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Changes in dye coupling of stomatal cells of *Allium* and *Commelina* demonstrated by microinjection of Lucifer yellow

B.A. Palevitz [1] * and P. K. Hepler [2]

[1] Botany Department, University of Georgia, Athens, GA 30602,
[2] Botany Department, University of Massachusetts, Amherst, MA 01003, and
Marine Biological Laboratory, Woods Hole, MA 02543, USA

"You can observe a lot by watching"
Lawrence Berra, as quoted in "Sports Illustrated", vol. 60 (No. 14), p. 94, 2 April 1984

Abstract. Lucifer yellow has been microinjected into stomatal cells of *Allium cepa* L. epidermal slices and *Commelina communis* L. epidermal peels and the symplastic spread of dye to neighboring cells monitored by fluorescence microscopy. Dye does not move out of injected mature guard cells, nor does it spread into the guard cells when adjacent epidermal or subsidiary cells are injected. Dye does spread from injected subsidiary cells to other subsidiary cells. These results are consistent with the reported absence of plasmodesmata in the walls of mature guard cells. Microinjection was also used to ascertain when dye coupling ceases during stomatal differentiation in *Allium*. Dye rapidly moves into and out of guard mother cells and young guard cells. Hovewer, dye movement ceases midway through development as the guard cells begin to swell but well before a pore first opens. Since plasmodesmata are still present at this stage, the loss of symplastic transport may result from changes in these structures well in advance of their actual disappearance from the guard cell wall.

Key words: *Allium* (stomatal cells) – *Commelina* – Dye coupling – Guard cell – Lucifer yellow – Plasmodesma.

Introduction

The guard cells of higher plants undergo dramatic turgor and shape changes as a result of ionic interchanges with adjacent cells (for review see MacRobbie 1981). The mechanisms that govern these ion movements are not well understood, but specific transport systems in the plasmalemma and tonoplast are probably involved. A number of ultrastructural studies have shown that plasmodesmata (Pd), though present in young guard cells of higher plants, disappear by the time cell differentiation is completed (Allaway and Setterfield 1972; Carr 1976; Willmer and Sexton 1979; Wille and Lucas 1984). The manner in which these channels are removed is perplexing, but their absence indicates that ion transport into and out of guard cells does not occur via a symplastic route but must traverse the plasmalemma. However, to our knowledge, a loss of symplastic movement has not been directly demonstrated in guard cells. Electrical coupling and the movement of solutes between cells have been investigated in animals and plants by electrophysiological methods (e.g. Overall and Gunning 1982) and by following the spread of injected dyes such as Lucifer yellow (LY), Procion yellow and fluorescein derivatives (Stewart 1981; Tucker 1982; Goodwin 1983; Erwee and Goodwin 1983, 1984; Warner et al. 1984). Lucifer yellow is of particular value in demonstrating dye coupling across gap junctions and Pd because of its relatively low molecular weight, high fluorescence intensity, and fixed negative charge that precludes leakage through the plasmalemma (Stewart 1981). In the work reported here, we have employed microinjection of LY to show that dye coupling ceases between guard cells and adjacent cells during stomatal differentiation.

* To whom reprint requests should be addressed

Abbreviations: DIC = differential interference contrast; GMC = guard mother cell; LY = Lucifer yellow; Pd = plasmodesmata

Materials and methods

Plants. Seeds of *Allium cepa* L. cv. White Portugal were obtained from the Joseph Harris Seed Co., Rochester, N.Y., USA and germinated in vermiculite moistened with distilled water. Epidermal slices were obtained from the cotyledons of 5-d-old

seedlings as previously described (Palevitz and Hepler 1974). Sprigs of *Commelina communis* L. were collected locally and epidermal peels were obtained from young leaves approx. 1–2 cm in length. Both slices and peels were gently pressed in a drop of distilled water between a slide and coverslip to remove trapped air and then transferred to a paraffin-lined well prepared on another slide. The ends of the slices and peels were secured to the slide surface with warm 5% agarose (low-temperature gelling type VII; Sigma Chemical Co., St. Louis, Mo., USA). After the agarose had gelled, the well was filled with distilled water plus or minus one or two drops of a solution containing 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.0, 5 mM KCl and 0.2 mM $CaCl_2$.

Lucifer yellow. Lucifer yellow CH was obtained from Sigma and working solutions (0.16–1.0 mg/ml) were prepared directly in distilled water or a solution containing 1 mM Hepes, pH 7.0, 1 mM KCl and 0.04 mM $CaCl_2$. The dye solution was filtered through Acrodiscs (0.2 μm pore size; Gelman Sciences, Ann Arbor, Mich., USA) immediately after preparation and again after storage in the refrigerator for 3–5 d. Fresh solutions were prepared regularly.

Micropipettes. Borosilicate-glass microcapillaries containing a single inner filament (1 mm outer diameter, 0.58 mm inner diameter; type 1B100F-4, World Precision Instruments, New Haven, Conn., USA) were cleaned in 10% nitric acid, washed in distilled water, rinsed twice in 100% acetone and air-dried on a hot plate. They were pulled into fine tips (impedance approx. 20 mΩ; outer tip diameter approx. 0.25 μm as determined by scanning electron microscopy) on a vertical pipette puller (Model 700D; David Kopf Instruments, Tujunga, Cal., USA) equipped with a platinum-iridium filament. The micropipettes were then backfilled with dye solution using a 1-ml tuberculin syringe fitted with a long 28-gauge needle.

Microinjection. Live cells were selected for microinjection by inspection with differential interference contrast optics (DIC; see below). Dye-filled micropipettes were mounted on a hydraulic micromanipulator (type MO-102; Narishige Scientific Instrument Laboratory, Tokyo, Japan) secured to the stage carrier of the microscope. A thin platinum wire lead from a homemade iontophoresis unit was then inserted in the rear of the pipette and a ground lead placed in the well fluid. Course approach to the cell was made using a 10X objective. Final approach and impalement were performed under a 40X, water immersion, long-working-distance objective used in the DIC mode. Current (approx. 0.3–2.0 nA) was then applied for less than 1 s, after which the cell was monitored in both the DIC and epifluorescence modes. Observations were intermittent to prevent damage to the cell from the light sources.

Microscopy and photography. Microinjection and subsequent examination of cells were performed on a Universal R microscope (Carl Zeiss, Oberkochen, FRG) equipped for epifluorescence and DIC optics). The cells in Fig. 1 were viewed on a Zetopan microscope set up for DIC (C. Reichert Optische Werke, Vienna). Lucifer-yellow fluorescence was stimulated with a 100-W mercury source, a filter combination providing broad-band blue excitation (390–440 nm), and an LP 520 barrier filter. Images were viewed with the aid of double intensified vidicon (model DV-2; Venus Scientific Inc., Farmingdale, N.Y., USA) and newvicon (model 65; Dage-MTI, Michigan City, Ind., USA) video cameras and black and white monitors (mainly model NV 5310; Panasonic, Atlanta, Ga., USA). A 640-nm short pass filter (Ditric Optics, Hudson, Mass., USA) was placed between the top exit port of the microscope and the video camera to suppress chlorophyll autofluorescence. Exposures were made directly from the video monitor using 35-mm Panatomic-X or Plus-X film (Eastman Kodak, Rochester, N.Y., USA) and developed in HC 110, dilution B (Eastman Kodak). Factor 8 (Min Max, North Hollywood, Cal., USA; company now defunct) was added to increase the sensitivity of Plus-X film to ASA 1000.

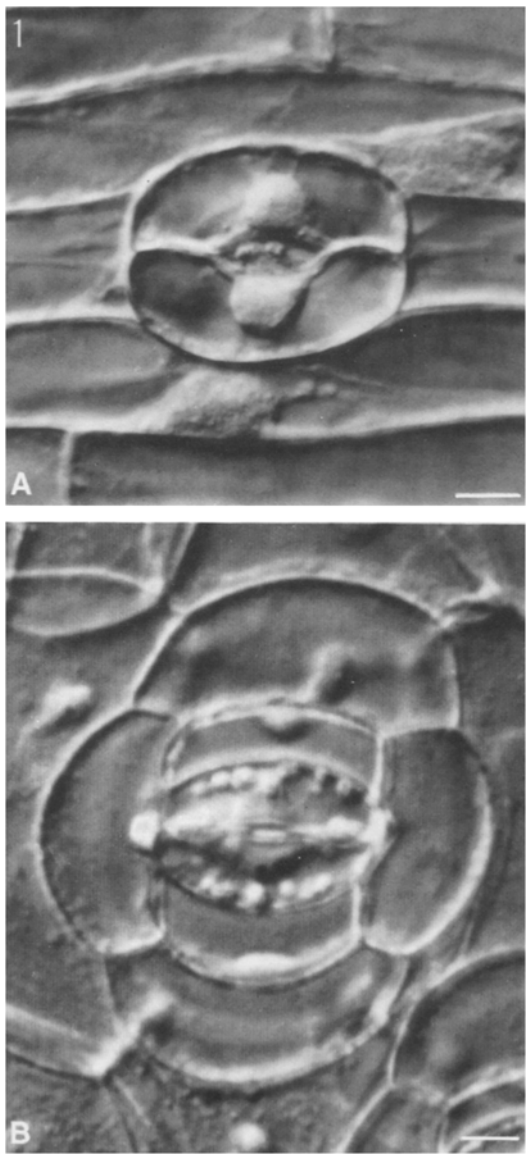

Fig. 1. A A stomatal complex in an epidermal slice taken from a cotyledon of *Allium cepa,* viewed with DIC. The epidermal cells contain transvacuolar strands of streaming cytoplasm and are thus intact and alive. ×775. **B** A stomatal complex in an epidermal peel taken from a young *Commelina communis* leaf. The guard cells are surrounded by four lateral and two terminal subsidiary cells. Only the guard and subsidiary cells are intact and alive. ×720. Figures 1A and B were viewed with a Reichert Zetopan microscope, a Dage-MTI newvicon camera and a Tektronix 634 monitor. Bars = 10 μm

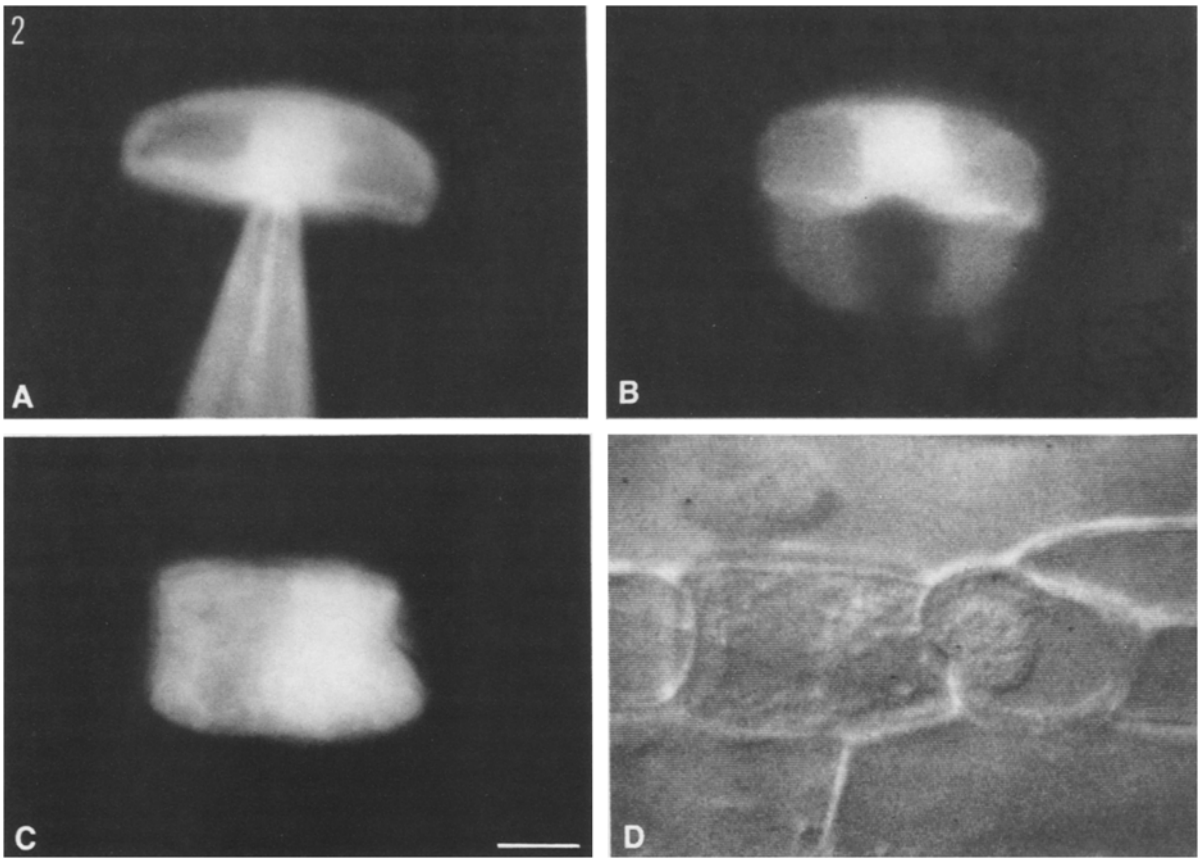

Fig. 2. A A mature guard cell of *Allium* approx. 8 min after microinjection with LY. An out-of-focus image of the micropipette is evident. Note that dye is present in the nucleus and cytoplasm of the guard cell, but has not spread to neighboring cells. This and all subsequent figures are ×1100; bars=10 μm. **B** A microinjected guard cell of *Allium*. The micropipette has been withdrawn. Note the bright LY fluorescence in the nucleus and cytoplasm. The much less intense vacuolar autofluorescence characteristic of *Allium* stomatal cells is evident in the lower, sister guard cell. Lucifer yellow has not spread to this cell (note that its nucleus is black) or to other epidermal cells 0.5 h after injection. **C** A proximal guard cell (of a proximal-distal pair) of *Allium* that has been injected with LY. Fluorescence is brightest in the cytoplasm and nucleus of the injected cell (compare with panel D). However, the cell contents are more evenly distributed than in the guard cells seen in panels A and B, and the fluorescence appears more uniform as a result. The cell was alive and the dye had not spread to adjacent cells 1.5 h after injection. **D** A DIC image of the same proximal-distal stomatal complex seen in panel **C**. The suboptimal quality of the figure is due to the use of the double intensified vidicon camera in this nonfluorescence format. Note that the distribution of cytoplasm and nucleus largely corresponds to areas of brighter fluorescence in panel C

Results

Effects of microinjection. Guard cells and subsidiary cells of *Allium* and *Commelina* could be routinely impaled with the tips of our micropipettes. The observations on dye coupling described below are based on the impalement of more than 50 stomatal cells of *Allium* and nearly 20 from *Commelina*. Guard mother cells (GMCs) and young guard cells are easier to impale successfully than mature ones, presumably because of their thinner walls. Nevertheless, mature cells can be successfully penetrated through the dorsal, ventral and paradermal walls. The guard cells of *Commelina* are easier to penetrate than those of *Allium*.

Some guard cells or GMCs collapse within 1 min, usually following a gross penetration, but such cells were not included in the results described here. Other cells lose turgor more slowly (after approx. 10 min), while many seem unaffected except for a cessation of cytoplasmic streaming. Guard cells or GMCs can form wound plugs around the pipette tip, regain turgor and resume streaming. Guard mother cells can also divide after impalement. No change in dye distribution has been seen in recovered cells monitored for as long as 1.5 h (e.g. dye remains confined to an injected guard cell). Moreover, there is no difference in dye distribution in relatively unaffected cells and those that exhibit delayed shrinkage. Since the spread of dye,

when it occurs, is usually rapid (1–3 min), this last observation is not surprising. We also note that compatible patterns of dye movement are seen when adjacent cells are injected and movement of LY *into* guard cells is ascertained. These last-named experiments serve as controls against mechanical injury being a factor in our results. It is also noteworthy that consistent results are obtained with *Allium* epidermal slices, in which most epidermal cells are alive and intact, and *Commelina* peels, in which most nonstomatal epidermal cells are damaged (Fig. 1). We thus belive that our cells are rather gently penetrated and the observations reported here are not complicated by injury artifacts. However, we did try to minimize the effects of impalement even further by the use of an external osmotium (0.3 M sorbitol) and by varying the Ca^{2+} concentration in the external and-or internal medium. These attempts did not improve the results, however.

All of the results described below were obtained with injections into the cytoplasm or nucleus. By carefully positioning the micropipette tip and patiently applying force, we have been able to avoid vacuole injections, except in a few instances. The latter cases are not included in the observations described below.

Injection of Lucifer yellow into mature guard cells of Allium. When mature guard cells of *Allium* (defined as fully elliptical in shape and bordering a newly opened pore; Fig. 1A) are iontophoretically injected with LY, the dye rapidly spreads throughout the cytoplasm and nucleus (Fig. 2A, B). This fluorescence is much brighter than and clearly distinguishable from the vacuolar autofluorescence characteristic of *Allium* guard cells (Fig. 2B; see also Zeiger and Hepler 1979; Palevitz et al. 1981). The LY fluorescence does not spread into sister guard cells or neighboring epidermal cells even 90 min after injection. The same lack of dye movement is also observed in the proximal-distal guard cells (Fig. 2C, D) that result from abnormal division sequences in this species (Palevitz and Hepler 1976).

Injection into stomatal complexes of Commelina. We have also found that mature guard cells are not dye-coupled to each other or to neighboring cells in *Commelina communis* epidermal peels. The stomatal complexes of this species differ from those of *Allium* by the presence of six subsidiary cells bordering the guard cells (Fig. 1B). The guard cells of *Commelina* also contain large, green chloroplasts. When a live (streaming) *Commelina* guard

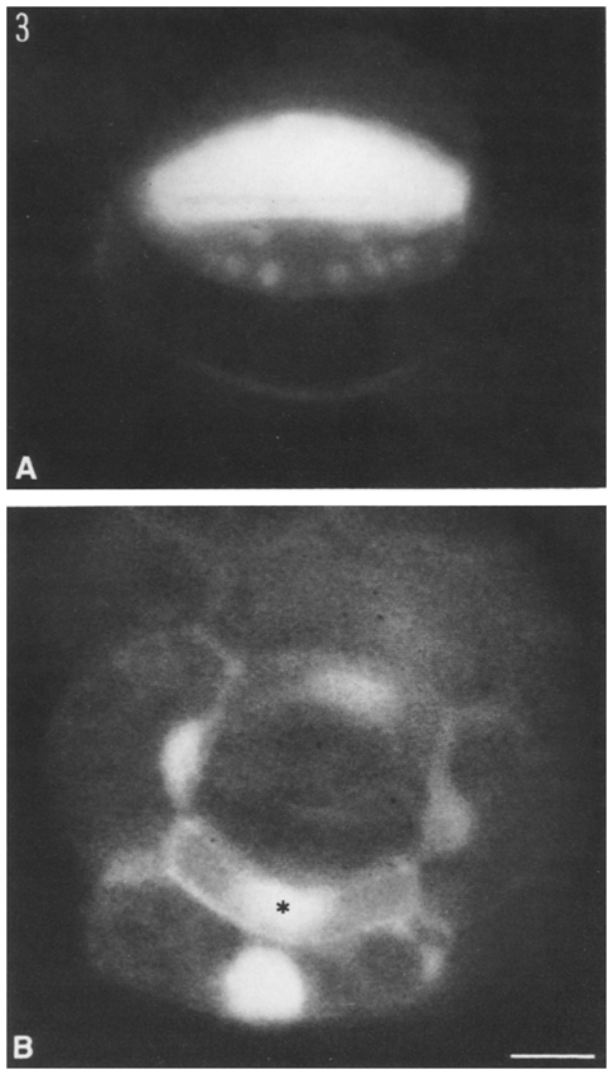

Fig. 3. A A mature stomatal complex of *Commelina*. One of the guard cells has been injected with LY and is still very fluorescent 24 min later. Dye has not spread to the subsidiary cells or to other cells in the epidermis. Plastids in the lower, sister guard cell exhibit dim autofluorescence. **B** A *Commelina* stomatal complex in which a subsidiary cell (*) has been injected with LY. Dye has quickly (1–3 min) spread to the other subsidiary cells (note fluorescent nuclei) but not to the guard cells in the center of the complex

cell is injected with LY, the cytoplasm and nucleus become very fluorescent, but none of the dye spreads to the sister guard cell or to surrounding subsidiary cells (Fig. 3A). In order to probe dye movement in these complexes further, lateral and terminal subsidary cells have been injected. Dye quickly spreads to the cytoplasm and nuclei of all other subsidiary cells, but not to the guard cells (Fig. 3B). In other experiments, a guard cell has been injected and, after ascertaining that dye has not spread to adjacent cells, a subsidiary cell is

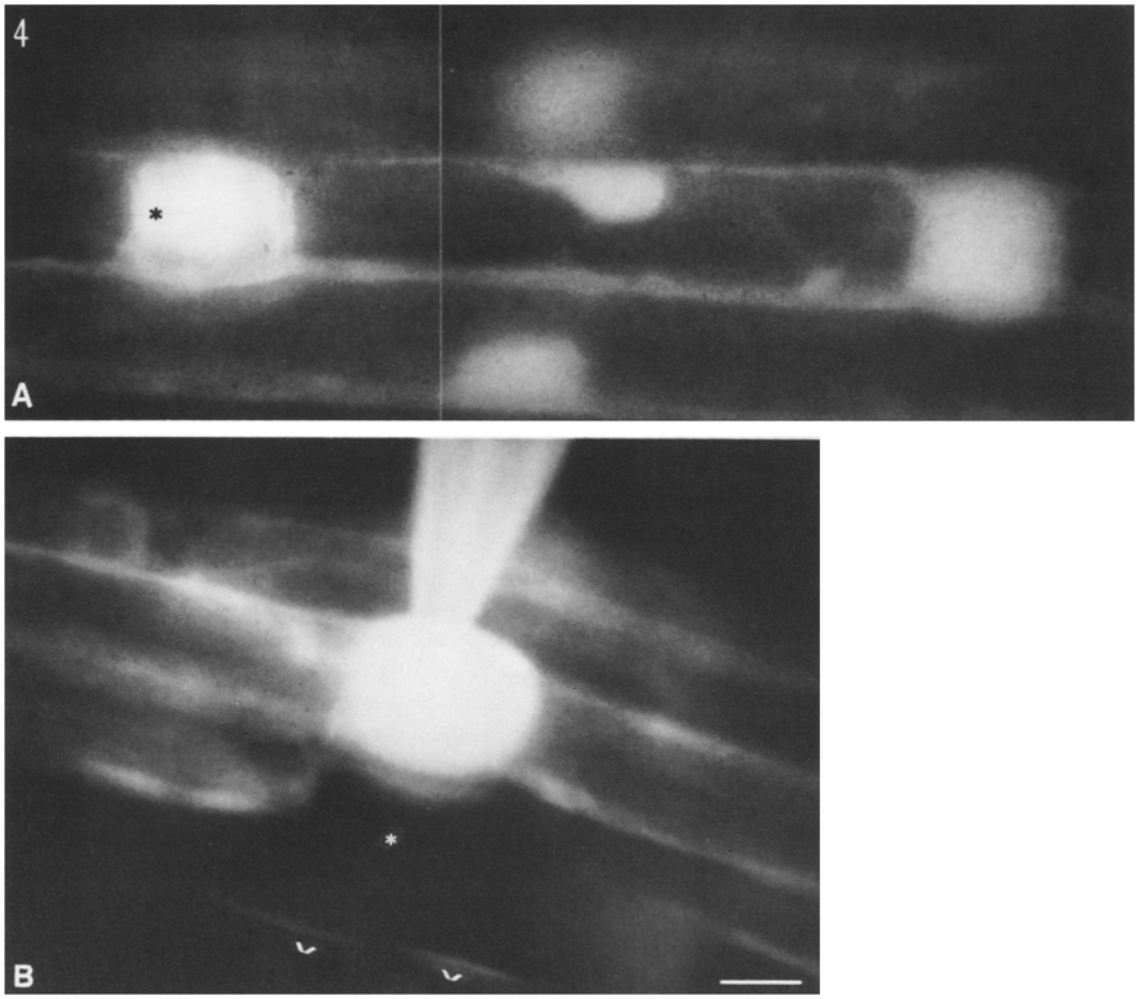

Fig. 4. A The *Allium* GMC (*) at the left of the figure has been injected with LY. Dye has spread to the cytoplasm (note fluorescent transvacuolar stands) and nuclei of adjacent intact epidermal cells and to another GMC at the right of the figure. **B** A young guard cell of *Allium* injected with LY (the micropipette is still present). Dye has spread to the sister guard cell and to the surrounding epidermal cells less than 5 min after injection. Because of the intensity of fluorescence, the two young guard cells cannot be distinguished. A complex containing more differentiated guard cells is located at the asterisk (*). Note that dye has not entered these cells, although some fluorescence is evident in epidermal cells on the other side of the complex (*brackets*). The micrograph was taken approx. 8 min after injection

then injected. Dye quickly spreads to the other subsidiary cells of the complex, but not to the uninjected guard cell. Thus, the lack of dye movement after a guard cell is injected is not a consequence of disruption of adjacent subsidiary cells. Although spread of LY from subsidiary cells to other epidermal cells outside the complex has not been observed, this is not unexpected given the disruption of the epidermal cells that occurs during the peeling process.

Injection into immature stomatal cells of Allium. The above results show that mature guard cells are not dye-coupled to each other or to neighboring cells. We now ask whether immature cells are dye-coupled, and if so, when coupling ceases during differentiation. Epidermal slices from *Allium* cotyledons have been used for these studies because they contain many live GMCs and young guard cells, in addition to the mature guard cells used above. The reader can refer to Palevitz and Hepler (1974, 1976) for a more complete description of these cells and illustrative DIC micrographs. When GMCs of *Allium* are injected, dye fluorescence quickly spreads to the nuclei and cytoplasm of adjacent epidermal cells (Fig. 4A). Furthermore, spread of the dye continues to successive epidermal cells and to other GMCs (Fig. 4A). Lucifer yellow injected into young, recently-formed guard cells also spreads rapidly, both into sister

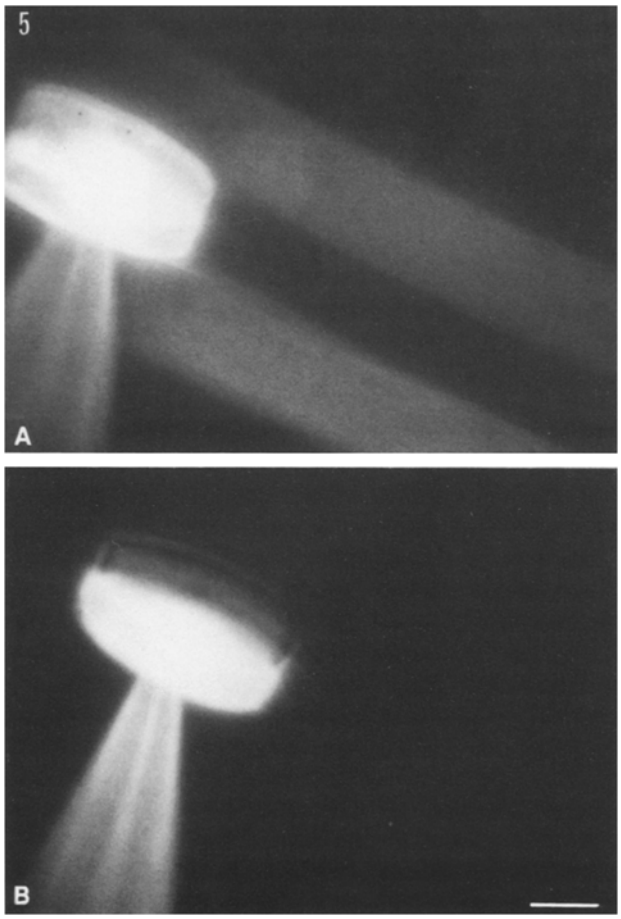

Fig. 5. A In this mid-differentiated complex of *Allium*, dye injected into the lower guard cell has spread to the sister cell and to flanking epidermal cells. The cells were photographed approx. 10 min after injection. **B** In this more mature complex (the guard cells are more swollen than those in panel A, but a pore has not yet formed between them), injected dye has not spread to other cells. The faint fluorescence associated with the sister guard cell is a focus artifact. Spread of dye did not occur by 15 min after injection, although this micrograph was taken earlier

guard cells and adjacent epidermal cells (Fig. 4B). Dye coupling appears to cease, however, near the middle of differentiation. In general, cells that have begun to swell noticeably are uncoupled (Fig. 5B), whereas somewhat younger cells that are not as swollen but have thickening walls remain coupled (Fig. 5A). However, this distinction is not clear-cut, since uncoupled cells with the latter appearance have also been found. The LY does not move out of older cells that are nearly elliptical in shape but do not as yet have open pores. We have never encountered a complex in which dye spreads to a sister guard cell but does not move into nearly epidermal cells, or vice versa. Thus, loss of coupling seems to occur between a guard cell and all adjacent cells at approximately the same time.

Other experiments in which dye is injected into a nearby cell reinforce these findings. Dye moves *into* GMCs and young to mid-stage guard cells, but not into more differentiated guard cells (Fig. 4A, B).

Discussion

A number of reports have shown that the walls of mature guard cells lack Pd (Allaway and Setterfield 1972; Carr 1976; Willmer and Sexton 1979; Wille and Lucas 1984), whereas those between subsidiary cells retain them (Carr 1976; Couot-Gastelier et al. 1984). Since symplastic transport is dependent on Pd (see Gunning and Robards 1976 for reviews; also Erwee and Goodwin 1984), their absence in guard cell walls indicates that the ion transport necessary for stomatal action occurs via the apoplast and is regulated in part by plasmalemma channels or pumps. This conclusion seems even more logical in light of the steep ion and electrical gradients between guard cells and surrounding epidermal cells (MacRobbie 1981; Edwards and Bowling 1984). Recently, potassium-selective channels have been identified in guard cell protoplasts of *Vicia faba* using patch-clamp procedures (Schroeder et al. 1984). Our observations show that microinjected dye does not pass between two mature guard cells or between a guard cell and adjacent epidermal or subsidiary cells in epidermal slices and peels. Dye movement is rapid, however, between subsidiary cells and between GMCs or immature guard cells and adjacent cells. These results thus provide direct evidence that guard cells become symplastically uncoupled from each other and from neighboring epidermal cells during development.

We cannot rule out the possibility that some solute species (e.g. ions) much smaller than LY are still transported through plasmodesmata between our dye-uncoupled cells. However, the movement of LY has been correlated with electrical and ionic coupling in animal cells that are linked by gap junctions (Stewart 1981; Warner et al. 1984). It is noteworthy that the upper size limit for movement through gap junctions is in the same range (<1000 Da) as that determined for transport through plasmodesmata (Tucker 1982; Goodwin 1983). Since the molecular weight of LY (457.2 Da) is well below this limit, its movement is undoubtedly indicative of the symplastic transport of at least some solutes.

Plasmodesmata are present in the walls of GMCs and young guard cells, but are somehow lost when the latter mature. The stage at which

this occurs and the mechanisms responsible are still unclear. Our results might indicate that Pd are lost as the cells become noticeably swollen but before the pore initially opens. However, our electron micrographs (e.g. see Palevitz and Hepler 1976) and those of Wille and Lucas (1984) show that Pd are still present (especially in the end walls) in clearly swollen guard cells of *Allium*. Indeed, only much older, mature cells seem to lack them (Allaway and Setterfield 1972; Palevitz and Hepler 1976). Wille and Lucas (1984) report that the Pd in *Allium* guard cells become truncated during development, but our micrographs indicate that at least some of them still traverse the walls of guard cells similar in appearance to those in which dye coupling has ceased. It may thus be valuable to consider an alternative explanation for the loss of dye coupling. That is, alteration of the Pd may begin as subtle changes which are not visible in the electron microscope but which alter the function of these structures well in advance of their removal from the wall. Erwee and Goodwin (1983, 1984) have found that aromatic amino acids and divalent cations such as Ca^{2+} reduce the symplastic transport of fluorescein conjugates and may normally play a specific role in uncoupling cells during differentiation. In this regard, it is noteworthy that developing guard cells accumulate various substances including K^+ and organic acids (Palevitz and Hepler 1976; Palevitz 1981; Palevitz et al. 1981). Recently, high levels of Ca^{2+} have also been detected in or around the stomatal complexes of oat coleoptiles (Dauwalder et al. 1983 and personal communication; Department of Botany, University of Texas, Austin, USA). Further investigations into the mechanisms responsible for the loss of dye coupling in the stomatal complex are clearly warranted.

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