14

Ultrastructural Mechanisms of Aposporous Embryo Sac Initial Cell Appearance and Its Developmental Process in Gametophytic Apomicts of Guinea Grass (*Panicum maximum*)

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1. Introduction

The ultrastructural mechanisms of aposporous embryo sac initial cell (AIC) appearance and its developmental process have been studied in gametophytic apomicts of guinea grass (*Panicum maximum* Jacq.), using ovary clearing treatment and Nomarski differential interference-contrast microscopy (DIC), and ultra-thin section and the transmission electron microscopy (TEM). For the observation of AIC appearance by DIC, AIC appears while megaspore degenerated in apomictic accessions, and most of the ovules contain several AICs, and the number of AICs increased as the ovary grew before anthesis. That is, several AICs in the same ovule did not differentiate synchronously, but instead, they seemed following a continuous course and appeared one by one during the period from after megasporogenesis to the first AIC-derived embryo sac maturity. It was also found that the higher the frequency of apospory was, the greater the number of AICs was, and the longer the duration of AICs appearance should be. For the mechanism of seed-forming embryo development in polyembryonic ovules in apomictic accessions observed by DIC, the first AIC is located dominantly in micropylar end, and the percentage of mature embryo sacs in micropylar end was higher than that in the other ends. The rates of the ovules contained developed embryo sacs in micropylar end at 4 days after anthesis were 33~91% comparable to 0~2% of that in the other ends. The embryo of the developed sac in micropylar end, in final, became a seed-forming embryo, and in contrast, the other sacs, were crowed out to chalazal end and degenerated at 10 or more days after anthesis. Form the results described here, in can be concluded that the AIC-derived embryo sac in micropylar end, in most of

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cases, has temporal dominant in embryo sac mature, and has the positional dominant in fertilization and subsequent development of embryo sac when compared with the cases in the other end in polyembryonic ovules.

For the AIC appearance and its development in apomictic accessions observed by TEM, the first sign observed is the degeneration in dyad stage, and accompanying it, the cell adjacent to the dyad in chalazal end, derived from nucellus began to increase its size of cell and change its organelles and shape, and thus, the cell become to the first AIC usually located in micropylar end. The AICs appear in order continently adjacent to the first AIC in chalazal end up to the stage of anthesis. For the AICs further development, they, as the substitute of megaspore, form four-nucleate embryo sac, containing one egg, one polar and two synergids at anthesis. The characteristics of reproductive cells formed in embryo sac derived from AIC are observed in detail, and the interdependent patterns of the nucellar cell and AIC, and their relationship are also discussed at subcellular level. These ultrastructural evidence obtained here provides important information to isolate AIC-specific genes (=apomixis-specific gene) and to understand the mechanism how the somatic nucellar cell changes into reproductive AIC by using single cell manipulation.

Apomixis is a reproduction mode that bypasses female meiosis and syngamy to produce embryos genetically identical to the maternal parent. In general, three major mechanisms of apomixis have been identified based on the origin and development of cells from which the embryo derives, and those are considered as important reproduction modes in plant breeding (Asker 1979, Asker and Jerling 1992, Bashaw and Hanna 1990). Apomixis phenomenon involves in many kind of plants in different types. Apomixis not only represents an interesting reproduction mode that can result in the reproduction of 4- and 8-nucleate embryo sacs simultaneously, i.e. in *Panicum maximum* (guinea grass), but also provides a method for cloning plants through seeds. From the unique reproduction mode, the significance of apomixis in two standpoints, i.e. macro and micro, can be considered. In the macro, apomixis can promise to bring the economic benefits to human being more than that of the "Green Revolution", by means of fixation of F1 varieties to cost down F1 production expense, fixation of hopeful breeding materials to shorten the times of breeding generation, and transformation of apomixis-specific gene into vegetative plants, like sweet potato and potato, to produce seeds to avoid the usage of seed tubers. And in micro, to understand the mechanism how the somatic nucellar cell changes into reproductive aposporous embryo sac initial cell (AIC) will take place another "Revolution of Reproduction" in plant. For example, somatic cell can be changed into sexual one like apomixis, and in the reverse, sexual cell can be the somatic one, by means of artificial biotechnology. In this meaning, the understanding and resolution of sexual reproduction can be expected not only in plant but also in human being.

In this chapter, we will focus on the appearance of AIC and its further developmental process in transmission electron microscopy (TEM) using the guinea grass as a model, based on the same observation in Nomarski differential interference-contrast microscopy (DIC). We will structure the wider discussion around the knowledge of apomixis we have accumulated from our study of *P. maximum*, which we established a model system of apospory.

2. Cytological mechanisms of AIC appearance and its developmental process using DIC

Up to now, three major mechanisms of apomixis, that is, apospory, diplospory and adventitious embryony, have been identified based on the origin and development of cells from which the embryo derives (Hanna and Bashaw 1987). Warmke (1954) firstly described that in guinea grass aposporous apomixis with pseudogamy is the mechanism of apomictic reproduction. Following that, researches on the reproduction mode in guinea grass have been performed to reveal the mode inheritance of apomixis in sexual (Smith 1972, Hanna et al. 1973) and in apomictic accessions (Warmke 1954, Savidan 1975, Savidan and Pernes 1982, Nakajima and Mochizuki 1983, Nakagawa 1990, Chen et al. 2000, 2001). And in the other species *Pennisetum*, co-inheritance of apomictic reproduction and two molecular markers has been demonstrated by Ozias-Akins et al (1993). Among the researches above, an important and key point is that by how many genes the apomixis is controlled, and the point has attracted many scientists working on it day and night for over a half century. They had attempted to clarify the genetic mode of apomixis using the traditional method of crossing hybridization (Daniel et al. 1998a, b). Unfortunately, the plant materials which appear apomictic figures are usually belonging to tetraploids, so that it is more difficult to find out the solution in tetraploids than that in diploids. However, the researches made by the scientists could be concluded with that apomixis may be mainly controlled by a single dominant or a few tightly linked genes (Savidan 1975, 1989, Nakajima and Mochizuki 1983, Chen and Kozono 1994a).

Although some researches have been reported on cytology and inheritance of apomixis in guinea grass, the period of apomictic gene expression, the key to cloning the apomixis genes has not been identified yet. When observing the differences between sexual and apomicts, the appearance of AIC, from which aposporous embryo sac is derived, should be considered the most relevant stage for the expression of apomixis genes, as these cells appear only in apomictic but not in sexual plants. Here, the problem is that the mechanism of AIC development is not well understood. And as a hypothesis that if an observable or measurable index could be found out through cytological evidence, it can be used to estimate the range of period of AIC appearance based on it the apomixis genes may be cloned. This study mainly describes cytological observation of AIC appearance and its development using DIC, and based on it provides information on the best timing for sampling materials in the program for apomixis gene isolation.

The application of DIC was carried out to study cytologically the mechanism of AIC appearance and its developmental process in guinea grass (*P. maximum*) using an improved method (Herr 1982). For obtaining the reliable data, seven facultively apomictic accessions and three obligately sexual accessions collected from Tanzania, Zambia and Japan were used in our study, and one hundred to 300 bus or flowers staged before and at thesis were collected per accessions for embryo sac analysis. The important point is that the pre-treatment of the samples be performed with FPA50 (formalin : propionic acid : 50% ethanol = 5:5:90) (Chen and Kozono 1994a) for one week at 4 °C. After the treatment of ethanol series, the samples are cleared in Herr (1982) fluid of benzyl-benzoate-four-and-half for over 2h at 0-4 °C. The observation of the samples was conducted suing DIC.

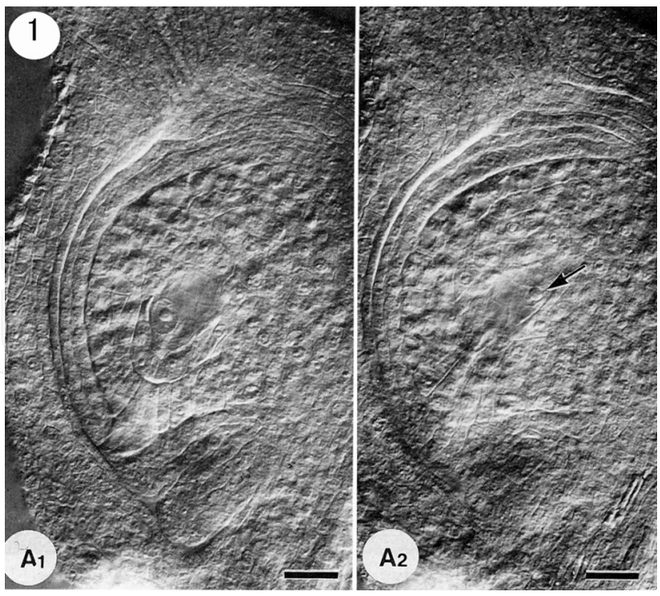

Fig. 1. Appearance of aposporous embryo sac initial cell (AIC) in facultative apomictic *Panicum maximum*. (A1) appearance of AIC in micropylar end and (A2) remaining of functional megaspore without nuclear membrane in chalazal end (arrow) in one ovule. Bar= 50μm.

Until megasporogenesis, differences between obligate sexual and aposporous accessions were not observed in ovule development. After that, the megaspore of sexual ovules showed a manner typical of the *Gramineae* family forming 8-nucleated embryo sacs as described by Hanna et al. (1973). In contrast, the same of apomictic ovules stopped its continuous development or degenerated. Usually at the same times that the megaspore lost its function, the unreduced (2n) nucellar cells around the degenerated megaspore appeared and enlarged their sizes, moved to the empty space and developed into a functional AIC (Fig. 1). The earliest AIC usually appears in ovule always located in where would turn toward and finally become micropylar end as the ovary grows. And then, the AIC divided twice and directly into 4-nucleated embryo sacs, containing one egg, two synergids and one polar nucleus. The AIC usually exists with megaspore, which often degenerated in the same ovule. Usually, several AICs appear in one ovule and their number increase as the ovary grows until anthesis. To clarify the mechanism of appearance of AIC, ovary length was selected as an index and measured when they were observed in different AIC appearance and its development. From the ovary length measuring result, it is understood that the AICs do not appear together in same time, instead, they seemed following a continuous course and appeared one by one during the period from megasporegenesis even to first embryo sac maturity. And accessions with higher frequency of apomixis have plural embryo sacs and also showed wider range of period of AIC appearance, when ovary length was measured. As another meaning, that is, their developmental stages of the ovary can be estimated using the ovary length as an index. The AIC appearance is a unique event in apospory different from the sexual, and may correspond with the time of gene expression of apomixis. Here, based on a hypothesis that the time of apomixis gene expression is just before the time of AIC appearance, it could be considered that the more the number of embryo sacs per ovule are, the longer the expression period will be. Therefore, as the materials for apomixis gene cloning, the accessions containing higher number of embryo sacs / higher frequency of apomixis, should be advantageous for their having wider duration of AIC appearance.

The ovary length of stages of first AIC was longer than that of functional megaspore in most accessions also indicates that apospory is initiated after megasporogenesis. And more, the ovary length staged in functional megaspore was wide and close to that staged in degeneration of embryo sac. These results also support that the development of sexual embryo sac is often terminated in many aposporous apomicts at the megaspore mother or megaspore stage, and the products of sexual process degenerate (Nogler 1984, Asker and Jerling 1992). However, as the limitation of DIC observation, the sexual termination in stage of megaspore mother cell was not observed in this study.

About the sexual embryo sac formation in ovules with plural embryo sacs in facultative apomictic plants, Nakajima and Mochizuki (1983) have reported that a few ovules had two sexual embryo sacs, and that in polyembryonic ovules having both sexual and apomictic embryo sacs, number of sexual embryo sacs was limited to be one. In this study, five types of embryo sac formation were recognized. That is, SS: only two 8- or 5-nucleate mature sexual embryo sacs in an ovule; S: only one 8-nucleate embryo sac in an ovule; An: one or more 4-nucleate mature apomictic embryo sacs; S+An: one 8- and one or more 4-nucleate embryo sacs in an ovule; SS+An: two 8- and one or more 4-nucleate embryo sacs in an ovule. Fore the case of ovules with one or two 5(8)-nucleate embryo sacs appeared with or without 4-nucleate ones in one ovule, two pathways could be considered that 1) the sexual embryo sac formation results from the direct division of one or two megaspore(s) though the AIC(s) appeared (or not) in the same ovules; and that 2) it is derived from AIC(s). In particular, as the ovules with two megaspores in chalazal end were not observed in this study while AIC(s) appeared in the micropylar end, the former pathway could be hardly considered as putative one. If try to explain the appearance of ovules containing one or two sexual embryo sacs with or without 4-nucleate apomictic ones, the later, however, seems reasonable based on that AICs develop into not only 4-nucleate but also, at a low frequency, 5-nucleate embryo sacs in *Panicum* (Nakajima and Mochizuki 1983), or 8-nucleate ones in *Hieracium* (Nogler 1984). For the 5-nucleate embryo sac formation, it could be imaged that after megaspore or AIC divided into two nuclei, only the micropylar nucleus continued to divide twice and to form 4-nuclei, the chalazal nucleus and one of the four micropylar nuclei pair with each other to form two polar nuclei. On the other hand, a rare case of 4-nucleate embryo sac divided from AIC, with an egg cell, one synergid cell and two polar nuclei was also depicted for *Panicum* by Bashaw and Hanna (1990). From the above observation of embryo sac formation reported in this study, it could be concluded that facultative apomictic guinea grass forms not only common and rare *Panicum*-type (4-nucleate), but also its typical type (5-nucleate), and in addition, *Polygonum*-type (8-nucleate), within the same one ovule.

Based on the observation of this study that the period of AIC appearance is the key to catch the apomixis genes, and ovary length can be used as an index to do sampling of the key period of AIC appearance, apomixis-specific gene-1 (*ASG*-1) and its family genes have been cloned from the key period of AIC appearance in facultative apomictic guinea grass using differential screening method (Chen et al 1999).

3. Cytological mechanisms of AIC-deriving egg cell and seed-forming embryo development

From the above observation of DIC, we have known that facultative apomicts of guinea grass usually form one more embryo sacs derived from AICs in the same ovule. While doing germination test of seeds, only one germinated from the facultative apomicts. There is an important problem in facultative apomicts that which one will be the seed-forming embryo

in progeny, and it is important for breeder to know the frequency of sexual or apomixis when doing crossing experiments. Therefore, an effective and repaid method for estimating the degrees of sexuality or apomixis is needed to be developed for their utilization in various plant breeding programs (Chen and Kozono 1994b). Up to now, two methods to estimate the degree of sexuality, i.e. embryo sac analysis and progeny test were often combined and used by many breeders (Warmke 1954, Smith 1972, Hanna et al. 1973, Savidan 1975, Savidan and Pernes 1982, Nakajima and Mochizuki 1983, Nakagawa 1990). Progeny test is impractical to provide direct estimation of frequencies of sexual and apomictic embryo sacs, and to observe entire variability in progeny (Nakajima and Mochizuki 1983). Embryo sac analysis permits it possible to identify the morphology of embryo sacs (Nakagawa 1990). Nakajima and Mochizuki (1983) had compared the two methods and indicated that two degrees of sexuality expressed as embryo sac type and frequency of off-type plants agreed mostly well in both of highly and lowly sexual accessions, but disagreed in the facultative apomicts. As two types of sexual and apomictic embryo sacs coexist in a same ovule, it remains unknown in facultative apomicts which embryo sac will become the seed-forming one. This study mainly focuses to demonstrate cytologically the events at the stages of embryo sac formation and seed-forming embryo development in polyembryonic ovules of facultative apomicts, to find out the relations between the stages and then, to provide reliable information for the estimation of the degree of apomixis or sexuality without progeny test.

To clarify the cytological mechanism of seed-forming embryo development in polyembryonic ovules in facultative apomictic guinea grass, the samples staged after anthesis of seven facultative apomicts were collected every day up to 10th day after open pollination (DAP), and observed using DIC and improved clearing method (Chen and Kozono 1994b). The continuous observation of the ovules indicate that, 1) the first AIC is located dominantly in micropylar end, and the percentage of mature embryo sacs in micropylar end was higher than that in the other end; 2) the rates of ovules containing embryo at 4 DAP was 90% in micropylar end higher than 2% in the other end; 3) the embryo in micropylar end, in final, became a seed-forming embryo, and in contrast, the others were crowded out to chalazal end and degenerated at 10 or days. The above results suggest that the degrees of sexuality or apomixis can be estimated based on the frequency in present generation, even without progeny test.

For the AIC appearance, Chen and Kozono (1994a) indicated that during the period from after megasporogenesis to anthesis, AIC appears one by one in facultative apomictic plants of *P. maximum*, according to a continuous course, and the first AIC appeared in the ovule always located dominantly in the micropylar end, forming mature 4-nucleate embryo sac . On the other hand, Nakajima and Mochizuki (1983) described that the most vigorous embryo sac, sexual or apomictic one, is considered to be representative of the polyembryonic ovule. In fact, however, it is difficult to decide which is the most vigorous one, especially, for the beginner while observation. To find out a solution of deciding which embryo sac will develop into seed-forming embryo, is very important not only in establishment of determination standard for all of observers but also in evaluation of sexuality or apomixis degree (Chen and Kozono 1994b). This study attempted to compare the percentage of mature sacs and the sizes of nucleoli of the embryo sacs positioned in micropylar end with those in the other ends, to determine the reproduction mode of the polyembryonic ovules. The result that the percentage of mature sacs and sizes of nucleoli of embryo sac in micropylar end were more advantageous than those in the other end, means that the AIC formed in micropylar end has the temporal dominant in formation and

maturity of the embryo sac when compared with the other sacs (Fig. 2). In other words, the dominantly matured sac in micropylar end has been ready for fertilization. This result was also supported by that if numerous embryo sacs exist in an ovule, the embryo sac in the favorable position, i. e., closest to the micropylar end of the ovule, is usually the one that the pollen tube enters (Koltunow 1993).

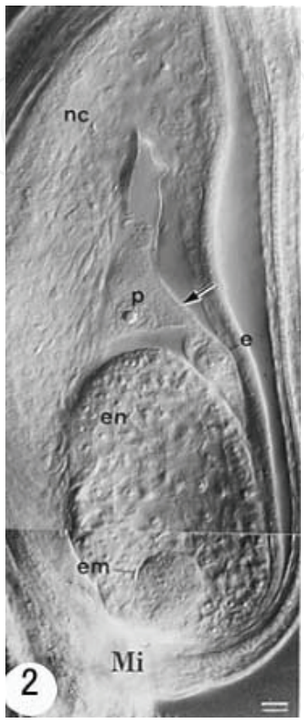

Fig. 2. Dominant development of AIC-derived embryo sac in micropylar end with pre-globular-stage embryo and well-developed endosperm at 3 DAP in polyembryonic ovule of facultative apomictic *Panicum maximum*. e = egg cell; en = free-nuclear endosperm; p = polar nucleus; em = embryo; nc = nucellar cell; Mi = micropylar end; bar = 50μm.

Accession	No. ovules observed	No. embryo sacs	E.s. in micropylar end				E.s. in the other end			
			Embryo and endosperm		Embryo only	Endo-sperm only	Embryo and endosperm		Embryo only	Endo-sperm only
	A		B	(B/A)			C	(C/A)		
T-75	46	66	42	(91%)	2	2	0	(0%)	0	0
T-41	47	144	34 (1)[1)]	(68%)	5 (1)[2)]	2	0 (1)[1)]	(0%)	2 (1)[2)]	0
N68/84-1-s-6	40	115	20	(43%)	0	0	0	(0%)	0	0
Natsukaze	54	63	18	(33%)	0	0	0	(0%)	0	0
N68/96-8-o-10	40	55	30	(75%)	0	0	0	(0%)	0	0
N68/96-8-o-11	102	235	78 (6)[1)]	(76%)	3	0	2 (6)[1)]	(2%)[3)]	0	0

[1)] Twin embryo sacs containing both of embryo and endosperm, respectively, in an ovule. [2)] Twin embryo sac containing one embryo but no endosperm, respectively, in an ovule. [3)] The embryo sac in the other end, instead o in the micropylar end, containing developed embryo and endosperm.

Table 1. Development of embryo sac (e.s.) in the flowers 4 DAP in facultative apomictic accessions of *Panicum maximum*.

Next, the fertilization degrees of different embryo sacs in an ovule were examined to certify that if the position of embryo sac located has the advantage in fertilization. The results were obtained that 33~98% of the ovule contained the developed sac in micropylar end in all of the 6 accessions tested, but in the other ends, 0% in 5 accessions and 2% in N68/96-8-o-11, at 4 DAP, respectively (Table 1). It strongly supports the above proposition. When observing the process of the embryo and endosperm developments, it is found out that the sac in micropylar end is usually the one that will be fertilized (or pseudogamy) dominantly (here, the fertilization means that when endosperm formation it is needed) and, just the egg develops automatically to embryo in the sac without fertilization, in final, became to the seed-forming embryo. On the other hand, the other sacs have no chance to receive entrance of pollen tube for fertilization, and be crowded out by the developed sac to chalazal end, in final, be completely degenerated after 10 DAP.

This study provides reliable data for effective embryo sac analysis. 1) If the matured embryo sac in micropylar end is apomictic one, apomictic seed-forming embryo should be formed, which can be evaluated at anthesis; 2) If the sac is sexual one, sexual seed-forming embryo should be formed, which can also be evaluated at anthesis. Therefore, it can be concluded that with this method mentioned here, it should be possible that using the frequency of apomictic and sexual embryo sacs in micropylar end at anthesis estimate the degree of sexual or apomixis, even without progeny test. It is interesting as a further project to compare the two degrees of sexuality or apomixis estimated by embryo sac analysis as described here and by progeny test. Although the same project had been conducted (Nakajima and Mochizuki 1983), a new conclusion will be expected using the method described here.

4. Ultrastructural mechanism of AIC appearance

Chen et al (1999) has reported that the *ASG*-1 gene was cloned using differential screening method based on ovary length as an index to sample the different developmental stages of facultative apomictic guinea grass. And then, the *ASG*-1 was used as a probe to do in situ hybridization in apomictic guinea grass, indicating that the signals of the gene expression were detected not only in the expected AIC and AIC-derived embryo sac but also in unexpected anther (Chen et al. 2005). The above result indicates that AIC appearance is most different event from sexual, so further characterization of real timing of AIC appearance means importance not only in clarification of the mechanism but also in cloning of aposporous gene.

For the observation of AIC in guinea grass, many efforts have been turned to this work for a long time in section method (Warmke 1954) and in embryo sac analysis method (Savidan, 1975, Nakajima and Mochizuki 1983, Nogler 1984, Nakagawa 1990, Chen and Kozono 1994a, b). They have made it clear that 1) the first difference between sexual and apomicts was degeneration of megaspore in facultative apomictic guinea grass; 2) after megasporogenesis, it is almost at the same time that megaspore degenerates and AIC appears; 3) when examining the degree of sexuality or apomixis in facultative or obligate apomictic ovary with polyembryonic ovules, the embryo sac located in micropylar end represents the fate of the ovary as it develops into the only one of seed-forming embryo (Chen and Kozono 1994b). Naumova and Willems (1995) firstly reported an ultrastructural characterization of aposporous megagametophytes in *P. maximum*, indicating that AIC usually differentiates

adjacent to degenerating tetrad, and megaspore or 1-nucleated sexual embryo sacs. And they also indicated that little is known about the processes that regulate cellularization and differentiation of aposporous megagametophytes.

In the section, we focus the ultrastructural features of the developing aposporous megagametophytes in guinea grass (*P. maximum*), in particular, on the timing of degeneration in process of megasporogenesis, AIC differentiation and AIC-derived embryo sac formation. And the interdependent patterns of the nucellar cell and AIC, and their relationship are also discussed at subcellular level.

The facultative apomicts of guinea grass, "Petrie" and "Gatton" of 2 varieties were used and the samples were collected from 3 classes of the stages 1) before and completion of megasporogenesis, 2) the AIC appearance and 3) formation of the AIC-derived embryo sac based on ovary length as an index (Chen and Kozono 1994a). The ovaries were fixed and the fixed samples were dehydrated by a graded ethanol series and embedded in Spurr's resin (Guan and Adachi 1997). Ultrathin sections were cut on an ultramicrotome using a glass knife and double stained with uranyl acetate and lead citrate. The sections were viewed with a HITACHI H-800 MV transmission electron microscopy (TEM) at 75 kV.

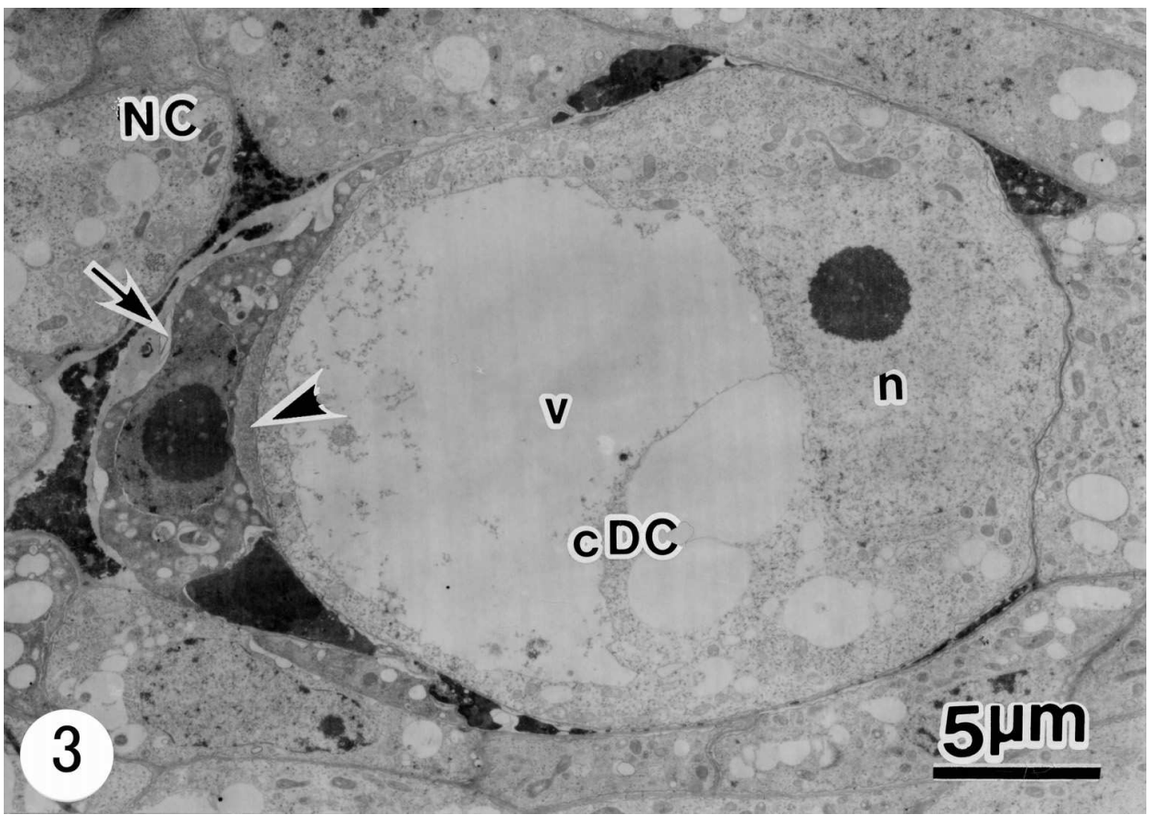

Fig. 3. A dyad stage of the megasporocyte displaying the degenerated dyad cell (arrow) in micropylar end and well-developed chalazal dyad cell (cDC) with the electron-dense material in the transverse wall (arrowhead). cDC = chalazal dyad cell; fn = free nuclear; n = nucleus; NC = nucellar cell; v = vacuole.

This study mainly focused on the timing and processes in degeneration of sexual cells and the appearance of AIC at ultrastructural level. It is found that the degeneration occurred as early as the stage of dyad. As first event, micropylar dyad was degenerated and chalazal dyad showed vacuoles in high degree during megasporocyte division (Fig. 3). The occurrence of vacuoles is normal phenomenon in megasporegenesis process (Guan and Adachi 1994), and it is also an auspice of cell degeneration (Guan and Adachi 1997) reported in *Fagopyrum esculentum*. The ultrastructure of dyad cells of *P. maximum* is comparable to that observed in other plants (Russel 1979, 1985). The vacuole appearance may be one of auspice of sexual cell degeneration in the case of *P. maximum*. The thickness of chalazal dyad cell wall between micropylar end and chalazal end is different, and there usually appeared thick layer of callose in micropylar cell wall. That some electron dense materials are present in the transverse wall of the dyad and tetrad was also observed in this study. It is bright comparison between that micropylar dyad degenerated completely and that there appeared electron-dense wall in chalazal dyad toward micropylar end. As Naumova and Willems (1995) indicated, the incomplete callose wall is probably an early sign of low activities, which will be followed by degeneration of the megaspore. As the appearance of both complete and incomplete callose in different ovules, the incomplete callose wall is probably not the reason for the onset of apospory, but a sign of apospory.

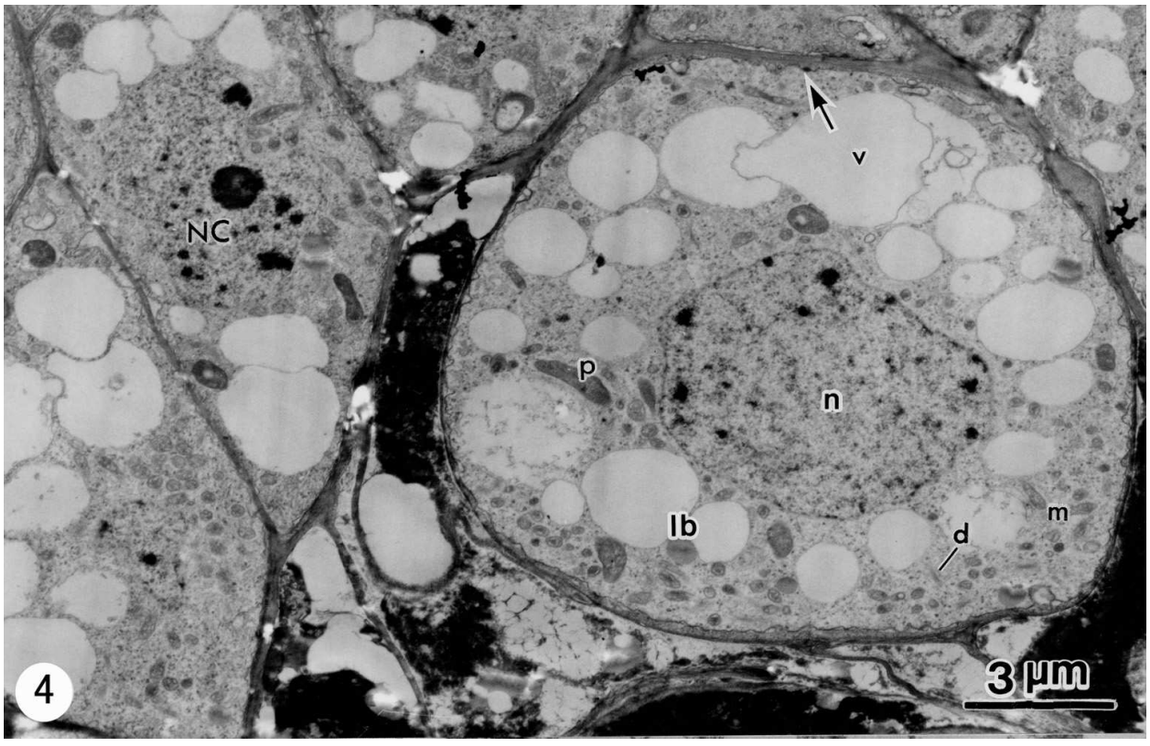

Fig. 4. A near round shape nucellar cell (NC) occurred adjacent to the degenerated cell located in lower side and to the other NC in other directions. The NC formed its original cell wall with two cell layers between the round NC and normal NC (arrow), and contains abundant contents with vacuole (v), lipid body (lb), dictyosomes (d), plastid (p), mitochondria (m) and a big nucleus (n) located in the center of the NC, that differs from the normal NC. Here, the newly formed round NC was named as AIC.

For the AIC appearance, it is found that the dyad generated, at the same time, the cell of nucellus showed subtle change, meaning appearance of AIC (Fig. 4). The stage of AIC appearance is earlier than tetrad stage reported by Naumova and Williams (1995). At the chalazal end of degenerated sexual cells, it is usually observed that some nucellar cells appear with bigger size than normal nucellar cells and with round shape. These cells have their independent cell walls. As ovule develops in early stage, the cells continue to enlarge their size, and show vacuole, a host of RES and high dense of nucleotides. These subtle changes described here mean the cell will develop into AIC. There are no differences observed between the nucellar cells around megasporocyte in ultrastructure. The nucellar cells have capability to form aposporous embryo sacs, and they depend upon and inhibit each other by the communication between thin cell wall and cytoplasm, with which the internal balance is kept. However, the capability to become AIC is very different according to the position of nucellar cells located in (Chen and Kozono 1994a, b, Chen et al. 2000, 2001, Naumova and Willems 1995). Although every nucellar cell has the chance to develop into AIC, only one or some cells break down the balance and inhibition among the nucellar cells, and after extreme competition, become the AIC to develop into AIC-derived aposporous embryo sac, in final.

And then, how the AIC develops into embryo sac will be described. The AIC will continues to differentiate and forms ellipse initial cell, and the size of which is 8.5 times of the round nucellar cells in volume. The AIC has uniformly thick and complete wall, and lack communication substance between cells. The thickness of cell wall increases, and plasmodesmata connections diminish leading to the end of symplastic transport and separating the cell from the other.

Chen et al. (1999) indicated that the *ASG*-1 gene isolated from stage of AIC appearance in facultative apomictic guinea grass, containing 1177 bp and 305 amino acids, shows the 45-48% positive similarity to polygalacturonase 1 beta chain precursor (Polyg 1) of *Lycopersicon esculentum* (Zhang et al 1992) that has a bifunctional plant proteins that interact with both structural components of the cell wall and catalytic proteins to localize and/or regulate metabolic activities within the cell wall. *ASG*-1 existing in AIC and AIC-derived embryo sac is also reported by in situ hybridization (Chen et al. 2005). *ASG*-1 with cell wall growing function is also supported by the ultrastructural analysis as described here. The structure of cell wall thickness means that AIC has already had the differentiation capability of self-complete and genetic system. The AIC, finally, results in formation of aposporous embryo sac.

In early stage of ovule development, around the AIC many degenerating cells at different stages of megasporogenesis with high density and black degenerated cytoplasm in center of the ovule. These degenerated cells could be considered as that 1) they are dyad, tetrad, megaspores, or degenerated sexual cells staged in sexual embryo sac formation; 2) some nucellar cells degenerated. As AIC appears, it competes with nucellar cells round it, and absorbs nutrients from them for its own differentiation. At the same time, as the volume of the AIC increases, it pushes and affects the other cell around it. Therefore, it is considered that when the AIC divides into embryo sac, it absorbs part nutrients from nucellar cells around it, and from degenerated sexual cells or sexual embryo sac.

Concluding the above mentions in ultrastructural analysis, it can be found that from the subtle change of organelles, volume and sizes, and shape of nucellar cells, aposporous development has already began while sexual cell, e. g. dyad, degenerated. Generally, sexual cells and nucellar cells are localized in balance. However, it is considered that nucellar cells located in chalazal end inherit and develop dominantly when they sense some signals from sexual ones which will be the fate of abortion and degeneration, and/or lose capacity of division. At the same time, one of nucellar cell-derived AIC cells appears, sexual cells and other nucellar cells provide nutrients for AIC differentiation (Guan et al. 2006, 2007, 2008). In some meanings, the life of the ovule (or ovary) will be kept by whatever either sexual or asexual (nucellar) cell becomes to embryo sac. In this way, that over 90% of ovules contain aposporous embryo sac, and lower 10% of megaspores in small ratio developed dominantly into sexual embryo sac, was observed in both of facultative apomictic guinea grass, 'Petrie' and 'Gatton' (Chen and Kozono 1994a, b).

5. Ultrastructural mechanism of AIC-derived embryo sac formation

For the AIC differentiation in facultative apomictic guinea grass, the above section has already given a clear description in ultrastructural level. However, the mechanism of AIC-derived embryo sac formation has not yet been described in ultrastructural level, although DIC observation of it had been reported by Chen and Kozono (1994a, b). In a practical meaning of breeding, when examining the degree of sexual or apomixis in facultative apomictic ovule with AIC-derived embryo sac in *P. maximum*, the method of progeny test, i. e. percentage of apomictic origin, was used, in general. After the ovule–clearing technique (so called embryo sac analysis) used, the most vigorous embryo sac is considered to be representative of multiple embryo sacs in ovule for estimation of apomixis degrees (Nakajima and Mochizuki 1983). However, the standard of estimation of the most vigorous embryo sac in the ovule should be different according to the observers. Using the improved ovule clearing technique, Chen and Kozono (1994b) have defined that, the embryo sac located in micropylar end represents the fate of the ovule as the sac develops into the only one of seed-forming embryo. And a new challenge for isolation of reproductive cells of aposporous embryo sac has been reported by Chen et al. (2005), indicating the differences of size, morphology and numbers between egg, synergid and polar in the sac. Little is still known about ultrastructural characteristics of reproductive cells in aposporous embryo sac in *P. maximum*. This study wants to make it clear why the AIC located in micropylar end develops dominantly, and what the functions of the reproductive cells are, as the characteristics of AIC in ultrastructural analysis may provide information to understand the overall functions of gametophytic apomict.

For the sample preparation, the facultative apomicts of guinea grass, “Petrie” and “Gatton” of 2 varieties were used and the samples were collected. The ovaries were fixed and the fixed samples were dehydrated by a graded ethanol series and embedded in Spurr’s resin (Guan and Adachi 1997). Ultrathin sections were cut on an ultramicrotome using a glass knife and double stained with uranyl acetate and lead citrate. The sections were viewed with a HITACHI H-800 MV transmission electron microscopy (TEM) at 75 kV.

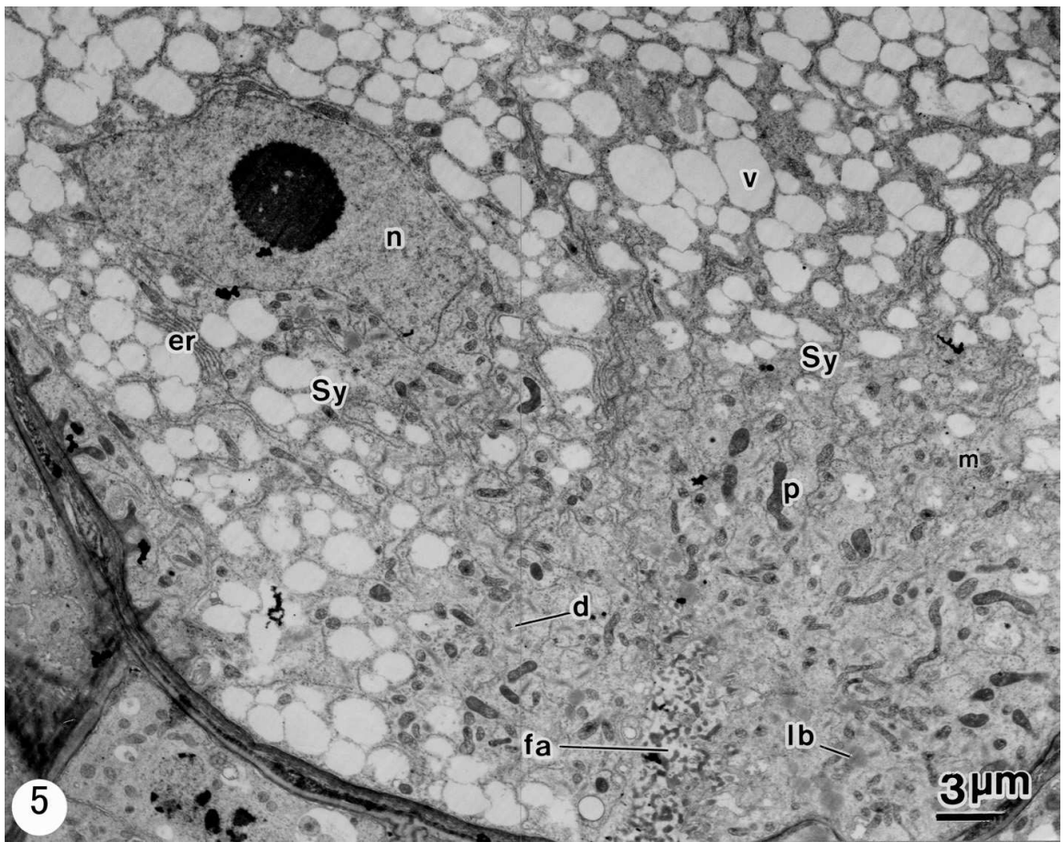

Fig. 5. Two synergids (Sy) with filiform apparatus (fa) in the micropylar end of the cell. v = vacuole; m = mitochondria; p = plastid; lb = lipid body; d = distyosome; er = endoplasmic reticulum.

For cloning of apomixis-specific gene, two strategies are mainly tried out, i. e. using genomic DNA (Ozais-Akins et al 1998), and using differential screening of mRNA of AIC (Chen et al 1999). In particular, when using mRNA to do gene cloning the examination of special developmental stage, which is considered as the target gene expression stage, will be important (Chen et al 2005). Moreover, for the developmental stage determination, ultrastructural data is stricter than that of observation of normal microscopy. However, ultrastructural data about apospory and other types of apomixis are scarce and not discussed in recent publication (Koltunow 1993). And thereafter, ultrastructural analysis of AIC appearance in *P. maximum* have been reported in KK-15 by Naumova and Willemse (1995), and in two varieties of 'Petrie' and 'Gatton' by Chen and Guan (2006), respectively. This study shows ultrastructural characteristics of the reproductive cells in AIC-derived embryo sac.

In sexual and aposporous *P. maximum*, the developmental process of gametophytes is generally reported as shown in Fig. 6, according to the previous reports (Warmke 1954, Savidan 1975, Chen and Kozono 1994a). And Chen and Kozono (1994a, b) has indicated that while the megaspore failed to divide, some nucellar cells enlarge and become the AIC to replace the megaspore to form 4-nucleate sac, based on the observation of embryo sac analysis by using DIC.

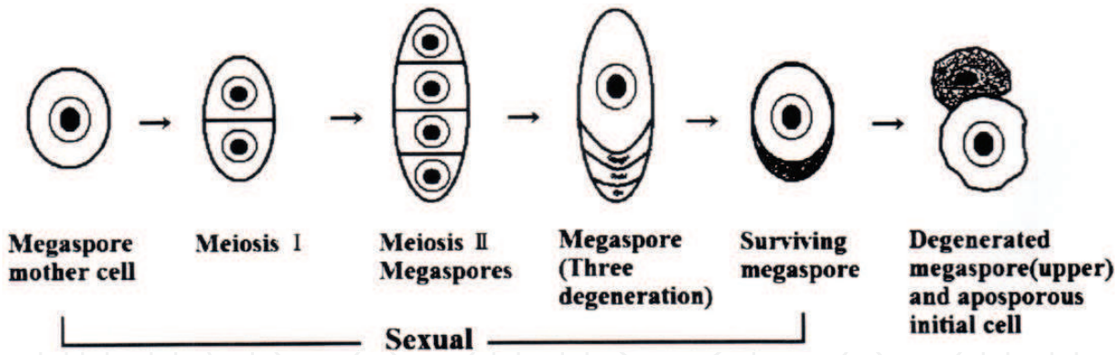

Fig. 6. The illustration of megasporogenesis in sexual and aposporous *P. maximum*.

In multiple aposporous embryo sac ovule of *P. maximum*, each sac displays difference from the other in size, reproductive cell number, cell location and cell developmental stage. In the same ovule, embryo sacs usually are located in the center of ovule, and every ovule has different number of sac, i. e. usually from two to four. As the number of the sac increases the difference in its sizes among the sacs becomes bigger. This result agrees with that AIC appears as ovary length grows (Chen and Kozono 1994a). The micropylar end of the sac displays half-sphere shape, and the chalazal end is irregular.

Here, different number of reproductive cells between the embryo sacs located in micropylar and the other end was found out by DIC and TEM. The sac located in micropylar end usually consists of four cells. And the sac develops dominantly from micropylar end so that the numbers of reproductive cells in the sac and the size of the sac are more and bigger than that located in chalazal end. At anthesis, the sacs in different developmental stages coexist, and later formed AICs develop to immature sacs containing 1-nucleate, coenocytes or incomplete developed cells. All of the sac have monopolarity and lack antipodals. This constitution of sac in aposporous *P. maximum* is different from another aposporous *Hieracium aurantiacum*, which have seven cells with eight nuclei same to sexual embryo sac (Bicknell 1997, Koltunow et al. 1998).

For the first aposporous embryo sac development, which is usually located in micropylar end of the ovule, there are the egg apparatus with two synergids and one egg cell arranged triangularly in the sac. The egg apparatus differentiates ultrastructurally with morphological characteristics similar to those found in sexually functional synergids and egg cell. The triangular organization of the egg apparatus is not necessarily conversed as the unreduced egg cell may appear in more lateral position with respect to one of synergids (Naumova and Vielle-Calzada 2006). The first aposporous embryo sac with 4 cells usually develops dominantly and becomes the only seed-forming embryo (Chen and Kozono 1994b). It can be considered that as the first aposporous embryo sac contains four cells and is located in micropylar end from where pollen tubes go through the filiform apparatus and enter into the synergid (Guan and Adachi 1997).

For the other sacs, they showed containing less than four cells, and feathers of degeneration. During development of the sacs, there are competition in space and nutrition among the sacs, so that some sacs may fail to develop. The degenerated embryo sac, in some meaning, will provide nutrition for aposporous embryo sac development. This degeneration often

takes place in multiple aposporous embryo sac ovule, indicating that the more the number of the sac are, the rates of degeneration are higher. From the points of structure and nutrition, the results that the sac located in micropylar end gives 76% of rates of embryo formation, but the other only 2% (Chen and Kozono 1994b), can be understood well. This result also indicates that the sac in micropylar end decides the fate of the seed formation in multiple sac ovule. This also supports the estimation method reported by Chen and Kozono (1994b) that the embryo sac in micropylar end represents sexuality of the ovary.

There showed clear deference between the both ends of the egg apparatus. That is, the micropylar end is separated by many normal cell walls of synergid, egg-synergid, synergid-polar, and egg-polar, and in the chalazal end, it is only surrounded by the plasma membrane. For the cell wall ingrowths, Chapman and Busri (1994) indicated that in facultative apomictic genotype in *Pennisetum*, many wall projections were observed in the micropylar region, but few ingrowths were found in the chalazal region of both sexual and apomictic megagametophytes. The results obtained in this study are also agreement with their observations.

For the development of synergids in the first sac, the cell wall between two synergids is expanded to form conveniently orientated filiform apparatus in the micropylar end. However, variational filiform apparatus presents in micropylar end of the synergid located in the other sac, where only one synergid formed. The content feature of the single synergid was not different from that of the two synergids. The synergids had characteristics of highly vacuolated cells and high localized cytoplasm containing a large and irregular shaped nucleus. The filiform apparatus formed only in first sac has conformity with that only one seed-forming embryo formed in multiple embryo sacs in ovule of guinea grass (Chen and Kozono 1994b). This fact also confirms indirectly the estimation to be correct, that is, in ovule with multiple embryo sacs, the first sac located in micropylar end represents the reproductive mode of the ovule (Chen and Kozono 1994b). According to the hypothesis of Savidan (1989), which postulates through a complete egg cell wall, the sperm-cell penetration cannot be taken place. However, such a complete wall in this study was not observed in the mature aposporous embryo sac.

For the ultrastructures of the aposporous embryo sac and the reproductive cell in the sac, they have own characteristics, respectively. Egg cell containing highly dense cytoplasm, and around its nucleus plastids and mitochondria are remarkable. Lipid bodies and rER and dictyosomes are few, and big vacuoles are distributed in cytoplasm. The polar cell is occupied almost by one big vacuole, the lower dense cytoplasm than that of egg cell wraps one to two nuclei and is distributed around egg apparatus. That only in the sac in micropylar end the cell wall of the polar exists with ingrowth of cell wall shows that this structure transports the nutrition for the embryo development. Synergid usually contains lowest dense of cytoplasm, and lipid bodies, plastids and mitochondria are distributed along to the filiform apparatus in micropylar end. Extreme rER and vacuoles distributed in chalazal end of the synergid might contain abundant inorganic substance and they are absorbed as a part of nutrition for the developing embryo (Fig. 5). The ultrastructural analysis of AIC and AIC-derived embryo sac formation described in this study will give information not only for gene cloning but also for single AIC manipulation in next step.

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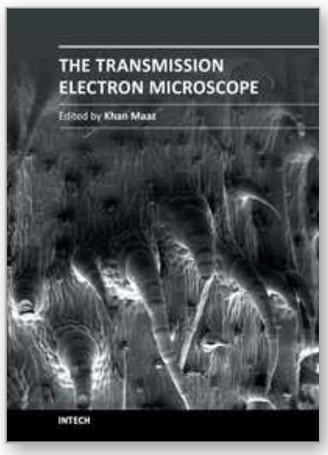

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The book "The Transmission Electron Microscope" contains a collection of research articles submitted by engineers and scientists to present an overview of different aspects of TEM from the basic mechanisms and diagnosis to the latest advancements in the field. The book presents descriptions of electron microscopy, models for improved sample sizing and handling, new methods of image projection, and experimental methodologies for nanomaterials studies. The selection of chapters focuses on transmission electron microscopy used in material characterization, with special emphasis on both the theoretical and experimental aspect of modern electron microscopy techniques. I believe that a broad range of readers, such as students, scientists and engineers will benefit from this book.

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