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THE APOMIXIS-SPECIFIC PHENOMENON: APPEARANCE OF APOSPOROUS EMBRYO SAC INITIAL CELL (AIC), AIC-DERIVED EMBRYO SAC FORMATION AND EMBRYOGENESIS WITHOUT FERTILIZATION IN APOMICTIC GUINEA GRASS (*PANICUM MAXIMUM*)

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ABSTRACT

Apomixis is a reproductive mode which bypasses female meiosis and syngamy to produce embryos genetically identical to the maternal parent. Apomixis is usually classified into three modes, apospory, diplospory and adventitious embryogeny. Here, we describe the mechanism of aposporous initial cell appearance in guinea grass (*Panicum maximum*), which belongs to aposporous mode. Based on the observation of young buds by Nomarski differential interference-contrast microscopy (DIC), there was no cytological difference between sexual and apomicts up to megasporogenesis. After that, however, the megaspore usually degenerated and in final, disappeared in the apomicts in contradistinction to that in sexual to form 8-nucleate embryo sac. At the same time, aposporous embryo sac initial cell (AIC), derived from nucellar tissue around the megaspore, appeared, which divides into 4-nucleate embryo sac. And before anthesis, the AIC appears one by one and each AIC become mature AIC-derived embryo sac (AES), i.e. one egg, two synergids and one polar cell. After anthesis, the egg cell develops into embryo automatically without fertilization with sperm cell, but with the stimulation of the fertilization between the one polar and sperm cell. To further clarify process of AIC appearance, ultrastructural observation was studied by transmission electron microscopy (TEM). The first degeneration was observed in dyad stage. Accompanying the degeneration of sexual cell, the cell adjacent to the dyad in chalazal side, 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 side. The AIC appeared in order continently adjacent to the first AIC in the chalazal side. The interdependent patterns of the nucellar cell and AIC, and their relationship are also discussed at subcellular level. At anthesis, the first AES located in micropylar end consists of egg apparatus with two synergids and one egg cell, and the central cell with one nucleus. The other AESs contain less than four cells. The egg cell contains high dense of cytoplasm, and which around the nucleus plastids and mitochondria are remarkable. There exist lipid bodies, few rough endoplasmic reticulum (rER), dictyosomes, and big vacuoles distributed in the cytoplasm. The polar cell is occupied almost by one big vacuole, the lower dense cytoplasm than that of egg cell wraps one or two nuclei and is distributed around egg apparatus. 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 (Chen and Kozono 1994b). The following results were understood. 1) the first AIC is located dominantly in micropylar 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. Based on cytological observation using ovary length as an index to sample the young buds, and differential screening method, AIC stage-specific genes were cloned. The gene analysis using in situ hybridization and transgenic plants carried out was also discussed.

INTRODUCTION

Panicum maximum Jacq. (Guinea grass) is an important tropical forage grass native to tropical Africa and cultivated widely in tropical, subtropical, and even in the temperate region in the world. Its reproductive mode belongs to one of apomixis, so-called apospory (Warmke 1954). Apospory is a form of apomixis in which sporophytic cells in the ovule give rise to unreduced female gametophytes (Gustafsson 1947). The autonomous division of aposporous egg cells generates viable embryos without fertilization. However, the majority of aposporous species is pseudogamous and requires fertilization of the polar nuclei for endosperm development (Nogler 1984). A cytological analysis of aposporous megagametophytes had

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early been conducted in *P. maximum* by using methods of section (Warmke 1954), embryo sac analysis (lactic acid: choral hydrate: phenol: eugenol: xylen = 2 : 2 : 2 : 2 : 1 by weight) (Savidan 1975, Nakajima and Mochizuki 1983), improved methyl salicylate clearing (Nakagawa 1990). Chen and Kozono (1994a, b) have developed a procedure of embryo sac observation, indicating that pre-treatment of samples using FPA50 solution, dehydration series using 70%~100% ethanol, and transparentness using Herr's clearing solution (Herr 1982) are necessary for fine clearing of ovary in *P. maximum*. An ultra structural characterization of aposporous mega gametophytes has firstly been carried out in *P. maximum* by Naumova and Willems (1995). They indicated that AIC usually differentiate adjacent to degenerating tetrad, and megaspores or one-nucleated sexual embryo sacs. In this chapter, we focus on the analysis of aposporous embryo sac initial cell (AIC) differentiation in apomictic guinea grass, using the levels of cytological observation with Nomarski differential interference-contrast microscopy (DIC), and ultra structural characterization with transmission electron microscopy (TEM). We structure the wider discussion around the knowledge of apomixis we have stored from our study of *P. maximum*, a model system of apospory we had established.

Cytological Characterization of Aposporous Reproductive Mode

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 pseudo gamy 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, 2001a, b, c). 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, 1983, 1989, Nakajima and Mochizuki 1983, Chen and Kozono 1994a). Although some researchers 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 pretreatment 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. 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. 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 megasporogenesis 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 1979, 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. For 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, Bicknell 1997, Bicknell and Koltunow 2004).

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).

Embryogenesis in AIC-Derived Embryo SAC

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. 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). 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. 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. Chen *et al* (2005), at first time, tried to isolate the viable egg cell, indicating that multiple embryo sac formation was observed with the egg number (more than one) in *P. maximum*.

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.

Ultra structural Characterization of Aposporous Reproductive mode

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). In grass of *Pennisetum*, Chapman and Busri (1994) reported the ultrastructural studies for the first time. In *P. maximum*, Naumova and Willems (1995) firstly reported an ultrastructural characterization of aposporous megagametophytes, 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 *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 ultra microtome 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. This study mainly focused on the timing and processes in degeneration of sexual cells and the appearance of AIC at ultra structural 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. The occurrence of vacuoles is normal phenomenon in megasporogenesis 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. 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. The stage of AIC appearance is earlier than tetrad stage reported by Naumova and Williams (1995), and Naumova and Vielle-Calzada (2006). 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 ultra structure. 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, 2001a, b, c; 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 ultra structural 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 ultra structural 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).

Apomixis-Apecific Gene Cloning and its Characterization

Apomixis provides asexual reproductive mode that produces offspring genetically identical to the maternal parent through seeds, meaning that it is expected to simplify the development of heterogeneity and the production of commercial hybrid seed when used in breeding program (Calzada *et al* 1996). Guinea grass is a tropical forage crop (2n=4x=32) that contains sexual and apospory reproductive modes (Chen and Kozono, 1994a, b). Based on ovary length used as index, *ASG* (apomixis-specific genes) cDNAs were cloned from cDNA library of aposporous initial cell (AIC) appearance-stage by using differential screening method (Chen *et al*. 1995). *ASG*-1 was analyzed with sequencing and homologue searching (Chen *et al*. 1999), and in situ hybridization (Chen *et al*. 2005; Chen and Guan 2006). We used improved differential screening and collected some *ASG* cDNAs by Northern hybridization. A surprised result was that they showed same sequences but different lengths among the collected clones. When they were used as probes for Southern hybridization, the results between sexual and apomictic guinea grass showed different band patterns.

The homologue search results of these clones also showed the same to that of *ASG*-1. When they used as RNA probes for in situ hybridization, strong signals were detected in AIC and AIC-derived from embryo sac with different developmental stages of apomictic guinea grass but sexual ones. When the *ASG* gene transferred into rice, some variants have been obtained with different morphology in reproductive organs from regenerated plants. These *ASG* genes are probably the same gene which may control apomixis if *ASG* genes were apomixis ones. According to the purpose of breeding, apomixis is often classified into three major mechanisms: adventitious embryony, apospory and diplospory. Among the three, apospory is considered as the most valuable reproductive mode in agriculture production as it occurs and develops without recombination in appearance of aposporous initial cell (AIC) when the megaspore degenerated (Chen and Kozono, 1994a). We have also found that the number of AIC increase and AIC-derived embryo sac becomes mature as ovary length grows until anthesis. After anthesis, the multiple

embryo sacs in an ovule form but only one embryo sac located in micropylar end can develop into seed-forming embryo (Chen and Kozono, 1994b). Based on the cytoembryological observation, *ASG*-1 (apomixis-specific gene, GenBank No. AB000809)) was cloned from *P. maximum* using differential screening method according to ovary length used as index (Chen *et al*. 1995, 1999). *ASG*-1 has 1177bp, in which it contains 305 amino acids. And it showed homologues of seed- and embryo-specific genes reported previously. The expression of *ASG*-1 was monitored during gametogenesis in obligate-sexual and facultative-apomictic genotypes of *P. maximum*. *ASG*-1 expression is strong and specific to AIC, and then continues through different stages of AIC-derived aposporous embryo sac development, indicating that the gene may play a role in this developmental process (Chen *et al*. 2005).

Functional analysis of *ASG*-1

Up to now, *ASG*-1 was tried to produce out transgenic plant for its functional analysis (Seo *et al* 2004; Chen *et al* 2013b). In order to quickly clarify the functions of *ASG*-1, an apomixis specific gene, the model plant of *Arabidopsis thaliana* was used to establish vital and realizable transformation system (Chen *et al*. 2013a). Using the combinations of the floral dip and inoculating methods, a binary pActnos/Hm2 vector, and the *Arabidopsis thaliana*, for transformation of *ASG*-1 establishes a suitable procedure for plant regeneration of transformants. For the plasmid construction, a binary vector was used to contain *ASG*-1, named as pActnos/Hm2. The resultant plasmid was mobilized into *Agrobacterium tumefaciens* strain EHA105. Using pipetteman to drop the bacteria on the flower buds, and or putting the bolts into the tube containing bacteria was used for transformation. For detection of *ASG*-1, DNAs of T1 plants were used for PCR, using the primers designed according to *ASG*-1, hygromycin and the primers from Actin and from *ASG*-1, in pActnos/Hm2 vector. The morphology of T1 plants was also observed. About 87 of T1 transgenic plants were obtained after selection with hygromycin B-supplemented medium, and when comparing them with the control no transformed plant, they showed 4 kinds of phenotypes with different morphological traits, named as Type A, Type B, Type C and Type D. For detection of *ASG*-1 in T1 transformants, the PCR products gave *ASG*-1 and hygromycin specific bands in different primer combinations, respectively. And the primers from Actin and *ASG*-1 indicated the expected specific band with 150 bp in T1 and T2 plants, respectively. The procedures using a binary vector pActnos/Hm2 containing *ASG*-1 revealed, as the first case, that *Agrobacterium*-mediated transformation system in *Arabidopsis thaliana* was established for the functional analysis of *ASG*-1, an apomixis specific gene isolated from apomictic guinea grass (*Panicum maximum*). The project on apomixis research is in progress.

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