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MULTIPORATE POLLEN AND APOMIXIS IN PANICOIDEAE

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Abstract

Pollen in Panicoideae has usually single pore. However, it was observed with higher frequencies of multiporate pollen (2-6 pores) in 14 species or cytotypes out of 6 genera. The observations indicated that all species with multiporate pollen showed multiple ploidy levels and all apomicts occur among multiporate pollen and multi-ploidy level species. Our studies on embryo sac and embryo development show that species diploid *Paspalum notatum* without multiporate pollen was engaged in normal sexual reproduction and multiple-ploidy species (*Paspalum distichium*, *P. commersonii*, *P. thunbergia* and *Bothriochloa ischaemum*) were engaged in apomixis including apospory and diplospory. There was no evidence of apomixis occurring in *Panicum repens* which also exhibited multiporate pollen, however, all its embryo sacs were deteriorated. The authors suggest that the occurrence of multiporate pollen originate from abnormality in microsporogenesis and is representive of morbidity and multiporate pollen may have some internal relativity with apomixis. So the presence of multiporate pollen may be used as a preliminary identification of apomixis.

Introduction

The family Poaceae includes some of the most important cereal crops and also possesses the largest number of known apomictic species. Because the offspring of an apomict is genetically identical to its mother plant, it has potential for fixing hybrid vigour of crops (Asker & Jerling, 1992; Hanna *et al*., 1996; Vielle-Calzada *et al*., 1996; Bhat *et al*., 2005), especially Gramineae which includes main grain crops. Consequently studies on apomixis have become a new focus of contemporary biological science. However the practical utilization of apomixis for grain crops appears problematic as many of the mechanisms and relationships relating to apomixis are not understood. This lack of understanding is attributable to a shortage of verifiable apomictic germplasm. More apomictic species need to be identified, especially obligate apomicts. Identification methods for apomixis is currently done through observation of megasporogenesis, development of the embryo sac and embryo, however microsporogenesis and pollen abnormality have received little attention. Usually the pollen in the Gramineae has only one pore (Yu *et al*., 2000). However multiporate pollen in the Gramineae has been previously reported. The earliest report on multiporate pollen in the Gramineae is the generation of a hybrid between rye and common wheat (Erdtman, 1944). Reports of multiporate pollen in other Gramineae species are sparse (Zucol, 1998). These reports concluded that multiporate pollen was related to abnormal plant development and that it represented morbidity characteristics. These studies combined with our own embryological studies have determined that all of them are apomicts (Chao, 1964; 1974; 1980; Ma *et al*., 2001a; 2004; 2007; Ma & Zhao, 2003). This aroused our interest and we posed the following questions: does multiporate pollen have some relationship with

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apomixis? To identify this relationship, we undertook a study to observe multiporate pollen in species that in previous reports have been identified as apomicts such as *Paspalum* spp., (Ma *et al*., 2003; 2004; Ma & Zhao, 2003), *Pennisetum* spp., (Sherword *et al*., 1994; Wen *et al*., 1998), *Panicum maximum* (Warmke, 1954; Nakagawa, 1990; Naumova & Willemse, 1995), *Apluda mutica* (Murty, 1973; Ma *et al*., 2002), *Heteropogon contortus* (Brown & Emery, 1958; Tothill & Knox, 1968). Given the vital importance of utilizing apomixis to facilitate cereal breeding, establishing better methods for identification of apomixis is needed. In this study our objective was to find a new and simple method to identify apomictic germplasm, based on the relationship between multiporate pollen and apomixis.

Material and Methods

Plant material included 14 species from 6 genera, including *Panicum*, *Paspalum*, *Pennisetum*, *Apluda*, *Heteropogon* and *Bothriochloa*. All species were collected from South China Botanical Garden and around Guangzhou (Table 1).

Cytological study: Plants were propagated by cuttings and seeds at South China Botanical Garden, Guangzhou. Mitosis was studied using root tips to check chromosome numbers and ploidy levels. Immature inflorescences (before heading) were fixed in Carnoy solution (100% ethanol- chloroform- glacial acetic acid, 6: 3: 1) to observe microsporogenesis. Meiosis of the pollen mother cells was examined by the aceto-carmine squash technique.

Pollen morphology: 1% Iodide potassium solution (I_2-KI) was used to establish pollen stainability and multiporate pollen characteristics under standard light microscopy during the plants flowering periods. For closer observation of pollen morphology, fresh pollen grains were scattered on a special metal platform and after a series of treatments the pollen grains were studied using scanning electron microscopy (SEM) (Ma *et al*., 2001a).

Development of embryo sac and embryo observation by paraffin section: Inflorescences (for observing the development of embryo sac and embryo) were fixed in FAA (70% ethanol- glacial acetic acid-37% formaldehyde, 18: 1: 1) for 2 days at room temperature and then transferred to 70% ethanol for storage at 4^{o}C until use. Ovaries in different developmental stages were taken from spikelets (except for some immature spikelets), stained with haematoxylin, dehydrated in an ethanol series, embedded in paraffin, and then vertically sectioned (6-8 µm thick) in a microtome and sections mounted on glass slides for observation.

Development of embryo sac and embryo observation clearing methods: For more acute observations of the development of embryo sacs and pre-embryos, clearing methods were used (Young *et al*., 1979; Nakagawa, 1990). The ovaries were transferred to the clearing solution (lactic acid: chloral hydrate: phenol: eugenol: xylene, 10: 10: 10: 10: 5) for 48 h then the embryo sac and pre-embryo development in the ovules were observed using an interference contrast microscope (Ma *et al*., 2004; 2007).

Table 1. Ploidy level, pollen viability and pollen multiporate in Panicoideae.

Species (Cytotypes)	Ploidy level	Pollen viability (%)	Pores numbers	Multiporate frequency
Apluda mutica	3	58.7	1-6	21.4
Bothriochloa ischaemum	4	78.5	1-4	21.4
Heteropogon contortus	4	63.7	1-2	3.4
Panicum maximum	4	67.5	1-6	8.7
P. repens	4	74.4	1-6	37.3
Paspalum commersonii	10	5.7	1-4	18.9
P. commersonii	8	56.3	1-6	14.2
P. commersonii	6	61.5	1-6	9.6
P. conjugatum	4	78.0	1-2	6.4
P. distichum	4	39.4	1-2	4.5
P. longifolium	4	74.6	1-3	6.3
P. notatum	2	86.6	1	0
P. orbiculare	4	65.0	1-3	18.5
P. thunbergii	4	21.9	1-6	12.7
Pennisetum squamulatum	6	58.8	1-2	3.5

Results

Identified ploidy levels ranged from 2X to 10X: *P. notatum* was diploid (Fig. 1A). However *A. mutica* was triploid (Fig. 1B). Tetraploidy was the most common ploidy level and included *Paspalum* (*P. thunbergii*, *P. conjugatum* and *P. longifolium*), *P. maximum*, *P. repens* and *H. contortus* (Fig. 1C, D, E, F). *H. contortus* contains aneuploids having between 44 and 46 chromosomes (Fig. 1E). *P. commersonii* was observed to be hexaploids, octoploidy and decaploid, respectively (Fig. 1G, H, I). The decaploid *P. commersonii* exhibited the highest ploidy cytotype of this particular genus (Table 1).

Pollen viability of different species or cytotypes ranged from 5.7 to 98% (Table 1). The species with higher pollen viability (>80%) included *P. notatum*. Species exhibiting lower pollen viability (<70%) included *P. thunbergii*, *P. distichum*, *P. commersonii*, *P. orbiculare*, *P. maximum*, *A. mutica* and *H. contortus.* The decaploid *P. commersonii* had the lowest pollen viability (5.7%).

Pollen in 14 species (cytotype) was multiporate (Table 1). Pollen usually has 2-6 pores and the frequency of multiporate pollen is between 3.5-37.4%, with some differences among the species (Fig. 2A-C). More pollen pores were evident in *A. mutica*, *P. commersonii*, *P. repens*, *P. thunbergia* and *P. maximum* while the higher frequencies of multiporate pollen occurred in the *A. mutica*, *P. orbiculare* and *P. repens*. However, in the species *P. notatum*, only normal single pore pollen was observed and multiporate pollen was not found.

Embryological studies the species showed no apomictic development. The megaspores develop normally to develop into typical 8-nucleate embryo sacs and none of pre-embryo or multiple embryo sac observed indicated that they engaged in a normal sexual reproduction (Fig. 3A).

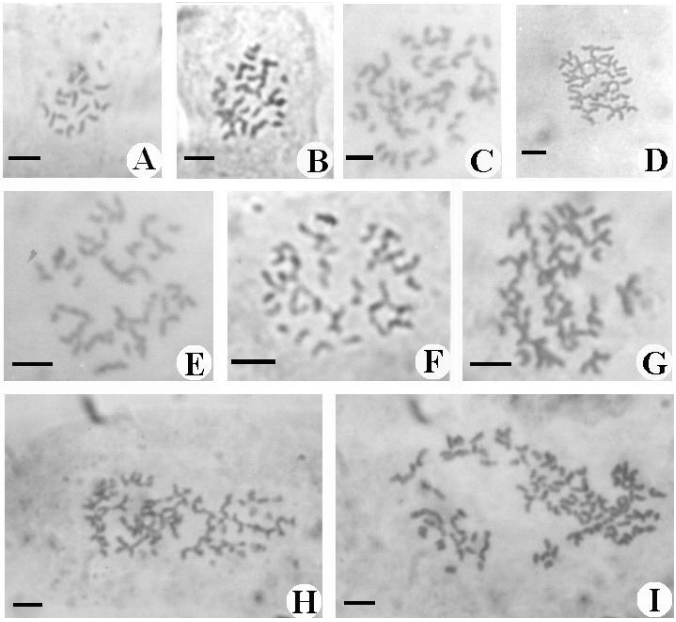

Fig. 1. Chromosome numbers of Panicoideae species (bar=10 μm)
A. *P. notatum* (20); B. *A. mutica* (30); C. *P. longiforium* (40); D. *P. thunbergii* (40); E. *H. Contortus* (44); F. *P. repens* (40); G, H, I. *P. commersonii* (60; 80; 100)

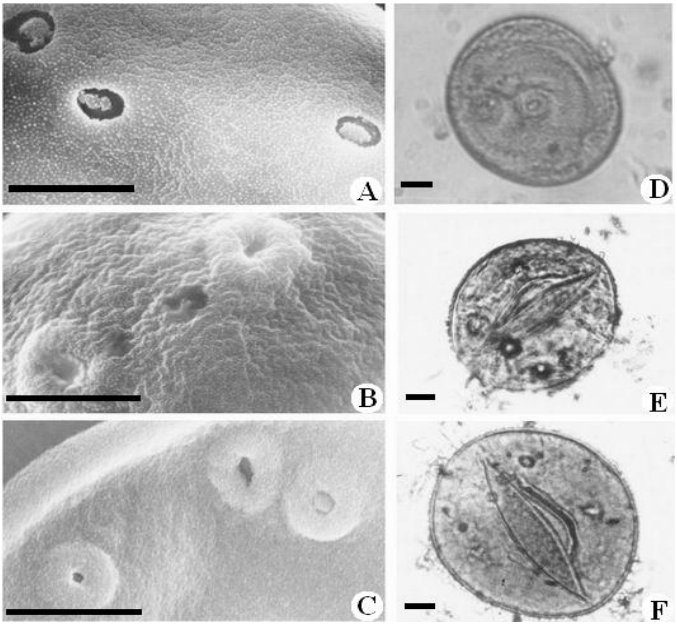

Fig. 2. Pollen multiporate observation in different Panicoideae species (Bar =10 μm).
A, D. *P. commersonii*; B, E. *P. thunbergii*; C, F. *B. ischaemum.*

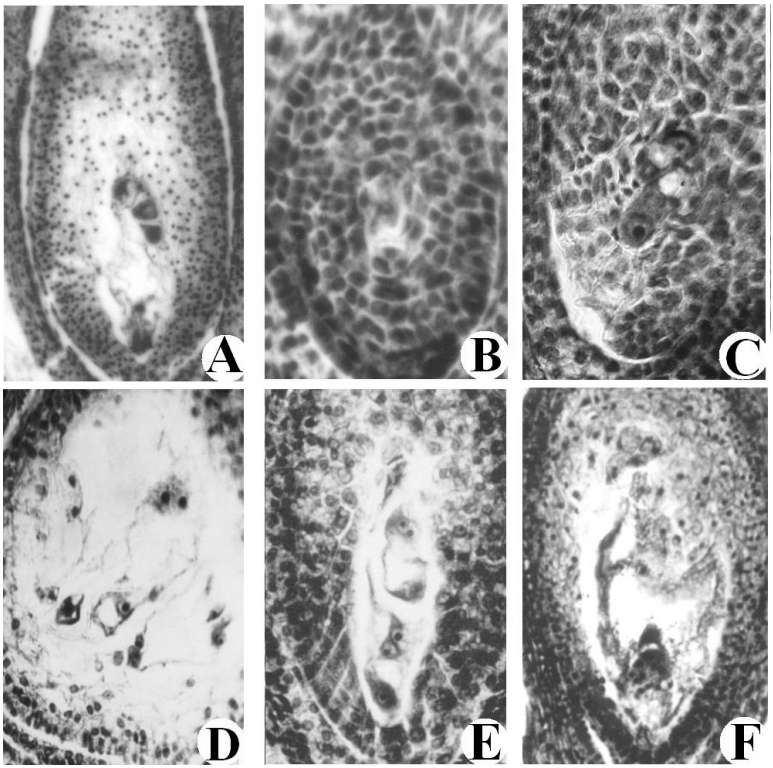

Fig. 3. Ovary section by paraffin section of different Panicoideae species.
A. Sexual embryo sac of *P. notatum*; B. Embryo sac of *P. repens*, after that the nucellar cells would deteriorate; C. Two 2-nucleus aposporous sacs in *B. ischaemum*; D. Multiple aposporous embryo sacs of *P. thunbergii*; E. Multiple aposporous embryo sacs of *P. distichium*; F. Pre-embryo sac of diplospory in *P. commersonii*.

The mother megaspore *of P. repens* developed into a tetrad with one cell closed to a chalaza and developed into an embryo sac (Fig. 3B). However, after showing a single nucleate embryo sac in the ovule, further development was not observed. At the same time, some nucellar cells and ovary tissue begin to deteriorate. Normal embryo sac development of the embryo sac or fertilization was not observed in more than 100 ovaries indicating that ovule development in *P. repens* may cause serious sterility.

In *B. ischaemum* 87.8% of observed embryo sacs were apomict. Typically 1-3 or more aposporous embryo sacs could be developed in a single ovule (Fig. 3C). The mature aposporous sac was usually characterized by an egg and only one polar nucleate. The egg could develop spontaneously into a large pre-embryo mass prior to anthesis. When several aposporous sacs occurred in the same ovary, usually 2 aposporous sacs were involved in pseudogamy and developed into separate endosperm masses in the same ovary. 13.5% twin-embryo seedlings could be obtained after mature seeds germinated. The data suggests that *B. ischaemum* is a facultative apomict.

As the megaspore of *H. contortus* develops to the tetrad, the sexual embryo sacs cease to develop, 1-4 nucellar cells begin to develop into aposporous embryo sacs. This is different from previous reports on *Paspalum* and *Bothriochloa* where the development of nucellar cells usually occurred simultaneously in the same sac cavity. However, development of the nucellar cells occurs in different sites of the ovule in different periods. Otherwise, the mature aposporous embryo sac was usually characterized by one egg and one polar nuclear. The egg could spontaneously develop into a pre-embryo before anthesis. However, the polar nucleus is involved in pseudogamy to form the endosperm. Sexual embryo sac of *H. contortus* has not been found indicating that is an obligate apomict.

For *P. thunbergii*, megasporogenesis was initially normal; however the megaspore deteriorates at the tetrad developmental stage. At this stage 1-5 specific nucellar cells become active and begin enlarging, develop into aposporous embryo sacs (Fig. 3D). The mature aposporous sacs usually showed 3 nuclei (one egg and two polar nuclei). The egg could develop spontaneously to form a pre-embryo prior to anthesis. When several aposporous sacs occurred in the same ovule, usually one sac near the micropyle was involved in pseudogamy while the other sacs were not involved. Twin-embryo seedlings were observed at a low frequency after seeds matured. Examination of three successive generations by paraffin section revealed that no sexual sac was observed, therefore *P. thunbergii* was considered to be an obligate apomict that reproduced by apospory.

Development of the aposporous embryo sac, embryo and pseudogamy were studied in the tetraploid *P. distichum*. In most cases, shortly after the megasporocyte developed into a tetrad, it began to deteriorate; meanwhile several nucellar cells became active and enlarged. Normally 1-3 nucellar cells developed into mature aposporous embryo sacs characterized by one egg and two polar nuclei (Fig. 3E). The egg could spontaneously develop into a pre-embryo prior to anthesis. However, the polar nuclei were involved in pseudogamy to develop the endosperm after heading and pollination. When several aposporous sacs occurred in the same ovule, only the sac near the micropyle developed endosperm while the other sacs did not. The frequency of twin-embryo seedlings from geminated mature seeds was low. A few sexual 8-nucleus embryo sacs as well as both sexual and aposporous sacs were found in the same ovule. It suggested that the tetraploid *P. distichum* is a facultative apomict reproducing by apospory.

We observed both pre-embryo and antipodal cells in the ovary of tetraploid and decaploid *P. commersonii*, determinating that both ploidy levels engage in diplospory (i.e. sextuple and decuple ploidy in *P. commersonii*) (Fig. 3F).

Embryo sacs, pre-embryos, embryo and endosperm could be clearly observed under the interference contrast microscope (Fig. 4A-F). For the aposporous embryo sac, the embryo sacs usually do not develop fully and only a few nuclei were observed in the embryo sacs. For *P. thunbergii* and *H. contortus*, only one egg and 1-2 polar nuclei were observed in the same embryo sac (Fig. 4D, E). In the aposporous species, some species often have several embryo sacs, such as *P. distichum*, *P. thunbergii* and *P. maximum* (Fig. 4A, B, E). Pre-embryos were often observed in the diplosporous embryo sacs of *P. conjugatum* and aposporous embryo sacs of *B. ischaemum* and *P. thunbergii* (Fig. 4C, D, F). In *P. conjugatum*, we could observe antipodal cell masses and the pre-embryo in the same ovule (Fig. 4F). It was a typical diplosporous embryo sac in the Panicoideae.

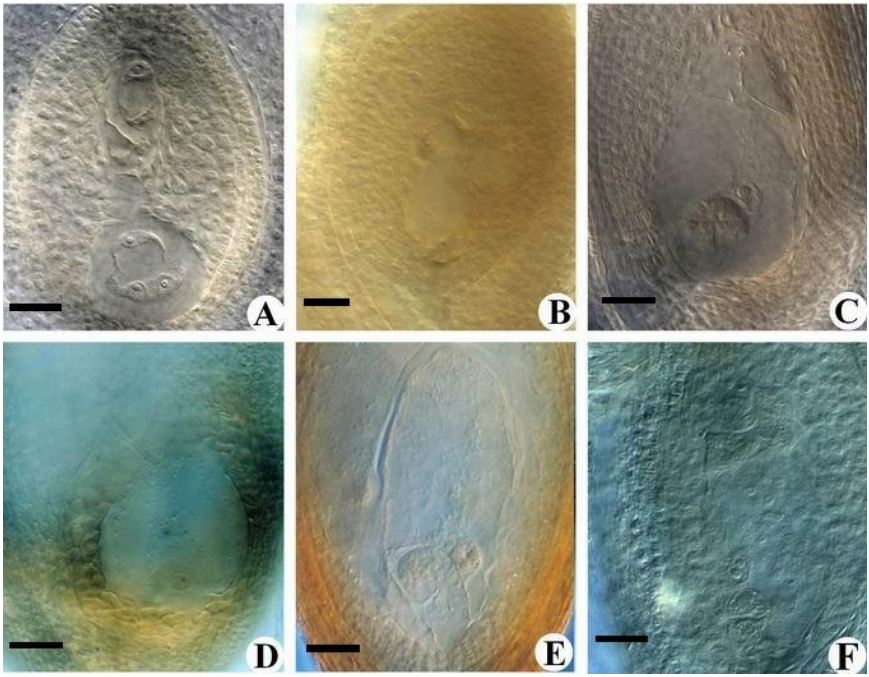

Fig. 4. Observation on ovaries in different Panicoideae species by clearing method under the interference contrast microscope (one bar= 20 μm).
A. Multiple aposporous sacs in *Paspalum distichum*; B. Multiple aposporous sacs *P. maximum*; C. Pre-embryo development in *B. ischaemum*; D. Aposporous embryo sac in *H. contortus*; E. Aposporous embryo sac and pre-embryo in *P. thunbergia*; F. Diplosporous embryo sac, pre-embryo and antipodal cell in *P. conjugatum*.

Discussion

Through observation of ploidy level, pollen viability and multiporate pollen we can begin to summarize some characteristics. Species with multiporate pollen are all polyploidy. In the case of diploids, virtually no multiporate pollen occurs.

In the Panicoideae, all apomicts occur among multi-ploidy level species (Table 2). Only two types of apomixis: diplospory and apospory have been reported in the Panicoideae (Ma *et al*., 2001b). In diplosporous species, meiosis in the megaspore appears abnormal in the sense that it is replaced by mitosis; in aposporous species, meiosis does occur. However, the tetrads deteriorate and do not develop into sexual embryo sac. Aposporous embryo sac develops from specialized nuclear cells. All apomixis is a means of reproductive compensation due to abnormal development of the megaspore and defeat of sexual reproduction. All species of apomicts studied here showed abnormal microspore formation in the mother cell (besides their abnormal megaspore development). In species such as *Calamagrostis hakonensis* and *Dichanthium aristatum*, sexual reproduction is not achievable due to sterile pollen, and therefore asexual reproduction by apomixis is a likely alternative (Harlan & de Wet, 1963; Carman & Hatch, 1982).

Table 2. Embryo sac development of apomixis in some species of the Panicoideae.

Species	Observation results	Frequency of apomixis (%)
B. ischaemum	Apospory	87.5
H. contortus	Apospory	100
P. repens	All embryo sacs deteriorate	0
P. commersonii (hexaploid)	Diplospory	3.5
P. commersonii (decaploid)	Diplospory	9.8
P. conjugatum	Diplospory	10.6
P. distitrium	Apospory	91.2
P. longifolium	Diplospory	2.4
P. orbiculare	Diplospory	1.8
P. thunbergii	Apospory	100
P. notatum	Normal sexual	0

The genus *Paspalum* contains the largest apomictic species group in the Gramineae (Ma *et al*., 2003). Quarin's (1992) summary of the characterization of apomixis in the genus *Paspalum* indicates that apart from only one tetraploid species, all other species where microspore mother cell mitosis is abnormal and chromosome pairing is confused have been reported to engage in facultative apomixis. Examples of this include such species as tetraploid *P. conjugatum*, *P. longifolium*, *P. orbiculare* and hexaploid *P. commersonii* (Brown & Emery, 1957; Chao, 1964; 1974; 1980; Ma *et al*., 2003). Apomixis in this latter group belongs to the diplosporous type. These are the only four species found in the genus *Paspalum* that engage in diplospory. Because the mature diplosporous embryo sac has the same 8-nucleus embryo sac as the normal sexual embryo sac, it is difficult to differentiate only by appearance. However the obvious difference between the diplosporous embryo sac and sexual embryo sac is whether they can develop past pre-embryo stage. Preliminary observations indicated that these four diplosporous species reproduce by a low frequency of diplospory due to low incidence of pre-embryo development. The diploid *P. notatum*, produced seeds by sexual reproduction. For the decaploid species *P. commersonii*, we observe both pre-embryo and antipodal cells in the embryo sac; therefore we conclude that it is engaged in diplospory similar to the sextuple *P. commersonii*. The authors suggest that the occurrence of multiporate pollen might originate from abnormality in mother cell mitosis and represent morbidity.

According to previous reports, *P. thunbergi*, *P. distichium*, *P. squamulatum*, *P. maximum*, *H. contortus*, *A. mutica* etc. reproduce by apomixis (Ma *et al*., 2002; 2004; Dujardin & Hanna, 1983; 1984; Murty, 1973; Savidan, 1983; Tothill & Knox, 1968). This work compares with our studies where all the known apomicts exhibit multiporate pollen.

All apomicts showed multiporate pollen in our studies. This showed that multiporate pollen has exactly higher relativity with apomixis. For decreasing the current difficulties in identifying apomictic species, we suggest that presence of multiporate pollen may be used as a preliminary identification of apomixis.

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