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# Sexual reproduction development in apomictic *Eulaliopsis binata* (Poaceae)

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**ABSTRACT.** Apomixis is a particular mode of reproduction that allows progeny formation without meiosis and fertilization. *Eulaliopsis binata*, a tetraploid apomictic species, is widely used for making paper, rope and mats. There is great potential for fixation of heterosis in *E. binata* due to autonomous endosperm formation in this species. Although most of its embryo sac originates from nucellus cells, termed apospory, we observed sexual reproduction initiation in 86.8 to 96.8% of the ovules, evidenced by callose deposition on the walls of cells undergoing megasporogenesis. However, only 2-3% mature polygonum-type sexual embryo sacs were confirmed by embryological investigation. Callose was not detected on aposporous initial cell walls. The aposporous initial cells differentiated during pre- and post-meiosis of the megaspore mother cell, while the sexual embryo sac degenerated at the megaspore stage. DNA content ratio of embryo and endosperm in some individuals was 2C:3C, based on flow cytometry screening of seed, similar to that of normal sexual seed. These results confirm that apomictic *E. binata* has conserved sexual reproduction to a certain degree, which may contribute to maintaining genetic diversity. The

 

finding of sexual reproduction in apomictic *E. binata* could be useful for research on genetic mechanism of apomixis in *E. binata*.

**Key words:** Apomixis; Callose; Embryology; Sexual reproduction; Flow cytometry screen seed

## INTRODUCTION

Although most flowering plants reproduce sexually, some species (over 40 plant families) form asexual seeds, a process termed apomixis in nature. Unlike sexual reproduction, seedlings arising from apomixis retain the genotype of the maternal parent. The hallmark components of apomixis include female gamete formation without meiosis (apomeiosis), fertilization-independent embryo development (parthenogenesis), and developmental adaptations to ensure functional endosperm formation (Koltunow and Grossniklaus, 2003). Thus, apomictic plants can stably produce seeds in an environment unfavorable for fertilization. However, apomixis is rare in food crops. Nogler (1984) proved that apomixis is under genetic control, although its expression could be affected by genetic modifiers or environmental conditions. The molecular mechanisms underlying apomixis would be critical for the efficient use of apomixis in a crop plant. If an apomixis gene could be used in plant breeding, it would provide a tremendous opportunity to maintain hybrid vigor over successive seed generations, and thereby accelerate the breeding process by enabling the stable combination of many traits (Koltunow and Tucker, 2008).

Apomictic seed production can differ among species. Our understanding of the cytological development of major apomixis features has come from different genera including: *Hieracium* spp (Koltunow, 1993), *Taraxacum palustre* (Richards, 1970), *Poa pratensis* (Matzk, 1991), and *Pennisetum squamulatum* (Wen et al. 1998). Many apomictic species are facultative, with a certain frequency of residual sexual reproduction, and produce meiotically reduced pollen grains (Koltunow and Grossniklaus, 2003). Malecka (1973) found some reduced egg cells that needed fertilization for viable seed set in the apomictic *T. palustre*. Thus, the existence of sexual reproduction in apomictic plants provides an opportunity to uncover the genetic basis and molecular mechanisms of apomixis. Molecular genetic analysis has been carried out with multiple types of molecular markers in sexual and apomictic genotypes to map the traits of apomixis. Ebina et al. (2005) used cross-pollination and the co-segregation of AFLP and RAPD to establish mapping markers with aposporous apomixis in *Panicum maximum*. Screening differentially expressed genes between ovaries of sexual and apomictic accession was an alternative strategy to elucidate candidate genes of apomixis (Rodrigues et al., 2003; Laspina et al., 2008). Therefore, the identification of sexual accession in apomicts is critically valuable for understanding the genetic background and molecular mechanisms in apomixis.

*Eulaliopsis binata* is a perennial grass belonging to the family Poaceae. Since the discovery of apomixis in *E. binata* (Zhang et al., 1996), the embryological and cytological studies of apomixis in *E. binata* have been investigated in recent years. Embryological investigation indicated that the developmental pattern of embryo sac formation in *E. binata* represents gametophytic apospory, and the embryo originates from an unreduced egg cell without fertilization. Particularly, its endosperm development is in an autonomous pathway (Yao et al., 2004b, 2007). The karyotype analysis and observation of chromosome behavior

 

during meiosis of pollen mother cells demonstrated that *E. binata* is a heterotetraploid plant with 40 chromosomes (Liu et al., 2007). The nine populations of *E. binata* collected from four provinces of China were classified into four biotypes according to their genetic diversity by RAPD, AFLP and SSR markers (Yao et al., 2004a; Liu et al., 2006, 2008). The studies in previous articles suggested that most populations of *E. binata* that we collected showed facultative apomixis (Yao et al., 2007; Liu et al., 2008). Although sexual reproduction is not completely eliminated in apomictic *E. binata*, the cytological evidence of sexual embryo sac development is lacking and sexual reproduction degradation is still uncertain. Furthermore, it is unclear whether developing sexual and aposporous structures of *E. binata* undergo any form of communication.

Our ultimate goal was the elucidation of the molecular genetic mechanisms leading to apomictic seed production in *E. binata*, and hence the identification of sexual reproduction accession was essential. We, thus, present a detailed observation of sexual reproduction development in facultative apomictic *E. binata*, in addition to considering the signaling between close neighboring cells in the facultative apomictic ovule.

## MATERIAL AND METHODS

### Plant materials

Four *E. binata* populations, Xingzi, Hengyang, Xixia, and Xichuan, were used in this study for cytoembryological analysis. Seeds from Xingzi, Hengyang and Xixia were used for flow cytometry analysis. The materials were from Guangdong, Hunan, Henan, and Shanxi Provinces of China and planted in the experimental field of Huazhong Agricultural University.

### Cytoembryological analysis

Inflorescences from four populations were collected at 3-5 developmental stages, using the morphological criteria described by Yao et al. (2004b, 2007). Inflorescences during megasporogenesis and mature embryo sac were collected and fixed in FAA (70% ethanol, glacial acetic acid, formaldehyde; 90:5:5) for 24 h. Pistils were dehydrated in a tertiary butyl alcohol series and embedded in paraffin. Samples were sectioned at 6-8 μm and stained in 0.1% (w/v) decolorized aniline blue (DAB) with 0.03 mM $K_3PO_4$ for 1 h at room temperature; slides were coverslipped and sealed with DAB. Samples were observed under UV light using an Olympus BX61 (Olympus, Japan) fluorescence microscope. At least 30 florets were examined over 3-5 stages for each individual, among 10 individual plants from each population.

Ovaries at different developmental stages were embedded in paraffin wax, sectioned at 6-8 μm and stained with Ehrlich's hematoxylin or toluidine blue to observe the process of sexual embryo sac formation and modes of reproduction for different populations.

### Flow cytometry analysis

Flow cytometry seed screens (FCSS) were performed according to the method of (Doležel and Göhde, 1995). Samples consisted of 15-20 seeds from different individual plants. The DNA content of nuclei (C-value) from seedling leaves was used as a standard and the rela-

tive DNA content of nuclei was measured using flow cytometry Ploidy Analyzer PA (Partec, Germany). Each run represented about 5000 nuclei, and three runs were read for each sample.

## RESULTS

### Callose (β-1,3-glucan) deposition pattern in *E. binata*

The synthesis and dynamic changes in callose wall in the megaspore mother cell and macrosporogenesis are a key cytological event during sexual embryo sac development, and deficiencies in callose deposition have been observed in apomictic plants. In order to study the callose deposition pattern of apomictic *E. binata*, the ovaries from four populations were collected for histological analysis at pistil development stage 3.

Callose was initially deposited at the micropylar end of the ovule; the cell with intense callose fluorescence is located in the nucellar epidermal cell inner layer (Figure 1A). Subsequently, the cell with callose deposition undergoes meiosis, which was usually seen in the development of the sexual megaspore mother cell and was not reported in mitosis. Thus, the nucellar cell with callose deposition must be megaspore mother cell (MMC) (Figure 1B). During meiosis, callose was deposited in the cell wall of megaspores, including the transverse walls and side walls. Figure 1C shows that the megaspores were surrounded by a wall with callose during dyad. Callose was also deposited in tetrad stages, in the walls of four megaspores and also in the chalazal wall (Figure 1E). This result suggests that the chalazal end megaspore would be selected for megagametogenesis. This is also typical of the callose distribution during sexual reproduction megasporogenesis. However, the selected megaspore did not expand after meiosis in normal apomixis ovules, and the callose persisted in the walls of four megaspores during tetrad degeneration (Figure 1E).

In contrast to the callose deposited in the sexual initial cell (MMC), callose was not detected in the walls of aposporous initial (AI) cells in apomictic *E. binata*. In most ovaries, the walls of sexual megaspores had obvious callose fluorescence, while the AI cells, which adjoin the sexual cells had no fluorescence (Figure 1C,D, indicated by arrows). However, in a few ovaries, no callose fluorescence was observed in the ovule, while fluorescence was seen in the MMC or microspore tetrad in the anther in the same flora (Figure 1G), suggesting that callose did not distribute in the wall of the initial cell, likely due to the happening of obligate apomixis. Figure 1H shows the control sections treated with distilled water both in anthers and in ovaries, with the lack of fluorescence under UV light during meiosis. Therefore, we could identify sexual reproduction and apomixis reproduction by the difference in callose deposited in early ovule development of *E. binata*.

In the four populations, 86.81 to 96.77% of the ovules examined showed deposited callose, which indicated that the sexual embryo sac initial cell is common in *E. binata* (Table 1). There were no differences between the different populations in the pattern of callose deposited, and the callose distribution observed showed no difference between transverse walls and side walls, or between micropylar pole and chalazal pole.

### Development of sexual embryo sac in *E. binata*

The developmental process of the sexual embryo sac in four populations of *E. binata*

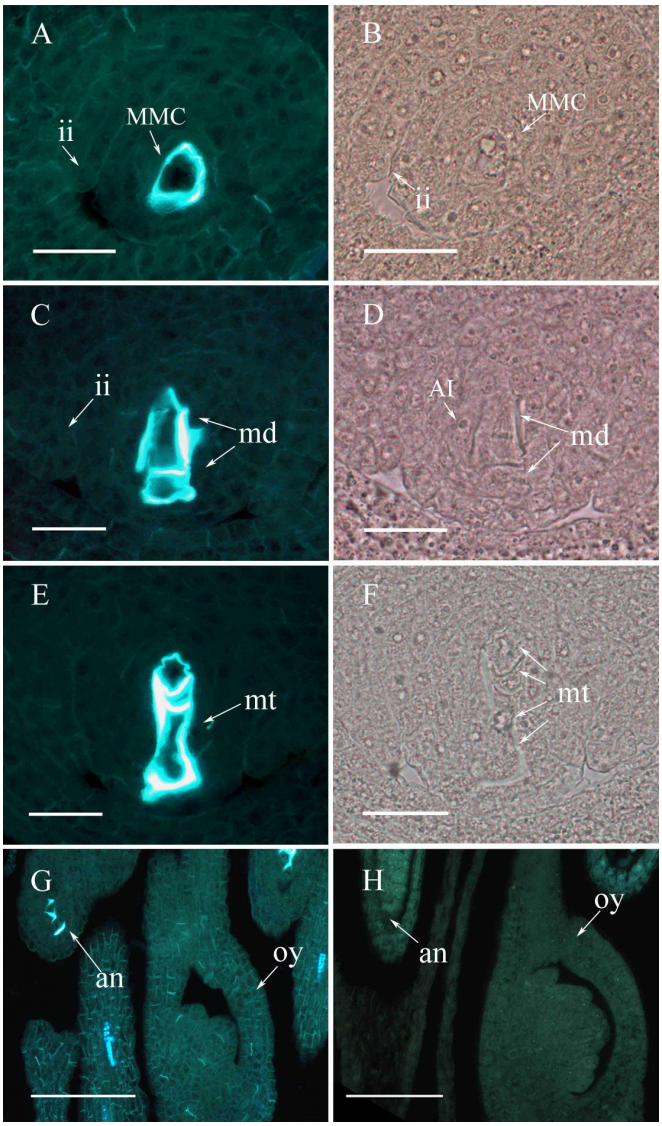


**Figure 1.** Patterns of callose deposition in *Eulaliopsis binata* during megasporogenesis. Callose fluorescence under UV light was detected using DAB in all panels except B, D and F, which were bright field images of A, C and D, respectively. **A.-F.** Images are oriented with the micropylar end of ovule at the top and chalazal end of the ovule at the bottom. **A. B. G.** Megaspore mother cell stage (arrow). **C. D.** Megaspore dyad stage. **E. F.** Megaspore tetrad. **H.** Control sections treated with distilled water. AI = aposporous initial cell; an = anthers; ii = inner integument; oy = ovary; md = megaspore dyad; MMC = megaspore mother cell; mt = megaspore tetrad. Bars = 30 μm (A-F); 100 μm (G, H).

was examined by cytoembryological observation. Ovaries were harvested from different stages of pistil development, fixed, sequentially sectioned, and stained with Ehrlich's hematoxylin or toluidine blue (Figure 2).

**Table 1.** The mode of reproduction of four *Eulaliopsis binata* populations.

| Population | No. of ovules (MMC to tetrad) | No. of ovules displaying callose | Percent | No. of mature sacs | No. of sexual sacs | Percent |
|---|---|---|---|---|---|---|
| Xixia | 93 | 90 | 96.77% | 115 | 2 | 1.74% |
| Xingzi | 91 | 79 | 86.81% | 95 | 2 | 2.11% |
| Hengyang | 101 | 96 | 95.05% | 99 | 2 | 2.02% |
| Xichuan | 96 | 89 | 92.71% | 93 | 0 | 0% |

MMC = megaspore mother cell.

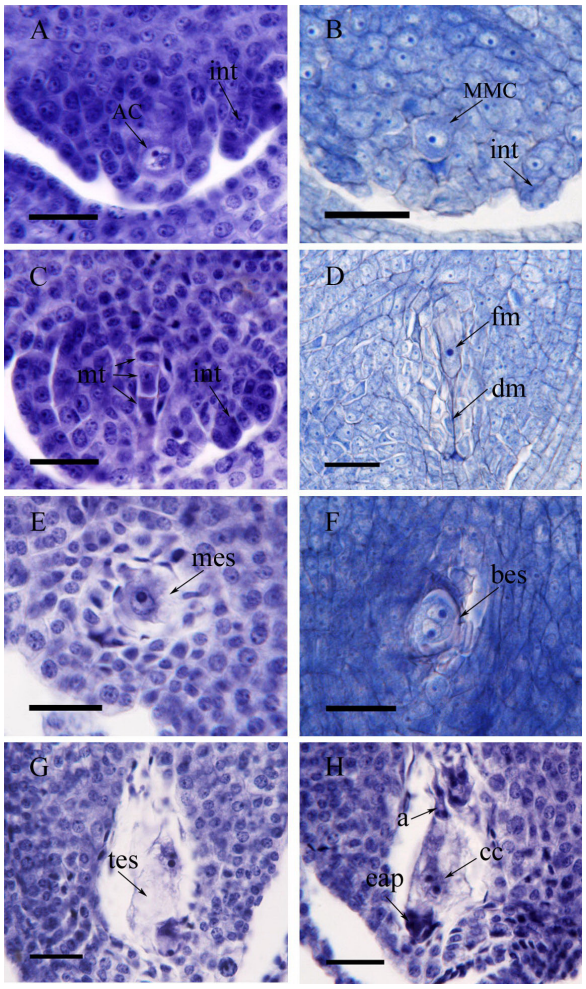


**Figure 2.** Cytoembryological observation of the sexual embryo sac development in *Eulaliopsis binata*. **A. C. E. G. H.** Stained with Ehrlich's hematoxylin. **B. D. F.** Stained with toluidine blue. All images are oriented with the micropylar end of ovule at the top and chalazal end of the ovule at the bottom. AC = archesporial cell; int = integument; MMC = megaspore mother cell; mt = megaspore tetrad; fm = functional megaspore; dm = degenerated megaspores; mes = monosporic embryo sac; bes = binucleate embryo sac; tes = tetranucleate embryo sac; eap = egg apparatus; a = antipodal cells; cc = central cell. Bars = 50 μm.

At the beginning of stage 3, an archesporial cell was observed located near the nucellar epidermal cells of the micropylar pole when the ovule integument appeared (Figure 2A). The specific cell could be easily distinguished from surrounding nucellus cells by its dense cytoplasm, large nucleus and cell volume. Subsequently, MMC was differentiated from the archesporial cell surrounded by a layer of nucellar epidermis (Figure 2B). At the end of stage 3, MMC divided via meiosis into a linear array of four megaspores, as the ovule integument had enveloped them (Figure 2C). The functional megaspore near the chalazal end enlarged in cell volume, while the three other megaspores close to the micropylar end degenerated rapidly (Figure 2D).

During megagametogenesis, the functional megaspore continued to enlarge and formed a monosporic embryo sac with a bigger cell volume, and meanwhile, the nuclei of the cells became denser (Figure 2E). The monosporic embryo sac underwent mitosis thrice; after the first mitosis, the mononucleus divided into two nuclei, forming a binucleate embryo sac (Figure 2F), and the two nuclei then moved to the micropylar end and chalazal end, respectively. The second mitosis resulted in a four-nucleate embryo sac (Figure 2G). Following the last mitotic division, an eight-nucleate embryo sac formed, with four nuclei at the two ends, respectively, and the nuclei from each end of the embryo sac then moved toward the center. At this time, a mature embryo sac of the *Polygonum*-type was present. Figure 2H showed the sexual reproduction embryo sac, which contained one egg cell, two synergids near the micropylar end, two polar nuclei localized in the center, and three antipodal cells, which were adjacent to the chalazal end.

Because the four-nucleate *Panicum*-type embryo sac lacks antipodal cells in apospory *E. binata*, we could identify the sexual sac and aposporous sac with the character of antipodal cells in the mature embryo sac. According to the observation of the mature embryo sac in the ovary, three populations (Xingzi, Hengyang, and Xixia) of the four tested populations exhibited a sexual *Polygonum*-type embryo sac, but the sexual reproduction rate was very low, only about 2-3% (Table 1).

## Aposporous initiation and the degeneration of sexual cells in *E. binata*

Although most ovules had a sexual initial cell based on the observation of the development of megasporogenesis in four *E. binata* populations (Figures 1 and 2), the number of mature *Polygonum*-type embryo sacs of sexual reproduction was very low. In order to understand aposporous initiation and the degeneration of sexual cells, the early stage of ovule development of *E. binata* was examined (Figure 3).

The AI cells with a large nucleus and large cell volume were distinguished from surrounding nucellus cells, and the feature of AI was similar to that of MMC at the light microscopy level. AI cells were first observed at the MMC stage (Figure 3A,B), but in fact, most AI cells initiated during MMC meiosis, and they were seldom observed after meiosis (tetrad stage) in the ovary (Figure 3C). The appearance of AI cells occurred after the differentiation of MMC during sexual embryo sac formation. In facultative apomictic *E. binata*, sexual and apomixis reproduction could have co-existed contingently in one ovule, and thereafter, sexual events aborted and degenerated soon after tetrad or megaspore selection, coinciding with the development of aposporous initial cells. Generally, AI cells expanded into the left cavity after sexual spore degeneration (Figure 3D), and finally formed a single embryo sac in this position as that of the sexual embryo sac. At the stage of mature embryo sac, more than 95% of ovules

only had a *Panicum*-type aposporous embryo sac. The phenomenon of a sexual *Polygonum*-type and *Panicum*-type aposporous embryo sac co-existing in one ovule was not observed in our study. In *E. binata*, although sexual reproduction initiated prior to apomixis reproduction, the aposporous embryo sac had obvious advantages during the competition between sexual and apomixis embryo sac development, and the development of the aposporous embryo sac led to the abortion of the sexual embryo sac.

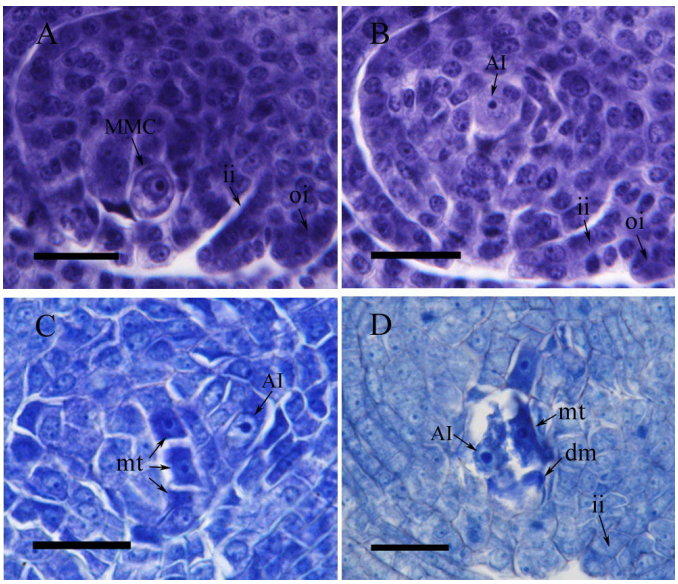


**Figure 3.** The time of aposporous initiated for *Eulaliopsis binata*. **A.** and **B.** Two serial sections of the same ovule stained with Ehrlich's hematoxylin. **C. D.** Stained with toluidine blue. All images are oriented with the micropylar end of ovule at the top and chalazal end of the ovule at the bottom. MMC = megaspore mother cell; mt = megaspore tetrad; ii = inner integument; oi = outer integument; AI = aposporous initial cell; dm = degenerated megaspores. Bars = 50 μm.

## Identification of mode of reproduction in *E. binata* by FCSS

FCSS was used for determining the mode of reproduction in *E. binata*. The leaf cells were used as a calibration standard, which showed a single peak representing 2C cell ploidy (Figure 4A). The seed samples from individuals of the Xingzi, Hengyang and Xixia populations were analyzed by FCSS. The C-value ratio of mature seeds in most individuals was 2C:3C:4C, where few individuals showed 2C:3C peaks (Table 2). The Xingzi 14 and Hengyang 11 had only two peaks representing a C-value of 2C and 3C (Figure 4B,C). The 2C peak may originate from unreduced apomictic embryos (2C), or fertilized sexual embryos (1C + 1C), or autonomous endosperm derived from a central cell composed of single polar nucleus (2C). The 3C peak could be from the endosperm that developed from the fusion of a central cell and one reduced sperm nucleus (2C + 1C), or embryo derived from fertilization of an unreduced egg (2C) with a reduced sperm (1C). If the endosperm automatically formed from an unfertilized central cell, the C-value would be 4C. Thus, in combination with the embryo-

logical observation, the C-value ratio of embryo and endosperm in mature seed indicated that Xingzi 14 and Hengyang 11 with 2C:3C peaks would be sexual reproduction individuals, and most of the other individuals, such as Xixia 3 with 2C:3C:4C peaks (Figure 4D), showed the facultative apomixis type. These results also showed that there were different modes of reproduction in one population.

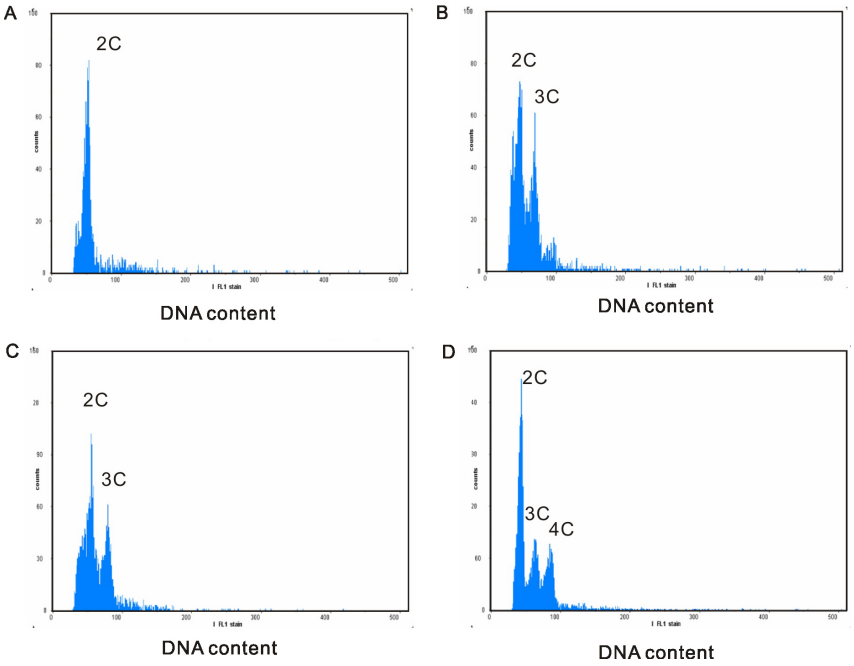


**Figure 4.** Cytometric histograms of seeds from individuals of Xingzi, Hengyang and Xixia populations of *Eulaliopsis binata*. **A.** DNA content distribution in leaf cell of *E. binata*. **B.** DNA content distribution in seed of Xingzi 14. **C.** DNA content distribution in seed of Hengyang 11. **D.** DNA content distribution in seed of Xingzi 3.

**Table 2.** DNA content distribution in seed of Xingzi, Hengyang and Xixia *Eulaliopsis binata*.

| Individuals | C-values in embryo and endosperm |
|---|---|
| Xingzi 14 | 2C:3C |
| Xingzi 3, 6, 7, 12 | 2C:3C:4C |
| Hengyang 11 | 2C:3C |
| Hengyang 5, 7, 2, 3, 10 | 2C:3C:4C |
| Xixia 3 | 2C:3C |
| Xixia 5, 6, 7 | 2C:3C:4C |

## DISCUSSION

### Identification of sexual reproduction in *E. binata*

In a previous study, Yao et al. (2007) found one case of mature sexual embryo sac in Red-haulm *E. binata* population (from Shanxi Yangxian population) by the whole-mount-cleared

technique. In this study, we provide cytoembryological evidence of sexual reproduction of MMC formation and sexual embryo sac development in four populations of *E. binata*. We not only detected the megasporegenesis of Xingzi, Hengyang, Xixia, and Xichuan populations, but also found three populations of *E. binata* producing a mature *Polygonum*-type embryo sac at a certain frequency (Table 1). These results revealed that there may be a certain rate of sexual reproduction in these populations of *E. binata*, although the rate of sexual embryo sac was only 2-3%.

The FCSS is based on estimations of the relative DNA content of the embryo and endosperm in mature seeds, which was used to analyze the mode of reproduction in natural apomictic species. Matzk et al. (2000) revealed the reproductive pathway of 32 species, identifying pure sexual or obligate genotypes from facultative apomictic species by FCSS. Subsequently, FCSS has been used in other apomictic species to identify reproductive mode based on the relative ploidy of embryo and endosperm, namely *Hieracium* (Bicknell and Koltunow, 2004), *Hypericum* (Matzk et al., 2003), *Taraxacum* (Martonfiova, 2006), *Arabis gunnisoniana* (Taskin et al., 2004), and *P. maximum* (Kaushal et al., 2008). Flow cytometry experiments were set up to determine the DNA content of experimental progeny and screen for C-values corresponding to seeds derived from sexuality or apomixis (Siena et al., 2008). *E. binata* is an autonomous apomictic species and involves fertilization independent of endosperm from two unreduced polar nuclei, giving a DNA content ratio between embryo and endosperm of 2C:4C, which is an index of obligate apomixis biotype, whereas another biotype containing the relative DNA content of 2C:3C:4C displays facultative apomixis (Yao et al. 2007). FCSS was performed to identify the sexual reproduction in individuals in this study. Seed samples of three individuals from Xingzi 14, Hengyang 11 and Xixia 3 showed a DNA content ratio between embryo and endosperm of 2C:3C, which is similar to the usual sexual reproduction seed. Recently, the cross-progeny plants between populations of *E. binata* were analyzed by SSR markers, and the results indicated that the seeds derived from Xingzi 14 crossed with Hengyang had paternal specific loci, and therefore, the maternal parent of Xingzi 14 may show sexual reproduction owing to its acceptance of the paternal pollen (Liu et al., 2008). Although we did not find the sexual reproductive population in *E. binata*, the sexual reproduction individuals were identified by embryological observation, FCSS testing and molecular marker analysis.

Although apomixis may prevent the vulnerabilities of sexual reproduction in agricultural systems associated with fertilization mechanisms stimulated by environment conditions, most apomictic plants retain a low frequency of sexual reproduction (Koltunow and Grossniklaus, 2003). This hint of sexual reproduction in apomictic plants is also critically important for the evolution of environmental adaptation. Even though the frequency of residual sexual reproduction is quite low, the hundreds of millions of seeds can accumulate enough genetically diverse progeny because of recombination for apomictic plants to develop adaptation to changing environmental conditions.

## Histological-cytological marker to identify the mode of reproduction in *E. binata*

In the majority of flowering plants, the accumulation of callose in the cell walls of the MMC and megaspores is one of the most distinguishing morphological features observed during megasporogenesis. Callose deposition patterns vary between plant species undergoing different modes of embryo sac formation (Rodkiewicz, 1970), but in most cases, callose accumulates at one pole of the mature MMC and then in the transverse walls that separate the

megaspores during meiosis. After the completion of meiosis, callose tends to persist in the part of the cell wall of the functional megaspore that is closest to the degenerating megaspores and diminishes elsewhere, possibly due to the activity of β-1,3-glucanase enzymes that target callose for degradation (Tucker et al., 2001; Levy et al., 2007). Callose degrades in the cell wall of the generative cell to guide the functional megaspore towards the gametophyte. Callose deposition pattern has been examined in other apomictic species, including *Elymus* (Carman et al., 1991), *Hieracium* (Tucker et al., 2001), *Tarasacum* (Leblanc and Mazzucato, 2001), *P. pratensis* (Leblanc et al., 1995) and *Vulgaris* (Shen et al., 2006). In diplospory apomicts, lacking or having reduced callose deposition during megasporogenesis is the result of abnormal meiosis and formation of an embryo sac by mitosis. In aposporous apomicts, the walls of MMC contain callose, but callose is absent from the walls of aposporous initial cells.

In this study, serial sectioning and decolorized aniline blue staining have been used to detect the presence of callose in the embryo sac initial cell wall in sexual reproduction and apomixis in *E. binata.* The cells undergoing megasporogenesis were surrounded by callose, and callose accumulated at the micropylar pole more than at the chalazal pole. This pattern of callose distribution resembled the megasporogenesis of apomictic *Hieracium* (Tucker et al., 2001), as well as corresponding to the *Polygonum*-type embryo sac. Interestingly, not only the three parallel transverse walls separating each of four megaspores by callose but also the lateral walls had intense callose fluorescence, which is different from the observation in *Hieracium* (Tucker et al., 2001). In contrast to sexual reproduction, callose was not detected in AI cells of facultative apomixis *E. binata* ovules (Figure 1). This result indicated that the pattern of callose deposition was different from sexual and apomictic reproduction, and whether callose is present or not in embryo sac initial cell may be a histological-cytological marker to identify the mode of reproduction in *E. binata*.

Whether the presence of callose or its deposition in a particular pattern around the megaspores influences their development remains unclear. In the ovule, callose may act as a molecular or nutritional filter decreasing the permeability of the cell wall, thus enabling the megaspores to embark upon an independent course of development compared with diploid sporophytic surrounding tissues (Bouman, 1984). Whether callose deposition leads to sexual termination in facultative apomictic remains unclear. Tucker et al. (2001) found that callose deposition and dissolution during megasporogenesis of *Hieracium* were unaffected when AI cells formed postmeiosis, indicating that other events may cause termination of the sexual pathway.

## Relationship of aposporous and sexual embryo sac development in *E. binata*

In aposporous apomicts, such as *Ranunculus auricomus* (Nogler 1984), *Pennisetum squamulatum* (Roche et al., 1999) and *Hieracium piloselloides* (Koltunow et al., 1998), the sexual process initiates typically with the differentiation of the MMC. Concurrent with these early stages of sexual development, one or more somatic cells in close proximity to the MMC or megaspores differentiate into AI cells, bypass meiosis and directly undergo mitosis (apomeiosis) to give rise to an embryo sac. AI cells can appear at different times and with different frequencies during ovule development. In apomictic *H. piloselloides*, for example, from one to four AI cells differentiate at the chalazal end of the ovule during late meiosis and megaspore selection (Koltunow et al., 1998, 2000). In apomictic *E. binata*, multiple AI cells differentiated in the late MMC meiosis stage, and occurred at the chalazal end of the ovule, which is similar

to that in *H. piloselloides*. Although the development of the apomictic embryo sac usually leads to the abortion of the sexual embryo sac in most facultative apomictic plants, sexual sac initiation is always earlier than apomictic sac. Tucker and Koltunow (2009) proposed that the initiation of sexual reproduction may be a prerequisite for apomixis. MMC of sexual reproduction first appeared in the ovule of *E. binata*, and MMC could undergo meiosis and form a megaspore tetrad. Whether sexual reproduction initiation is necessary for aposporous embryo sac development or not needs more evidence to address the question in *E. binata*.

In apomictic *E. binata*, the embryo sac initiated by sexual and apomictic processes within an ovule, sexual development was terminated in most ovules, and the percentage of *Polygonum*-type sexual embryo sac development was only 2-3%. Moreover, the 4-cell aposporous embryo sac and 8-cell *Polygonum*-type embryo sac did not co-exist in one ovule of apomictic *E. binata*. This phenomenon was different from that in apomictic species of *Brachiaria*, where both sexual and aposporous embryo sacs have been found in one ovule, thus resulting in the production of seeds containing both hybrid and maternal embryos (Araujo et al., 2005).

Therefore, in apomictic *E. binata*, communication between aposporous and sexual cell types is likely to influence the fate of the sexual process; termination of the sexual process may be promoted by AI cells by unclear mechanisms. In other aposporous apomictic species, such as *Pennisetum* and *Hieracium*, the development of the sexual embryo sac usually terminates at the MMC or megaspore stage, and is replaced by the development of the aposporous embryo sac (Bray, 1978; Koltunow et al., 1998). However, termination of sexual process and degeneration of megaspores are not simply due to mechanical displacement (Tucker et al., 2001). Studies showed that megasporogenesis was completed in at least 96% of the ovules from *H. piloselloides*, which sets >97% apomictic seed, suggesting an active mechanism induced by the presence of AI cells, which is likely to influence the fate of the sexual megaspores in *Hieracium* (Tucker and Koltunow, 2009). The proximity of AI cells to the sexual structures can influence the fate of both cell types, possibly by a mechanism that involves cross-talk and changes in expression of genes that regulate megaspore fate. Therefore, further molecular evidence will be provided in the future to address the question in *E. binata*.

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