Sex Plant Reprod (1990) 3:98–102

Sexual Plant Reproduction

Relatively high calcium is localized in synergid cells of wheat ovaries

Rajendra Chaubal and Bonnie J. Reger
USDA, ARS, Richard B. Russell Agricultural Research Center, P.O. Box 5677, Athens, GA 30613, USA

Summary. Using energy-dispersive X-ray microanalysis of mature, unpollinated wheat ovaries fixed by freeze-substitution, we show here that the synergid cells store a relatively high concentration of calcium. Based on our results and other indirect evidence, we suggest that supraoptimal levels of calcium in synergids may regulate: (1) correct orientation of the pollen tube, by forming a calcium gradient in the vicinity of the synergids, and (2) arrest and rupture of the pollen tube to release the sperm near the egg.

Key words: Synergids – Calcium – Chemotropism – Fertilization – Energy-dispersive X-ray microanalysis.

Introduction

Fertilization in angiosperms requires that a pollen tube deliver sperm to the vicinity of the egg and central cell within the embryo sac of an ovule. Accomplishment of this feat involves a sequence of processes which, in most angiosperms, appear to be mediated by one of two synergid cells flanking the egg at the micropylar end of the embryo sac. After penetration of the stigma, a pollen tube in the gynoecial tissue grows toward the ovule; passes successively through the placenta, micropyle, and the nucellus; and finally, depending upon the plant species, penetrates either an intact or a degenerating synergid cell (see reviews by Jensen 1973, 1974; Van Went and Willemse 1984). It is assumed that the synergids secrete substances that chemotropically guide the pollen tube, especially in the final stages of its growth (also see Mogensen 1972, 1978; Tilton 1981; Wilms 1981; Jensen et al. 1985). Following pollen tube entry, the penetrated synergid degenerates rapidly. Once the pollen tube is inside the synergid, its growth is arrested; the tip or a region near it ruptures; and two sperm, a tube nucleus, and some tube cytoplasm are discharged. The mechanisms of these synergid-mediated processes remain unknown.

The pollen tube-synergid interactions exhibit some striking similarities to calcium ion effects on pollen tubes in vitro. Pollen tubes elongate by cytoplasmic streaming-mediated vesicular exocytosis leading to formation of new wall at the tip (Heslop-Harrison 1987). Considerable evidence indicates that these processes are controlled by optimal calcium and that supraoptimal calcium arrests pollen tube growth and disrupts their tips (Brewbaker and Kwack 1963; Herth 1978; Reiss and Herth 1982; Picton and Steer 1982, 1983, 1985). Also, the pollen tubes of *Antirrhinum*, *Narcissus*, and *Clivia* spp. grow directionally toward increasing calcium gradients in vitro (Mascarenhas and Machlis 1962, 1964). Information on the calcium levels of the synergids may, therefore, provide important clues to the sperm-delivery mechanisms of angiosperms. Such information is lacking owing largely to the technical difficulty of combining the fixation of water-soluble components of the embryo sac and the specific detection of elements in situ. To date, only inorganic ash has been visualized in vacuoles and cytoplasm of the synergids by dark-field microscopy of microincinerated sections of excised, freeze-dried, and paraffin-embedded cotton ovules (Jensen 1965). This ash, which was largely absent from the egg and micropyle, is assumed to contain calcium (Jensen 1973, 1974; Jensen et al. 1985). Here we report the detection and relative distribution of calcium within the synergids and other cells of mature, unpollinated, and freeze-substituted wheat ovaries by energy-dispersive X-ray microanalysis.

Materials and methods

Plant material

Wheat (*Tritium aestivum* L., cv Chinese Spring) plants were grown in a glasshouse and maintained with approximately 14-h

Offprint requests to: R. Chaubal

photoperiod at 20–25° C day and 10–15° C night temperatures and >70% relative humidity. The pistils used were collected only from the primary and secondary florets (exhibiting pale green, undehisced, and approximately 3-mm-long anthers) from the middle region of flowering spikes that had completely extended beyond the flag leaves.

Freeze substitution

Unpollinated pistils consisting of an ovary and two short styles with feathery stigmas were excised at the ovary base and immediately plunged, ovary-base first, into rapidly stirred liquid propane at −189 to −191° C. The cryofixing apparatus and techniques were essentially similar to those described by Hoch (1986). In some instances the ovaries split into two unequal parts after immersion in propane, with one part retaining an intact ovule. Frozen ovaries or parts retaining the ovules were dried by substitution in cold acetone (−80° C for 48–72 h, −20° C for 4 h, 4° C for 1 h, and finally room temperature for 30 min), critical-point dried from liquid CO_2, and mounted on carbon stubs using adhesive carbon cement. Samples were viewed under a dissecting microscope and sectioned sagittally with a single-edge razor blade to expose the embryo sacs. All samples were coated with carbon and observed with a Philips 505 scanning electron microscope (SEM).

X-ray microanalysis

Elemental analysis of selected samples was performed using a energy-dispersive X-ray analysis system (Tracor Northern TN 5500 MCA) fitted to the SEM. The microscope was operated at an accelerated voltage of 10 kV and the stage-tilt was adjusted to obtain a take-off angle of about 48.9°. Electron-induced X-rays were detected by a Si(Li) spectrometer detector (Tracor Northern 3.0-mm^2 Microtrace), with a 7.5 μm-thick beryllium window placed 20 mm from the specimen. In preliminary surveys, X-ray spectra were collected for 200 s each from different regions (about 40 μm^2) of the ovaries. In all these regions, peaks of Mg, P, S, Cl, K, and Ca were detected. Calcium peaks were always very high (Ca K-alpha peaks clearly distinguished from K K-beta peaks) in spectra collected from the micropylar region, which included the synergids (Fig. 1).

Color-coded maps depicting relative distribution and concentration of the elements in selected regions of the specimen were generated using the Image Processing Program of the Tracor Northern. X-ray spectra acquired from the micropylar end of the embryo sac were used to set up X-ray energy ranges of interest (Fig. 1). For reference, a region from the background spectrum (BS) was included, and a secondary electron image (SEI) of the scanned area was simultaneously acquired. The X-ray image intensity in these maps reflected the concentration of the element with regions of higher concentration appearing brighter.

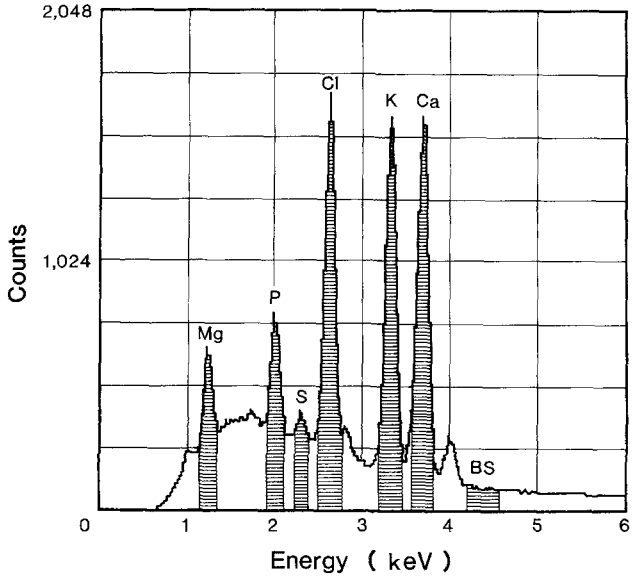

Fig. 1. A typical X-ray spectrum acquired from the micropylar end of the embryo sac of a rapid-frozen, acetone-substituted, and sagittally sectioned wheat ovary. Resolvable element peaks were detected in the X-ray energy range of 0–5 keV. *Cross-hatching* represents energy ranges used to generate distribution maps of *Mg, P, S, Cl, K, Ca*, and a part of the background spectrum (*BS*). (See Figs. 2 and 3)

Results and discussion

Mature wheat ovaries are about 2 mm long and each contains a large, bitegmic, and anatropous ovule, a reduced basal placenta, and a region of the pollen tube transmitting tract. The ovule encloses a definite nucellus and a polygonum type embryo sac with distinct synergids, egg, central cell, and antipodals. These features make wheat ovaries particularly well suited for investigating the distribution of elements within different cells of a typical angiosperm ovary by the techniques employed in this study.

Among the different compartments within an ovary, a localized region at the micropylar end of the embryo sac consistently displayed the highest calcium concentration. Some calcium was also detected in the nucellus. In the micropyle, ovary wall, central cell, and the antipodals, calcium levels did not appear to be above the background (Fig. 2). Maps acquired from the micropylar end of the embryo sac revealed that the high calcium was restricted to the synergids. The egg contained mainly potassium (Fig. 3). Calcium was not detected above background levels in the egg or the micropylar region. The synergids also contained magnesium and potassium (Fig. 3). Considering that some of the magnesium X-rays, being low energy, may have been lost due to absorption by the specimen and the detector, the synergids appear to store abundant magnesium. However, of all the elements detected in the synergids the calcium concentration was the highest (Fig. 3).

Mature synergids from a wide variety of plant species display characteristically similar structural organization. The cells are rich in cytoplasmic organelles and are only partly surrounded by a cell wall, which has characteristic thickenings (filiform apparatus) at the micropylar end (Jensen 1973, 1974; Willemse and Van Went 1984). High levels

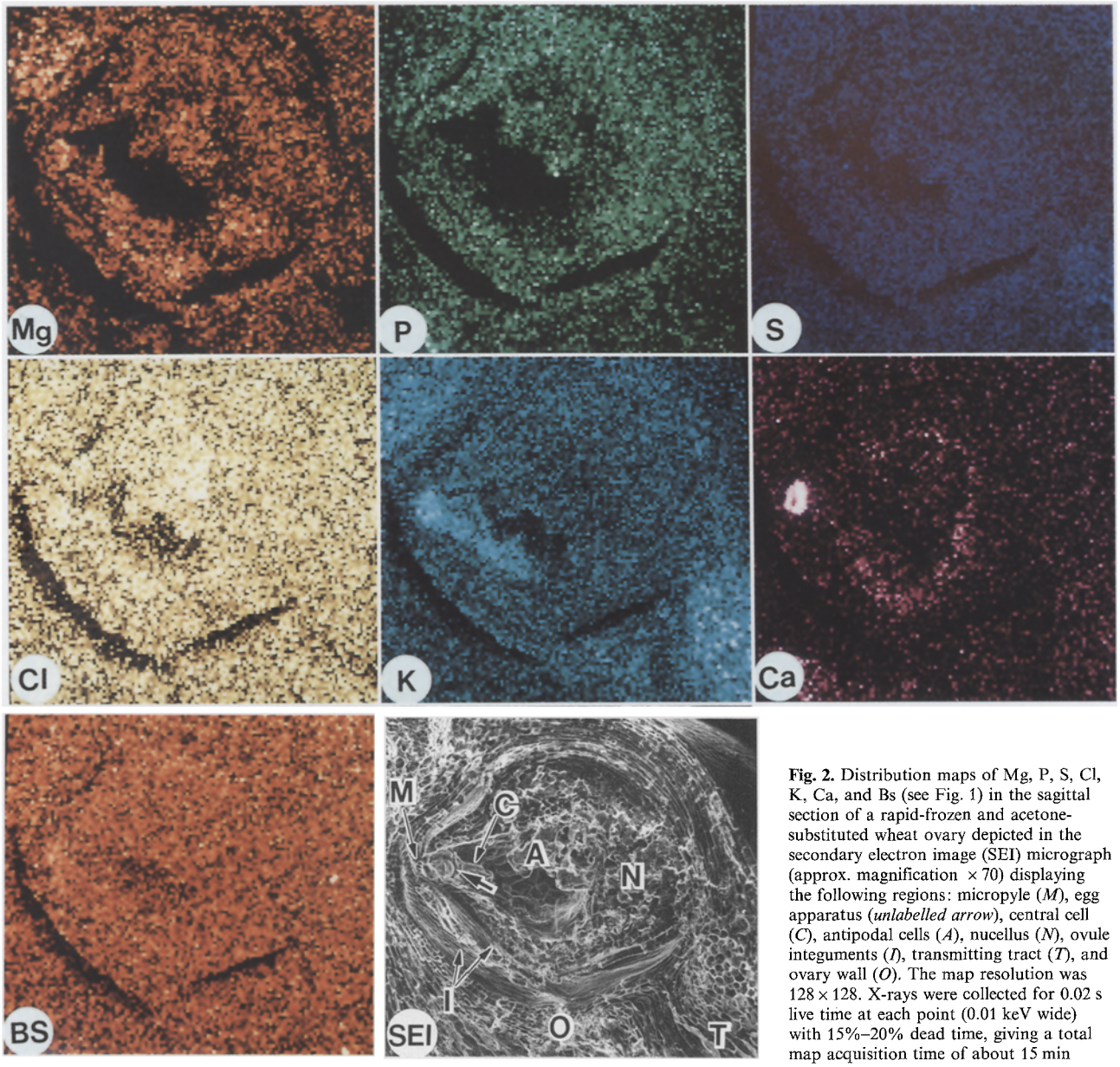

Fig. 2. Distribution maps of Mg, P, S, Cl, K, Ca, and Bs (see Fig. 1) in the sagittal section of a rapid-frozen and acetone-substituted wheat ovary depicted in the secondary electron image (SEI) micrograph (approx. magnification × 70) displaying the following regions: micropyle (*M*), egg apparatus (*unlabelled arrow*), central cell (*C*), antipodal cells (*A*), nucellus (*N*), ovule integuments (*I*), transmitting tract (*T*), and ovary wall (*O*). The map resolution was 128 × 128. X-rays were collected for 0.02 s live time at each point (0.01 keV wide) with 15%–20% dead time, giving a total map acquisition time of about 15 min

of calcium in the synergids may, therefore, be a general phenomenon in angiosperms. In support of this idea, we have also found that within mature, unpollinated wheat (cvs Hope and Little Club), pearl millet, rye, and maize ovaries the synergids contain the highest concentration of calcium (unpublished observations). The functional role of this calcium is not known.

Previous investigations attempting to assess a chemotropic role of calcium failed to find continuously increasing calcium gradients from the stigma to the ovule in *Antirrhinum* and *Oenothera* (Mascarenhas and Machlis 1964; Glenk et al. 1971). In *Antirrhinum*, not even restricted gradients from the placenta to the ovule, a region where pollen tubes exhibit sharp turns toward the ovule, could be demonstrated (Mascarenhas 1966, 1978). These negative findings, however, do not satisfactorily discredit the putative chemotropic role of calcium in vivo, for three reasons. First, calcium levels of whole ovules rather than the synergids were compared with the calcium levels of the placenta. Second, the restricted calcium gradients were investigated by light microscopy of ovary sections, which do not clearly display the synergids. Third, because only unpollinated gynoecia were used in these studies, the possibility of a restricted gradient being formed by pollination-triggered synergid degeneration, as suggested by Jensen et al. (1985) remains open.

You and Jensen (1985) reported that in chemically fixed unpollinated wheat ovaries both syner-

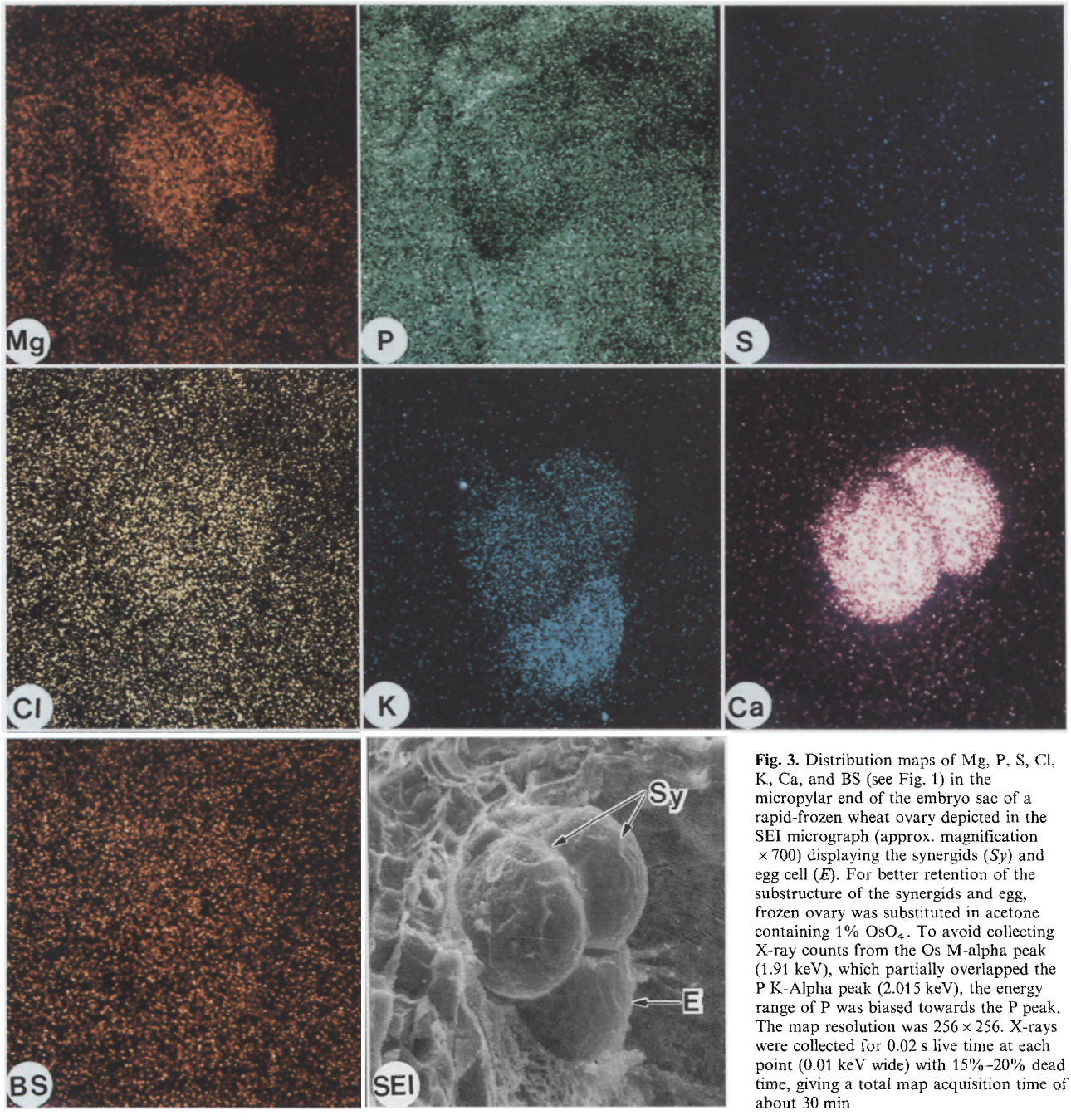

Fig. 3. Distribution maps of Mg, P, S, Cl, K, Ca, and BS (see Fig. 1) in the micropylar end of the embryo sac of a rapid-frozen wheat ovary depicted in the SEI micrograph (approx. magnification $\times 700$) displaying the synergids (*Sy*) and egg cell (*E*). For better retention of the substructure of the synergids and egg, frozen ovary was substituted in acetone containing 1% OsO_4. To avoid collecting X-ray counts from the Os M-alpha peak (1.91 keV), which partially overlapped the P K-Alpha peak (2.015 keV), the energy range of P was biased towards the P peak. The map resolution was 256×256. X-rays were collected for 0.02 s live time at each point (0.01 keV wide) with 15%–20% dead time, giving a total map acquisition time of about 30 min

gids may partially degenerate; complete degeneration of a synergid is delayed until pollen tube entry. Although we did not detect a calcium gradient from the micropyle to the synergid in the unpollinated wheat ovaries used in this investigation, the presence of a weak gradient in this region cannot be ruled out because of the detection limits set by the instrument and the uneven specimen topology.

Based on our results and other evidence from the literature, we propose the following. (1) Continuous calcium gradients from the placenta or the micropyle to the synergid in angiosperm ovaries may be formed by calcium secretion from the synergids. The resulting gradient could chemotropically guide a pollen tube growing in either of these regions into the synergid. Such gradients, however, may be only detectable in pollinated ovaries of species in which a synergid cell completely degenerates before the pollen tube arrives at the ovule. (2) Pollen-tube arrest and rupture of the tip inside the synergid may be regulated by the supraoptimal calcium concentration of this cell. Essentially similar postulates have been made by Jensen et al. (1985).

A few plant species belonging to the family *Plumbaginaceae* possess reduced embryo sacs, which lack synergids. In these plants, the egg wall at the micropylar end is modified to resemble the filiform apparatus. Also, the egg exhibits increased metabolic activity comparable to that of the synergids (Cass and Karas 1974). A pollen tube entering through the micropyle penetrates the egg wall at the micropylar end, but not the egg membrane, and deposits its discharge between the egg and the central cell (Russell 1980, 1982). Examination of these exceptional plants by the techniques described in this paper should prove useful in determining the role of calcium in sperm delivery processes of angiosperms.

Very little is known about the molecular events that occur after sperm delivery into the synergid. Generally only naked sperm nuclei fuse with the egg and the central cell; the rest of the discharge remains trapped in the milieu of the degenerating synergid (Mogensen 1988). During the final stages of fertilization in animals, the processes of sperm and egg activation, fusion, and development of the zygote are apparently triggered by calcium (Epel 1982; Schackmann and Shapiro 1982; Yanagimachi 1988). Extracellular calcium surrounding the plant reproductive cells (which resemble animal cells to the extent that they either lack or are not fully enclosed by cell walls) prior to their fusion may, therefore, be suspected of performing some similar functions.

References

Brewbaker JL, Kwack BH (1963) The essential role of calcium ion in pollen germination and pollen tube growth. Am J Bot 50:859–865

Cass DD, Karas I (1974) Ultrastructural organization of the egg of *Plumbago zeylancia*. Protoplasma 81:49–62

Epel D (1982) The physiology and chemistry of calcium during the fertilization of eggs. In: Cheng WY (ed) Calcium and cell function, vol 2. Academic Press, New York London, pp 355–383

Glenk HO, Wagner W, Schimmer O (1971) Can Ca^{++} ions act as a chemotropic factor in *Oenothera* fertilization? In: Heslop-Harrison J (ed) Pollen development and physiology. Butterworths, London, pp 255–261

Herth W (1978) Ionophore A-23187 stops tip growth, but not cytoplasmic streaming, in pollen tubes of *Lilium longiflorum*. Protoplasma 96:275–282

Heslop-Harrison J (1987) Pollen germination and pollen-tube growth. Int Rev Cytol 107:1–78

Hoch HC (1986) Freeze-substitution of fungi. In: Aldrich HC, Todd WJ (eds) Ultrastructure techniques for microorganisms. Plenum Press, New York, pp 183–221

Jensen WA (1965) The ultrastructure and histochemistry of the synergids of cotton. Am J Bot 52:238–256

Jensen WA (1973) Fertilization in flowering plants. Bioscience 23:21–27

Jensen WA (1974) Reproduction in flowering plants. In: Robards AW (ed) Dynamic aspects of plant ultrastructure. McGraw-Hill, New York, pp 481–503

Jensen WA, Ashton ME, Beasley CA (1985) Pollen tube-embryo sac interaction in cotton. In: Mulcahy DL, Ottaviano E (eds) Pollen: biology and implications for plant breeding. Elsevier Biomedical, New York Amsterdam Oxford, pp 67–72

Mascarenhas JP (1966) The distributution of ionic calcium in the tissues of the gynoecium of *Antirrhinum majus*. Protoplasma 62:53–58

Mascarenhas JP (1978) Sexual chemotaxis and chemotropism in plants. In: Hazelbauer GL (ed) Receptors and recognition. Chapman and Hall, London, pp 171–203

Mascarenhas JP, Machlis L (1962) Chemotropic response of *Antirrhinum majus* pollen to calcium. Nature 196:292–293

Mascarenhas JP, Machlis L (1964) Chemotropic response of the pollen of *Antirrhinum majus* to calcium. Plant Physiol 39:70–77

Mogensen HL (1972) Fine structure and composition of the egg apparatus before and after fertilization in *Quercus gambelii*: the functional ovule. Am J Bot 59:931–941

Mogensen HL (1978) Pollen tube-synergid interaction in *Proboscidea louisianica* (martineaceae). Am J Bot 65:953–964

Mogensen HL (1988) Exclusion of male mitochondria and plastids during syngamy in barley as a basis for maternal inheritance. Proc Natl Acad Sci USA 85:2594–2597

Picton JM, Steer MW (1982) A model for the mechanism of tip extension in pollen tubes. J Theor Biol 98:15–20

Picton JM, Steer MW (1983) Evidence for the role of Ca^{2+} ions in tip extension in pollen tubes. Protoplasma 115:11–17

Picton JM, Steer MW (1985) The effects of ruthenium red, lanthanum, fluorescein isothiocyanate and trifluoperazine on vesicle transport, vesicle fusion and tip extension in pollen tubes. Planta 163:20–26

Reiss H-D, Herth W (1982) Disoriented growth of pollen tubes of *Lilium longiflorum*. Thunb. induced by prolonged treatment with the calcium-chelating antibiotic, chlorotetracycline. Planta 156:218–225

Russell SD (1980) Participation of male cytoplasm during gamete fusion in an angiosperm, *Plumbago zeylanica*. Science 210:200–201

Russell SD (1982) Fertilization in *Plumbage zeylanica*: entry and discharge of the pollen tube in the embryo sac. Can J Bot 60:2219–2230

Schackmann RW, Shapiro BM (1982) Calcium and metabolic activation of spermatozoa. In: Cheung WY (ed) Calcium and cell function, vol 2. Academic Press, New York London, pp 339–353

Tilton VR (1981) Ovule development in *Ornithogalum caudatum* (liliaceae) with a review of selected papers on angiosperm reproduction. IV. Egg apparatus structure and function. New Phytol 88:505–531

Van Went JL, Willemse MTM (1984) Fertilization. In: Johri BM (ed) Embryology of angiosperms. Springer, Berlin Heidelberg New York, pp 273–317

Willemse MTM, Van Went JL (1984) The female gametophyte. In: Johri BM (ed) Embryology of angiosperms. Springer, Berlin Heidelberg New York, pp 159–196

Wilms HJ (1981) Pollen tube penetration and fertilization in spinach. Acta Bot Neerl 30:101–122

Yanagimachi R (1988) Mammalian fertilization. In: Knobil E, Neill J (eds) The physiology of reproduction. Raven Press, New York, pp 135–185

You R, Jensen WA (1985) Ultrastructural observations of the mature megagametophyte and the fertilization in wheat (*Triticum aestivum*). Can J Bot 63:163–178