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Quantitative three-dimensional study on the position of the female gametophyte and its constituent cells as a prerequisite for corn (*Zea mays*) transformation

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Summary. The position of the embryo sac in the spikelet and of the embryo sac's constituent cells within the sporophytic tissues of *Zea mays* was localized by scanning electron microscopy, serial thick sectioning, and computer three-dimensional reconstruction. Within certain limits, the embryo sac is consistently oriented in the same position inside of the spikelet. This information is a prerequisite for successful microinjections into the in situ female cells of *Zea mays*.

Key words: Biotechnology – Embryo sac – Microinjection – Micromanipulation

Introduction

The embryo sac in most angiosperms, including *Zea mays*, is located deep within the ovule which is, in turn, located within the ovary (Randolph 1936; Kiesselbach 1949; Diboll and Larson 1966; Van Lammeren 1986a). Since the female cells are not readily accessible, genetic transformations have been accomplished through gross injections of exogenous DNA into the general region of the ovules (Zhou et al. 1983; Zhou 1986; De la Pena et al. 1987), or even by application of foreign DNA to severed pollinated styles (Luo and Wu 1988). Although such approaches circumvent the necessity of in vitro regeneration, which is not yet possible on a reliable basis in *Zea mays* (Rhodes et al. 1988), serious problems such as the repeatability of the experiments and the lack of genetic homogeneity of the progeny still remain.

In the present study we measured the position of the embryo sac in the spikelet (the reduced flower of grasses) and quantified the location of the constituent cells within the embryo sac for the purposes of microinjection. Use of the method by experimenters should result in repeatable, homogeneous transformations in the absence of in vitro regeneration.

Materials and methods

Zea mays L., hybrid line (A 632), was grown in an experimental garden in Lyon from July to September. Plants were detasseled and the inflorescences were bagged prior to silk emergence to ensure an unpollinated condition. Spikelets were selected from inflorescences with silks that had emerged 3 cm from the ear.

Position of the embryo sac

Spikelets for scanning electron microscopic observations were fixed in 5% glutaraldehyde in Na-cacodylate buffer (0.1 *M*, pH = 7.0) at room temperature for 2 h, then rinsed in the same buffer for 1 h (three changes). One median slice of the spikelet was made by hand with a razor blade. Post-fixation was in 2% buffered osmium tetroxide for 2 h at ambient temperatures. Following three rinses in distilled water (1 h total), the material was dehydrated in an acetone series and then critical-point dried. [This preparation procedure gave the least possible shrinkage, 1%–9% in total volume, with corn embryonic tissues (Van Lammeren 1986b)]. Material was observed at a 90° angle with a calibrated Joel 35 scanning electron microscope. Micrographs were taken of entire spikelets in which the embryo sac was sectioned longitudinally. Of the over 200 bilaterally symmetric spikelets examined in this study, 48 were cut at a median angle, which allowed for the embryo sac to be viewed in longisection. Measurements were obtained from enlarged photomicrographs.

Position of constituent cells

After partial removal of the carpel tissue surrounding the ovule, fixation and dehydration were accomplished as described above

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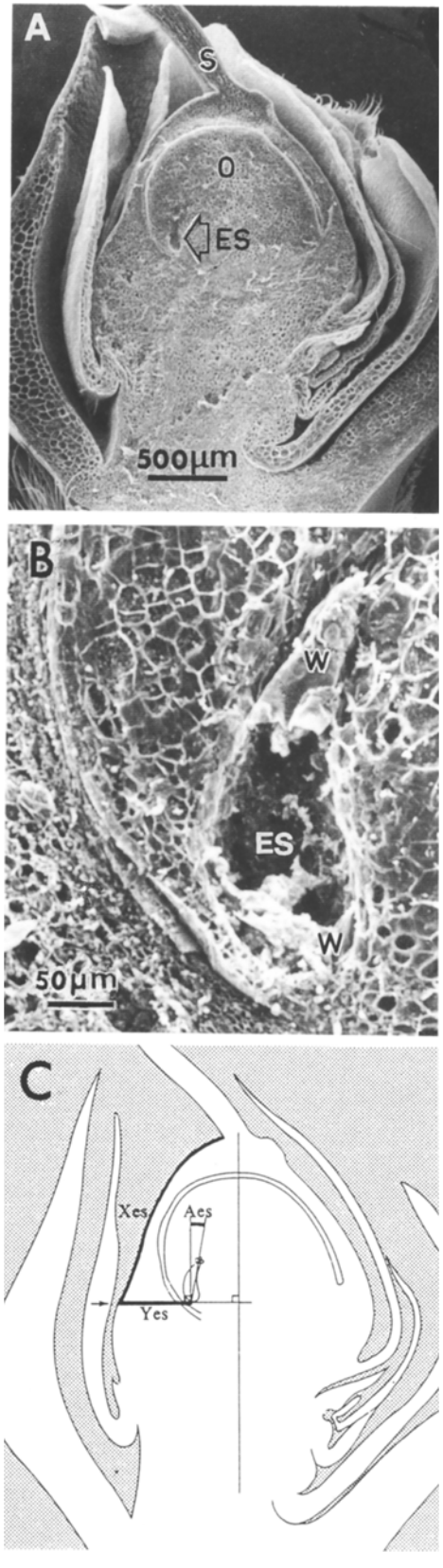


and the ovules were then embedded in Spurr's low viscosity resin. Serial sagittal thick sections (4–5 µm) of the ovule were cut with a glass knife and observed with an interference contrast microscope. Qunatification of cellular position was achieved by tracing the outlines of the ovule, containing the embryo sac and its constituent cells, onto plastic sheets from photographic reprints of over approximately 200 serial thick sections for each of the six ovules studied. Fiducial points were deciphered from nearby debris, specific structures on the ovules, and the section edge. Measurements were made from the plastic sheets, using triangulation to correct for the angle of sectioning. Three-dimensional reconstructions were produced by the use of a computer-aided system described elsewhere (Mogensen and Wagner 1987).

Results

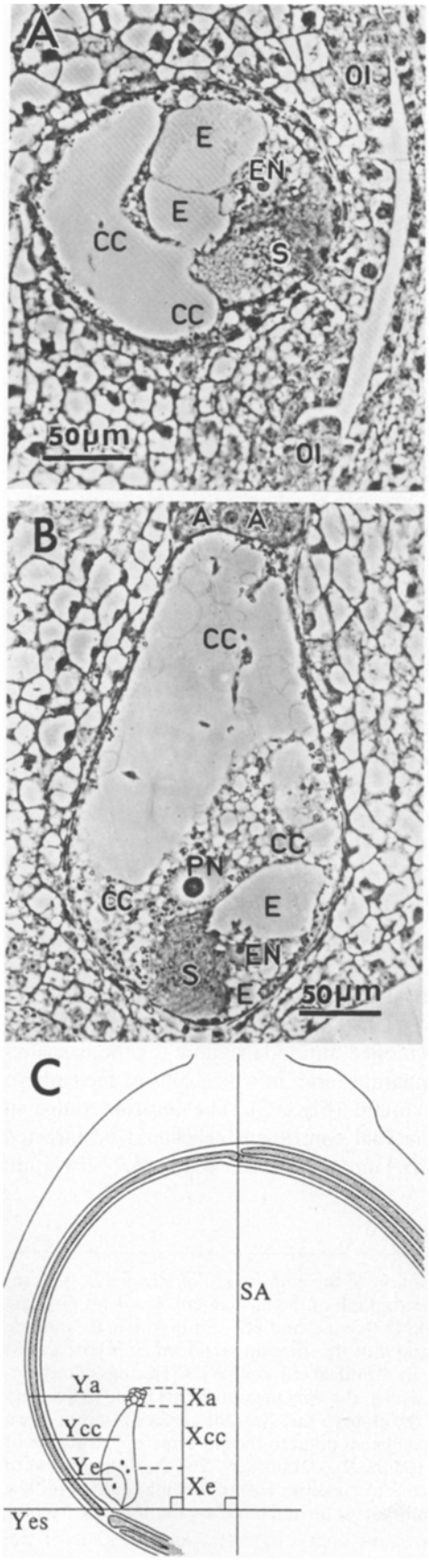
Position of the embryo sac

The embryo sac is positioned in the median plane of the ovule (Fig. 1 A) and oriented on the side facing the rachis of the inflorescence. The female gametophyte can be easily identified by the boundaries of its pear-shaped cell wall (Fig. 1 B). Three quantitative measurements of the embryo sac's micropyle position within the spikelet were conducted as described in Fig. 1 C. The average surface distance down from the silk attachment point (Yes) was found to be $x = 1{,}456$ µm (sd = 83 µm, size of range = 420 µm) and the distance from the surface to the embryo sac (Xes) was determined to be $x = 478$ µm (sd = 51 µm, size of range = 255 µm). The angle of the embryo sac in reference to the axis of the spikelet (Aes) is $x = 1.5°$ (sd = 4.2°, size of range = 15.5°).

Position of the constituent cells

Serial thick section observations of six ovules allowed for the quantification of the egg, central, and the several antipodal cells of the embryo sac (Fig. 2 A and B). The micropylar-positioned egg cell, the central cell, and the chalazally positioned antipodals allow for the measurement of the separate zones in which cells of the embryo sac could be found (Fig. 2 C). The location range in which an individual constituent cell could be targeted (Xe'egg' = 0–85.9 µm; Xcc'central cell' = 85.9–204.0 µm;

Fig. 1 A–C. Position of the embryo sac of *Zea mays*. **A** Scanning electron micrograph of the median-cut *Zea mays* pistillate spikelet. S – Silk, O – Ovule, and ES – Embryo sac. **B** Scanning electron micrograph of the median-sectioned embryo sac (ES) revealing the easily identified cell wall (W). **C** Tracings of median-cut *Zea mays* spikelet, showing measurements made to quantify the position of the embryo sac. Xes, the surface distance down from the silk attachment point to the most micropylar region of the embryo sac (on the abaxial surface). Yes, the distance inward from the outer edge of the ovary wall to the embryo sac. Aes, the angle of the embryo sac in reference to the long axis of the spikelet



Xa'antipodals' = 204.0–268.8 µm) and the distance from the adaxial surface of the ovule to the center of a particular cell (Ye'egg' = 81.4 µm; Ycc'central cell' = 144.8 µm; Ya'antipodal' = 200.1 µm) are given in Table 1.

The orientation of the embryo sac (Fig. 3 A) and its constituent cells within the ovule (Fig. 3 B) are revealed in three-dimensional reconstructions. The paired polar nuclei are found near the micropylar end of the central cell in close proximity to the egg. The egg nucleus is found centrally located inside the pear-shaped egg cell. The synergid pair is positioned between the egg and polar nuclei (Fig. 3 B).

Discussion

The results presented here quantify the position of the female gametophyte within the sporophytic tissues of *Zea mays* in order to allow for direct acccess by microinjection. Although an intra-individual variation was detected in our measurements, the quantification of the embryo sac's position can only lead to an increased probability of a successful microinjection into the female gamete. The observed variance is not surprising since inter- and intra-individual ovular variations have been reported in other plant species (Scagel et al. 1985; Maze et al. 1986, 1987). Intra-species differences in embryo sac position have been previously observed between two inbred lines of *Zea mays* (Van Lammeren 1986a). The variation may be due to different developmental states of spikelets found on the same inflorescence (Kiesselbach 1949; Van Lammeren 1986a).

Alteration of the material of this study was kept to a minimum during specimen preparation procedures. We used a preparation procedure that was previously shown to cause a 1%–9% change in total volume, considerably less in diameter measurements, as tested on early stages of embryonic tissues in *Zea mays* (Van Lammeren 1986b). Ovules used in this experiment were controlled before and after processing, with almost no measurable

Fig. 2 A–C. Position of the constituent cells of the embryo sac. **A** Light microscopical cross-section of the corn embryo sac in the ovule. OI – outer integument, E – egg cell, EN – egg nucleus, CC – central cell and S – synergids. **B** Phase contrast micrograph of a longitudinal section of the *Zea mays* embryo sac in the ovule. The constituent cells and their nuclei are easily identified. E – egg cell, EN – egg nucleus, CC – central cell, PN – polar nuclei, S – synergid, and A – antipodals. **C** Tracings of sagittal section of the maize ovule, showing measurements made to quantify the position of the constituent cells of the embryo sac. The three zones in which an individual constituent cell could be targeted using Yes as a reference (Xe – egg, Xcc – central cell and Xa – antipodals), and the distance to the center of a constituent cell (Ye – egg, Ycc – central cell, Ya – antipodals). Note the spikelet axis (SA) through the core of the spikelet

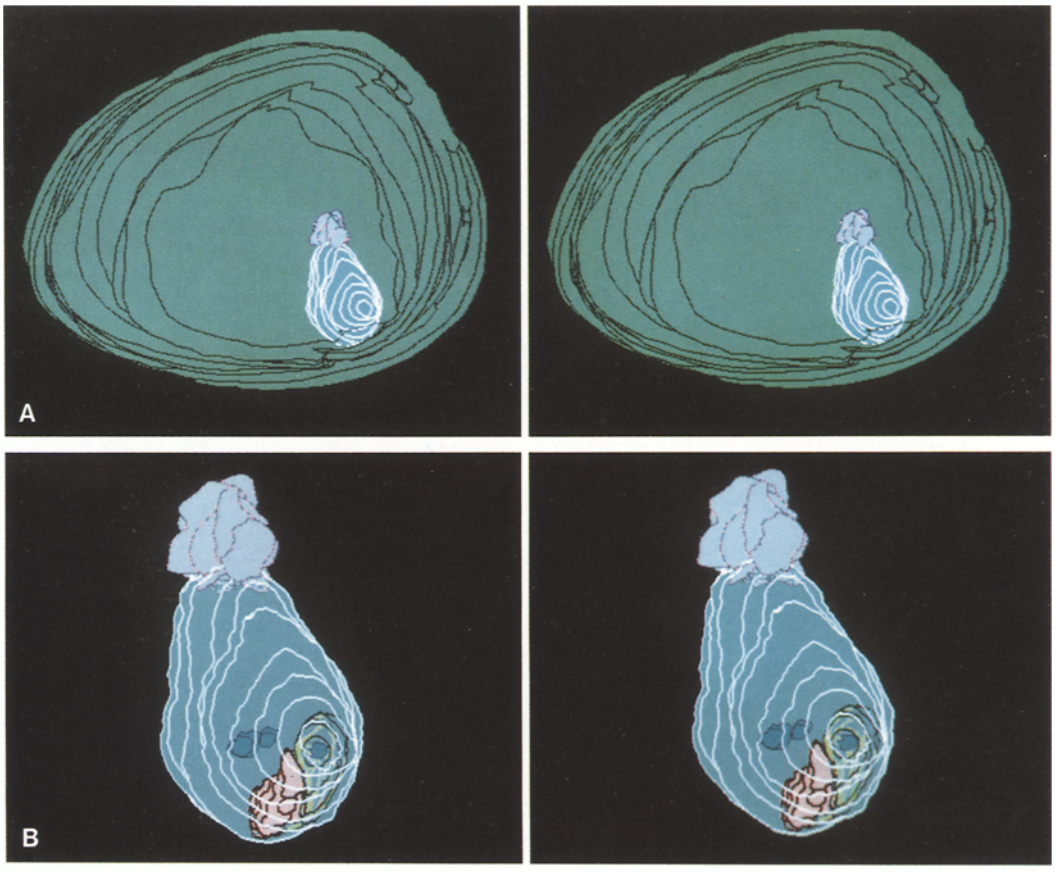


Fig. 3 A and B. Three-dimensional reconstruction of embryo sac and its constituent cells. **A** Computer-generated stereoscopic pair of the embryo sac (blue with white outline) in the ovule (green). The placenta-chalazal region of ovule attachment to the ovary is oriented toward the bottom of the photograph. Reconstruction based on serial sagittal thick sections from central portion of the ovule. **B** Reconstruction based upon same series as Fig. 3A, but enlarged and showing the cellular components of the embryo sac. Note the central cell (blue with withe outline) and its polar nuclei (dark blue); egg cell (yellow) and its nucleus (blue); and accessory cells, the synergids (pink) and antipodals (gray)

Table 1. Two types of measurements were made to quantify the position of constituent cells of the embryo sac as described in Fig. 2C: the size range of the zones in which an individual constituent cell could be targeted (Xe, Xcc, Xa), and the distance to the center of a constituent cell (Ye, Ycc, Ya). All measurements were made from serial thick sections and reported in μms

ES	Xe	Xcc	Xa	/	Ye	Ycc	Ya
1	0–87.8	87.8–209.2	209.2–276.1	/	83.7	159.0	209.2
2	0–81.0	81.0–234.0	234.0–297.0	/	84.3	143.2	185.0
3	0–84.9	84.9–207.0	207.0–301.3	/	93.3	161.2	254.6
4	0–90.0	90.0–216.0	216.0–270.0	/	76.9	139.8	181.7
5	0–88.2	88.2–140.1	140.1–216.8	/	66.4	125.8	174.7
6	0–83.8	83.8–216.6	216.6–251.6	/	83.8	139.7	195.6
X=	0–85.9	85.9–204.0	204.0–268.8	/	81.4	144.8	200.1
SD=	(3.3)	(32.6)	(31.3)		(9.0)	(13.3)	(29.3)

change in diameter observed. After preparation, the tissues of the spikelets were undamaged and the individual cells were of expected size and shape.

Transformation by intraovarian injection of exogenous DNA holds considerable potential for crop improvement. A small number of transgenic plants, verified by molecular techniques, have already been achieved through injections into pre-pollinated or post-pollinated ovules (De la Pena et al. 1987; Zhou et al. 1983; Zhou 1986). However, problems with the repeatability of the

experiments and lack of genetic homogeneity of the progeny remain to be resolved.

A more precise microinjection in a controlled plant system should prove to be superior, especially in terms of repeatability. *Zea mays* makes an excellent candidate for such an approach since its spikelets can be dissected from the plant, microinjected, and cultured in a developed in vitro system (Gengenbach 1977; Higgins and Petolino 1988), where the timing of pollination to fertilization events is well documented (Dupuis and Dumas 1989). With this method, microinjections of exogenous DNA can be timed to near-fertilization events when the embryo sac may be most receptive to a transformation, thus avoiding chimeric embryo production if the introduction of DNA is made during embryo development.

We believe that these results shall allow for successful microinjection into the embryo sac and even into the individual constituent cells. Preliminary results using a microinjection (Hepher et al. 1985) into the quantified position of the maize embryo sac have demonstrated that any injury due to micropipette penetration does not interfere with normal embryo and seed development (E. Matthys-Rochen, personal communications). Presently, we are trying to determine the success rate of a microinjection into the embryo sac with the fluorescent dye, Lucifer yellow (Steinbiss and Stabel 1983; Steinbiss et al. 1985) and the results shall be reported in a forthcoming paper.

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