Bud Initiation, Microsporogenesis, Megaspurogenesis, and Cone Development in *Platycladus orientalis*

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Additional index words. bud initiation, microsporogenesis, megaspurogenesis, cone development, *Platycladus orientalis*

Abstract. As a native tree species with a strong adaptability, *Platycladus orientalis* is a species of choice for afforestation and landscaping in northern China. However, it develops mostly male cones and few female cones. In addition, its reproductive characteristics are not yet clear, which limits further breeding work. To systematically clarify the reproductive biology characteristic and fertilization mechanism of *P. orientalis*, the present study comprehensively investigated the process of micro and macro-sporogenesis in male and female cones from bud initiation to fertilization, and seed development. The specific time in each developmental stage, including bud initiation, microsporogenesis, megaspurogenesis, and cone and seed development, was determined, and the abortive phenomenon during development was discovered in both male and female cones. In addition, this research showed that the microspore mother cells were dormant in winter at meiosis stage, and the male gametophyte started to develop when dormancy ended. The tapetum developed normally and belonged to the secretory type. The optimal treatment time for male and female cones transformation by artificial induction was from late June to mid-July. This finding provided a theoretical basis for hybridization, breeding, improvement of seed yield and quality, and artificial induction of male and female cone transformation in *P. orientalis*.

Plants in the Cupressaceae family, which are widely distributed in the southern and northern hemispheres, are the only gymnosperms distributed worldwide (Zheng and Fu, 1978). *P. orientalis*, a monotypic genus belonging to Cupressaceae, is one of the most commonly used trees worldwide. Moreover, as one of the city trees in Beijing, it is an important local tree species for afforestation, border trees, and landscaping unique to

northern China. Its wood has moderate hardness and strong corrosion resistance, and its seeds, roots, leaves, and bark can be used as traditional Chinese medicine. As a monoecious tree species, *P. orientalis* produces a very large amount of pollen, which easily causes allergic symptoms in spring; however, a low number of female cones limits the breeding process. Therefore, it is necessary and meaningful to conduct a comprehensive and detailed study on the reproductive biology characteristics and ontogeny to provide further information for production and cultivar breeding of *P. orientalis*.

At present, there have been some studies about the embryo development, palynology, and leaf structure of Cupressaceae plants, but the micrographs were unclear due to the relatively long time frame and the limitations of microscopy over which the studies were conducted. Most of these studies focused on morphological research and plant classification (Gadek and Quinn, 1985, 1988; Kumrann, 1994; Sugihara, 1992). In *P. orientalis*, research had mainly focused on breeding, cultivation techniques, physiological and ecological characteristics, diseases, and insect pests. There were a few reports on the reproductive development of *P. orientalis*; however, they were not comprehensive. Cone initiation, development, and phenology of *P. orientalis* were reported by Dong et al. (1992). Cao et al. (1997) studied the mi-

crosporogenesis and formation of the male gametophyte in *P. orientalis*. The female cone and ovule development were detected by Zhang et al. (2000). There were no reports on the reproductive biology of *P. orientalis* in the past 20 years, and parts of the development processes were lacking, such as the detailed date, internal and external morphological characteristics of cone initiation, sporogenesis and seed formation process, and so on. Owens and Marje (1977), Owens and Molder (1974, 1980), and Owens and Pharis (1967) reported the sexual reproduction process of *Thuja plicata* and *Chamaecyparis nootkatensis* in detail; in addition, gibberellin-induced cone formation was studied. There is a lack of comprehensive research on the bud initiation, cone development, and sexual reproduction of *P. orientalis*, and even less research at the gene level.

The cone development and reproductive biological characteristics in pine and Chinese fir were studied comprehensively and in detail in gymnosperms in recent years. Morphologic and anatomical observations in the process of ovulate strobilus generation and development were researched in *Pinus tabulaeformis* (Zhang et al., 2017). Embryonal development revealed the annual cycle of ovulate cone development in *Pinus sibirica* in the western Sayan Mountains (Tretyakova et al., 2004). An embryological study revealed the systematic significance of the primitive gymnosperm *Ginkgo biloba* (Wang et al., 2011a). Morphological and structural changes during female and male cone development in *Cunninghamia lanceolata* were reported in detail by Jiang et al. (2017) and Zhu et al. (2018). Furthermore, recent investigations of gene level in *P. tabulaeformis* had been researched deeply. High-throughput gene expression profile chip in male and female cones were detected, and the specifically expressed genes and expression pattern were analyzed in different cone development stages (Niu et al., 2013). Analysis of hermaphrodite in *P. tabulaeformis* provided the basis and new ideas for molecular biology research on the cone development of coniferous species (Niu et al., 2016).

To understand the reproductive biology and ontogeny more systematically, and to explore the mechanisms of pollination and fertilization, the present research comprehensively and systematically reported the cone initiation, formation, and development processes and the phenomenon of megaspore and microspore abortion in *P. orientalis* by using a paraffin section method. This study provides a theoretical basis and precondition for improving seed yield and quality, achieving gender transformation, hybridization, and breeding in *P. orientalis*.

Materials and Methods

The new cultivar of *P. orientalis* ‘Dieye’, used in the present research, was from a coniferous plant resource nursery at the Beijing Academy of Forestry and Pomology Sciences, Beijing, China. The tree was

Received for publication 29 Sept. 2020. Accepted for publication 6 Nov. 2020.

Published online 9 December 2020.

This study was funded by The Youth Research Foundation of Beijing Academy of Agricultural and Forestry Sciences of China (No. QNJJ201923) and The Science and Technology Innovation Ability Construction Projects of Beijing Academy of Agricultural and Forestry Sciences of China (No. KJCX20200207, KJCX20200114).

We are grateful to senior engineer Jin Bai for identification of experimental materials in this research.

T.L. and J.C. conceived and designed the experiments; T.L. and G.L. performed the experiments; L.G. and Y.W. collected the samples of different stages; Y.Y. took photos in field; T.L. analyzed the data and photos; and T.L. wrote the manuscript. J.C. is the corresponding author. E-mail: caojun@baafs.net.cn.

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≈15 m high and 20 years old, and was situated on alkaline soil (pH 7.1–8.2) and subjected to a typical subhumid continental monsoon climate. The mean annual temperature was 11 to 13 °C, and the mean annual rainfall was ≈626 mm. Rainfall occurred primarily from June to August, with 80% of the annual precipitation recorded during this period. There were ≈180 to 200 frost-free days in the year.

Male and female cones at different developmental stages were collected from bud initiation to fertilization, and female cones up to seed maturity, which was from June 2018 to the following Aug. 2019. Depending on the different developmental stages, samples were taken once a week from June to Aug. 2018, every 2 weeks from Sept. to Nov. 2018, and once a month from Dec. 2018 to Aug. 2019. The samples were fixed in FAA fixing solution, which was composed of 5 mL formalin, 5 mL glacial acetic acid, and 90 mL 70% alcohol, for at least 24 h, and then stored at 4 °C. Bud initiation, micro and megasporogenesis, and development of male and female cones were observed using a paraffin section method with slight modifications (Li, 1978). The materials were dehydrated in an alcohol series and embedded in paraffin with a 58 to 60 °C melting point. The sections were cut at a thickness of 10 µm by using a microtome (Leica, Wetzlar, Germany) and stained with hematoxylin-eosin Y and toluidine blue. Observation and photomicroscopy of the sections were carried out by using a BX-51 microscope (Olympus, Tokyo, Japan). Field photographs of cone development were taken with an EOS 77D camera (Canon, Tokyo, Japan). Changes during bud initiation were photographed by stereoscopic microscopy (Olympus SZ2-ILST).

Results

Bud initiation. The vegetative branches of *P. orientalis* began to transform to reproductive branches in late April, presenting a flat yellow-green color. In mid-June, before bud initiation, the tip of the male cone showed no difference from the vegetative bud, and the apical meristem could be seen in longitudinal section, with a conical growth point in the middle and a pointed apex. There were leaf primordia on both sides, with young leaves on the periphery. A group of vigorous protoplasts located at the top of the growth point produced central mother cells, which were larger than the epidermal cells, by continuous pericycle division. The central mother cell constantly divides downward to produce the pith (Fig. 1A). In appearance, the male cone was consistent with the vegetative buds (Fig. 1B and K). In early July, the initiation of male cones was first observed. At the beginning of initiation, the site where the leaf primordium was produced at the growth point had very active cell division, and the two bulges on both sides of the growth point were raised to form the first pair of microsporophyll primordia (Fig. 1C). At this point, the male cones were indistin-

guishable from the vegetative buds in appearance and morphology (Fig. 1D and L). Subsequently, the subepidermal cells at the base of the distal axial plane divide continuously to produce the microsporangium primordium (Fig. 1E), at which point the initiation of the male cones was complete. When the microsporangium primordium first appeared, the male cones were still indistinguishable from the vegetative buds in appearance and morphology (Fig. 1F and M). With the growth of the microsporangium, in mid to late July, the male cones expanded gradually and developed from a flat shape to a round ball, which was significantly different from the development of vegetative branch tips (Fig. 1G–J). The initiation of male cones was not simultaneous and was observed from early July to early August.

The initiation of female cones was also transformed from the vegetative branch tip, and there was no difference between the vegetative branch tip and the male cones before initiation in mid-June (Fig. 1N). The initiation of the female cones could be observed as early as the end of July. After bulges in both sides of the growth point, a short and round ovule primordium appeared. Then, the nucellus and the integument were observed. The macrosporophyll and ovuliferous scales initially overlapped (Fig. 1O), and the initiation of the female cones was completed.

Microsporogenesis and male cone development. In *P. orientalis*, the male cones developed between August and October. In mid-August, the boundary between the epidermal cells and sporogenous tissue was obvious, with a constantly expanding microsporangium (Fig. 2A). In late August, the middle layer and tapetum cells were distinct from the epidermal cells under morphological observation. (Fig. 2B). In September, the microsporangium continued to grow, and the male cones developed rapidly. The male cones were pink and round, with a slightly curved and pendulous apex (Fig. 2C, D, and K). Until mid-October, the cytoplasm of the middle layer and tapetum cells were relatively less developed, with a distinct boundary with sporogenous tissue (Fig. 2E). From mid-October to mid-November, the microsporangium was markedly enlarged. In early November, with the transformation of the sporogenous tissue into microspore mother cells (MMCs), the tapetum cells became plump and changed from mononuclear cells to binucleated cells, forming a thin layer of rectangular cells with rare cytoplasm. The middle layer cells were gradually absorbed and attached to the inner surface of the epidermal cells. In addition, a large number of MMCs, which were squeezed into irregular cell shapes with obvious edges and corners, had been formed and lined up tightly (Fig. 2F). During the development of MMCs, some degenerated, and a large cavity could be found inside the microsporangium (Fig. 2F).

After MMC formation, the male cones entered the dormant period; at this point, the

male cones were round, with a light brown apex (Fig. 2L). From mid-November to mid-December, the MMCs underwent meiosis. The nucleoli were particularly prominent with low condensed chromatin. No cell wall formed between the two nuclei, whereas the tapetum cells were still flat, and some had two nucleoli (Fig. 2G and H). In mid-January of the next year, tetrahedral tetrad shapes were observed by meiosis of the MMCs, and the tapetum cells near the sporogenous tissue started to disintegrate, after which mononuclear pollen grains formed (Fig. 2I). The male cone morphology before pollen dispersal showed obvious microsporophylls; this was significantly different from the morphology observed during the dormant period (Fig. 2M). In mid-March, the middle layer and tapetum cells were all absorbed and disintegrated, and the pollen ripened (Fig. 2J). In late March, the microsporophylls opened naturally, and the male cones dispersed mature pollen (Fig. 2N). In late April, the pollen of *P. orientalis* was released completely, and the male cones dried out (Fig. 2O).

Megasporogenesis and female cone development. During the initiation and development of female cones, the ovule primordium was formed in early August (Fig. 3A). With the continuous division of the ovule primordium, the nucellar tissue was formed in the central region, and the tubular structures on both sides formed the integument. The integument grew continuously, and finally, a tubular micropyle was formed at its end in late September (Fig. 3B). At this point, the macrosporangium was formed. The female cones stopped growing in late October or early November, entered a dormant period (Fig. 3C), and then continued to develop in late March of the following year. On the next 29 Mar., abortive ovules were observed in some of the female cones, with abnormal development of the integument and nucellus (Fig. 3D–G). By the next late March, the megaspore mother cells had formed in some ovules, and their nuclei were larger than those of other surrounding cells (Fig. 3H). On the next late April, the ovule pollinated and entered the early stage of embryo development: the embryonic sac began to expand, producing a small number of free nuclei. At the same time, abortive ovules also could be observed. In the abortive ovules, some of the integument developed normally with abortive nucellus, whereas both the integument and nucellus were abortive (Fig. 3I–L). The ovule continued to expand, and more free nuclei were produced in the embryonic sac from the next May to June. The outer integument developed normally, separating from the inner integument, and the nucellus was abnormal in the aborted ovules (Fig. 4A–F). After that, the embryo developed rapidly, and the mature embryo first appeared at the end of next August (Fig. 4G–J).

The apparent morphology and transverse and longitudinal diameters of *P. orientalis* female cones during the development stage

Fig. 1. Bud initiation process in *Platycladus orientalis*. (A) Longitudinal section showing the stage before the initiation of male cones on 15 June. (B) Bud under a stereoscopic microscope on 15 June. (C) Male cones began to differentiate on 1 July. (D) Bud under a stereoscopic microscope on 1 July. (E) Male cones in initiation on 15 July. (F) Bud under a stereoscopic microscope on 15 July. (G) The initiation of the male cones was completed on 22 July. (H) Male cones under a stereoscopic microscope on 22 July. (I) Microsporangium was formed on 29 July. (J) Male cones under a stereoscopic microscope on 29 July. (K) Bud morphology on 15 June. (L) Bud morphology on 1 July. (M) Bud morphology on 15 July. (N) Longitudinal section showing the period before the initiation of the female cones on 15 June. (O) Female cones in initiation on 29 July. CC = central mother cell; I = integument; L = leaf primordium; MC = male cone; MP = microsporophyll primordium; MSP = microsporangium primordium; N = nucellus; OS = ovuliferous scale; PI = pith.

were shown in Figs. 5 and 6. On the next 8 Apr., the apex of the female cones had not yet dehisced (this process occurred 1 week later) and dark brown megaspore leaves that spread

outward were observed. On the next 22 Apr., the female cones expanded and turned gray-green with a purple halo on the outside. On the next 29 Apr., the female cones continued

to grow, three to four pairs of ovuliferous scales could be observed, and the tip of the outermost pair of ovuliferous scales turned outward. On the next 6 May, the female cones

Fig. 2. Microsporogenesis and male cone development process in *Platycladus orientalis*. (A–E) Longitudinal section showing the growth of microsporangium on 15 Aug., 31 Aug., 15 Sept., 29 Sept., and 15 Oct., respectively. (F) Longitudinal section showing the microsporangium on 7 Nov. and the formed microspore mother cells (MMCs). (G) Longitudinal section showing the microsporangium on 15 Nov., with the MMCs undergoing meiosis. (H) Longitudinal section showing the microsporangium on 15 Dec., with the MMCs still undergoing meiosis. (I) Longitudinal section showing the microsporangium on the following 15 Jan., when the MMCs were forming a tetrad through meiosis and the tapetum had been absorbed and disintegrated. (J) Mature pollen was observed on the following 15 Mar. (K) External morphology of male cones on 15 Sept. (L) Dormant male cones on the following 4 Jan. (M) Photo showing the male cones before anther dehiscence on the following 15 Feb. (N) The male cones were dispersing pollen on the following 29 Mar. (O) The dried male cones on 30 Mar., after pollen dispersal. CS = cavity structure; EC = epidermal cell; MC = male cone; ML = middle layer cell; MMC = microspore mother cell; MS = microsporangium; P = pollen; TC = tapetum cells; T = tetrad.

continued to increase in volume, and the purple halo disappeared in some of them. At the end of next May, the ovuliferous scales

closed into ball shapes and developed into young cones with green surfaces covered by white powder. The young cones had four

pairs of megasporophyll scales, and the average transverse and longitudinal diameters of the cones were ≈ 11.40 and 12.05 mm,

Fig. 3. Megasporogenesis and female cone development process in *Platycladus orientalis*. (A) Ovule primordium formed on 4 Aug. (B) Longitudinal section showed the ovule primordium on 29 Sept. (C) Dormant megasporangium on the following 4 Jan. (D) A normal ovule and an abortive ovule were observed on the following 29 Mar. (E) Enlarged view of (D), with arrows showing a normal ovule on the following 29 Mar. (F) Enlarged view of (D), with arrows showing the abortive integument in ovules on the following 29 Mar. (G) Abortive ovule with nucellus and integument aborting on the following 29 Mar. (H) The developing female cone, with an arrow showing the megaspore mother cell on the following 29 Mar. (I) A normal ovule and an abortive ovule were observed on the following 29 Apr. (J) Enlarged view of (I) showing a normal ovule on the following 29 Apr., arrow showed the free nuclei. (K) Enlarged view of (I) showed an abortive ovule on the following 29 Apr. (L) The abortive ovule on the following 29 Apr. AO = abortive ovule; FN = free nuclei; I = integument; M = micropyle; MMC = megaspore mother cell; N = nucellus; NO = normally developed ovule; OS = ovuliferous scale.

respectively. By the end of next June, the female cones continued to grow; at this point, the seeds were formed, and the seed scales began to lignify. Throughout the developmental period of the female cones, the cone volume increased significantly from the next April to September, with the largest increase rate in the next May and June, reaching the maximum volume in

mid to next late August. At this time, the average transverse and longitudinal diameters of the cones were ≈ 21.07 and 21.30 mm, respectively. In the next early September, the female cones became mature and began to dehisce, cracks could be observed, and the fruit scales gradually turned brown. By the next middle of October, the female cones were all dehisced and

became dry and brown; finally, the seeds fell off easily.

Discussion

In the present research, the anatomic formation and development of male and female cones of *P. orientalis* were studied in detail. The process of cone development in *P.*

Fig. 4. The embryonic development of female cones. (A) The normal developing ovules on the following 6 May. (B) Enlarged view of A showed the normal ovule on the following 6 May, arrow showed the free nuclei. (C) The abortive ovule on the following 6 May, arrow showed the abortive nucellus. (D) The normal developing ovules on the following 10 June. (E) Enlarged view of (D) showed the normal ovule on the following 10 June, arrows showed the free nuclei and vacuole. (F) The abortive ovule on the following 10 June. (G) The embryo on the following 29 July. (H) The embryo on the following 29 Aug. (I) The embryo on the following 29 Sept. (J) The mature embryo on the following 12 Oct. AO = abortive ovule; C = cotyledon; FN = free nuclei; NO = normal development ovule; V = vacuole.

orientalis was summarized in Table 1. The morphological initiation of buds was influenced by both internal and external factors, such as light, temperature, water, nutrients, and endogenous hormones (Jackson, 1970; Sanyal and Bangerth, 1998). From the earliest observed period of bud initiation, the male cones of *P. orientalis* started differentiating in early July, whereas the initiation of the female cones occurred relatively late, beginning in late July. The initiation process of cones was short, but due to unsynchronized development, different stages of cones could be observed in the same tree. After initiation finished, the development of sporophyll be-

gan. Compared with *T. plicata*, *Tsuga heterophylla*, and *Chamaecyparis nootkatensis*, the buds of *P. orientalis* differentiated later. The determination of the critical transformation point of bud initiation laid an important foundation for the transformation of male and female cones. For *P. orientalis*, the best treatment time should be between late June and mid-July if male and female cones need to be transformed through artificial regula-

After initiation, the microsporophyll began to develop. The process of tetrad formation by meiosis of the MMCs, which develop into mature pollen grains in gymnosperms,

could be roughly classified into three types: a) MMCs entered a dormant stage in winter and underwent a meiotic period after dormancy broke the following spring, such as *Pinus sylvestris* (Luomajoki, 1982), *Picea abies* (Luomajoki, 1982), and *Picea glauca* (Owens and Molder, 1979); b) MMCs formed mature pollen grains through meiosis before dormancy, for example *Larix gmelinii* (Luomajoki, 1982), *Chamaecyparis obtusa* (Owens et al., 1980), and *Taxus chinensis* (Pennell and Bell, 1986); and c) the MMCs underwent meiosis before dormancy and completed division after dormancy. For example, the MMCs of *Pseudotsuga menziesii* (Owens

Fig. 5. Development process and external morphology of female cones. (A–G) The female cones on 8, 15, 22, and 29 Apr.; 6 and 30 May; and 30 June of the second year. (H) The seeds on 30 June of the second year. (I–N) The female cones on 15 and 29 Apr., 30 May, 30 June, 15 July, and 17 Aug. of the second year. (O) The mature female cones about to crack on 1 Sept. of the second year. (P) The dehiscent female cones on 15 Oct. of the second year.

and Molder, 1971) and *Larix decidua* (Ekberg et al., 2010; Luomajoki, 1982) passed through dormancy in the diplotene stage, whereas *T. plicata* (Owens and Molder, 1971) and *T. heterophylla* (Owens and Molder, 1971) passed through dormancy in a pachytene stage. It was found in *L. gmelinii* (Rupr.) Kuzen. that over-

wintering with meiosis in the pachytene or diplotene stage could increase resistance to severe cold. The developmental process of *P. orientalis* showed that the MMCs were formed in early November and then entered into the meiotic stage immediately, followed by dormancy, suggesting that this species belonged to

the last type mentioned previously. According to the present research, with the meiosis of the MMCs, the tapetum cells started to degenerate. In the process of degeneration, the inner tangential wall of the cells disintegrated and disappeared, and the nutrients in the tapetum were released into the microsporangium

Fig. 6. The transverse and longitudinal diameters of female cones. Different letters in the same graph indicate statistically significant differences ($P < 0.05$).

Table 1. Summary of the development period of *Platycladus orientalis*.

Date	Male cone	Female cone
July	Buds differentiate, microsporangium produced	Buds differentiate
August	Development	Ovule primordium produced
September	Development	Megasporangium formed
October	Development	Development
November	MMCs formed, dormancy	Dormancy
December	MMCs division, dormancy	Dormancy
The following January	Tetrads formed	Dormancy
The following February	Mature pollen formed	Dormancy
The following March	Pollen becomes powder	Megaspore mother cell formed
The following April	Become dry	Ovules pollinated
The following May to August	—	Fruits and seeds developed
The following September to October	—	Cones cracked, seeds fall off

MMCs = microspore mother cells. Dashes mean the male cone had dropped out, and there was no male cone.

through the intracellular membranes, so the tapetum cells of *P. orientalis* were secretory.

This research also found that during the formation of MMCs, a large cavity sometimes formed inside the sporangium. This was consistent with the phenomenon observed in *P. orientalis* mentioned by Cao et al. (1997) and Wang et al. (2011b). This might be because of nutrient deficiencies in the MMCs. When MMCs were formed, a large number of nutrients were needed to enter the meiotic stage. However, MMCs could not meet the large demand at this time, so some MMCs needed to be degraded to maintain the normal development of other MMCs. In addition, this cavum phenomenon was also reported in Gentianaceae plants, in which some sporogenic cells became sterile because lacking of a well-developed tapetum to provide nutrients for the developing microspores (Hu, 1982). It was worth mentioning that no other abnormal phenomenon of pollen abortion was observed from the development process of male cones in *P. orientalis*.

According to this research, the initiation of the female cones occurred slightly later than that of the male cones. The number of female cones was small, and it was difficult to determine the location of female cones before initiation due to phenotype. When the female cones began to develop in April of the following year, they could be easily distinguished from their external morphology. There were fewer strobile in *P. orientalis*

might be because of fewer female cones, which was similar to the research of the tung tree (Li et al., 2020). In addition, during the development of female cones, some of the ovules were aborted, including the integument, and nucellar cells were disintegrated. Some ovules were able to develop normally into seedcoats, but the inner integument and nucellus were aborted. This abortive phenomenon was more common in angiosperms and less frequently reported in gymnosperms. Research on *Camellia oleifera* showed that the rates of ovule abortion were 85.5% and 46.3% under self-pollination and cross-pollination, respectively, which was mainly due to the late self-incompatibility in ovary (Liao et al., 2014). There were 10 to 16 seeds in the female cones of *P. orientalis*, and nutrition supply and competition existed among seeds during the development process, which resulted in embryo abortion in seeds.

In conclusion, the present research confirmed the accurate time, overwintering type, and tapetum development type of cones in *P. orientalis* through the anatomic study of the whole process of bud initiation, microsporogenesis, megasporogenesis, and male and female cone development. This provided a basis for subsequent hybridization, breeding, and artificial induction of male and female cone transformation in *P. orientalis*.

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