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A histological comparison of the development of pollen and female gametophytes in fertile and sterile *Cryptomeria japonica*

Received: 14 March 2005 / Accepted: 17 June 2005 / Published online: 2 August 2005
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Abstract To determine a possible mechanism causing male and female sterility in *Cryptomeria japonica* male and female cones were collected from a *C. japonica*, tree, ShinDai2, that lacks pollen release and fertile seeds and specimens were processed to examine the development of pollen and female gametophytes using light microscopy and field emission scanning electron microscopy. Pre-meiotic development proceeded normally, but the formation of aberrant meiotic products was observed in cones of both sexes. In sterile microsporangia, heterogeneous microspore populations ranging from monads to polyads gave rise to mature pollen grains of non-uniform size. These pollen grains were covered with an amorphous layer and adhered to each other. In addition, they remained in the microsporangia and were not released even after the onset of pollen dissemination from fertile trees. In the ovules of sterile female cones, megaspores with abnormal shapes, numbers, and sizes formed, and the development of female gametophytes was arrested at the free nuclear or archegonium formation stages. These gametophytes collapsed, and no fertile embryo was generated. Results indicate that meiotic defects are important in the sterility mechanism.

Keywords *Cryptomeria japonica* · Female gametophyte · Meiosis · Pollen · Sterility

Introduction

Cryptomeria is a coniferous genus in the Taxodiaceae, which is distributed throughout Japan and in areas of China. *Cryptomeria japonica* is one of the most important conifers for forestry in Japan because of its straight stem development, excellent growth rate, and wood quality. This species comprises 45% of the cultivated forests in Japan.

Allergic diseases are increasing worldwide (Ishizaki et al. 1987; Wüthrich 1989; Wüthrich et al. 1995). In particular, an increase in *C. japonica* pollinosis is becoming a social problem in Japan. *C. japonica* is wind-pollinated or anemophilous, so abundant pollen is released from male cones. The pollen of *C. japonica* is a major early-spring aeroallergen and affects nearly 10% of the exposed human population (Ishizaki et al. 1987). A recent epidemiological study found that 20% of people living in urban areas display significant symptoms in response to this allergen (Nishihata et al. 1999). Two major allergens in *C. japonica* pollen were identified as Cry j1 and Cry j2 (Yasueda et al. 1983; Sakaguchi et al. 1990; Sone et al. 1994).

In *C. japonica*, male cone primordia form in the axes of small branches from late June to late August. Microsporocyte meiosis begins in September, and microspores are formed from late October to late November. In December, the generative cell and the tube cell are formed. Staminate cones then remain in an arrested state of development until the following spring when pollen grains are released (Hashizume 1962; Nagao et al. 1989). In recent years, developing male-sterile *C. japonica* that do not release pollen has received interest as a potential measure to reduce allergenic problems associated with the release of pollen. Propagation and afforestation of male-sterile trees

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is expected to reduce the amount of pollen released in the future. In 1992, a male-sterile *C. japonica* tree was discovered for the first time in Toyama, Japan; in which microspores enlarged, fused, and ultimately collapsed (Saito et al. 1998). This male sterility was controlled by a pair of recessive nuclear genes (Taira et al. 1999).

Recently, another suspected male-sterile *C. japonica* was found in Niigata, Japan. This male-sterile tree, identified as ShinDai2, was observed for a period of 3 years, during which time it released no pollen and produced seeds that did not germinate. Therefore, this tree is suspected to be both male- and female-sterile. Female cone primordia form in the apices of branchlets from late July to early August in *C. japonica*. Ovules with developed integuments and nucelli are formed in late October, and the megaspore mother cell in the nucellus develops in the following spring (Yokoyama 1975; Takaso and Tomlinson 1989). After pollination, meiosis I of megaspore mother cells results in dyad cells of which the upper does not divide, while the lower undergoes meiosis II forming a megaspore triad (Singh and Chatterjee 1963). Although we have some anatomical knowledge of female cone development, few reports have documented female sterility in this species (Yokoyama 1997). Male and female sterility was identified in ShinDai2 based on the gross morphology of cones and seeds. However, the histological mechanisms causing sterility have not been investigated.

In this study, we histologically examined sterile male and female cones of *C. japonica*. Male and female cones were collected at fixed time intervals from a fertile and a sterile tree, and the development of pollen and female gametophytes was observed using light microscopy and field emission scanning electron microscopy (FE-SEM). The differences between fertile and sterile cones were investigated, and a possible mechanism causing sterility was examined.

Materials and methods

Plant materials

Histological samples were collected from the adult *C. japonica* trees from a 40-year-old plantation forest in Irihiro, Niigata, Japan. We examined ShinDai2, which is suspected to be both male- and female-sterile, in comparison with a fertile tree nearby.

Ten or more male cones were collected weekly from the beginning of August 2001 until the beginning of April 2002 to observe pollen development from the early pre-meiotic stage to maturation. In this area, pollen release begins in late March and lasts until middle May. Five to ten female cones were collected every 10 days from the time of pollination drop emergence in early April until the completion of embryogenesis in mid-August 2002.

Light microscopy

Male and female cones were fixed overnight in formalin-acetic acid-alcohol (FAA: 5:5:90, by volume) at 4°C. Fixed cones were dehydrated using a graded ethanol and *n*-butyl alcohol series and embedded in paraffin. Longitudinal serial sections approximately 8-μm thick were cut from the embedded blocks using a sliding microtome (ESM-100L; ERMA, Tokyo, Japan). Good serial sections were obtained using this microtome instead of a rotary microtome in this study. Sections were stretched in a distilled water bath, serially mounted, and affixed on glass microscope slides, and then dried by heating on a hot plate overnight at 45°C. After removal of the paraffin in xylene, sections were stained with Delafield's premixed aluminum hematoxylin (Schneider 1981) and 1% eosin in 70% ethanol, and mounted in EUKITT Mounting Medium (Electron Microscopy Sciences, Hatfield, PA, USA). Several male cones fixed in FAA were squashed and stained with 1% acetocarmine in 45% acetic acid to observe microsporocyte and pollen grain morphology. Micrographs were taken using a light microscope (BX-60; Olympus, Tokyo, Japan). Approximately 50 microsporangia and ovules at all developmental stages were examined.

Field emission scanning electron microscopy (FE-SEM)

A portion of the mature male cones collected from both the trees was fixed in 2.5% glutaraldehyde in 0.07 M phosphate buffer (pH 7.4) overnight at 4°C. Cones were dehydrated using a graded ethanol series and processed using the *t*-butyl alcohol freeze-drying method (Inoue and Osatake 1988). Dried cones were cut longitudinally with a knife and coated with a layer (approximately 3.5-nm) of platinum-palladium using an ion sputter coater (E-1030; Hitachi, Tokyo, Japan). Pollen grains in the microsporangia were observed using an FE-SEM (S-4500; Hitachi) at an accelerating voltage of 1.5 kV and a working distance of 10 mm. FE-SEM is a desirable way to examine biological samples for topographical information because it provides high-resolution images with negligible electrical charging of the samples at a low accelerating voltage.

Results

Pollen development

In both the fertile and the sterile tree, the external appearance of male cones was similar until the time of pollen release (Fig. 1a). In male cones collected from the sterile tree in August, microsporangia were filled with angular pollen mother cells surrounded by the tapetum. The appearance of the pollen mother cells in sterile male cones rarely differed from that in fertile male cones

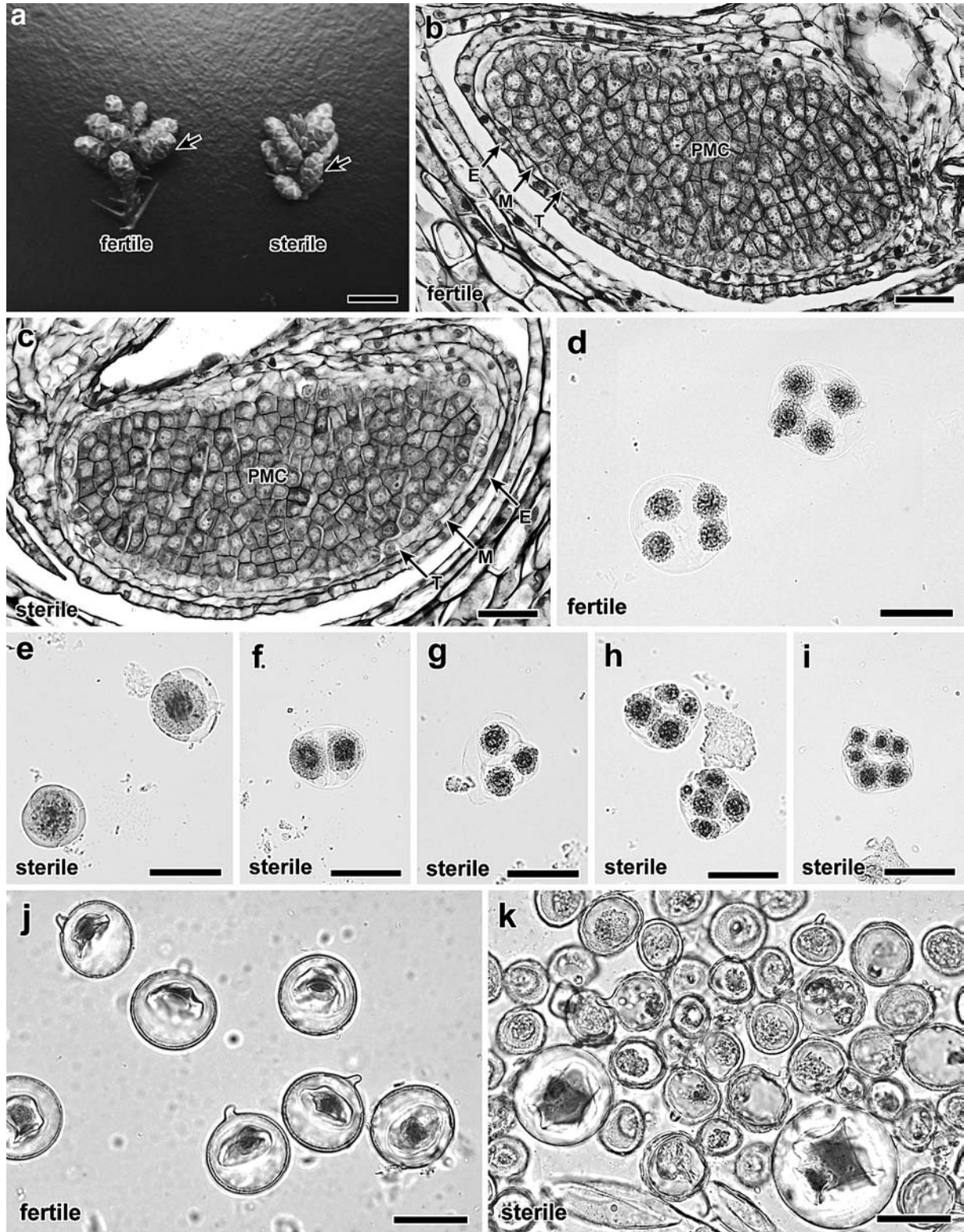


Fig. 1 External appearances of male cones and light micrographs (LM) of pollen development in fertile and sterile *Cryptomeria japonica*. **a** Mature male cones (arrows) of fertile and sterile trees. **b** LM of microsporangium of a fertile male cone. *PMC* pollen mother cells, *T* tapetum, *M* middle layer, *E* epidermis. **c** LM of sterile microsporangium with pollen mother cells (*PMC*), tapetum (*T*), middle layer (*M*) and

epidermis (*E*). **d** Isolated tetrads with fertile, uniformly sized microspores. **e-i** Examples of sterile microspores of varying sizes (same developmental stage as fertile microspores in **d**) showing monads (**e**), dyads (**f**), triads (**g**), tetrads, pentads (**h**) and hexads (**i**). **j** Fertile mature pollen grains. **k** Sterile mature pollen grains of varying sizes that adhered to each other. Scale bars indicate **a** 5 mm; **b, c** 50 µm; **d-k** 30 µm

(Fig. 1b, c). At the tetrad stage from September to October, tetrads of similar-sized microspores were observed in fertile male cones (Fig. 1d). However, sterile male cones had heterogeneous microspore populations arranged in monads, dyads, triads, tetrads, and polyads in the same microsporangium. Figure 1e–i illustrates some of the aberrant microspores of different sizes in the sterile microsporangia. Tetrads of similar-sized microspores were rarely seen, and most of them consisted of microspores of different sizes. In November, microspores separated and formed free spores in both fertile and sterile cones. Uniformly sized mature pollen grains formed in fertile cones in December (Fig. 1j). In contrast, pollen grains of various sizes formed in sterile cones, and some grains aggregated and adhered to each other (Fig. 1k). The sterile male cones did not dehisce and these abnormal pollen grains remained in the microsporangia when pollen dissemination from fertile trees began in April. Because the removal of undamaged pollen grains from sterile male cones was extremely difficult, we were unable to perform a pollen germination test.

Morphology of mature pollen using FE-SEM

Mature pollen grains from fertile male cones were spherical and 25–35 μm in diameter (Fig. 2a). The germinal zone had a distinctly smooth surface, whereas the body zone had a granular surface. The papilla was observed in the center of the germinal zone, and its tip was slightly curved. Small spherical orbicules adhered to the pollen surface (Fig. 2b). The shape of mature pollen grains from sterile male cones was similar to that of pollen from fertile cones, but displayed a wide range of diameters from 10 to 50 μm (Fig. 2c). The border between the germinal and body zones was indistinct, and pollen grains were covered with a layer of amorphous material (Fig. 2d) that appeared to contribute to the adherence of pollen to the interior of the microsporangium. Tapetum inside the microsporangium disappeared within the mature male cones of both fertile and sterile trees (Fig. 2a, c), and the thickness of the microsporangium walls was similar in fertile and sterile male cones. Amorphous material, however, was not seen on the inner surface of sterile microsporangium walls and pollen grains did not adhere to the walls (Fig. 2c).

Female gametophyte development

Pollination drops emerged, and cross pollination was observed in sterile female cones in early April. Viable pollen grains taken into the micropyle shed their outer layer (exine) and adhered to the nucellus (Fig. 3a). After pollination, pollen germinated and entered the nucellar tissue. At this stage, megaspore mother cells were observed in the ovules of sterile cones, and their appearance was similar to those in the ovules of fertile cones

(Fig. 3b). In the fertile ovules, three linear megaspores were observed after meiosis of megaspore mother cells in early May (1 month after pollination; Fig. 3c). However, more than three megaspores with distorted shapes and two megaspores of different size classes were observed in the sterile ovules at the same stage of development (Fig. 3d, e).

The development of female gametophytes within pollinated ovules in fertile trees reached the free nuclear stage, and pollen tubes began to grow through the nucellar tissue in early June (2 months after pollination; Fig. 4a). The functional megaspore underwent mitosis and created a coenocytic female gametophyte with a large, central vacuole. In contrast, female gametophytes of the sterile tree did not reach the free nuclear stage; and normal free nuclei were not seen. Instead, nuclei with different sizes were crowded within the ovule and a large central vacuole did not form (Fig. 4b). In the fertile tree, archegonia developed in the female gametophyte during late June to mid-July, after which fertilization occurred and embryogenesis was initiated (Fig. 4c). In the sterile tree, female gametophytes aborted and then degenerated and disappeared (Fig. 4d). In a very small number of cases, normal free nuclear gametophytes developed in sterile ovules, but the archegonia were small and had distorted shapes (Fig. 4e). Embryogenesis was completed in the fertile cones in mid-August. Mature embryos displayed well-differentiated cotyledons and hypocotyls and were surrounded by layers of female gametophytic cells with nutrient storage reserves (Fig. 4f). No normal embryos were found in sterile cones. Most female gametophytes were filled with yellowish-brown materials, indicative of abortion, and the remainder had already disintegrated (Fig. 4g). Because the abortion of female gametophytes and accumulation of yellowish-brown materials occurred very rapidly in sterile cones, this process could not be observed in detail.

Discussion

Micro- and megasporogenesis consist of three sequential stages, i.e., pre-meiosis, meiosis, and post-meiosis, which culminated in the production of gametes. Development of male and female cones was similar in the fertile and sterile *C. japonica* trees until the formation of pollen mother cells or megaspore mother cells. This observation indicates that pre-meiotic developments proceed normally in cones of both sexes of the sterile tree Shin-Dai2.

In male cones of this sterile tree, polyads with various numbers and sizes of microspores were formed at the normal tetrad stage, suggesting meiotic and/or cytokinetic defects in pollen mother cells. It is inferred that monads are caused by nuclear fusion or defects in chromosome segregation. The formation of microspores from dyads to polyads seems to result from abnormal chromosome segregation and cytokinesis. Defects in

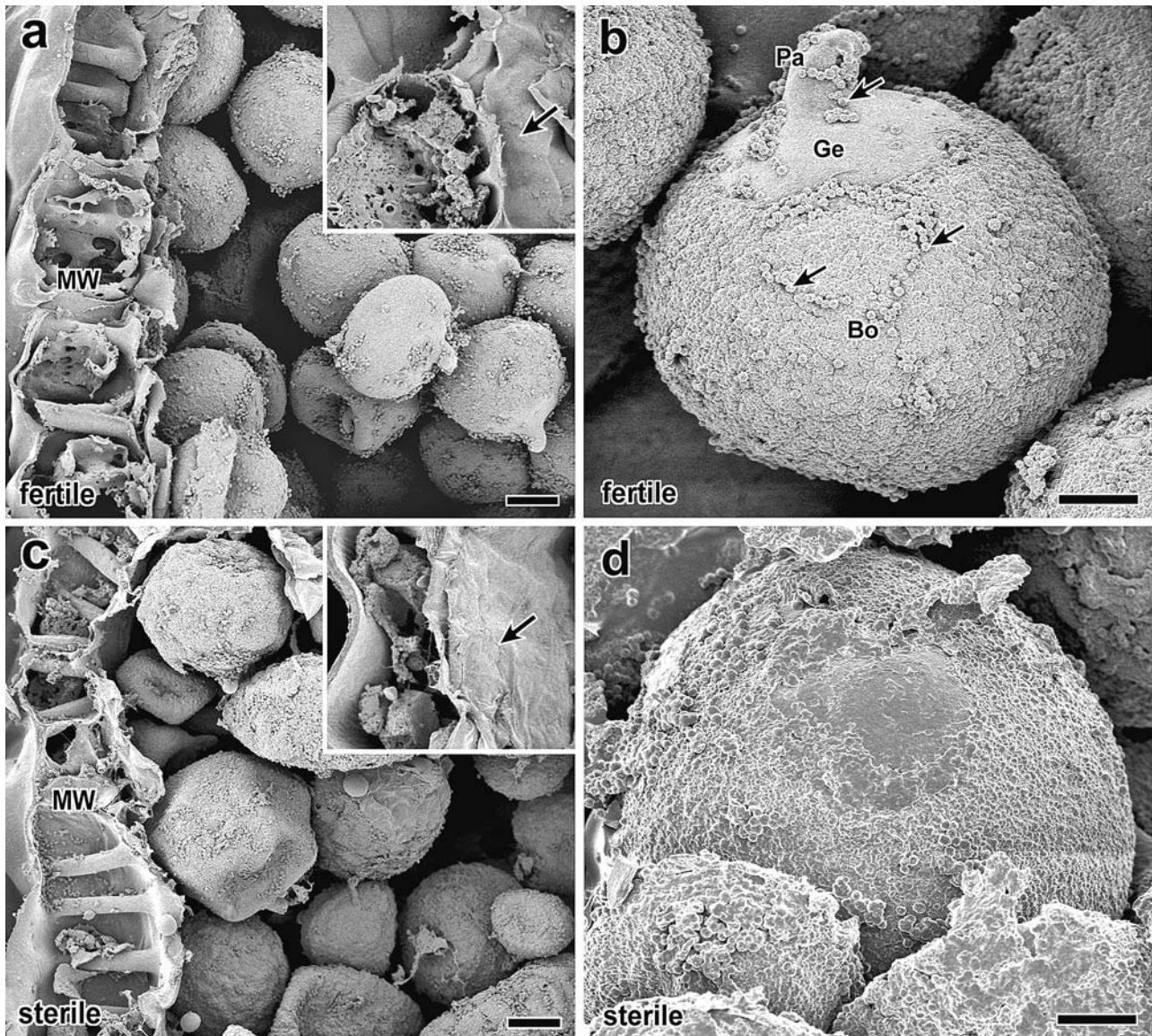


Fig. 2 Scanning electron micrographs of mature pollen grains in microsporangia of fertile and sterile *C. japonica*. **a** Fertile microsporangia containing uniformly sized pollen grains. **MW** microsporangium wall. The *inset* shows the inner surface of the microsporangium wall (*arrow*). **b** Fertile mature pollen showing the bent papilla (*Pa*), smooth surface of the germinal zone (*Ge*) and granular surface of the body zone (*Bo*). *Arrows* show orbicules. **c** Sterile microsporangia containing pollen grains of varying sizes. **MW** microsporangium wall. The *inset* shows the inner surface of the microsporangium wall (*arrow*). **d** Sterile mature pollen grain covered with an amorphous layer. *Scale bars* indicate **a**, **c** (including *insets*) 10 µm; **b**, **d** 5 µm

chromosome segregation and cytokinesis have been observed in the conifers *Picea abies* (Andersson 1947) and *Pinus silvestris* (Runquist 1968). In addition to the defects mentioned above, nuclear fusion was reported in *Cupressus dupreziana* (El Maâtaoui and Pichot 2001). Similar defects have been observed in some angiosperms such as *Zea mays* (Staiger and Cande 1993) and *Paspalum regnellii* (Pagliarini et al. 1998). In angiosperms,

mutations causing failure of the spindle apparatus have been reported as the main cause of meiotic defects (Bretagnolle and Thompson 1995). Similarly, meiosis of the male-sterile *C. japonica* may also be affected by mutant genes acting during meiotic division.

Based on the surface ornamentation, two morphological areas, i.e., a body zone and a germinal zone, can be recognized on pollen grains of the Taxodiaceae. The body zone has a fine, granular surface, and the center of the germinal zone supports a small protuberance called a papilla (Kurmann 1990; Uehara and Sahashi 2000). The basic morphology of pollen grains from fertile male cones of *C. japonica* was consistent with this description. However, pollen grains from sterile male cones were covered with an adherent amorphous layer, that appeared to cause adhesion between the pollen grains. The tapetum disappeared normally, but the amorphous material remaining on the pollen surface in sterile microsporangia, did not appear to be remnant tapetal cell

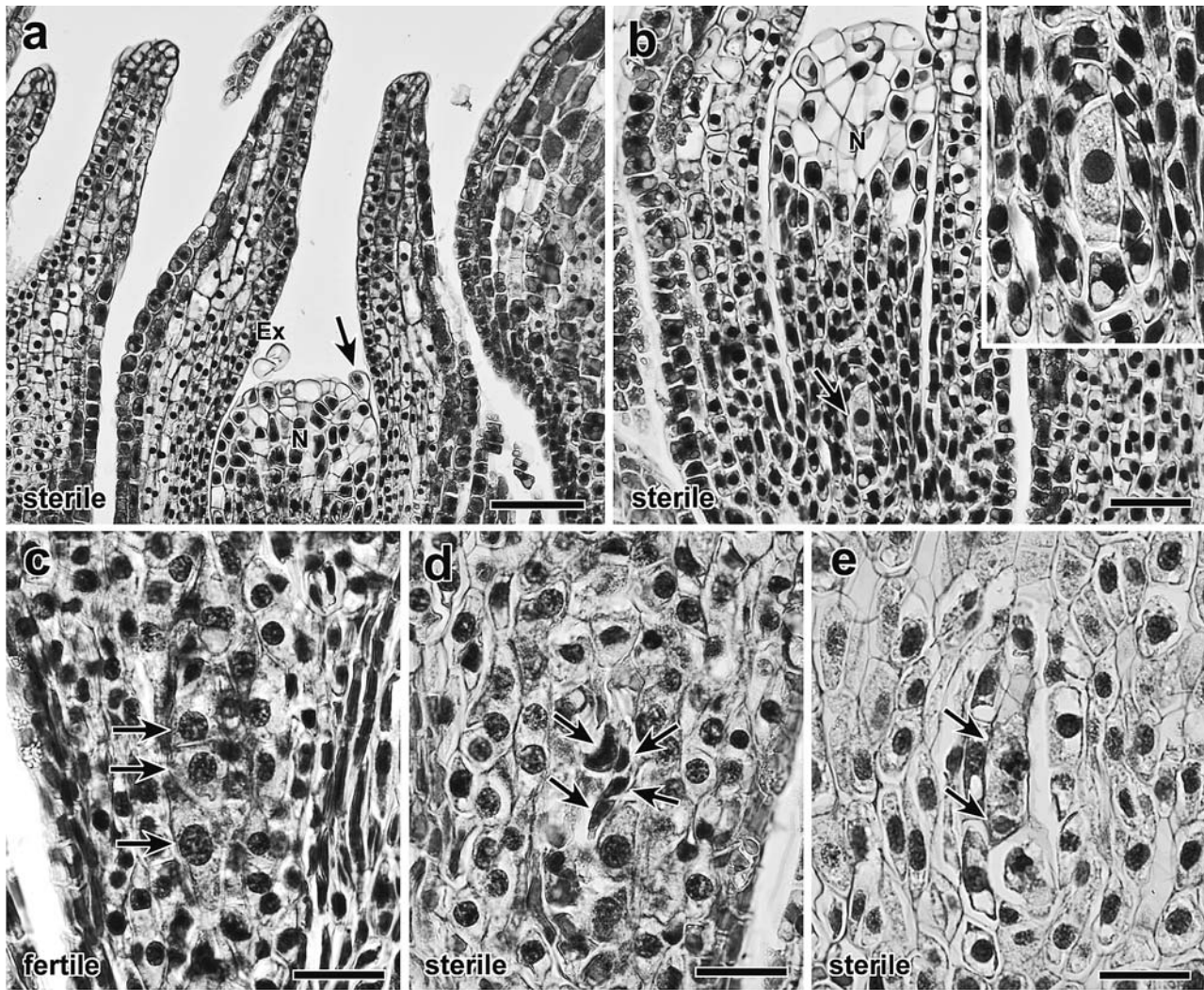
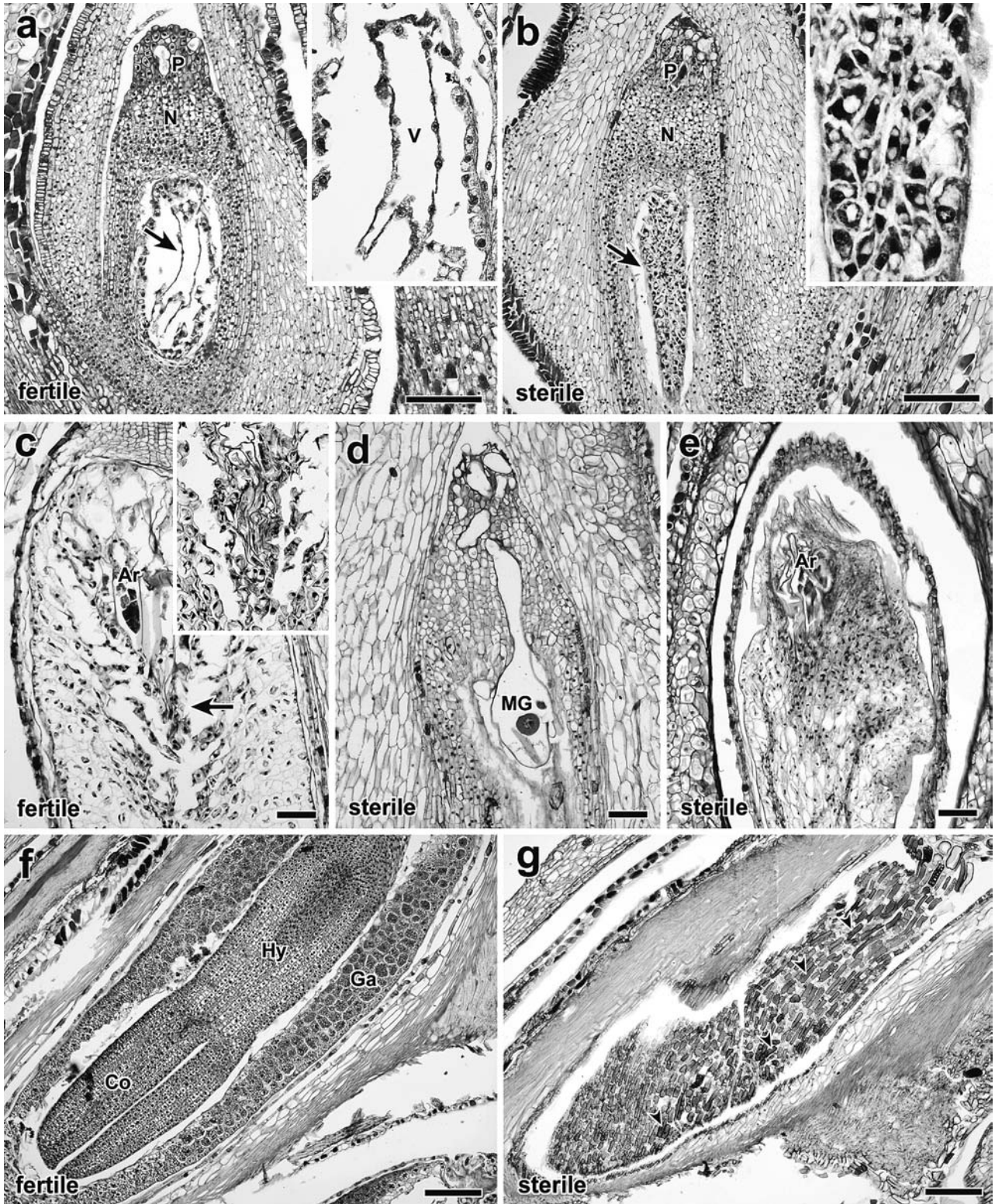


Fig. 3 Light micrographs of ovules in female cones of fertile and sterile *C. japonica*. **a** Pollinated ovule in a sterile female cone showing a pollen grain (arrow) that shed the exine (Ex) and contacted the nucellus (N). **b** Megaspore mother cell (arrow) in a sterile ovule. N nucellus. The inset shows the megaspore mother cell at higher magnification. **c** Linear megaspores (arrows) in the nucellus of fertile ovules. **d, e** Examples of sterile megaspores with abnormal shapes, numbers, and sizes (arrows). Scale bars indicate **a** 100 µm, **b** 50 µm, **c–e** 30 µm

Male sterility has been reported in several conifer species, including *Picea abies* (Andersson 1947), *Pinus monticola* (Wilson et al. 2003), and *C. japonica* (Saito et al. 1998). Saito et al. (1998) indicated that male sterility occurred because of microspore collapse, presumably caused by microspore abortion, after separation of the microspores from the tetrad in sterile *C. japonica*. However, our results differ, indicating instead that male

cytoplasm, but a more resistant material arising from imperfect exine formation in the callose wall stage. The tapetum surrounding the microspores synthesizes and secretes materials involved in pollen wall formation and normally degenerates as the pollen matures (Chapman 1987; Mascarenhas 1990). In the case of sterile pollen, apparently tapetal cell malfunctions may have resulted in abnormal deposition of pollen walls. Such changes in the function of tapetal cells may arise from timing defects or mutations in biochemical pathways of wall synthesis. The adhesion of pollen grains to the inner surface of sterile microsporangium walls was not seen, suggesting that the lack of pollen release from sterile cones is caused by defects in the walls themselves.

Fig. 4 Light micrographs of female gametophyte development in female cones of fertile and sterile *C. japonica*. **a** Fertile ovule with female gametophyte at the free nuclear stage (arrow). P pollen tube, N nucellus. The inset shows free nuclei and a large vacuole (V) at higher magnification. **b** Sterile female gametophyte (arrow) at the same developmental stage as the fertile female gametophyte in **a**. P pollen tube, N nucellus. The inset shows crowded nuclei with different sizes at higher magnification. **c** Archegonia (Ar) and developing embryos (arrow) in fertile ovules. The inset shows developing embryo at higher magnification. **d** Sterile ovule in which female gametophyte has disappeared. MG male gamete. **e** Aberrant archegonia (Ar) in sterile ovules. **f** Fertile embryo with cotyledons (Co) and hypocotyls (Hy). **g** Sterile megagametophyte filled with yellowish-brown material (arrow-heads). Scale bars indicate **a, b** 200 µm; **c–e** 100 µm; **f, g** 500 µm



sterility in the *C. japonica* ShinDai2 is caused by pollen wall deposition defects at a later stage that secondarily contribute to inability of the pollen to disseminate.

Little information is available on female sterility, especially with regard to conifers. Orr-Ewing (1977)

found that female sterility in *Pseudotsuga menziesii* was the result of chromosomal aberrations, gene action, or cytoplasmic effects that caused abortion or upset development. Recently, both male and female sterility were reported in *Pinus monticola*. In this species, meiosis of the

megaspore mother cell results in the formation of normal megaspores, but development is arrested at the functional megaspore stage (Wilson et al. 2003). Yokoyama (1997) found that female sterility in *C. japonica* was associated with ovule abortion, female gametophyte abortion, lack of pollination, or collapse of the embryo. Our results confirmed that female sterility in ShinDai2 is caused by abortion during female gametophyte development.

In *C. japonica*, meiosis I of megaspore mother cells results in dyad cells of which the upper cell does not divide, while the lower cell undergoes meiosis II, resulting in a linear triad rather than tetrad of megaspores. The megaspore closest to the chalazal end becomes the functional megaspore and develops into the female gametophyte (Singh and Chatterjee 1963). In the sterile tree, female cones developed megaspores that were abnormal in shape, number, and size, indicating defects in female meiosis. Irregularities in chromosome segregation and cytokinesis during meiosis seem to result in the formation of such abnormal megaspores. Ploidy of female gametophytes can sometimes be identified by size (Stelly and Peloquin 1985). Differences in cell and nuclear sizes of megaspores may also reflect different nuclear DNA contents, and aberrant nuclear DNA contents in functional megaspores, which may contribute to the abortion and disintegration of sterile female gametophytes.

Several genes and mutations that affect male meiosis have been recently reported in *Arabidopsis thaliana*. The *Arabidopsis skp1-like1* mutation (Yang et al. 1999) and mutations in *MEI1* (He and Mascarenhas 1999) are defective in chromosomal separation during meiosis in the anther, resulting in the formation of polyads with abnormal numbers, sizes, and karyotypes at the normal tetrad stage. Compared with genes controlling male meiosis, few genes or mutations affecting female meiosis have been identified. Mutations in the *SYN1/DIF1* gene disrupt chromosomal segregation during both male and female meiosis (Bai et al. 1999; Bhatt et al. 1999). Recently, a mutant called *dyad* has been identified that results in female-specific arrest of meiosis at the dyad stage (Siddiqi et al. 2000). Mutations of genes confirmed to be active in the sporophytic tissue, and specifically in the tapetum, have also been shown to cause male sterility in maize (Cigan et al. 2001), *Arabidopsis* (Wilson et al. 2001), and soybean (Ilarslan et al. 1999). In conifers, genes involved in male and female sterility have rarely been studied. We expect that the *C. japonica* tree ShinDai2 will contribute to the understanding of the genetics and molecular biology of sterility in conifers in the future through molecular analysis of genes differentially expressed between fertile and sterile cones.

The use of male-sterile *C. japonica* trees in the forestry cycle could help to reduce *C. japonica* pollinosis, which has become a nationwide problem in Japan. The vegetative propagation of male-sterile trees, such as ShinDai2, by cutting propagation and afforestation would contribute to alleviating problems related to pollen allergens.

Acknowledgements This study was supported by funds from the Research Project for Utilizing Advanced Technologies in Agriculture, Forestry and Fisheries. We wish to thank Dr. M. Yoshida, Graduate School of Bioagricultural Sciences, Nagoya University, for technical assistance with light microscopy and field emission scanning electron microscopy. We are also grateful to S. Akasaka and S. Miura, Faculty of Agriculture, Niigata University, for assistance in preparing samples for microscopic observation.

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