophore A23187 reduces the length of the critical dark period. It has also been shown that caffeine, which induces the release of Ca^{2+} from intracellular stores in animals, has a promotive effect on floral induction in *P. nil*. Moreover, the calcium chelator ethylene glycol-O,O′-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA), when applied to the apex, inhibited flowering in both *P. nil* (Tretyn et al. 1990) and *S. alba* (Havelange and Bernier 1993). It was proposed that stomata present on the adaxial surface of *P. nil* cotyledons are targets for calcium action (Tretyn et al. 1994). During a floral-inductive dark period an obscure substance is released via stomata to the cotyledon surface (Tretyn et al. 1994). Here we present cytological and histochemical characterization of structures from which this substance originates and call them cotyledonary bodies. These bodies are derived from single cells and are the same structures as previously described by Wada et al. (1981) as giant oil cells in *P. nil* and other members of the Convolulaceae.

Material and methods

Plant material

Seeds of *Pharbitis 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 W/m² cool-white fluorescent) at 25 °C.

Digestion of cotyledons

Both 6-day-old light-grown seedlings and plants of the same age but treated with a 12 h long dark period were used. Cotyledons, excised from seedlings, were cut into small pieces and incubated (ca. 2 g/10 ml) in 1.5% (w/v) cellulase "Onozuka" RS, 0.3% (w/v) macerozyme R-200 (Yakult Honsha Co., Ltd, Tokyo, Japan), 0.1% (w/v) pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd, Tokyo, Japan), 0.05% (w/v) zymolyase 20T (Seikagaku Co., Tokyo, Japan), 0.1% (w/v) bovine albumin (fraction V), 0.1% (w/v) polyvinylpyrrolidone (MW 360000), 1 mM KL-dithiothreitol (DL-DTT), 0.35 M sorbitol in 5 mM 2-(N-morpholino)ethanesulphonic acid (Mes), 5-amino-2-hydroxylmethyl-1,3-propanediol (Tris) buffer (all compounds from Sigma Chemical Company, St. Louis, MO, U.S.A.), pH 5.5 for 4 h at 22 °C. The digested tissues were then filtered through a 150 mesh sieve and subsequently through a 100 mesh sieve. The bodies which were retained on the sieve were washed a few times with Mes/Tris buffer and depending on the experiment re-suspended in distilled water or in absolute methanol. Yield was 90–95% and no damage was observed during washing of the bodies with low osmotic solutions. In the case of light-grown plants all manipulations and digestion procedures were performed in the light. However, digestion and purification of the bodies from cotyledons of dark-treated plants were carried out under a dim green safe light.

Light microscopy and vital staining

Cotyledons and isolated bodies were observed using an Olympus IMT-2 inverted light microscope. Fluorescence of examined bodies was studied with the aid of an Olympus BH2 fluorescence microscope using both UV (wide band) and violet (narrow band, 405 nm) excitation filters. Both microscopes were fitted with an automatic exposure photomicrographic system (Olympus, model PM-10AK). Isolated bodies were stained with 0.1 mM aqueous solutions of the following dyes: neutral red, methylene blue (both from Wako Pure Chemical Industries, Ltd., Japan), toluidine blue, methyl blue, calcofluor white M2R, and 1% (w/v) osmium tetroxide (all from Sigma). The DNA content of the bodies was stained with 4′,6-diamidino-2-phenylindole (DAPI). For identification of phenolic substances the bodies were treated either with $FeCl_2$ or vanillin-hydrochloric acid (Sigma) reagents (Ragan and Craigie 1978).

Scanning electron microscopy

For observation in the scanning electron microscope (SEM) the cotyledons were cut with razor into small pieces. Isolated bodies or pieces of cotyledon were fixed for 1 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 material was then treated for 1 h with 2% (w/v) tannic acid in the buffer at 35 °C (Tretyn et al. 1994). After washing in phosphate buffer, the examined material was post-fixed in 1% (w/v) osmium tetroxide (12 h, 4 °C), dehydrated in ethanol and propanol and freeze dried in a Hitachi ES 2020 freeze dryer. Samples were then coated with platinum using a Hitachi E 102 ion sputter and observed in a Hitachi S-2350 scanning electron microscope.

Fluorimetry and spectrophotometry

Cotyledons from about 40–50 seedlings were digested and the bodies were isolated as described above. After isolation they were resuspended in 5 ml of methanol. Excitation and emission of fluorescence of methanolic solutions were examined using a Hitachi fluorescence spectrophotometer (model F-3010). Spectrophotometric analysis was performed using a Beckman (Fullerton, CA, U.S.A.) DU-50 spectrophotometer.

Gas chromatography/mass spectrometry

Gas chromatography/mass spectrometry (GC/MS) analyses were carried out with a JEOL Automass JMS-AM150 mass spectrometer connected to a Hewlett-Packard 5890A-II gas chromatograph. Isolated cotyledonary bodies were treated for 2 h with absolute methanol. After centrifugation the supernatant was freeze dried. Before analysis freeze-dried samples were treated with 20 µl of N-methyl-N-trimethylsilyltrifluoroacetamide at 70 °C for 30 min. Analyses were conducted under the following conditions: ionization, EI (70 eV); column, fused silica capillary TC-1 (0.25 mm × 15 m, 0.25 µm film thickness); column temperature, 80 °C for 1 min, elevated to 320 °C at 30 °C/min, and then maintained at 320 °C. The identification for phenolic compounds was based on the direct comparison with authentic specimens. Fatty acids were tentatively identified by library search of the JEOL data system (Lucy version 2.30).

Fluo-3 loading and confocal microscopy

Fluo-3 was dissolved in anhydrous dimethyl sulfoxide (DMSO) and kept as a 5 mM stock solution. In all experiments 20 µM Fluo-3 in 5 mM 2-(N-morpholino) ethane-sulfonic acid/Tris (hydroxymethyl)-aminomethane (Mes/Tris) buffer pH 6.0 was used. Isolated cotyle-

donary bodies were incubated in darkness at room temperature (22–24 °C) with gentle shaking. After 1 h of treatment they were washed with Mes/Tris buffer and examined for Fluo-3 fluorescence. The Bio-Rad MRC-600 confocal scanning laser microscope (CSLM) was used for localization of fluorescence. Excitation of Fluo-3 was performed at 488 nm by a multiline argon ion laser. Each experiment was repeated 5 times with similar results and the figures show representative images from a single experiment.

Results

Using SEM we previously showed the secretion of unknown material from within the cotyledon to the adaxial surface occurs in *P. nil* during the 8th hour of a floral-inductive dark period. Droplets of different size were frequently observed in the proximity of open stomata (Tretyn et al. 1994). Here we have studied the location from where this unknown material originates. Observation of intact cotyledons using the light microscope, revealed semi-transparent structures, ca. 70–100 μm in diameter, randomly distributed over the cotyledons (Fig. 1 A). These structures have been previously described by Wada et al. (1981) as giant oil cells and we call them cotyledonary bodies. After 1 h of fixation of cotyledons in 2% glutaraldehyde these structures appear balloon-like (Fig. 1 B). Under a fluorescence microscope (UV excitation) they appear as blue fluorescent spots (data not shown). On the cross sections of fixed and dehydrated cotyledons examined using an SEM many well-like structures, similar in size to the bodies observed under the light microscope (after glutaraldehyde treatment) were seen (Fig. 1 C). Most of them were close to the adaxial surface of cotyledons (Fig. 1 C, D) and in the proximity of stomata (Fig. 1 D).

All further experiments were performed on enzymatically isolated bodies. After a 3 h treatment of cotyledons with a mixture of cell-wall digestive enzymes (see Material and methods) a suspension of mesophyll protoplasts and much larger and easily sedimentable bodies was obtained. It was therefore easy to separate the bodies from protoplasts. Under the light microscope they are seen as ca. 100–150 μm in diameter bodies with a dense core surrounded by a transparent zone and a thick, shell-like envelope (data not shown). These three components of the body were more clearly visible after vital staining with different dyes. The internal core easily absorbed osmium tetroxide, methylene blue (data not shown) and toluidine blue (Fig. 2 A), as well as neutral red (Fig. 2 B). After staining, especially with neutral red it was possible to see that the core is less dense in the center compared to its periphery. Moreover, in the cleft between the core and an envelope what appeared to be gas-like vesicles were observed (Fig. 2 B).

We confirmed that each cotyledonary body develops from a single mesophyll cell during development. They first appear in the green embryonic cotyledons about 2–3 weeks after pollination. Bodies isolated from embryos had a well-developed envelope (Fig. 2 C), which showed a blue fluorescence under UV light excitation (Fig. 2 D). After treatment with calcofluor white M2R, a dye which is commonly used for staining cellulose, only small spots of fluorescence were observed (data not shown). No fluorescence after treatment of isolated bodies with methyl blue, a stain used for callose cell wall localization, has been detected (data not shown). Bodies isolated from green embryos were quite different from those isolated from 6-day-old seedlings, containing many differently sized vesicles (Fig. 2 C). Bodies with higher numbers of the vesicles were usually the largest (Fig. 2 C). During development, fusion of the vesicles occurred resulting in smaller bodies with a uniform core (Fig. 2 C–D). The bodies isolated from dry and imbibed seeds showed the same structures present in 6-day-old seedlings (data not shown).

A study of the detailed structure and nature of the bodies was conducted using SEM (Fig. 3) and light microscopy (data not shown). After treatment with osmium tetroxide the structure of whole bodies, as well as their core and envelope was well preserved. Figure 3 shows the appearance of intact bodies (Fig. 3 A, C), and separately their cores (Fig. 3 A, B) and an envelope (Fig. 3 D). In some cases, it was possible to see the ghosts of mesophyll cells which surrounded the body inside intact cotyledons (Fig. 3 C). A typical body is surrounded by 50–80 mesophyl cells (see Fig. 3 C).

Freshly isolated bodies were resistant to treatment with some organic solvents, e.g., ethanol and acetone. However, treatment with methanol led to a loss of the hydrophobic contents. A few seconds after the treatment of the bodies with methanol they swell, their envelope breaks, and the methanol soluble contents explode out. After such treatment, empty, slightly fluorescent (under UV) shells were obtained which retained a little methanol insoluble material which was slightly fluorescent (data not shown). This material was stainable with DAPI, a dye commonly used for identification of DNA, and presumably represents residual nuclear material.

Methanol-soluble substances extracted from cotyledonary bodies were studied by means of GC/MS and

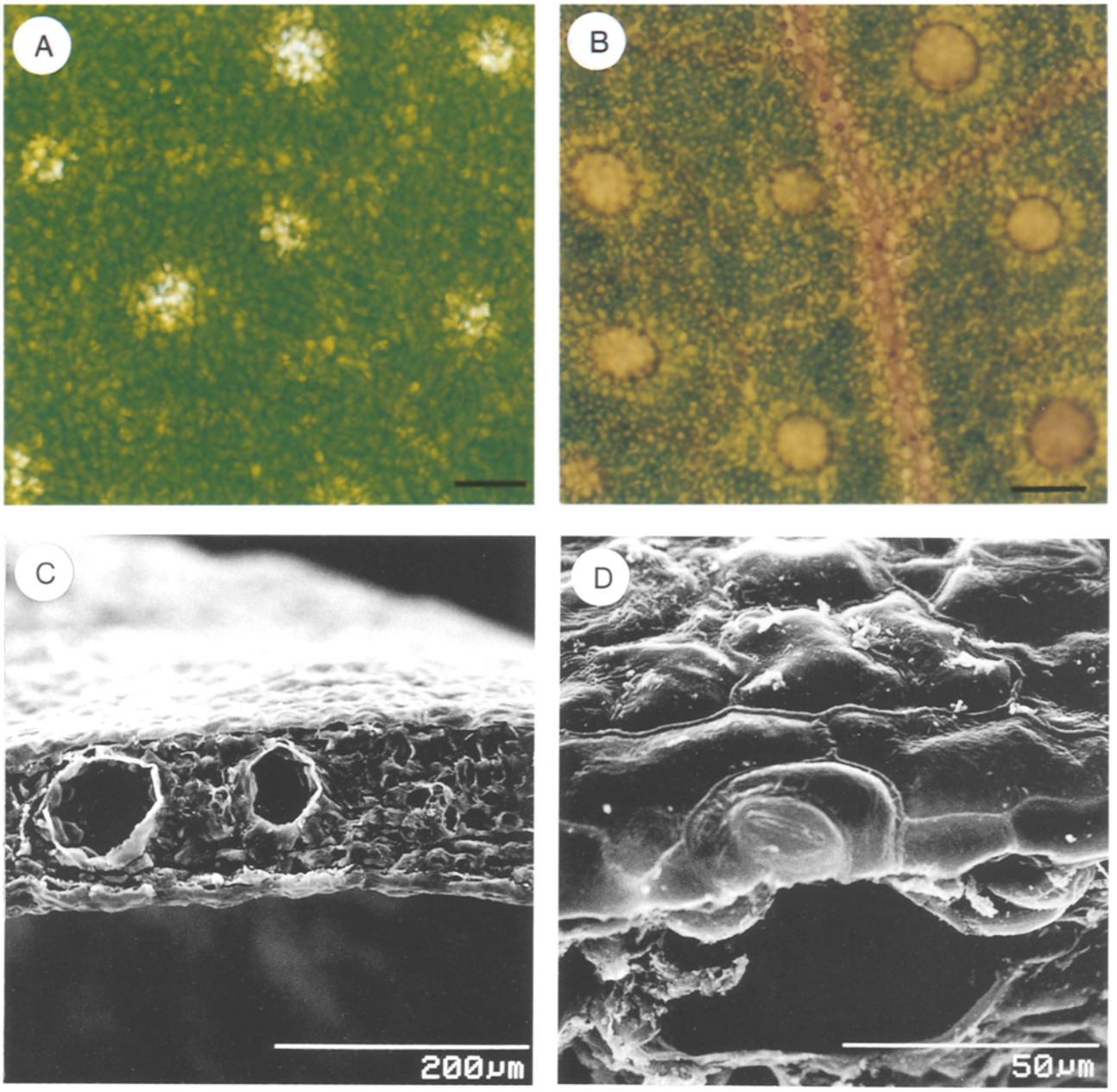

Fig. 1. Light (**A** and **B**) and scanning electron microscope (**C** and **D**) pictures showing appearance of *P. nil* cotyledons. In intact cotyledons, many semi-transparent structures are visible under a light microscope (**A**), which have a regular, balloon-like shape after a 1 h treatment of the tissue with 2% glutaraldehyde (**B**); bars: 100 µm. These structures, observed in a scanning electron microscope, were shown to be close to the adaxial surface of the cotyledons (**C**), frequently in a contact with stomata (**D**)

shown to be a complex mixture of fatty acids and phenolic compounds (data not shown). Relatively high amounts of palmitic acid, as well as linolenic and linoleic acid were tentatively identified by a library search (data not shown). By means of the direct comparison with authentic specimens *p*-coumaric, ferulic, and caffeic acids were identified. The most abundant phenolic compound found in extracts was *p*-coumaric acid (data not shown).

Methanolic extracts of the bodies were studied spectrophotometrically (Fig. 4). These extracts (diluted 10 times in methanol, HPLC grade) absorb light strongly with a broad peak from 260 to 360 nm. Two absorption maxima at 288 and 311 nm were recorded (Fig. 4

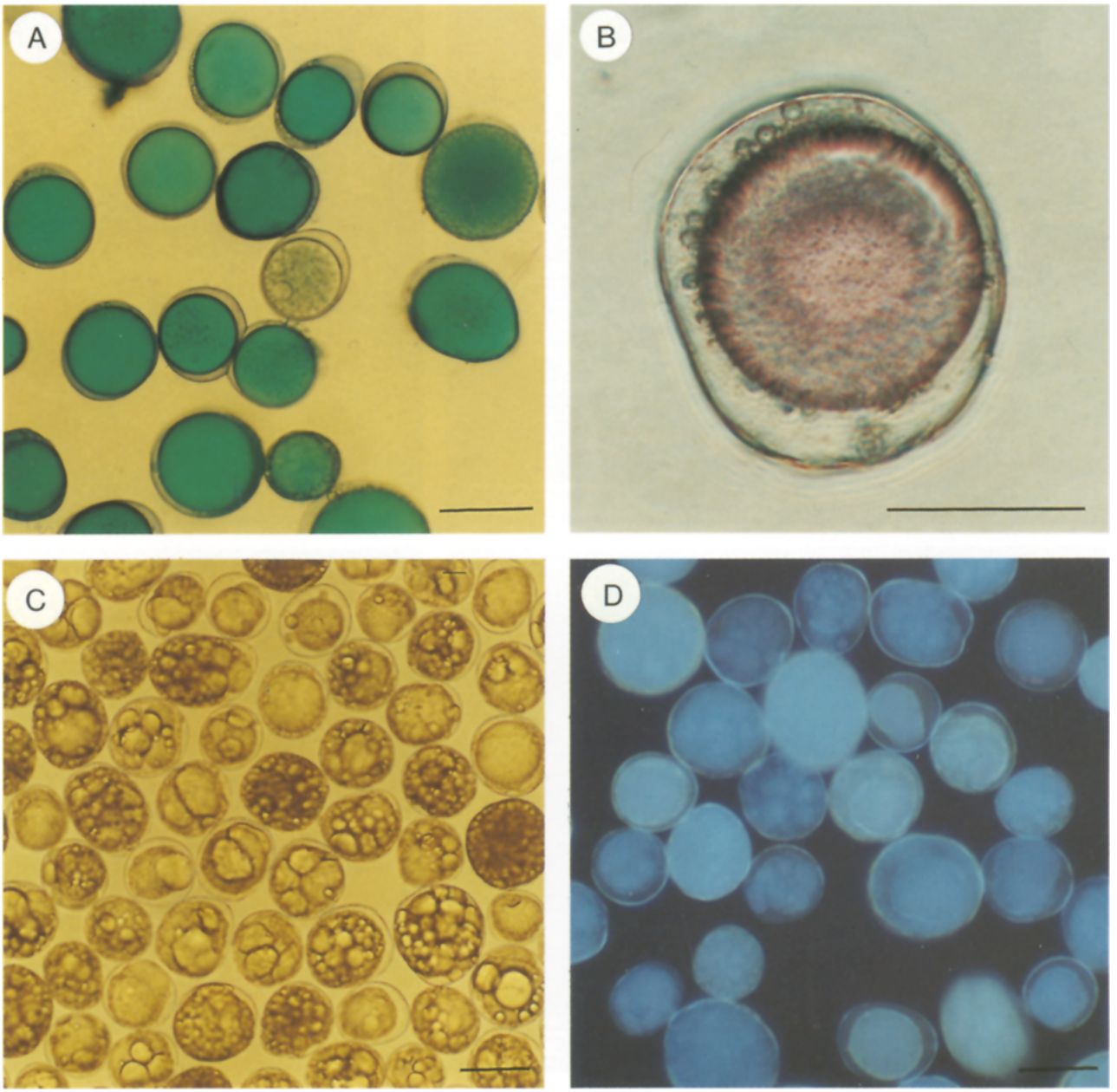

Fig. 2. Light (**A**–**C**) and fluorescence (**D**) microscope pictures showing appearance of globular cotyledonary bodies isolated from cotyledons of 6-day-old seedlings (**A** and **B**) or from embryos of *P. nil* (**C** and **D**). The bodies were stained with toluidine blue (**A**) or neutral red (**B**). Unstained isolated bodies from embryonic cotyledons of *P. nil* (**C**) and their fluorescence under UV excitation (**D**). Bars: 100 μm

A). The same extract showed high fluorescence (see Fig. 4 B, C). Two excitation and emission maxima were determined. When fluorescence was excited at 366 nm (the first maximum, quite broad), a mixture of blue, green, and yellow fluorescence was recorded (Fig. 4 B). Excitation at 405 nm led to narrow band, red fluorescence (Fig. 4 C).

These preliminary fluorescence studies were extended to intact, isolated bodies. The bodies were isolated either from cotyledons of 6-day-old light-grown seedlings (Fig. 5 A–C) or from the same age seedlings which had been treated with a 16 h, long dark period, which is sufficient for induction of flowering (Fig. 5 D–F) before starting digestion. Fluorescence of bodies was determined with both UV (wide band) and violet (narrow band, 405 nm) excitation filters. Figure 5 A–C shows the light (A) and fluorescence microscope images of the same sample (B, C). Most

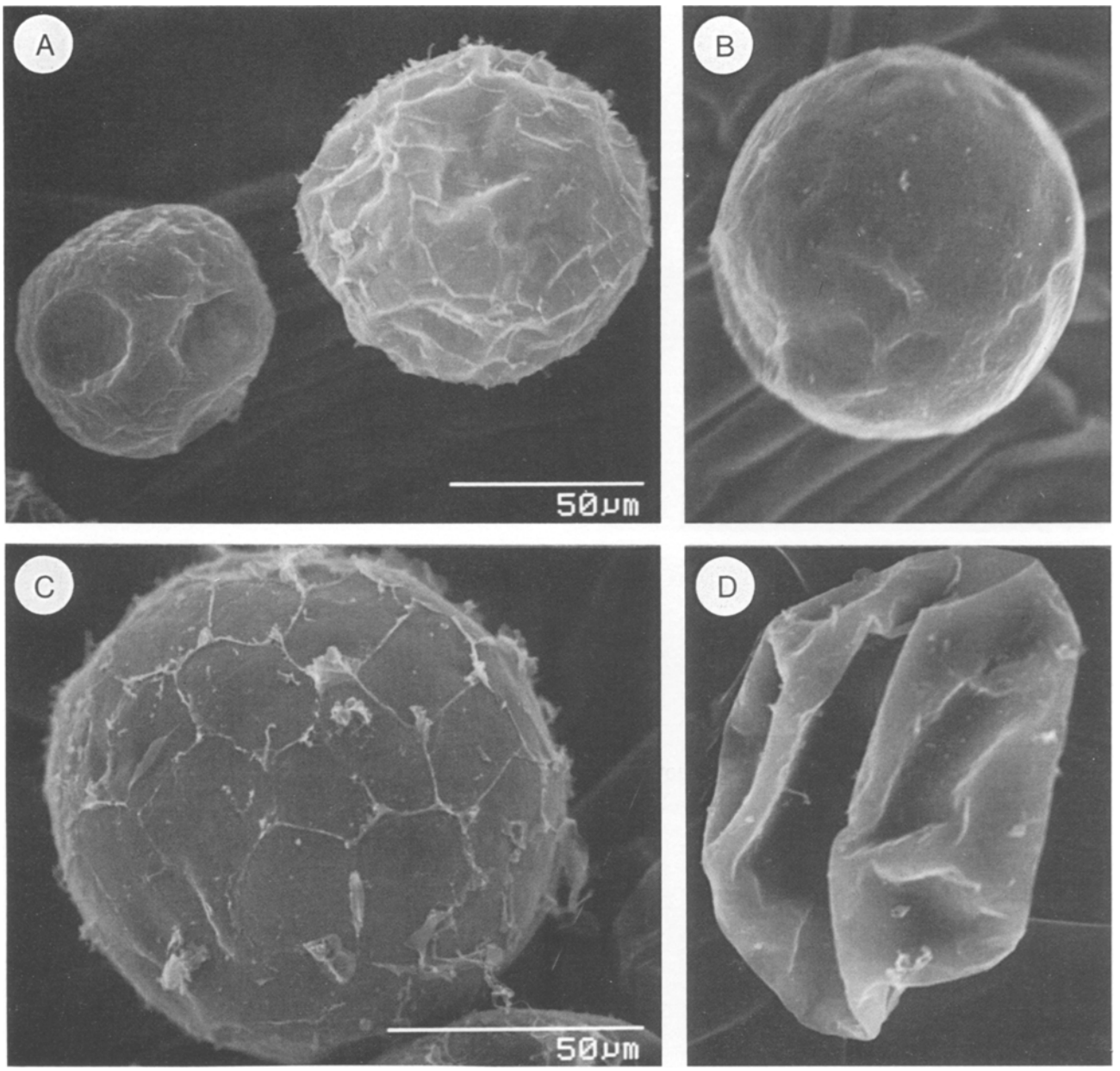

Fig. 3 A–D. Cotyledonary bodies isolated from 6-day-old, light-grown *P. nil* seedlings in a scanning electron microscope. Appearance of whole (**A**, right side, and **C**) bodies, the internal content (**A**, left side, and **B**) and a shell (**D**). B and D to the same scale as C

of the bodies illuminated with UV emitted a bright- to dark-blue fluorescence. Only some, usually the smallest bodies had pink-blue fluorescence (Fig. 5 B). Fluorescence of these smaller bodies, excited at 405 nm was yellow-orange, whereas most of the bodies exhibited a greenish fluorescence (Fig. 5 C). Cotyledonary bodies isolated from dark-treated (12–16 h after starting of the dark period) plants were the same size and appearance as those isolated from light-grown plants (compare Fig. 5 A and D). However, their fluorescence emission was significantly different. Most of the bodies irradiated with UV had a pale violet, dark blue, or magenta fluorescence emission (compare Fig. 5 B with E). The same structures excited with violet light (405 nm) exhibited a red fluorescence (Fig. 5 F, compare with Fig. 5 C). This difference in fluorescence was lost after 2–3 days of continuous white light (data not shown).

When excited at 488 nm only very weak autofluorescence of cotyledonary bodies isolated both from 6-

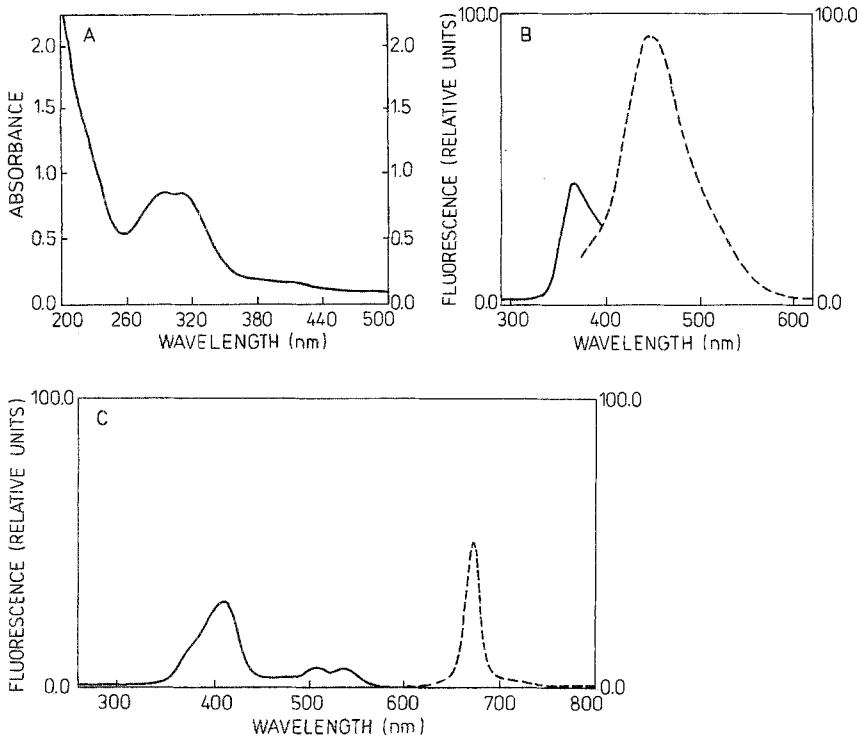

Fig. 4. Absorption (**A**) and fluorescence (**B** and **C**) spectra of methanolic extract from the cotyledonary bodies isolated from 6-day-old *P. nil*. In **B** and **C**, solid lines show excitation and dashed lines emission spectra

day-old *P. nil* seedlings treated with single 14 h dark period (Fig. 6 A) or grown under continuous white light (Fig. 6 C) was observed in CSLM. Most of cotyledonary bodies isolated from both light-grown and dark-treated seedlings were easily loaded with the fluorescent calcium-sensitive dye, Fluo-3. However, their fluorescence pattern was significantly different (Fig. 6 B, D). Small, but significant differences in $[Ca^{2+}]$ have been found inside cotyledonary bodies isolated from light-grown (Fig. 6 D) and dark-treated (Fig. 6 B) seedlings of *P. nil*. The level of free calcium was relatively higher in cotyledonary bodies isolates from dark-treated plants (compare Fig. 6 B with Fig. 6 D). The free calcium concentration gradually increased inside the bodies isolated from older light-grown as well as dark-treated plants.

Discussion

The chemical nature of the floral stimulus transmitted from the cotyledons to the apex during floral induction of *P. nil* remains unknown. Recently, we have shown that during a 14 h long sub-inductive dark period an unknown substance, which is synthesized inside the cotyledons, is transported to their adaxial surface (Tretyn et al. 1994). In the present paper we provide evidence that this substance originates from the cotyledonary bodies previously called giant oil cells (Wada et al. 1981 and reference therein).

Fully developed cotyledonary bodies consist of (*i*) a thick shell-like envelope, (*ii*) a transparent, hydrophilic zone, (*iii*) a hydrophobic core. At early stages of cotyledonary body differentiation the envelope appears similar in composition to an ordinary cell wall (it is stainable with calcofluor white). However, during further development changes in its chemical composition take place. Envelopes of bodies isolated from mature seeds or seedlings react neither with calcofluor white (used for cellulose staining) nor with methyl blue (dye used for callose cell wall localization). However, after treatment with methanol they have similar fluorescence to xylem cells. Therefore, we speculate that during embryogenesis the primary cellulose-like cell wall undergoes some additional modifications. However, contrary to xylem, the cell wall surrounding cotyledonary bodies is water permeable. Isolated bodies easily dehydrate, but can be rehydrated. On this basis we believe that cotyledonary bodies could function in cotyledons as a kind of osmoregulatory system, taking part in the control of water balance inside mesophyll tissue.

Cotyledonary bodies can be easily stained with osmium tetroxide and different vital dyes, including neu-

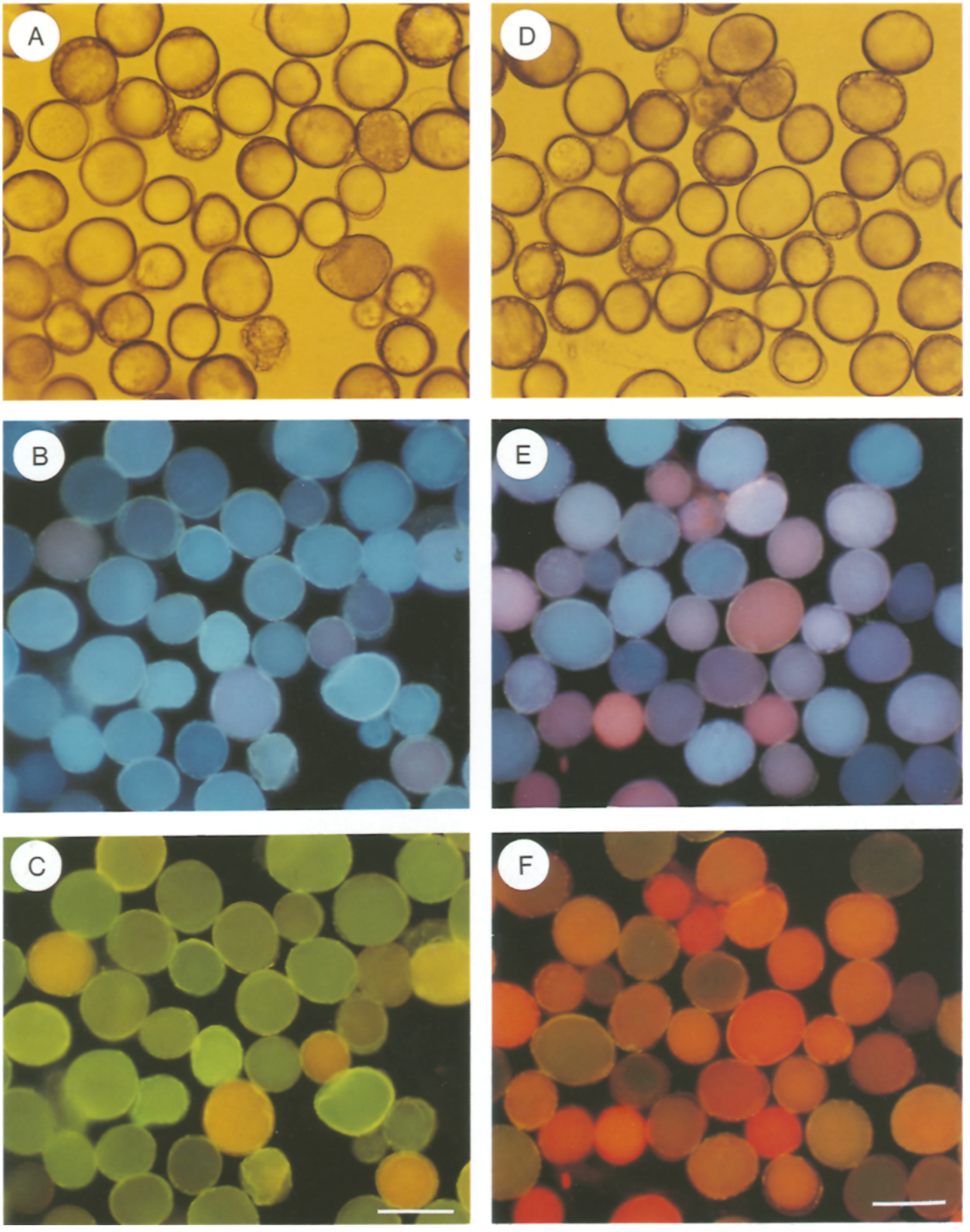

Fig. 5. Isolated cotyledonary bodies from 6-day-old *P. nil* seedlings grown under continuous white light (**A–C**) or treated with a single floral inductive, 16 h dark period (**D–F**) observed in a light microscope (**A** and **D**) or the same bodies observed under UV (**B** and **E**) and violet light excitation (405 nm) (**C** and **F**) in a fluorescence microscope. Representative results of one out of five independent experiments. Bars: 100 μm

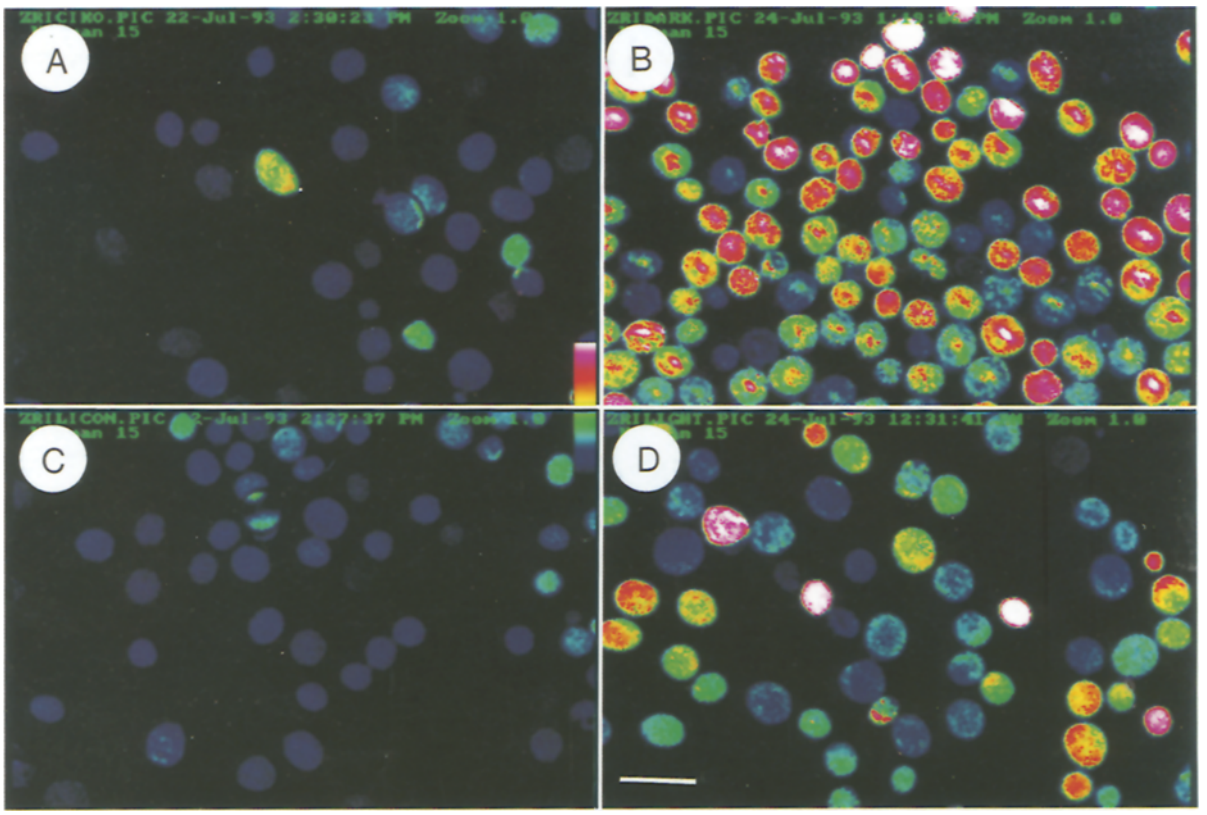

Fig. 6. Confocal laser scanning microscope images of cotyledonary bodies isolated from 6-day-old *P. nil* seedlings treated with a single floral inductive, 16 h dark period (**A** and **B**) or grown under continuous white light (**C** and **D**). Autofluorescence of the bodies excited at 488 nm (**A** and **C**). The bodies loaded with the fluorescent calcium indicator dye Fluo-3 were excited at 488 nm (**B** and **D**). The inserted scale shows the relative level of $[Ca^{2+}]$ (white represents the highest and blue the lowest). Representative results of one out of five independent experiments. Bar: 100 μm

tral red and toluidine blue. A similar staining pattern was found for an intracellular inclusion observed in some green (Grolig and Wagner 1989, Tretyn et al. 1992) and brown algae (Ragan 1976). These globular structures, known as physodes (Ragan 1976, Tretyn et al. 1992) contained phenolic compounds (Ragan 1976, Grolig and Wagner 1989). In isolated cotyledonary bodies treated with reagents commonly used for polyphenolics identification ($FeCl_2$, and vanillin-hydrochloric acid mixture; Ragan and Craigie 1978) a slight positive reaction was detected. Moreover, cotyledonary bodies were stainable with sudan III (data not shown), a compound used for identification of fatty substances. Based on these observations we speculate that an osmophilic substance which occurs inside cotyledonary bodies is a mixture of fatty acids and phenolic compounds. Presence of these substances in methanolic extracts isolated from cotyledonary bodies was partially confirmed by GC/MS analysis. The most abundant phenolic compound observed was *p*-coumaric acid.

The physiological function of fatty acids and phenolic compounds present inside cotyledonary bodies remains obscure. It is uncertain whether the fatty acids present inside cotyledonary bodies can be taken up by the surrounding mesophyll cells. On the other hand, phenolic compounds which are more soluble in water may be more easily exchanged between cotyledonary bodies and neighboring cells. Floral induction of *P. nil* under both poor nutrition (Hirai et al. 1993) and high-fluence illumination (Shinozaki et al. 1994) is closely correlated with the accumulation of some phenolic derivatives inside cotyledons of the plant. We believe that these compounds are stored inside cotyledonary bodies.

It was proposed that the main physiological function of physodes in green and brown algae is the regulation of intracellular free calcium concentration (Tretyn et al. 1996). We have found that there are some similarities between algal physodes and cotyledonary bodies of *P. nil*. As well as similar staining patterns with different vital dyes, cotyledonary bodies were

also slightly stainable with alizarin red (data not shown), a compound used for cytological Ca^{2+} localization (Miller and Kotenko 1987). Using Fluo-3 we have shown that cotyledonary bodies isolated from *P. nil* seedlings accumulate Ca^{2+}. Presence of this element inside cotyledonary bodies in situ was supported by X-ray micro-analysis (data not shown). Because these structures are dead it seems that the level of $[Ca^{2+}]$ inside them is regulated by neighboring cells. We believe that dependent upon the light treatment these cells can either extrude Ca^{2+} to or take it up from the bodies. We postulate that both hydroxyl and carboxyl groups of phenolic compounds and fatty acids present in hydrophobic zone of the bodies take part in the arrangement of co-ordination binding sites for Ca^{2+}.

Some authors, including Friedman et al. (1989), Tretyn et al. (1990, 1994), and Takeno (1993), have shown that calcium is involved in floral induction of *P. nil*. Recently, we have provided evidence that cotyledonary stomata are targets for calcium and its modulator action (Tretyn et al. 1994). Ruiz et al. (1993) have pointed out that the amount of calcium delivered by the transpiration stream to points of evaporation needs to be regulated if interference with stomata behavior is to be avoided. They also postulated that this regulation is likely to occur in mesophyll. Because mature guard cells have no plasmodesmatal connections with their neighbors apoplastic calcium may directly regulate their movement. We believe that cotyledonary bodies in *P. nil* are involved in this process.

Besides changes in $[Ca^{2+}]$ inside cotyledonary bodies we have also found that the fluorescence patterns of these bodies isolated from plants grown under continuous white light, non-inductive for flowering, and from plants treated with a floral-inductive dark period are quite different. We speculate that these changes taking place within the cotyledonary bodies could be involved in floral induction.

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