

The simple ultrastructure of the maize kinetochore fits a two-domain model

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Abstract. Light microscope observations suggest there are two discrete biochemical domains in the plant kinetochore, an inner domain containing structural proteins, and an outer domain containing proteins involved in motility. We analyzed the ultrastructure of maize meiotic kinetochores following high pressure freezing and freeze substitution, a method that provides excellent sample preservation. Data from meiosis II support previous descriptions of plant kinetochores as diffuse, nearly invisible domains, sometimes nesting in a cup of darkly staining chromatin. The ultrastructure is similar in meiosis I but there are two sister kinetochores that each protrude away

from the chromosome and form their own distinct kinetochore fibers. Microtubules terminate within kinetochores where their ends are splayed in a cone-shaped configuration suggestive of microtubule disassembly. We could not detect any significant substructure within the kinetochore proper. We suggest that the diffuse structure classically defined as the kinetochore represents only the outer domain of a two-domain organelle. The inner domain, known to contain chromatin-binding proteins, probably extends into the electron-dense chromatin of the primary constriction.

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The plant kinetochore is classically defined as the organelle that interacts with the centromere and guides chromosome movement. However, as kinetochore proteins have been identified and characterized, the distinction between kinetochore and centromere has begun to blur. For instance Centromere-specific Histone-H3 (known as CENH3 or CENP-A) is a histone variant that binds directly to centromeric DNA throughout the cell cycle (Henikoff et al., 2001). Other proteins such as CENP-C interact closely with CENH3 in a higher-order chromatin configuration (Ando et al., 2002). Such structural proteins are referred to as inner kinetochore proteins or centromere proteins. In contrast, many other kinetochore proteins are “passengers” that are only present and necessary during chromosome segregation (Yu et al., 2000; Houben and Schubert, 2003). Examples are Mitotic Arrest Defective 2 (MAD2), a pro-

tein that regulates the onset of anaphase (Yu et al., 1999), and Centromere Protein E (CENP-E), a kinesin-like protein involved in several aspects of chromosome movement (Hoopen et al., 2002). In both animals and plants, passenger proteins tend to localize to an outer domain of the kinetochore. This is particularly evident in maize, where CENH3 and CENP-C occupy a discrete inner domain, while MAD2 and another phosphoprotein involved in regulating chromosome movement (the 3F3/2 antigen) lie in an outer domain (Yu et al., 1999).

Despite recent evidence for biochemical subdomains in the plant kinetochore, reports on the ultrastructure of the kinetochore provide no support for a subdomain organization. Microtubules terminate in a diffuse irregular structure that is less electron-dense than chromatin, and which sometimes appears to contain granules (Wilson, 1968) or fibrils (Braselton and Bowen, 1971). The term “ball in a cup” has been used to describe the plant kinetochore, with the emphasis being more on the “cup” of chromatin than the “ball” within (Bajer and Mole-Bajer, 1972). By contrast, animal kinetochores are quite striking when observed in the electron microscope (Brinkley et al., 1989). They are often regarded as having four domains, three discrete layers each defined by differing electron density, and a fibrous corona extending away from the kinetochore.

Interestingly, data from a recent report on animal kinetochores preserved using high pressure freezing contradicts the

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three-layer model, suggesting that there is only one electron dense domain with an associated corona (McEwen et al., 1998). The authors argued that the trilaminar structure is an artifact of poor fixation. Intrigued by this observation, we use high pressure freezing to revisit the structure of plant kinetochores with the hope that more detail might be revealed. Nevertheless the results strongly confirm the original descriptions; in fact our analysis shows kinetochores to be essentially indistinguishable from the cytoplasm. In plants the kinetochore can only be definitively identified by a collection of microtubules that terminate at a focus on the chromosome. We suggest that the diffuse structure often described as the complete organelle is only half of it – the outer domain. The inner domain containing chromatin proteins would most likely appear as chromatin. Strands of deeply staining material that extend into the kinetochore proper are likely the ultrastructural manifestations of the inner kinetochore.

Materials and methods

Electron microscopy

Anthers roughly 1.5 mm in length (meiosis I) or 1.75 mm in length (meiosis II) were removed from tassels of the maize inbred W23 and placed into buffer A (Hiatt et al., 2002). The anthers were then bisected with a razor blade and placed in either aluminum or brass planchettes with enough 15% (w/v) dextran (MW 40,000) in water to displace the air surrounding each sample. Samples were frozen using a Balzer's HPM 010 High Pressure Freezing Machine, freeze substituted, and processed for study with TEM according to published procedures (Mims et al., 2003). Briefly, after 4 days in substitution fluid at -80°C , samples were transferred to a -20°C freezer for 3 h, a 4°C refrigerator for 2 h and brought to room temperature in the hood for 30 min. Samples were separated from the planchettes, rinsed 3 times in anhydrous acetone, and slowly infiltrated with Araldite/Embed 812 resin. Following polymerization in Permaxox petri dishes, anthers at specific stages of development were selected under the light microscope for sectioning. Thin sections were cut with a diamond knife, picked up on slot grids and allowed to dry on formvar-coated aluminum racks (Rowley and Moran, 1975). Sections were post-stained for 3 min each with a saturated solution of uranyl acetate followed by lead citrate (Reynolds, 1963) and examined using a Zeiss EM 902A transmission electron microscope operating at 80 kV.

Light microscopy

Maize anthers were fixed in formaldehyde and incubated with a combination of directly-labeled anti-MAD2 and anti-CENP-C antisera (Yu et al., 1999; Fig. 1C), or indirectly labeled anti-CENH3 and anti-tubulin antisera

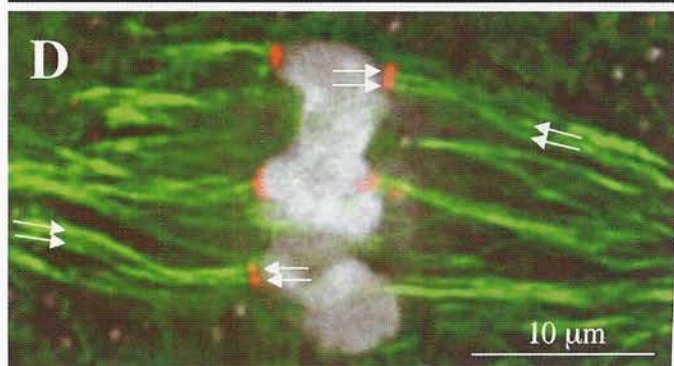
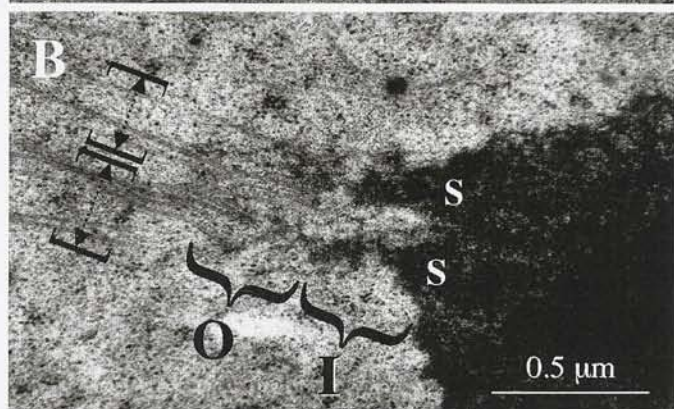
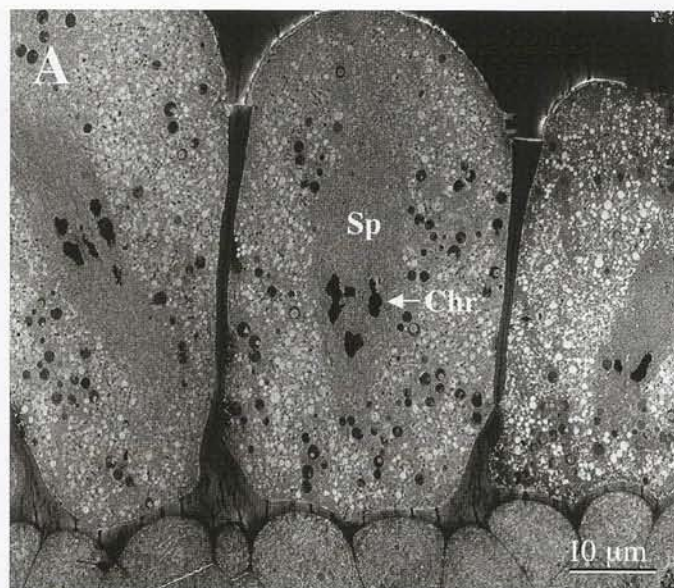


Fig. 1. The maize meiosis I kinetochore. **(A)** Three prometaphase I cells and their associated tapetal cells (lower line of cells). The chromosomes (Chr) and spindle (Sp) are indicated. **(B)** Higher magnification view of the separation of sister kinetochores (S) at prometaphase I. The inner (I) and outer kinetochores (O) are indicated with brackets. The boundaries of what appear to be two independent kinetochore fibers are indicated with bi-directional arrows terminated with bars. **(C)** Light microscope view of a kinetochore at prometaphase I. The inner kinetochore (I) protein CENP-C is shown in green and the outer kinetochore (O) protein MAD2 is indicated in red. Note the clear separation of domains despite the extensive stretching of the kinetochore at this stage. This image was provided by Hong-Guo Yu. **(D)** Light microscope image of the separation of sister kinetochore fibers at prometaphase I. Kinetochores are stained with antibodies to CENH3 (red) and tubulin is labeled in green. Arrows indicate the clear separation of both the kinetochores and the associated bundles of microtubules.

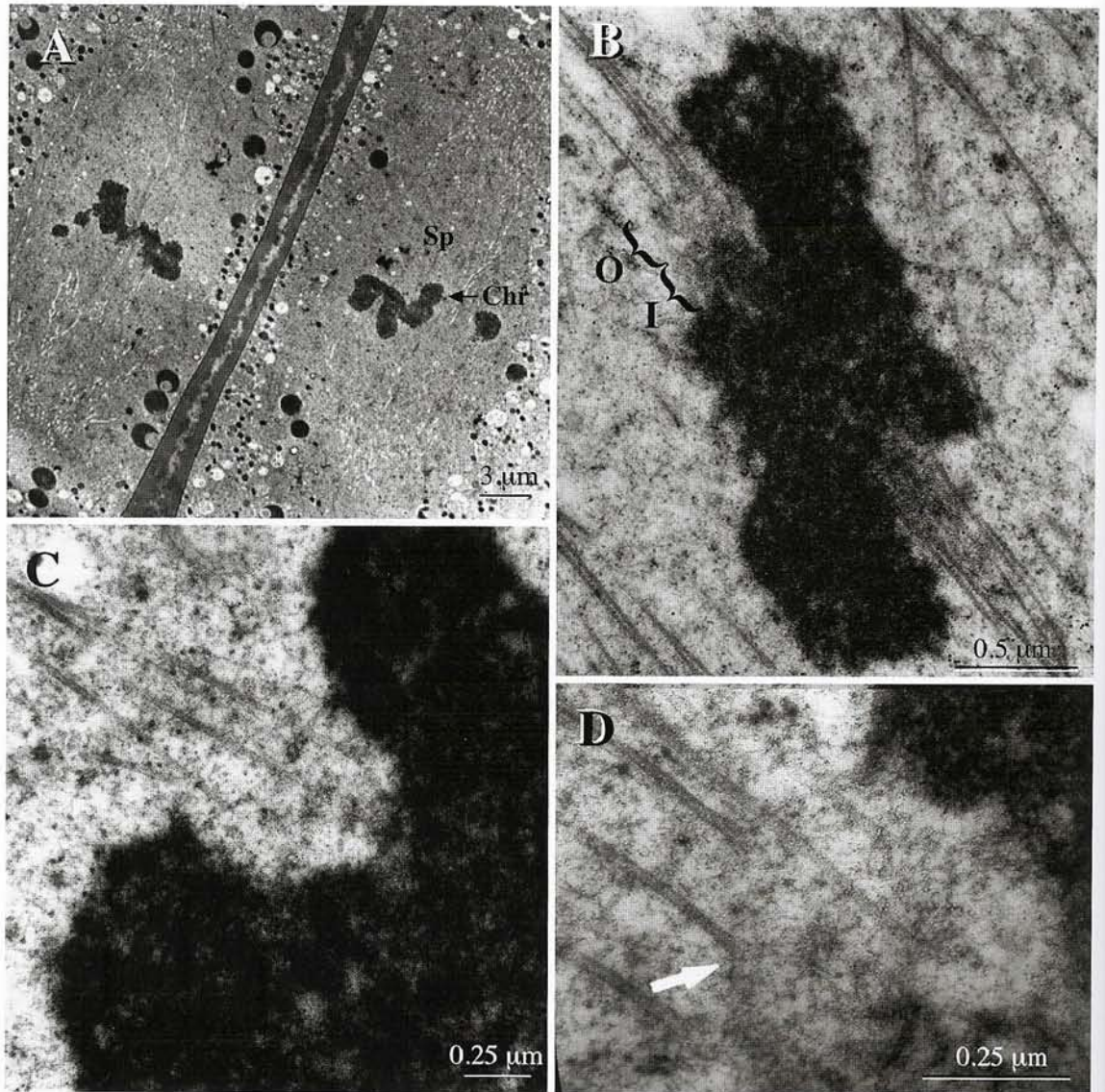


Fig. 2. The maize meiosis II kinetochore. **(A)** Two prometaphase II cells, recently separated from each other at meiosis I, are shown. Chromosomes (Chr) and spindle (Sp) are indicated. **(B)** A prometaphase II chromosome (black) showing both kinetochores and associated microtubules. The inner (I) and outer domain (O) of one kinetochore is indicated with brackets. **(C)** Another prometaphase II cell showing the “ball in cup” morphology. A cup of chromatin harbors a kinetochore that is nearly indistinguishable from the surrounding cytoplasm. **(D)** A high magnification view of microtubule ends in a prometaphase II kinetochore. The apparent splaying of protofilaments is indicated with an arrow.

(Zhong et al., 2002; Fig. 1D). Cells were then analyzed using an Applied Precision deconvolution-based 3D light microscopy system. Images were processed using software supplied by the manufacturer.

Results

General morphology of maize meiotic spindle and kinetochores

After high pressure freezing and freeze substitution, maize meiocytes were well preserved, showing dense microtubule-filled spindles that exclude most cytoplasmic organelles

(Figs. 1A, 2A). Meiotic kinetochores could only be identified by the cluster of well-preserved microtubules that terminated within them. We found no evidence of granules of any particular structure or size, fibrils, or even a characteristic electron density that would allow us to reproducibly identify the boundaries of the kinetochore. As described previously (Braselton and Bowen, 1971; Wilson, 1968), meiosis I kinetochores tend to protrude from the surface of the chromosomes (Fig. 1B), while meiosis II kinetochores tend to be sunken within the chromatin mass (Fig. 2B, C). This diffuse, ill-defined region classically defined as the kinetochore is referred to here as the “kinetochore proper”.

In several cases we observed what appeared to be strands of chromatin stretching out into the kinetochore proper. This is particularly evident in EM images of meiosis I (Fig. 1B, arrow), though at the light microscope level the inner and outer domains remain clearly distinguishable (Fig. 1C).

Sister kinetochores and their associated kinetochore fibers are distinct at prometaphase I

Data from both animals and plants suggest that the meiosis I kinetochore can be differentiated into two distinct sister kinetochores at or just before the time of microtubule attachment. As prometaphase progresses into metaphase, the separation of sister kinetochores becomes more and more distinct, such that they can often be fully resolved from each other in the light microscope at anaphase (Goldstein, 1981; Suzuki et al., 1997; Dawe, 1998; Dawe et al., 1999). Sister kinetochore separation at meiosis I has never been demonstrated at the electron microscope level in plants. As shown in Fig. 1B, our data revealed this phenomenon, and suggest that each kinetochore has its own kinetochore fiber (the bundle of microtubules attached to the kinetochore is known as the kinetochore fiber).

We could better resolve parallel sister kinetochore fibers by processing cells for light microscopy and labeling them with antibodies to tubulin and CENH3 (Zhong et al., 2002). The effect was most obvious at prometaphase I. As exemplified in the cell shown in Fig. 1D, at this stage sister kinetochores as well as their associated kinetochore fibers are split in two. In several cells we observed kinetochore fibers that stayed visibly separate all the way to the spindle poles. As the meiocytes progress from prometaphase I through metaphase I and anaphase I, the separation of fibers becomes less evident (data not shown).

Microtubules are splayed at the kinetochore interface

In one of the earliest studies of plant kinetochores, the authors described "structural irregularities" of the microtubules as they contacted the kinetochore (Wilson, 1968). The irregularities appeared as thickened microtubules and an apparent breakdown of the polymer. A similar change in microtubule morphology was described in kangaroo rat kinetochores (in an abstract without images: Mastronade et al., 1997). Similarly, we found that microtubules within the maize kinetochore broadened substantially at the ends (Fig. 2D). The shapes of microtubule ends resembled cones cut crosswise, as if the protofilaments were splaying apart.

Discussion

The plant kinetochore has few distinguishing ultrastructural features beyond serving as a focal point for microtubule attachment

Our goal was to document the ultrastructure of plant kinetochores under conditions that preserved the structure as effectively as possible. We adopted high pressure freezing/freeze substitution as the most suitable method available, since it has been shown to be superior in a number of applications (Dahl and Staehelin, 1989; McDonald, 1999).

Our observations of well-preserved kinetochores confirm and extend the results of a variety of previous publications. Studies in *Tradescantia*, wheat, *Lilium*, *Allium*, and *Zephyranthes* (Wilson, 1968; Braselton and Bowen, 1971; Bajer and Mole-Bajer, 1972; Wagenaar and Bray, 1973; Esponda, 1978; Hanaoka, 1981) all suggest that plant kinetochores have little or no discernable substructure. In some cases the kinetochore appears more electron dense than the surrounding cytoplasm (Bajer and Mole-Bajer, 1972; Wagenaar and Bray, 1973), but even this feature is inconsistent in our hands. In fact no attribute of the striking ultrastructure of animal kinetochores (Brinkley et al., 1989) is discernable in plants, suggesting that animal and plant kinetochores differ considerably.

Perhaps the single most distinguishing feature of plant meiotic kinetochore is the morphology of the microtubule ends. Consistent with previous data (Wilson, 1968; Mastronade et al., 1997), we show here that the microtubules in plant kinetochores can be splayed at their ends, as if the protofilaments are drawing away from each other. Since a splaying or curling of protofilaments is characteristic of depolymerizing microtubules (Mandelkow et al., 1991), our data may suggest that microtubules are shortening within the kinetochores. Animal kinetochores contain a microtubule-depolymerizing kinesin (Mitotic Centromere Associated Kinesin; MCAK) suggesting that this may indeed be the case in some species (Maney et al., 2000). Alternatively, the splayed appearance of microtubule ends may represent a specialized morphology involved in attaching the spindle to the kinetochore (Mastronade et al., 1997).

A new interpretation of the kinetochore ultrastructure with respect to a two-domain model

The results shown here represent the first study of kinetochore ultrastructure in maize, a species where a substantial amount of information on the centromere/kinetochore complex is already available (Yu et al., 2000; Houben and Schubert, 2003). Based on previous data, we had reason to believe that the maize kinetochore is composed of two subdomains, each with different functions. CENP-C and CENH3 both lie close to centromeric DNA, while MAD2 and the 3F3/2 antigen lie well outside of centromeric DNA and the inner kinetochore proteins (Yu et al., 1999). How can we reconcile data showing the existence of non-overlapping biochemical subdomains in the kinetochore with the fact that there is no evidence for subdivision at the ultrastructural level?

We suggest that the diffuse kinetochore proper represents only the outer domain as we defined it biochemically (Fig. 3). Supporting this view is the fact that microtubules appear to terminate throughout the kinetochore in maize (Fig. 2C, D) and other plants (e.g. Wagenaar and Bray, 1973; Hanaoka, 1981). The two known outer kinetochore proteins in maize, MAD2 and the 3F3/2 antigen, overlap perfectly and lie outside of the centromeric chromatin at prometaphase (Yu et al., 1999), suggesting that the outer domain is a large and relatively uniform structure. We further suggest that the inner domain lies entirely within the electron-dense chromatin. The idea that the inner kinetochore has properties of chromatin is strongly supported by recent evidence. Centromeric Histone H3 (CENH3) is a

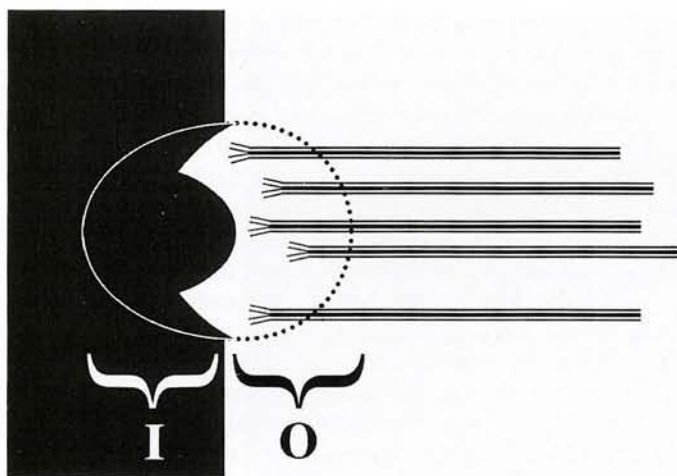


Fig. 3. A model of the plant kinetochore. The inner kinetochore (I), bounded here by a white line, does not stain differently from the rest of the chromatin (black). Occasionally chromatin extends into the outer domain (O). The outer kinetochore has no distinguishing staining characteristics under the electron microscope (after staining with uranyl acetate and lead citrate) but can be readily identified under the light microscope using antibodies to MAD2 (Fig. 1C). Microtubules terminate throughout the outer kinetochore, where the ends appear splayed.

known histone H3 variant and a key element of the inner kinetochore (Henikoff et al., 2001). Likewise, CENP-C is a constitutive protein of the inner kinetochore (Tomkiel et al., 1994) that co-immunoprecipitates with CENH3 (Ando et al., 2002), indicating that it lies within a higher-order centromeric chromatin complex. Several more inner kinetochore proteins have been

recently identified in animals, one of which is CENP-H. Antibodies to CENP-H can be used to immunoprecipitate centromeric DNA (Saffery et al., 2003), indicating that it too is a component of chromatin.

In previous reports we made special note of the strong demarcation between the inner and outer kinetochore at the light microscope level (Fig. 1C, Yu et al., 1999, 2000; Yu and Dawe, 2000). Similarly, at the ultrastructural level the demarcation between chromatin and the kinetochore proper is striking (Figs. 1B, 2B–D). The strong demarcation is easier to understand if the inner kinetochore is interpreted as a specialized chromatin domain on the continuous DNA molecule that links the centromere to chromosome arms. In contrast, the outer domain of the kinetochore may be better viewed as an autonomous organelle (Brinkley et al., 1988). Several lines of evidence indicate that the outer domain of animal kinetochores can be detached from chromosomes and still interact with microtubules on schedule (Wise, 1999).

The model in Fig. 3 suggests operational definitions of the inner and outer kinetochore. In general we expect antibodies to inner kinetochore proteins to localize to centromeres throughout the cell cycle and to immunoprecipitate centromeric DNA. In contrast outer kinetochore proteins are likely to localize outside of the centromeric DNA, to be found transiently at the kinetochore, and to be distant enough from DNA that chromatin immunoprecipitation would not reveal an interaction. To date, all known kinetochore proteins fall into either one of these categories.

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