Development of Pollen and Female Gametophytes in *Cryptomeria japonica*

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ABSTRACT

Cryptomeria japonica (Taxodiaceae) is a tall evergreen conifer that is native to Japan and China. It is important as an ornamental tree and as a source of lumber; thus, it has been planted in many regions of Japan. The pollen of *C. japonica* causes pollinosis, which is becoming a serious problem in Japan. Male-sterile *C. japonica* that release no pollen have recently gained interest as a potential measure to alleviate this problem. *C. japonica* is monoecious, and male and female cones usually occur on different branchlets. Both male and female cones begin to develop in summer. In male cones, meiosis of pollen mother cells occurs in October, and mature pollen grains form from late October to late November. Pollen grains are then disseminated in the following spring after winter dormancy. In female cones, ovules with developed integuments and nucelli form in late October, and megaspore mother cells undergo meiosis at the time of pollination. Fertilization occurs in June, and embryo development is completed in August. A better understanding of this reproductive process will contribute not only to breeding and genetic research, but also to measures against pollinosis caused by this species. This mini-review describes the current information available on the process of pollen and female gametophyte development and introduces recent studies of sterility in *C. japonica*.

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INTRODUCTION

Cryptomeria is a coniferous genus that belongs to the ancient family Taxodiaceae. *Cryptomeria japonica*, the sole species in this genus, is a tall evergreen tree with a conical crown and bluish-green foliage that is native to Japan and China. The straight trunk supports wide-spreading branches with drooping branchlets. The awl-shaped leaves are 0.5-1 cm long, spirally arranged, and point forward, while their bases clasp the twigs. The bark is reddish-brown, fibrous, and peels off in vertical strips. The wood has a pleasant color and scent, and is strongly rot resistant, easily processed, and used for buildings, bridges, furniture, and utensils (Ohba 1993; Fu *et al.* 1999). *C. japonica* has been used for various purposes such as an ornamental tree, a windbreak, and a source of lumber. In particular, it is one of the most popular and commercially valuable trees in Japan. It has also been widely introduced into other temperate areas

outside Japan and China (Page 1990).

    The reproductive organs of *C. japonica* are male and female cones. *C. japonica* is a monoecious tree, and male and female cones usually occur on different branchlets. Male cones arise in clusters near the apex of young shoots. Each cone is a small ovoid or ellipsoid, and arises in the axil of a bract. Female cones are almost globular and formed at the terminus of branchlets. Pollination occurs from February to April, and seeds mature in October. The seeds are dark brown, irregular ellipsoid with a somewhat winged margin, and 4-6 mm long. Seed germination is the epigeal type, which is usual for conifers. *C. japonica* is wind-pollinated or anemophilous, and abundant pollen is released from male cones. The pollen of *C. japonica* causes pollinosis, which is becoming a serious problem in Japan.

    C. japonica has long been planted throughout Japan; plantation forests now cover 4.5 million ha. Currently, approximately 12% of the total land in Japan is covered with
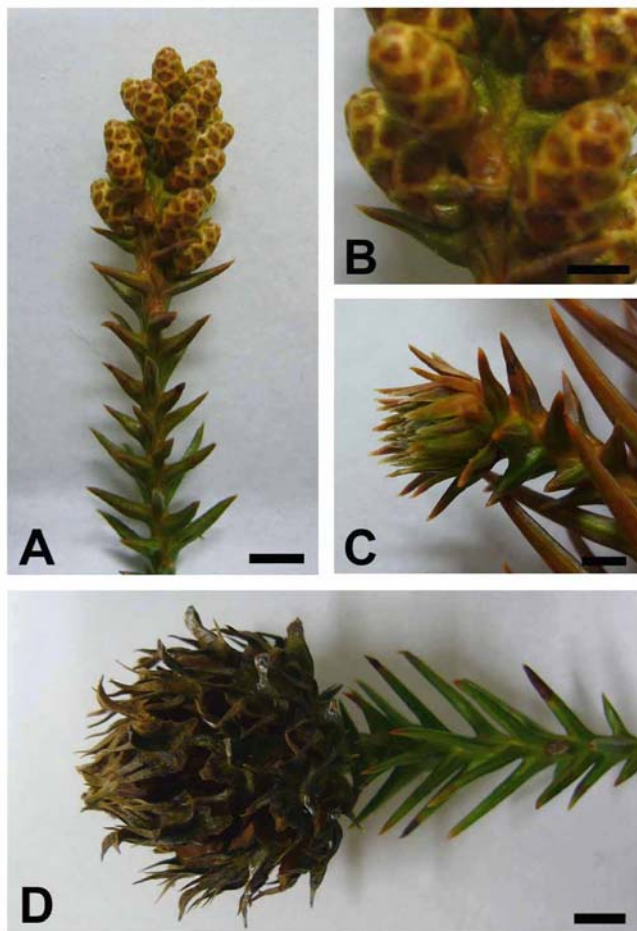
Invited Mini-Review

Fig. 1 External appearances of male and female cones of *Cryptomeria japonica*. (**A**) A cluster of mature male cones. (**B**) Higher magnification of mature male cones. (**C**) A young female cone before pollination. (**D**) A mature female cone. Scale bars indicate (**A**), (**D**) 5 mm; (**B**), (**C**) 2 mm.

this species. The pollen of *C. japonica* is a major source of aeroallergens in early spring and causes rhinitis and conjunctivitis. The prevalence of *C. japonica* pollinosis has increased during recent decades, and more than 16% of the Japanese population currently suffers from this allergy (Okuda 2003). Male-sterile *C. japonica* that releases no pollen has recently gained interest as a potential measure to alleviate allergenic problems associated with pollen release. Propagation and afforestation of male-sterile trees are expected to reduce the amount of airborne pollen in the future.

Knowledge of the reproductive process of *C. japonica* would be useful for breeding, genetic research, and measures against pollinosis. This mini-review describes information available on the process of pollen and female gametophyte development and introduces recent sterility studies in *C. japonica*.

MORPHOLOGY OF MALE AND FEMALE CONES

Male cones arise in the axils and form clusters near the apex of branchlets (**Fig. 1A**). A cluster consists of numerous cones (usually 10-20 cones), and each cone is sessile, oval or ellipsoidal, and approximately 5 mm long (**Fig. 1B**). Male cones turn from yellow-green to plum red when they mature. A male cone contains many spirally arranged microsporophylls (Fu *et al*. 1999). Each microsporophyll has a stalk that is almost perpendicular to the axis of the cone. It bears three or four microsporangia on the lower part of its abaxial surface.

Female cones are formed in the apices of branchlets. The young female cone has a curved stalk (**Fig. 1C**). The cone emerges from a rosette of leaves and is nearly 5 mm in diameter. At the time of pollination, it has a flat top, but

becomes almost globular within 1 month. A mature female cone has a tapering apex and is 1-2 cm in diameter (**Fig. 1D**). It contains 20-30 spirally arranged scales. Sharp pointed scales give the cone a spiny appearance. Each scale bears three or four ovules whose micropyles point away from the cone axis (Singh and Chatterjee 1963).

POLLEN DEVELOPMENT

Genes involved in the formation of male cones

In angiosperms, genetic studies using floral homeotic mutants have led to the ABC model of flower development. This model proposes that the combined action of three classes of homeotic genes specifies the identity of four types of floral organs (Bowman *et al*. 1991; Coen and Meyerowitz 1991). In brief, A-class genes specify sepals, A- and B-class genes specify petals, B- and C-class genes specify stamens, and C-class genes specify carpels. In *Arabidopsis*, *APETALA1* and *APETALA2* are A-class genes, *PISTILLATA* and *APETALA3* are B-class genes, and *AGAMOUS* is classified as a C-class gene. Molecular cloning has revealed that most of these ABC genes encode a well-conserved DNA binding domain, the MADS box (Schwarz-Sommer *et al*. 1990; Yanofsky *et al*. 1990; Jack *et al*. 1992; Mandel *et al*. 1992; Goto *et al*. 1994).

MADS-box genes encode a large family of transcription factors. Two MADS-box genes expressed specifically in male cones have been isolated in *C. japonica*: *CjMADS1* and *CjMADS2* (Fukui *et al*. 2001). These genes are homologous to B-class genes which determine the identities of petals and stamens in angiosperms and are thought to be involved in the formation of male cones. *CjMADS1* is expressed in male cones throughout their development, while *CjMADS2* is transiently expressed during male cone development. The expression of these genes is regulated by factors that are sensitive to environmental signals (Fukui 2003). However, the localization of transcripts and protein products of these genes at the tissue or cellular level has not been clarified. In addition, no other ABC-type gene has been isolated from *C. japonica*. Further isolation and functional analysis of homologs of angiosperm floral homeotic genes would increase our understanding of the details of floral initiation and development in *C. japonica*.

Morphology of pollen grains

The pollen of the Taxodiaceae is generally spherical and granular without air bladders. Several authors have examined the morphology of mature *C. japonica* pollen (Xi and Wang 1989; Kurmann 1992). Mature *C. japonica* pollen grains are spherical and 25-35 μm in diameter (**Fig. 2A**). Based on the surface ornamentation, two morphological areas, i.e., a germinal zone and a body zone can be recognized (Uehara and Sahashi 2000; Hosoo *et al*. 2005). The germinal zone has a distinct smooth surface, whereas the body zone has a granular surface. The papilla is in the center of the germinal zone and has a slightly curved top. Small spherical orbicules adhere to the pollen surface (**Fig. 2B**). The pollen wall consists of a single layer of outer wall (exine) and four sub-layers of inner wall (intine; Miki-Hirosige *et al*. 1994). The exine consists of an outer granular extexine and an inner lamellate endexine. The details of pollen wall development in *C. japonica* were investigated by Uehara and Sahashi (2000) using scanning and transmission electron microscopy.

Microsporangium and microsporogenesis

Male cone primordia are formed from late June to late August. Before meiosis, the microsporangium is filled with angular pollen mother cells with large nuclei (**Fig. 3A, 3B**). The microsporangium wall consists of the epidermis, the middle layer, and the tapetum, which is the innermost surface (Hosoo *et al*. 2005). The epidermis acquires annular
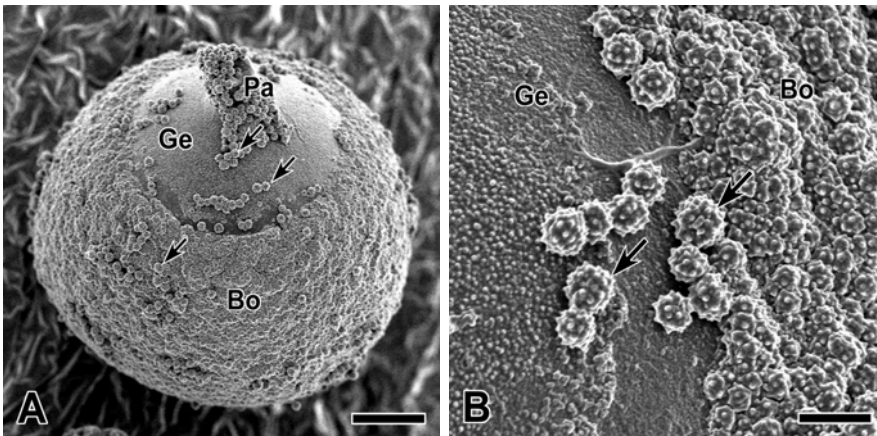
Fig. 2 Scanning electron micrographs of mature pollen grains of *Cryptomeria japonica*. (**A**) Mature pollen showing the bent papilla (*Pa*), smooth surface of the germinal zone (*Ge*), and granular surface of the body zone (*Bo*). (**B**) Higher magnification of mature pollen. *Ge* germinal zone, *Bo* body zone. *Arrows* in (**A**) and (**B**) indicate orbicules. Scale bars indicate (**A**) 5 µm; (**B**) 1 µm.
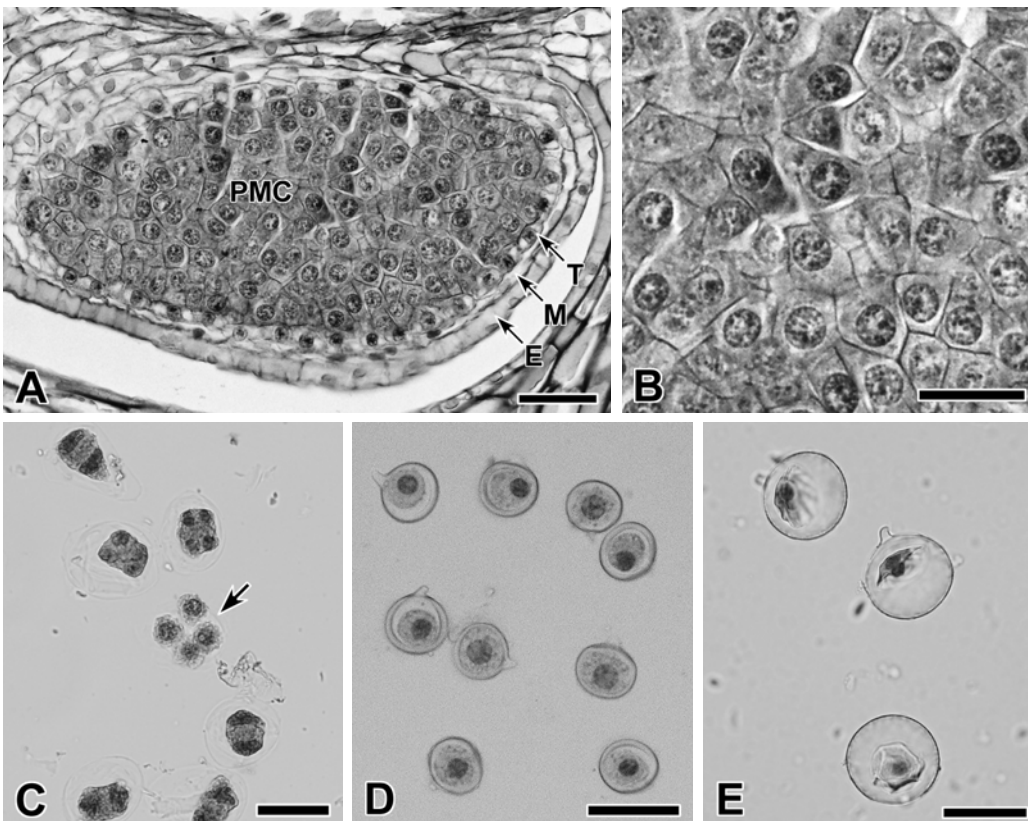

Fig. 3 Light micrographs of pollen development in *Cryptomeria japonica*. (**A**) Microsporangium with pollen mother cells (*PMC*), tapetum (*T*), middle layer (*M*), and epidermis (*E*). (**B**) Pollen mother cells at higher magnification. (**C**) Pollen mother cells during meiosis and microspore tetrads (*arrow*). (**D**) Microspores after separation from tetrads. (**E**) Mature pollen grains. Scale bars indicate (**A**) 50 µm; (**B**) 20 µm; (**C**)-(**E**) 30 µm.

thickenings during the maturation of pollen. The tapetum acts not only as a nutrient supply, but also synthesizes and secretes materials involved in pollen wall formation (Chapman 1987; Mascarenhas 1990). The tapetal cells degenerate as the pollen matures. Individual pollen mother cells then separate from each other. They become circular and are enclosed by a callose wall during meiosis (Uehara and Sahashi 2000).

Meiosis of pollen mother cells actively occurs and tetrads of similar-sized microspores are formed in October (**Fig. 3C**). The callose wall encloses the tetrads and separates each microspore cell within the tetrad. The exine begins to form just after meiosis and becomes thicker. The papilla protrudes, and its tip bends during the tetrad stage (Uehara and Sahashi 2000). The orbicules, formed in the tapetum, appear at the end of the tetrad stage. Microspores separate from the tetrads after dissolution of the callose wall (**Fig. 3D**). They become vacuolated and increase in volume. The exine is obviously increased in thickness, and the orbicules adhere to the entire pollen grains at this stage. Mature pollen grains are formed from late October to late November (**Fig. 3E**). In December, the generative cell and the tube cell are formed. Male cones then remain in an arrested state of development until the following spring when

pollen grains are disseminated (Nagao *et al*. 1989).

Pollen allergens

The existence of numerous allergens in the pollen of *C. japonica* has been suggested (Ito *et al*. 1986; Kawamoto *et al*. 2002), and several antigens have been identified. Cry j 1 is a major allergen homologous to the short ragweed allergen, Amb a 1 (Yasueda *et al*. 1983; Sone *et al*. 1994). Analysis of amino acid sequences and enzymatic analysis revealed that Cry j 1 has pectate lyase activity (Sone *et al*. 1994; Taniguchi *et al*. 1995). Cry j 2 is the second major allergen, possessing polymethylgalacturonase activity (Sakaguchi *et al*. 1990; Komiyama *et al*. 1994; Namba *et al*. 1994; Ohtsuki *et al*. 1995). CJP-6 is another important allergen that is homologous to isoflavone reductase family (Kawamoto *et al*. 2002). Fujimura *et al*. (2005) identified CJP-4 as a novel class IV chitinase allergen.

Transcripts of the *Cry j 1* gene are abundant in pollen but are not detectable in other tissues such as leaves, stems, roots, and female cones (Wang *et al*. 1998). The *Cry j 1* gene is expressed in male cones at the time of microspore formation and pollen maturation (Fukui *et al*. 2001). Localization of the Cry j 1 protein in *C. japonica* pollen was in-

vestigated using an immunocytochemical method (Miki-Hiroshige *et al*. 1994). Cry j 1 is localized on the exine and orbicules in microspores and mature pollen grains. This allergenic protein is also found in the tapetal materials remaining in immature microsporangia. Cry j 1 is likely synthesized in the tapetum and accumulates on the exine and orbicules during pollen maturation. Localization of transcripts and protein products of other allergen genes in the male cone are still poorly understood. Further studies will help elucidate the mechanisms of synthesis and accumulation of pollen allergens in *C. japonica*.

DEVELOPMENT OF FEMALE GAMETOPHYTES

Ovule

Female cone primordia form from late July to early August, nearly 1 month after the male cones. Ovules with developed integuments and nucelli are formed in late October. Development of the female cone and the ovule was investigated using scanning electron microscopy (Takaso and Tomlinson 1989). The ovule has a massive nucellus, and the integument is free from the nucellus to its base (**Fig. 4A**). Female cones then enter winter dormancy and their development is resumed in the following spring. The micropyle is wide open before pollination and points upward. Pollination drop related to the pollen capture mechanism that facilitates the introduction of pollen to vicinity of the ovule has been observed in many species of Cupressaceae and Taxodiaceae (Takaso 1990; Tomlinson and Takaso 1998). Pollen enters the pollination drop, the exine is cast off, and the intine swells enormously (Tomlinson and Takaso 1998). Pollination drop on the ovule is also seen in *C. japonica* at the time of pollination. Soon after pollination, the epidermal and subepidermal cells start elongating inward and close the micropyle (Singh and Chatterjee 1963).

Megasporogenesis

At the time of pollination, the megaspore mother cell differentiates in the nucellus (**Fig. 4B**). Megaspore mother cells then undergo meiosis to produce three linear megaspores (**Fig. 4C**). Meiosis I results in dyad cells; the upper cell does not divide, while the lower cell undergoes meiosis II and forms a megaspore triad (Singh and Chatterjee 1963). The charazal megaspore functions while the others soon degenerate. As the megaspore enlarges, the surrounding nucellar cells form spongy tissue. The nucellar cells in the
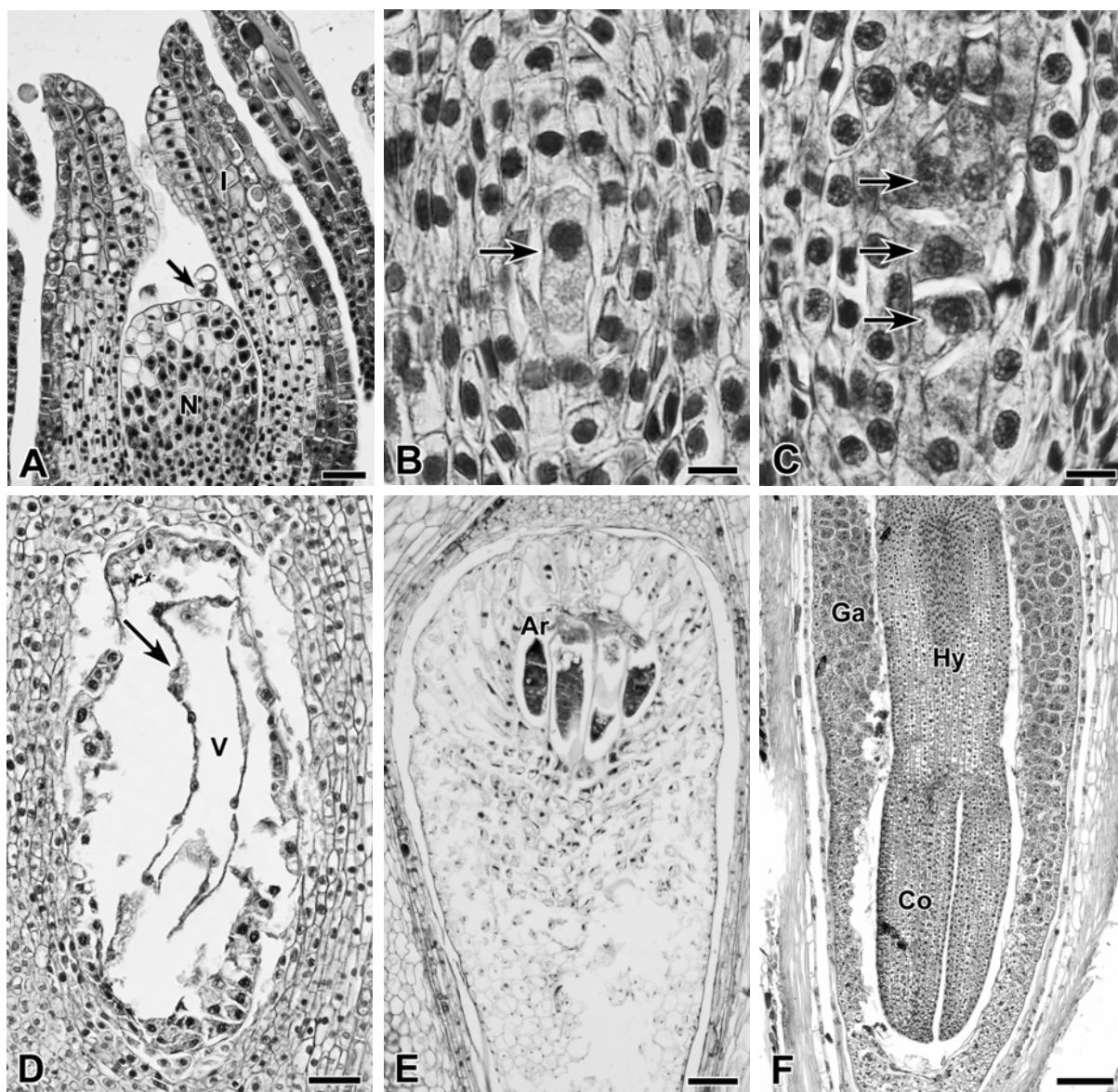

Fig. 4 Light micrographs of ovules and female gametophyte development in female cones of *Cryptomeria japonica*. (A) Pollinated ovule in a female cone showing a pollen grain (*arrow*) that contacted the nucellus (*N*). *I* integuments. **(B)** Megaspore mother cell (*arrow*) in the nucellus. **(C)** Linear megaspores (*arrows*) in the nucellus. **(D)** Female gametophyte at the free nuclear stage (*arrow*) with a large vacuole (*V*). **(E)** Archegonial complex containing many archegonia (*Ar*) at the micropylar end of the female gametophyte. **(F)** Matured embryo with cotyledons (*Co*) and hypocotyls (*Hy*). *Ga* gametophytic cells. Scale bars indicate **(A)** 50 µm; **(B), (C)** 10 µm; **(D), (E)** 100 µm; **(F)** 200 µm.

immediate vicinity of the spongy tissue degenerate during development of the free nuclear gametophyte.

Female gametophyte

The functional megaspore undergoes mitosis and creates a coenocytic gametophyte 2 months after pollination (Hosoo *et al*. 2005). A vacuole appears in the center, and the free nuclei are arranged in a peripheral layer of cytoplasm. Wall formation in the gametophyte then takes place and a fully cellularized gametophyte is formed. At the same time, the archegonial initials differentiate at the micropyler end of the gametophyte. The development of the archegonium was described in detail by Singh and Chatterjee (1963). A mature archegonial complex consists of many archegonia (**Fig. 4E**).

Fertilization and embryogenesis

Fertilization occurs and embryogenesis is initiated in July, and embryogenesis is completed in mid-August (Hosoo *et al*. 2005). The development of the embryo proceeds quickly and the ovule shows several elongated suspensor tiers within 1 week after fertilization. Fertilization and embryogenesis in *C. japonica* was studied by Singh and Chatterjee (1963) and Dogra (1966), but no detailed study has been performed since then. Further histological, cytochemical, and ultrastructural studies are necessary to fully understand these complex processes. A mature embryo has well-developed cotyledons and hypocotyl and is surrounded by layers of gametophytic cells filled with starch grains and fatty materials (**Fig. 4F**).

MALE AND FEMALE STERILITY

Male sterility is a desirable characteristic not only for studies on pollen development, but also for anti-pollinosis measures. In the early 1990s, a male-sterile *C. japonica* tree was discovered for the first time in Toyama, Japan. Saito *et al*. (1998) examined the anatomical details of male sterility in this tree. Pre-meiotic development and meiosis proceeded normally, but microspores enlarged, fused, and finally collapsed after they separated from the tetrads. Male sterility was controlled by a recessive allele at a single gene locus and expressed only in homozygotes (Taira *et al*. 1999). Saito *et al*. (2005) showed that *C. japonica* trees possessing the male-sterility gene may be widely distributed. Later, a *C. japonica* tree possessing the male-sterility gene as a heterozygote was found (Saito and Taira 2005).

Another *C. japonica* tree that is both male- and female-sterile was found in Niigata, Japan. Hosoo *et al*. (2005) investigated the development of pollen and female gametophytes in this sterile tree using light and scanning electron microscopy. Pre-meiotic development proceeded normally, but aberrant meiotic products were formed in the cones of both sexes. In male cones, heterogeneous microspore populations ranging from monads to polyads gave rise to pollen grains of various sizes. In female cones, megaspores with abnormal shapes, numbers, and sizes were formed, and female gametophytes collapsed. These results indicate that meiotic defects are important in the sterility mechanism. To date, developmental defects leading to sterility have been confirmed histologically in *C. japonica*. To understand the mechanisms causing sterility in detail, further studies on the development of pollen and female gametophytes in the sterile trees at the molecular level are necessary. Molecular analysis of genes differentially expressed in fertile and sterile cones should reveal the genes or mutations involved in sterility.

There is growing interest in identifying the mechanisms of male and female sterility in forest tree species. This interest is fuelled by the need for genetically engineered trees to be sterile, to prevent the release of engineered genes into natural or feral populations when engineered trees are released into the environment (Strauss *et al*. 1991).

Gene recombination technology was recently developed in *C. japonica*, and breeding using gene transduction is currently being established. The *C. japonica* tree that is both male- and female-sterile (Hosoo *et al*. 2005) could be valuable material for molecular breeding of *C. japonica* trees by transduction of useful genes in the future.

CONCLUDING REMARKS

Cryptomeria japonica is a conifer used as an ornamental and timber tree. However, pollinosis caused by *C. japonica* pollen is a serious health problem in Japan. The development of pollen and female gametophytes has been studied using morphological and histological approaches. Further knowledge of the process has been gained through analyses of sterile trees. However, many areas of the reproductive process of this species remain unclear. In particular, there is scant information available on the processes that occur in female cones, including megasporogenesis, female gametogenesis, and embryogenesis. Further histological and cytochemical analyses will reveal details of the reproductive process.

Pollen allergens have been identified and characterized, and the molecular basis of male cone development has recently been studied in *C. japonica*. Molecular analysis of the development of pollen and female gametophytes will be important in future studies. Cloning and characterization of genes involved in sterility would help to elucidate the molecular mechanisms controlling sporogenesis and gametogenesis. A better understanding of the reproductive process in *C. japonica* will contribute not only to breeding and genetic research, but also to anti-pollinosis measures.

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