LOW RESISTANCE CONNECTIONS
BETWEEN CELLS IN THE
DEVELOPING ANTHER OF THE LILY

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ABSTRACT

Low resistance junctions were demonstrated between cells in anthers from young buds of *Lilium longiflorum* Croft by standard electrophysiological techniques. Electrodes containing a dye were used to stain impaled cells for later histological identification. Electrical coupling is widespread; germinal cells are coupled to one another; coupling is also observed between somatic elements, and germinal and somatic cells are similarly interconnected. Cytoplasmic bridges are implicated in the first case; plasmodesmata are probably responsible for the interactions in the other two. Although the physiological role of the low resistance junctions shown here and present in embryonic animal tissues is unknown, the possible function of this form of intercellular communication in the development of the anther is discussed.

INTRODUCTION

The interiors of some neurons have been convincingly demonstrated to be connected by low resistance junctions (1, 8, 24). Such connections between like cells coordinate their signaling activity by permitting the passage of electric currents presumably carried by inorganic ions. In neuroglia and epithelia such as the liver and other glands, cells of the same kind are in electrical communication with one another, although these cells do not generate action potentials and there is as yet no evidence of electrical signaling between them (16–19, 21, 25). Even cells that are unlike one another in their developmental potential and in their recognizable morphology are connected in embryos, such contacts remaining until late stages of differentiation (9, 15, 28, 32, 33). Again no rapid signaling activity has been detected.

The demonstration that many types of animal cells, both embryonic and mature, possess specialized contacts permitting the intercellular transfer of ions has been well correlated with the presence of special regions of membrane contact (tight or "gap" junctions) seen with the electron microscope (for recent reviews of the rapidly expanding literature see references 2, 9, 18). There is little doubt that these regions of membrane specialization are the site of ionic exchange. The observation that small dye molecules injected into cells pass from cell to cell is consistent with the passage of substances larger than inorganic ions (9). Highly modified areas of cell contact which allow ions to pass between the interiors of cells without entering the extracellular space make the direct, nonsecretory passage of larger substances possible. Conclusive evidence that the cell-to-cell passage of dye is by way of the specialized membranes of tight or gap junctions is lacking, however.[1] The presence of widespread coupling in embryos and between cells exhibiting contact inhibition (20) has raised the possibility that low resistance junctions play a role in control of growth and differentiation.

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[1] *Note Added in Proof:* A paper dealing with this problem has recently appeared: Payton, B. W., M. V. L. Bennett, and G. D. Pappas. 1969. *Science (Washington).* 166:1641.

The possibility of direct interaction between cytoplasms of adjoining cells, now receiving increasing attention from animal physiologists, has been of interest to plant physiologists ever since the investigation of plasmodesmata with the electron microscope (3, 22, 23, 27, 29). Several early studies suggested that a tubule of endoplasmic reticulum provides intercellular continuity through the plasmodesma (e.g. reference 3). It has been shown that electrically excitable plant cells also manifest electrical coupling. The internodal cells of the brittlewort *Nitella translucens*, separated by a single nodal cell, have been demonstrated to be coupled (34). Action potentials generated in one internodal cell cause the passage of sufficient ionic current across the nodal cell to bring the next internodal cell to threshold and trigger another action potential. The only observed connections between the cells are plasmodesmata, and it is very likely that the passive spread of current occurs through these channels.

As in the case of animal tissues, it is of interest to see if the direct spread of substances can be demonstrated in plant tissues which interact inductively or exchange nutrients. A particularly interesting case in this regard is the development of the male gametophyte or pollen grain (microspore) in angiosperms, generally involving the differentiation of several tissues. Within each of the four roughly cylindrical lobes of an anther the parenchymal cell gives rise to two separate cell types in a characteristic relationship to one another: (a) a central core of microsporocytes which will undergo meiosis, and (b) a sheath of surrounding tapetal cells. It has been suggested that the tapetal cells serve an inductive or supportive role in the differentiation of the germinal cells (35, 39); the connections between these two elements form the subject of this investigation. The lily is particularly well suited to examination because the differentiation occurs synchronously in a large number of cells over a relatively long period of time (14, 38). Bud length is well correlated with the stage of development (6, 14, 38), and, as a result, it is relatively simple to obtain material to examine the relationship between the two elements at any stage in their maturation.
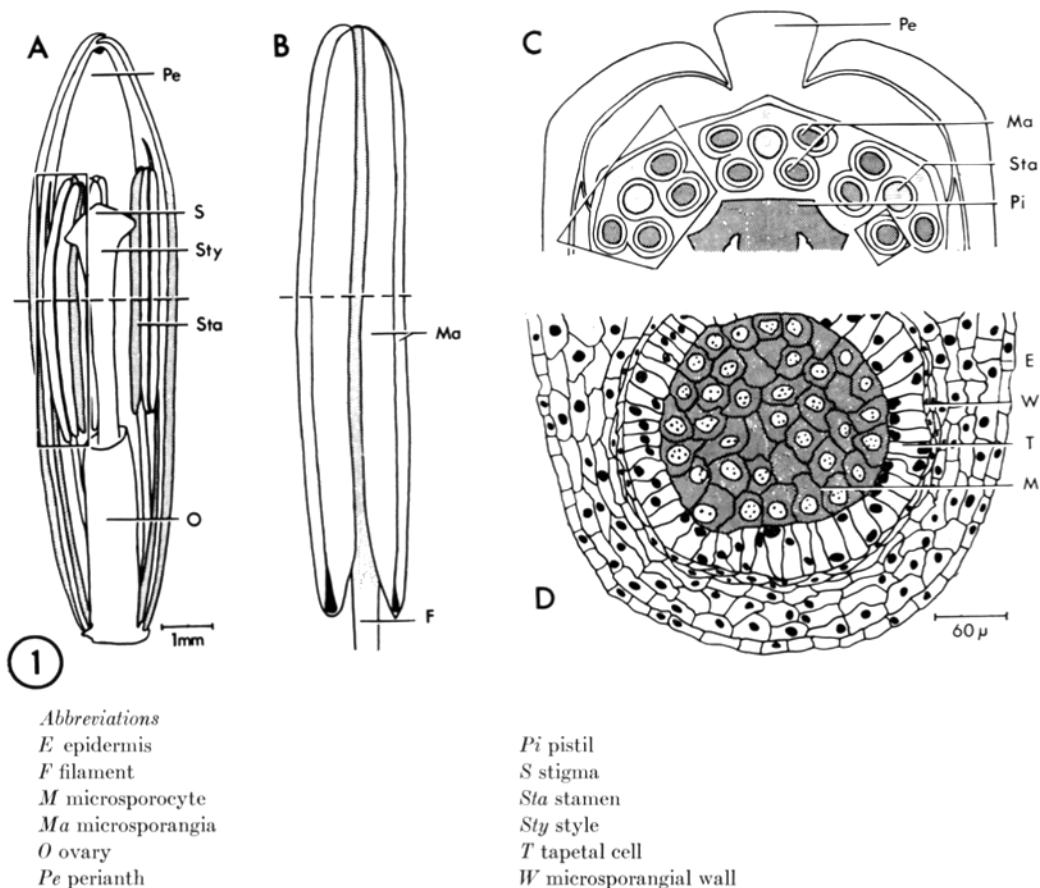
MATERIALS AND METHODS

Budding plants of *Lilium longiflorum* Croft were obtained locally from a commercial greenhouse. Experiments were performed over a 10 day period during which the plants were kept at 4°C in a dark room by night and exposed to approximately 8 hr of sunlight at room temperature by day. A bud of appropriate stage of development was selected and cut from the plant at its base. The length from tip to receptacle was measured, and later histological examination of all buds confirmed the generally accepted relationship between bud length and stage of development (6, 14). Thin, longitudinal sections (200-500 μ) of the whole bud were cut freehand with a razor blade and placed in a plastic Petri dish containing a physiological saline (see below) at room temperature (25°C). The sections were pinned with small stainless steel needles to a layer of clear plastic (Sylgard, Dow Corning Corp., Midland, Mich.) on the bottom of the dish. From each bud several favorable slices were obtained in which intact germinal and tapetal cells were exposed at the cut edge of a lobe of an anther. The anatomy of the preparation is best seen in cross-sections; Fig. 1 D illustrates a cross-section of a microsporangium prepared in this manner. Using both dark- and bright-field illumination with a dissecting microscope, it was possible during the experiment to see the central cylinder of germinal cells and the tapetal cells on either side (see Fig. 2).

The saline employed was White's modified solution as used by Ito and Stern (14), omitting vitamins and all components present in less than 10 mg per liter ($MgSO_4 \cdot 7H_2O$, 750 mg per liter; $Ca (NO_3)_2 \cdot 4H_2O$, 300; Na_2SO_4, 200; KNO_3, 80; KCl, 65; $NaH_2PO_4 \cdot H_2O$, 19). Sucrose was present at a concentration of 0.3 M and the pH was adjusted to 5.6-5.8.

Standard electrophysiological procedures were used to determine coupling between cells. Two glass micropipettes (10-15 MΩ when filled with 3 M KCl) containing a 3.5% aqueous solution of a negatively charged blue dye (Niagara sky blue 6B [Chicago Blue]; mol wt 993) were employed; each was capable of passing current or recording potential changes differentially (see Fig. 3). The current was measured across a resistor between the bath electrode and ground and displayed on one beam of an oscilloscope. The voltage changes were fed into a high input impedance amplifier and displayed on another channel of the oscilloscope. The electrodes were positioned above the desired cell types and then lowered until an abrupt negative change in the potential recorded from each indicated that the tips were intracellular. First, a rectangular current pulse was passed through one electrode, and the concurrent changes in the potential of the inside of the other impaled cell were measured. In all experiments the roles of the stimulating and recording electrodes were next reversed so that the spread of current in the opposite direction across the junction could be tested. To show that the recorded voltage changes were being measured across the cell membranes, the recording electrode was withdrawn from the cell that it impaled so that the tip was

Abbreviations

E epidermis	*Pi* pistil
F filament	*S* stigma
M microsporocyte	*Sta* stamen
Ma microsporangia	*Sty* style
O ovary	*T* tapetal cell
Pe perianth	*W* microsporangial wall

FIGURE 1   Diagram of bud anatomy and preparation: A, longitudinal bisection of lily bud; B, stamen, enlarged from rectangle in A; C, transection of A (dotted line), trapezoid encloses area seen upon transection of B (dotted line); D, higher power view of a cross-section of the experimental preparation, a single anther lobe or microsporangium (square in C). Scale at left for A; right scale for D.

just extracellular, and the current pulse was passed again. Hyperpolarizing currents were used throughout in order to deliver the negatively charged dye from the electrodes into the impaled cells; the rapid staining of a small and sharply bounded area of the tissue confirmed that the electrode tip was intracellular.

When the current electrode was just extracellular, a current pulse produced a small puff of blue dye at its tip which disappeared within a second; the tissue was not stained. At the conclusion of this procedure the positions of the two stained cells were noted on a map of the tissue and two other cells were selected. After moving from one end of the anther slice to the other, the experiment was terminated and the tissue was prepared for histological examination. Although as many as eight attempts were made, difficulties in obtaining resting potentials, passing dye out of both electrodes, and achieving good histological preserva-

tion led to an average of only two successes per slice.

The sections were fixed overnight at 2°C in a Sandborn fixative (30) modified as follows: glutaraldehyde, 6.5%; acrolein, 2%; 0.1 M acetate buffer, pH 4.0; the final osmolarity was 1050 milliosmols. The low pH was necessary for retention of the dye in the tissue (28). After dehydration in methanol in a graded series of dilutions with acetate buffer and clearing in propylene oxide, the sections were embedded in a thin disc of Epon. The stained cells could be identified in the block with the use of a compound microscope, and their position could be correlated with a map constructed during the experiment (see Fig. 2). Serial 8-μ sections of those cell pairs which were well stained and for which good recordings had been obtained were then cut with a steel knife (cross-sections as in Fig. 1 D). Recognition of stained cells was facilitated by the red fluorescence of the dye when the sections were examined with
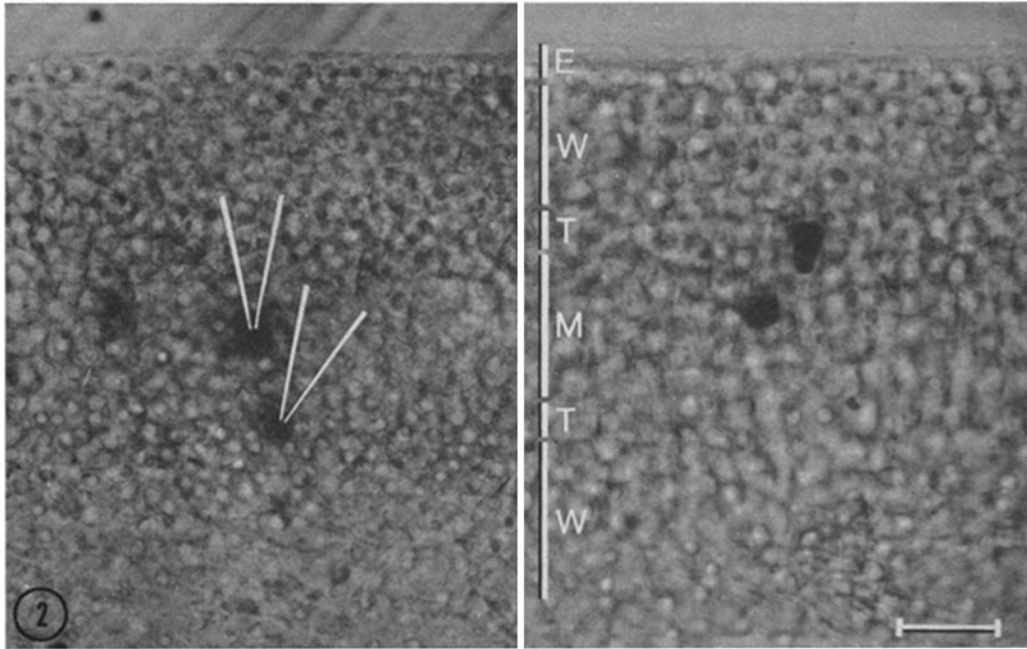
FIGURE 2 Anther slices flat-embedded in Epon blocks. The different tissues (legend as in Fig. 1) were as distinguishable during the experiment as here in the blocks. Note two tapetal-germinal cell pairs stained by electrophoretic injection of dye in two experiments. The positions of the microelectrodes are indicated on the left, where the tapetal cell is the lower of the two; on the right the tapetal cell is the upper one. The stain is well localized in the injected cells. Calibration 100μ. ×135.
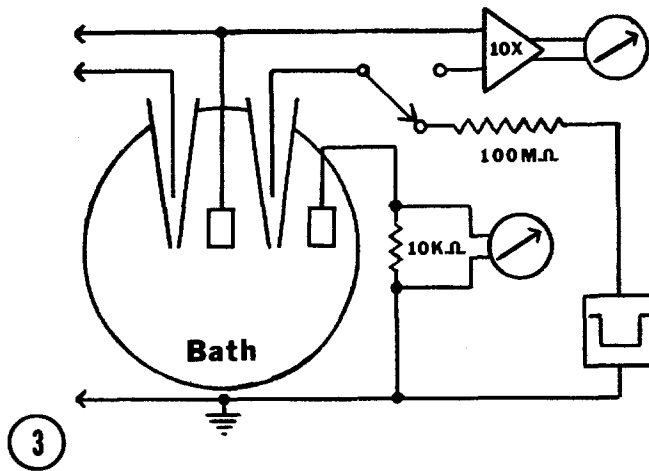

FIGURE 3 Diagram of the circuit for alternately recording membrane potentials and passing current with each electrode. Only one side of the symmetrical circuit is presented; both bath electrodes served both sides of the circuit.

dark-field illumination. Identification of cell type was straightforward.

RESULTS

13 buds, from four plants, ranging in length from 7 mm (early preleptotene) to 22 mm (diplotene)

(14) were studied. 26 examples of coupling in the lily anther were obtained.

Upon penetration of a cell, an abrupt voltage shift was observed, usually of about 5 mv and occasionally as large as 20 mv. This potential shift was seen in reverse in about half the cases when
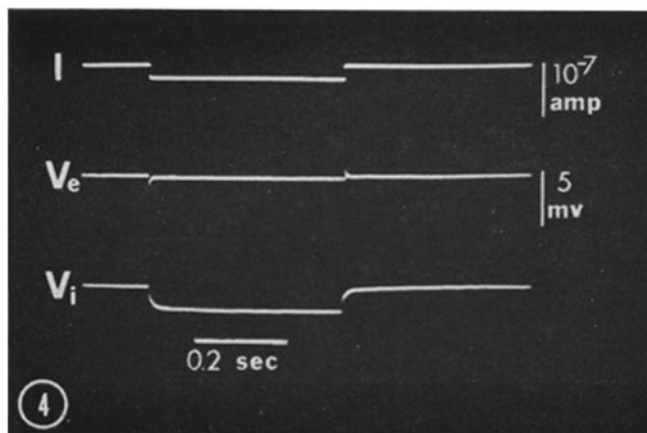
FIGURE 4 Electrical records for Fig. 5 show I, current pulse; V_e, voltage recorded extracellularly; V_i, voltage recorded intracellularly. No resting potential was measured upon withdrawal of one of the electrodes.

the electrodes were withdrawn after staining the impaled cells; in the other cases the potentials measured were smaller. These potential shifts were of interest in this study only as evidence that the electrodes were intracellular and should not be interpreted as accurate values of the resting potentials.

Coupling between Tapetal and Germinal Cells

In 15 instances a tapetal and a germinal cell constituted the impaled pair. Fig. 4 illustrates such a finding. The electrical records are taken from two successive sweeps of the beam across the face of the oscilloscope. During the first, the recording electrode was intracellular and a voltage change was recorded (V_i). The electrode was then placed just extracellularly, and on the second sweep only a very small potential shift was noted (V_e). The failure to record a resting potential in one of the cells at the end of the experiment reflects damage to the cell by the impalement and decline of this potential during maintained penetration, but does not detract from the fact that the interior of the cell was included in the circuit of ionic current flow. The cells were 600 ± 30 μ apart (Fig. 5; all distance measurements were made on fixed tissue; the error in measurement of center-to-center distance is equal to twice the average cell radius). It is significant that the two cells lay at a distance of many cell diameters from each other, because this eliminated the possibility that both electrodes were in the same cell and necessitated the spread of current through many intervening cells. The ratio of potential change in one cell to the current injected into another provides a rough measure of the strength of coupling; in this case the ratio,

which may be referred to as the transfer resistance, was 5 × 10^4 Ω. Fig. 6 illustrates coupling between tapetal and germinal elements (microsporocytes) that are much closer together; both stained cells appear in the same section (220 ± 30 μ apart). As expected, the coupling is greater and the transfer resistance is larger than in the previous example (1 × 10^5 Ω). Observations of coupling between these two elements were made consistently, and it is likely that any tapetal cell is well coupled to a nearby germinal cell. There were four further cases of coupling between cellular elements which could not be positively identified as tapetal-germinal cell pairs by their morphology or location in the anther. In all experiments in which current spread from one cell to another was observed, the transfer resistances in each direction were equal and the system thus appears to be linear; no rectification was seen.

Coupling between Germinal Cells

Clear records of coupling between germinal cells were obtained in four instances, one of which is illustrated in Fig. 7. Only one of the two injected cells is shown; the other lies in a different section 400 ± 30 μ away. In this bud the germinal cells are in the diplotene stage of meiotic prophase, and coupling between them is quite evident; the transfer resistance is 1.8 × 10^4 Ω. The spread of dye to a contiguous cell, which occurred to varying degrees in nearly half of the experiments, is considered in the Discussion.

Coupling was also observed between tapetal cells and microsporangial wall cells, and, in several experiments, between pairs of wall cells. The epidermal cells could not be impaled success-
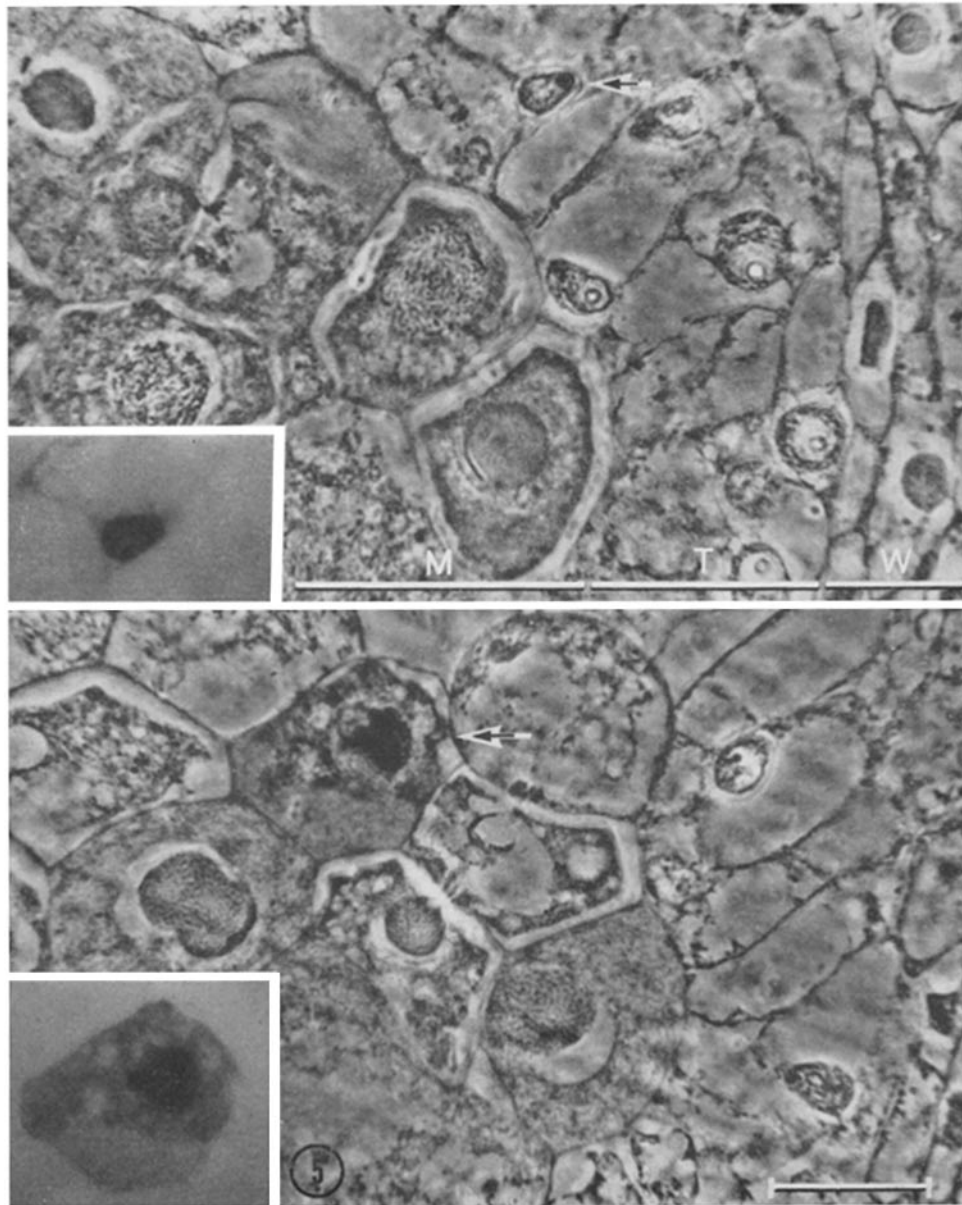
FIGURE 5   Electrical coupling between two cells: phase-contrast photomicrographs of stained tapetal cell (above) and stained germinal cell (microsporocyte) to which it was coupled (below; arrows). Material from an 18 mm bud; the tissues are labeled as in Fig. 1. *Inserts:* stained cells photographed with bright-field optics which reveal only the dye. The dye is clearly confined to a single cell in each case. Calibration 30μ. ×700.

fully. Since germinal cells ultimately develop into distinct pollen grains, or microspores, they must at some point become electrically uncoupled from each other and from tapetal cells. However, the cells were seen to be coupled even in late buds whenever they appeared in contact, and the integrity of the tissue was not disrupted (see Discussion).
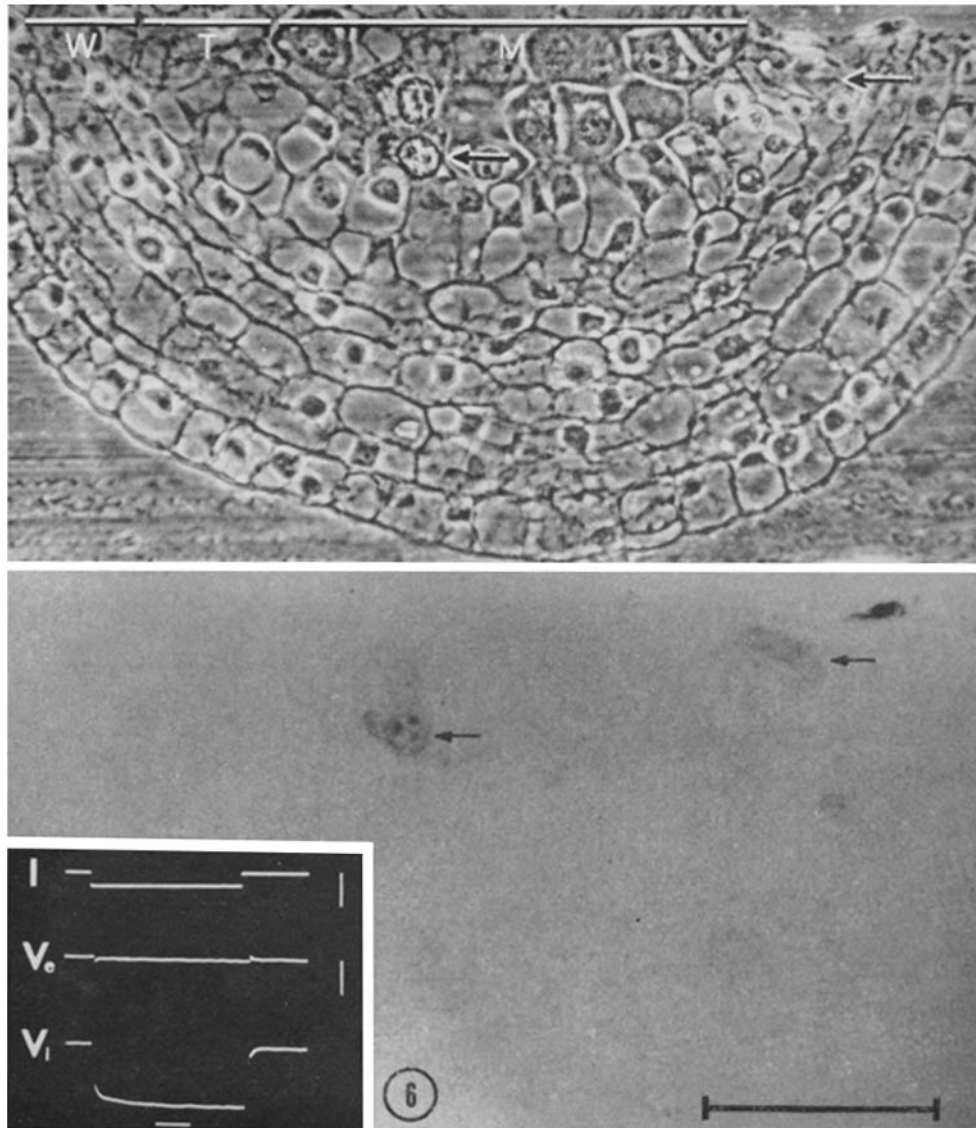
FIGURE 6 Both stained cells between which coupling was observed appear in same section (arrows), seen in phase contrast (upper) and with bright-field optics (lower). Material from a 12 mm bud; tissues labeled as in Fig. 1. Calibration 100μ. ×310. *Insert:* electrical records labeled as in Fig. 4. Calibration for I is 2×10^{-7} amp.; for V, 5 mv; time is 0.2 sec.

DISCUSSION

There were no special problems associated with the electrical measurements made on these developing plant cells; the techniques used were those employed in investigating commonly studied animal cells. The cell walls of the germinal, tapetal, and wall cells offered no particular barrier to

impalement with microelectrodes. On the other hand, in common with many electrically inexcitable animal cells (e.g. glia), these cells often failed to form a tight seal around an impaling electrode, and the resting potential declined during the duration of the impalement.

The observation that ionic current supplied to the inside of one cell gives rise to a voltage change
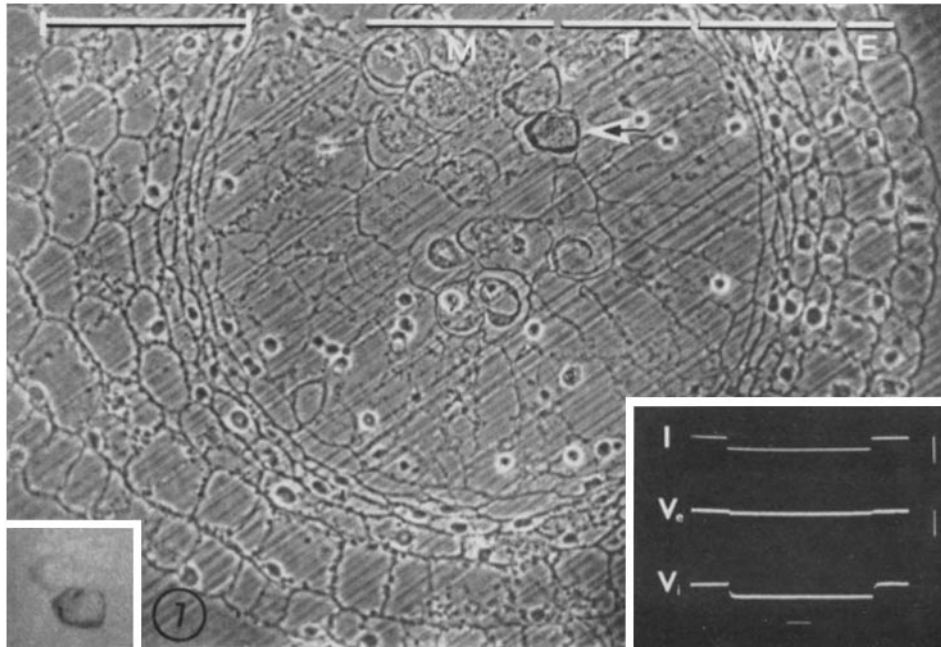
FIGURE 7 Coupling between two microsporocytes. Phase-contrast photomicrograph of one stained cell (arrow); other impaled cell lies in a different section. Material from a 21 mm bud; labeling as previously indicated. Calibration 100μ. ×280. *Left insert:* bright-field optics reveal dye in two germinal cells (see Discussion). *Right insert:* electrical records; calibration for I is 2×10^{-7} amp.; for V, 2 mv; time is 0.2 sec.

in an adjacent (or distant) cell is most readily explained by supposing that there are low resistance pathways connecting the interiors of the cells. The evidence for the anatomical basis for such low resistance paths is discussed below. Alternatively, a similar voltage drop could be produced if there were a large resistance between a point just outside the cell membrane and the bath electrode. This might result if there existed a sheath of high resistance enclosing the tissue or if the extracellular resistance between closely packed cells were very large in relation to the transmembrane resistance. These possibilities are not likely, because the potentials disappeared when the recording electrode was just outside the cell in extracellular space. Moreover, many instances of coupling were observed between cells at or near the surface of the anther slice where there is no visible structure which could provide such a resistance. One seems justified in concluding that ions moved from one cell to the next by a specialized, low resistance pathway.

The possibility that coupling is a peculiar attribute of this preparation must be considered, since the observations were made on relatively thin sections of tissue in which numerous cells had been damaged or brought in contact with an artificial environment. The question of an artificially created low resistance pathway arises. While this possibility cannot be finally dismissed until a way can be found to impale the intact anther, it seems highly unlikely in view of the cases in which coupling was observed between elements that lay several cell diameters below the cut edge of the anther slice and were often widely separated from each other. In these cases ions passed through cells that had not been directly traumatized and were several cell layers away from the saline solution; the coupled cells were not noticeably pushed together or pulled apart in the process of preparing the tissue for experimentation. These observations also help to eliminate the possibility that coupling is an artifact arising from damage incurred by cells during penetration with microelectrodes.

In this regard it is reassuring that there is extensive morphological evidence for specialized

intercellular connections in the anther which may provide the paths for the flow of ions between the interiors of cells. It has been known since 1911 that the microsporocytes of the lily are interconnected by cytoplasmic bridges (roughly 1 μ in diameter) that organize this tissue into a syncytium in early meiotic prophase (12, 13, 35). This phenomenon has been observed in the development of germinal cells in animals as well (7). Since large cytoplasmic organelles (mitochondria, endoplasmic reticulum) have been seen in these bridges, it is not surprising that small ions can pass relatively freely from one cell to another, as demonstrated in records of electrical coupling. The synchronous development of the germinal cells within a single microsporangium may be at least partly the result of these interconnections.

Smaller intercellular channels, the plasmodesmata, have been observed to connect elements of many plant tissues; these have been the object of some electron microscopic study and are morphologically quite distinct from cytoplasmic bridges. In cases in which they have been carefully examined, it has been demonstrated that they have a diameter of 200–500 A and are lined with a plasma membrane that often appears continuous with those of the cells on either side of the cell wall (22, 23, 29). There has been some disagreement about the nature of the tubular structure that runs through the plasmodesma and its relation to the endoplasmic reticulum in each cell; recent evidence supports the hypothesis that it may be a spindle fiber and not endoplasmic reticulum (23, 29). It seems clear that these channels have visibly structured contents, in contrast to the amorphous appearance of the ground substance seen in cytoplasmic bridges. The plasmodesmata in algae may be rather selective pathways, as indicated by the results of Spanswick and Costerton. Their electrical resistance suggests that there exists some barrier to diffusion, perhaps a plasma membrane (34). The presence of plasmodesmata between tapetal cells, wall cells, and these two different cell types has been clearly demonstrated (5, 11, 13). Presumptive germinal cells are linked to tapetal cells in this manner in several species (11); although the existence of plasmodesmata between tapetal and germinal cells in the lily has apparently not been reported, it is very likely that they are present and constitute the structural correlate of the observed coupling. Such specialized junctions may also play an important role in

mediating synchronous division of the tapetal nuclei (36).

Anatomical evidence indicates that the germinal cells of the lily gradually lose all connection with each other, starting at the diplotene stage of development, and are isolated by the end of meiotic prophase (12). The tapetal cells degenerate and disappear prior to the release of the microspores (36). Since tapetal and germinal cells are connected by low resistance junctions as late as the pachytene stage of the latter (Figs. 4 and 5), it would be of interest to determine the time at which these cells become electrically uncoupled.

At present, the degree to which the various tissues interact with each other is unknown. The function of the tapetal cells is not well understood. Their arrangement around the germinal cells suggests that they play a nutritive or supportive role. It seems clear that products of the degradation of tapetal cell deoxyribonucleic acid (DNA) do not contribute to the synthesis of DNA by the microsporocytes (36). However, the tapetum may produce materials for the wall formation of microspores (4, 37). Recently, Ito and Stern (1967) have been able to culture coherent filaments of germinal cells (without their tapetal cell sheaths) from a number of varieties of lily, including the Croft variety. When the cells were explanted at certain stages, they underwent meiosis on a chemically defined medium (14). In explants taken from some varieties at earlier stages the presumptive germinal cells which would have divided meiotically underwent mitotic divisions instead,[2] or failed to survive. One possibility is that determination of germinal cell development is controlled by the tapetal cell environment. These findings are consistent with the hypothesis that an essential role of the tapetum consists of triggering the meiotic development of germinal tissue.

Extrusion of a coherent filament of microsporocytes in which the syncytial organization was well preserved was a necessary requirement for the normal development of the cells when explanted prior to metaphase I. Single cells explanted before this time survived poorly or developed abnormally. The bridges between germinal cells may thus mediate some buffering against the environment and perhaps the sharing of nutrients.

The physiological role, if any, played by plasmodesmata is completely speculative. Perhaps the

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[2] H. Stern. Personal communication.

most conservative possibility is that only small ions can pass from one cell to the next and, again, that buffering of ion concentrations or sharing of a pumping load is achieved. It is possible that ion concentrations are important parameters for normal development (31). An additional speculation is that some trigger molecules, such as auxins, are transported from cell to cell by plasmodesmata (10, 23, 26, 39). In this regard, comment should be made of the occasional observation of the spread of dye from one germinal cell to another (Fig. 7) and between tapetal cells. This result is reasonable for germinal cells connected by cytoplasmic bridges, but more intriguing in the case of spread between tapetal cells, presumably via plasmodesmata. One cannot place much weight on these results, since it is difficult to be certain that only a single cell was injected and that no spread of dye occurred during subsequent histological procedures of fixation, dehydration, and embedding. Further, spread between germinal cells connected by bridges was not a consistent finding (see lower left insert, Fig. 5). Chicago Blue binds strongly to proteins, and dyes in general are fairly toxic; their failure to pass consistently from one cell to another could result from any one or combination of several reasons.

The involvement of coupling beyond detectable electrical signaling in plants is an extension that was made some time ago for animal tissues. It

seems quite likely, as suggested by Spanswick and Costerton (34) for *Nitella*, that the plasmodesmata are the low resistance paths between these cells. Thus, there are morphological specializations other than tight or gap junctions and cytoplasmic bridges underlying electrical coupling. Since nearly all cells of many adult plants are linked by plasmodesmata (40), including very differently specialized, contiguous elements, it may be of interest to study the size of molecules that can reliably traverse these channels. The demonstration of electrical coupling in embryonic tissues of plants as well as animals, and perhaps of many Metazoa, suggests that this widespread form of intercellular communication may play a role in their development.

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